Coactivation of μ- and κ-Opioid Receptors May Mediate the Protective Effect of Testosterone on the Development of Temporomandibular Joint Nociception in Male Rats

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Aims: To investigate whether the protective effect of testosterone on the development of temporomandibular joint (TMJ) nociception in male rats is mediated by the activation of central opioid mechanisms. **Methods:** Experiments were performed on 156 male Wistar rats. A pharmacologic approach was used to assess the ability of opioid receptor antagonists infused into the dorsal portion of the brainstem and adjacent to the caudal component (subnucleus caudalis) of the spinal trigeminal nucleus to block the protective effect of testosterone in male rats. The TMJ injection of 0.5% formalin was used as a nociceptive stimulus. One-way or two-way ANOVA was used for data analyses. **Results:** The injection of 0.5% formalin into the TMJ induced a significant nociceptive behavior in gonadectomized male rats ($P < .05$), but not in naïve, sham, and testosterone-replaced gonadectomized rats, confirming that testosterone prevents the development of TMJ nociception. The injection of either the nonselective opioid receptor antagonist naloxone (15 μg) or the simultaneous injection of the μ-opioid receptor antagonist Cys2, Tyr3, Orn5, Pen7amide (CTOP, 30 μg) and the κ-opioid receptor antagonist Nor-Binaltorphimine (Nor-BNI, 90 μg) significantly increased the 0.5% formalin-induced behavioral response in sham and testosterone-replaced gonadectomized rats ($P < .05$) but had no effect in gonadectomized rats. However, the injection of each selective opioid receptor antagonist alone or the simultaneous injection of μ- or κ- and δ-opioid receptor antagonists had no effect. **Conclusion:** These findings indicate that the protective effect of endogenous testosterone on the development of TMJ nociception in male rats is mediated by the activation of central opioid mechanisms. Furthermore, the coactivation of central μ- and κ-opioid receptors is necessary for testosterone to protect male rats from developing TMJ nociception. *J Oral Facial Pain Headache 2016;30:61–67. doi: 10.11607/ofph.1298*

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Temporomandibular disorders (TMD) is a term generally applied to conditions characterized by pain and/or dysfunction in the temporomandibular joint (TMJ) and masticatory muscles. Its characterization has been difficult because of the large number of symptoms and signs attributed to TMD, which are 1.5 to 2 times more prevalent in women than in men—80% of patients treated for this disorder are women.[1–3] It has been previously proposed that the lower prevalence of TMJ pain in males may result, at least in part, from a protective effect of endogenous testosterone reducing their risk of developing TMJ pain.[4] This has been suggested by findings showing that a low concentration of formalin (0.5%) injected into the rat's TMJ induces nociceptive behavior in gonadectomized (Gx) male but not in naïve male rats. However, the mechanisms underlying the protective effect of endogenous testosterone on the development of TMJ nociception remain to be elucidated.

In addition, at a supraphysiologic dose, testosterone decreases TMJ nociception induced by a high concentration (1.5%) of formalin in male rats[4] through the activation of the endogenous opioid system in the trigeminal subnucleus caudalis region.[5] Therefore, the aim of

this study was to investigate whether the protective effect of testosterone on the development of TMJ nociception in male rats is mediated by the activation of central opioid mechanisms. To address this question, a pharmacologic approach was used to test the ability of opioid receptor antagonists infused in the dorsal portion of the brainstem and adjacent to the caudal component (subnucelus caudalis) of the spinal trigeminal nucleus in blocking the protective effect of endogenous testosterone in sham Gx rats and of exogenous testosterone in testosterone-replaced Gx rats. To assess nociception development, the behavioral response induced by a TMJ injection of 0.5% formalin was used.

Materials and Methods

Subjects

The Committee on Animal Research of the University of Campinas approved the experimental protocols, which conformed to IASP guidelines for the study of pain in animals.[6] Experiments were performed on 156 male Wistar rats (200–300 g). Animals (six per group) were maintained (five per cage) in a temperature-controlled room (23°C ± 1°C) on a 12:12 light cycle, with food and water available ad libitum.

Drugs

All drugs were obtained from Sigma-Aldrich. The following drugs were used: formalin (an aqueous solution of 37% of formaldehyde dissolved in 0.9% NaCl to a concentration of 0.5%), the nonselective opioid receptor antagonist naloxone hydrochloride (naloxone, 15 μg),[5,7] the selective μ-opioid receptor antagonist Cys2, Tyr3, Orn5, Pen7amide ([CTOP], 0.2, 10, or 30 μg),[8] the selective κ-opioid receptor antagonist nor-binaltorphimine dihydrochloride ([Nor-BNI], 15, 45, or 90 μg),[8] and the selective δ-opioid receptor antagonist naltrindole hydrochloride ([naltrindole], 10, 30, or 90 μg)[8]; the opioid antagonists were dissolved in 0.9% NaCl and injected into the subarachnoid medullary space.[9]

Gonadectomy

The procedure was carried out in 45-day-old male rats under anesthesia induced by an intramuscular injection of a mixture of ketamine (55 mg/kg) and xylazine (5.5 mg/kg), through a single scrotal incision, as previously described.[10,11] The testicular bundles were ligated and removed, and the skin was sutured. Sham-operated rats underwent a surgical procedure similar to that of gonadectomized rats, except that the gonads were not removed. The efficacy of gonadectomy was confirmed by postmortem observation of atrophy of the prostate and seminal vesicles.

Testosterone Replacement

Testosterone (17β-Hydroxy-3-oxo-4-androstene) was obtained from Sigma-Aldrich and diluted in propylene glycol. The testosterone replacement protocol consisted of a daily subcutaneous injection of testosterone propionate (2 mg/kg) for 3 days.[12,13] Nociceptive testing was performed on the next day. Serum testosterone level was determined by radioimmunoassay using a specific kit (DSL-4100) from Diagnostic System Laboratories.

Subarachnoid Medullary Injection

Total volume injection in all experiments was 10 μL. All injections were performed at a rate of 1 μL/s. The injection of opioid antagonists or vehicle (0.9% NaCl) into the dorsal portion of the brainstem and adjacent to the caudal component (subnucleus caudalis) of the spinal trigeminal nucleus was performed as previously described.[9] Rats were placed in a facedown prone position and briefly anesthetized by inhalation of halothane. A small skin area overlying the high cervical region was shaved with an electric razor. A 30-gauge needle connected to a 50-μL Hamilton syringe by a polyethylene cannula was first inserted below the occipital bone up to 2 mm and slightly inclined in a cranial direction. The needle was advanced 2 mm more to perforate the atlanto-occipital membrane and reach the medullary subarachnoid space where the drugs were injected.

Because it has been reported that Nor-BNI may not be selective for κ-opioid receptors until several hours after its administration,[14,15] Nor-BNI was administered one day prior to the experiment. Naloxone, CTOP, naltrindole, or vehicle was administered 10 minutes before the TMJ injection of formalin.

TMJ Injections

The injection (30 μL) of 0.5% formalin or its vehicle (0.9% NaCl) into the TMJ region was performed as previously described.[16] A 30-gauge needle was introduced into the TMJ of rats briefly anesthetized by inhalation of halothane. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μL). Total injection volume in all experiments was 30 μL. Animals regained consciousness approximately 30 seconds after discontinuing the anesthetic. After the conclusion of the nociceptive behavioral testing, animals were anesthetized by an intraperitoneal injection of a mixture of urethane (1 g/kg) and α-chloralose (50 mg/kg). Evans blue dye (30 mg/kg) was injected systemically and 40 minutes later the animals were transcardially perfused with 0.9% NaCl. Because this dye binds to plasma protein, the correct site of injection was indicated by the observation that the plasma extravasation induced by the TMJ injection of formalin was restricted to the TMJ region.[16]

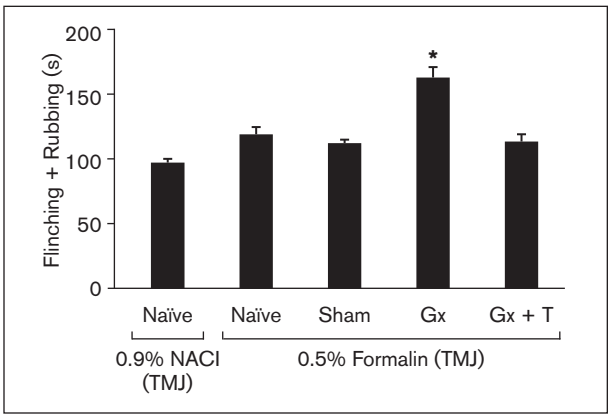

Fig 1 The protective effect of testosterone on the development of TMJ nociception. The TMJ injection of 0.5% formalin in gonadectomized (Gx) rats induced a behavioral response significantly greater than that of other groups as indicated by the symbol * ($P < .05$, one-way ANOVA, Tukey test). The TMJ injection of 0.5% formalin in naïve, sham Gx, and in testosterone-replaced (Gx + T) male rats induced a behavioral response similar to that induced by 0.9% NaCl. In this and in subsequent figures, data are expressed as mean ± SEM of behavioral score (flinching + rubbing), six rats per group.

Nociceptive Assay

Rats did not have access to food or water during the test, and each animal was used once. Before the experiments, each rat was briefly handled each day for 7 days so as to be habituated to the experimental manipulation. Nociceptive behavioral testing was performed in a quiet room maintained at 23°C during the light phase (between 9:00 am and 5:00 pm).[17] On the day of the experiment, each rat was individually placed in a test chamber (30 × 30 × 30-cm mirrored wood chamber with a glass at the front side) for a 15-minute habituation period to minimize stress. After the TMJ injection, rats immediately returned to the test chamber for counting behavioral nociceptive responses during a 45-minute observation period. This response was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period, as previously described.[16] Since head flinches followed a uniform pattern of 1 second of duration, each flinch was expressed as 1 second.[16] The recording time was divided into nine blocks of 5 minutes. When the behavioral response induced by formalin was significantly higher than that induced by its vehicle (0.9% NaCl), the behavioral response was characterized as nociceptive, as previously described,[16] and when it was not, it was characterized as nonnociceptive.

Data Analysis

The sum of the flinching and rubbing behaviors recorded during the entire duration of the experiment was used in the statistical analysis. One-way analysis of variance (ANOVA) was used to determine if there were significant differences among the groups. Groups with significant main effects were further analyzed by the Tukey post hoc test. Two-way analysis of variance (ANOVA) with two between-subject factors (ie, treatment with two levels and hormonal status with three levels) was used to determine if there were significant differences among the groups. This analysis tests the main effect of treatment, the treatment × hormonal status interaction, and the main effect of hormonal status for significance. The main effects of treatment and hormonal status were significant in this study. Newman-Keuls post hoc test was used to determine which groups were significantly different. A P value less than .05 was the accepted level for statistical significance. Data are presented in figures as means ± SEM. GraphPad Prism 6 software was used for statistical analysis.

Results

The Protective Effect of Testosterone on the Development of TMJ Nociception

The TMJ injection of 0.5% formalin in naïve, sham Gx, and testosterone-replaced Gx male rats induced a behavioral response similar to that induced by 0.9% NaCl in naïve male rats. In contrast, the behavioral response induced by the TMJ injection of 0.5% formalin was significantly greater (one-way ANOVA, Tukey post hoc test, $P < .05$) in Gx rats than in all other rats (Fig 1). These findings indicate that testosterone in naïve, sham Gx, and testosterone-replaced Gx male rats prevented the development of TMJ nociception, confirming its protective effect.

The total serum level of testosterone was significantly lower in Gx rats than in sham Gx rats and testosterone-replaced Gx rats (0.60 ± 0.04 vs 4.66 ± 0.58 and 2.87 ± 0.54 ng/mL, respectively, six rats per group, one-way ANOVA, Tukey post hoc test, $P < .05$), but not significantly different between testosterone-replaced Gx rats and sham Gx rats (one-way ANOVA, Tukey post hoc test, $P > .05$). These findings, taken together with the behavioral findings, suggest that the total serum level of testosterone in testosterone-replaced Gx males was sufficient to restore the protective effect of testosterone by preventing the development of TMJ nociception.

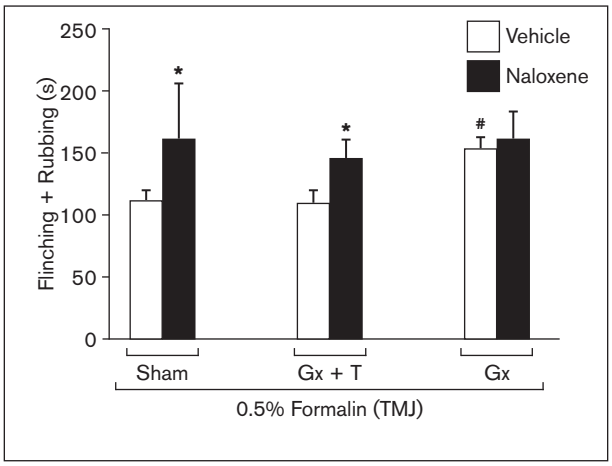

Fig 2 The protective effect of testosterone on the development of TMJ nociception is mediated by a central opioid mechanism. The subarachnoid injection of the nonselective opioid antagonist naloxone (15 μg) significantly increased TMJ formalin-induced behavioral response in sham Gx and in testosterone-replaced Gx (Gx + T) male rats, but not in Gx male rats. The symbols * and # indicate a behavioral response significantly greater than that of vehicle-treated sham Gx and Gx + T male rats ($P < .05$, two-way ANOVA, Newman-Keuls test). Gx = gonadectomized.

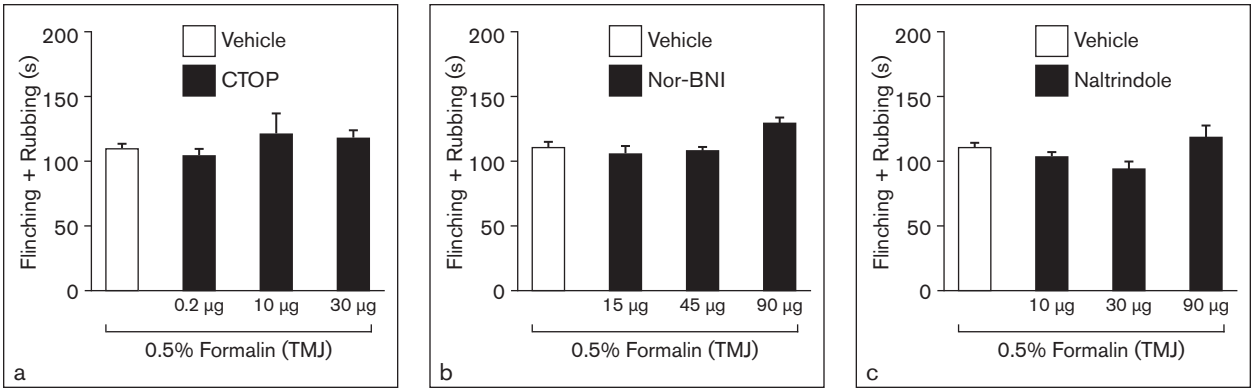

Fig 3 The protective effect of testosterone on the development of TMJ nociception is not mediated by the activation of only one central opioid receptor. The subarachnoid injection of **(a)** CTOP (selective μ-opioid receptor antagonist 0.2, 10, and 30 μg); **(b)** Nor-BNI (selective κ-opioid receptor antagonist, 15, 45, and 90 μg) or **(c)** naltrindole (selective δ-opioid receptor antagonist 10, 30, and 90 μg) did not affect the behavioral response induced by the TMJ injection of 0.5% formalin ($P > .05$, one-way ANOVA, Tukey test).

Effect of Opioid Receptor Blockade on the Protective Effect of Testosterone on the Development of TMJ Nociception

Further evidence of the protective effect of testosterone on the development of TMJ nociception was that the behavioral response induced by the TMJ injection of 0.5% formalin was significantly greater in vehicle-treated Gx rats than in vehicle-treated sham and testosterone-replaced Gx rats (Fig 2; two-way ANOVA, treatment × hormonal status interaction: $F [2, 30] = 2.341$, $P = .113$; main effect of hormonal status group: $F [2, 30] = 5.52$, $P = .009$, Newman-Keuls post hoc test, $P < .05$; two-way ANOVA, treatment × hormonal status interaction: $F [2, 30] = 11.58$, $P = .0002$, main effect of hormonal status group, $F [2, 30] = 14.74$, $P = .0001$, Newman-Keuls post hoc test, $P < .05$).

The injection of the nonselective opioid receptor antagonist naloxone (15 μg) into the medullary subarachnoid space inhibited the protective effect of testosterone, indicating that this effect is mediated by central endogenous opioids (Fig 2; two-way ANOVA, treatment × hormonal status interaction: $F [2, 30] = 2.341$, $P = .113$; main effect of treatment: $F [1, 30] = 18.60$, $P = .0002$). Post hoc analyses showed that naloxone significantly increased the TMJ formalin-induced behavioral response in sham Gx and testosterone-replaced Gx rats ($P < .05$), but not in Gx rats ($P > .05$).

CTOP (μ-opioid receptor antagonist; 0.2, 10, or 30 μg; Fig 3a), Nor-BNI (κ-opioid receptor antagonist; 15, 45, or 90 μg; Fig 3b) and naltrindole (δ-opioid receptor antagonist; 10, 30, or 90 μg; Fig 3c) failed to affect the protective effect of testosterone on the development of TMJ nociception (one-way ANOVA, $P > .05$) because animals receiving each one of these opioid antagonists had a behavioral response similar to that of animals receiving their vehicle. These findings, taken together with the finding that the nonselective opioid receptor antagonist naloxone blocked the protective effect of testosterone, suggest that coactivation of opioid receptors may be necessary for

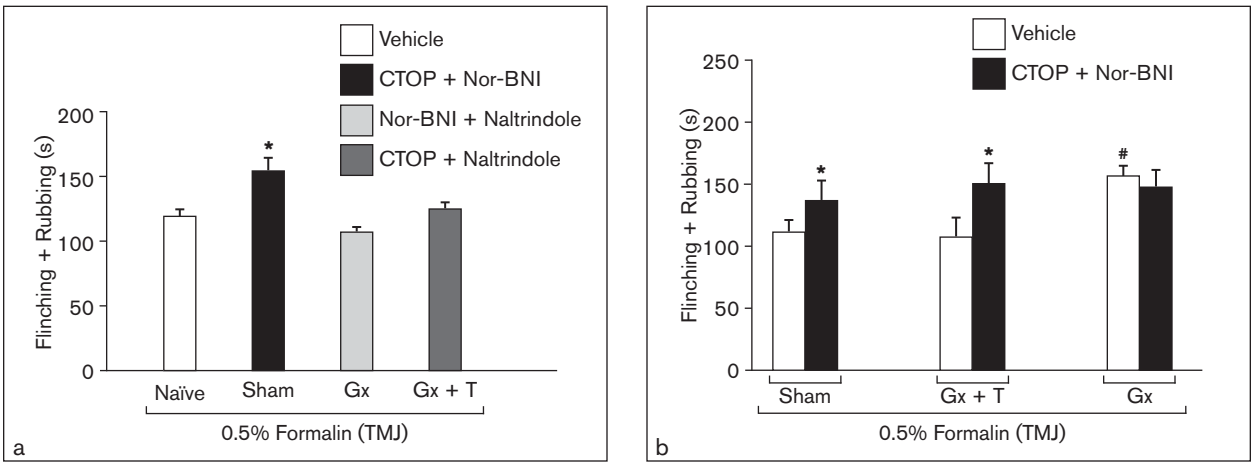

Fig 4 The protective effect of testosterone on the development of TMJ nociception is mediated by μ- and κ-opioid receptor cooperativity. **(a)** The subarachnoid injection of CTOP (30 μg) plus Nor-BNI (90 μg), but not that of naltrindole (90 μg) plus CTOP or Nor-BNI, significantly increased the behavioral response induced by the TMJ injection of 0.5% formalin in naïve rats. The symbol * indicates a behavioral response significantly greater than that of other groups ($P < .05$, one-way ANOVA, Tukey test). **(b)** The subarachnoid injection of CTOP plus Nor-BNI significantly increased TMJ formalin-induced behavioral response in sham Gx and in testosterone-replaced Gx (Gx + T), but not in Gx male rats. The symbols *, # indicate a behavioral response significantly greater than that of vehicle-treated sham Gx and Gx + T male rats ($P < .05$, two-way ANOVA, Newman-Keuls test). Gx = gonadectomized.

testosterone to protect males from the development of TMJ nociception. Therefore, to determine if coactivation of two opioid receptors is sufficient for this effect, selective opioid receptor antagonists were injected into the medullary subarachnoid space of naïve rats in combination (Fig 4a). Because the highest dose of each selective opioid antagonist was ineffective when administered alone, it was used in the combinations of the selective opioid receptor antagonists.

The ability of the combination of CTOP (30 μg) and Nor-BNI (90 μg) to block the protective effect of testosterone on the development of TMJ nociception was indicated by two findings. The first finding was that the combination of these antagonists significantly increased the behavioral response induced by the TMJ injection of 0.5% formalin in naïve male rats (Fig 4a; one-way ANOVA, Tukey post hoc test, $P < .05$). The second finding was that the combination of these two opioid antagonists prevented the reestablishment of the protective effect of testosterone in testosterone-replaced Gx rats (Fig 4b; two-way ANOVA, treatment × hormonal status interaction: $F [2, 30] = 11.58$, $P = .0002$; main effect of treatment: $F [1, 30] = 22.15$, $P < .0001$). Post hoc analyses showed that the combination of CTOP and Nor-BNI significantly increased the TMJ formalin-induced behavioral responses in sham Gx and testosterone-replaced Gx rats ($P < .05$), but not in Gx rats ($P > .05$). In contrast, the combination of CTOP or Nor-BNI and naltrindole (90 μg) had no effect (Fig 4a; one-way ANOVA, $P > .05$). Taken together, these findings indicate that the coactivation of central μ- and κ-opioid receptors is necessary for testosterone to protect male rats from developing TMJ nociception.

Discussion

This study has shown that testosterone protects male rats from the development of TMJ nociception via an opioid-dependent mechanism mediated by the coactivation of central μ- and κ-opioid receptors. The confirmation that testosterone protects male rats from developing TMJ nociception was shown by the findings that the TMJ injection of formalin at a concentration (0.5%) that was ineffective in inducing a TMJ nociceptive behavioral response in naïve and in sham Gx rats was effective in Gx rats, and that this effect in Gx rats could be blocked by testosterone replacement in the Gx rats. The protective effect of testosterone in the nociceptive system of males has also been indicated by clinical data showing that a testosterone deficit can contribute to the development and maintenance of some pain conditions.[18]

The injection of the nonselective opioid receptor antagonist naloxone into the medullary subarachnoid space blocked the protective effect of testosterone on the development of TMJ nociception in sham Gx and testosterone-replaced Gx rats, which provides evidence that the protective effect of testosterone is mediated by a central opioid mechanism. Indeed, the

finding that after the blockade of opioid receptors, the TMJ injection of formalin induced a similar nociceptive response in these rats and in Gx rats suggests that testosterone may activate the endogenous opioid system. Although it is well known that testosterone interacts with the opioid system in different brain areas,[19] the present study has provided the first demonstration that this interaction can decrease the risk of the development of nociceptive behavior.

The interaction of testosterone with the opioid system to protect male rats from developing TMJ nociception requires the coactivation of μ- and κ-opioid receptors. This is indicated by the finding that the simultaneous injection of selective antagonists of μ- and κ-opioid receptors into the medullary subarachnoid space was necessary to block the protective effect of testosterone in naïve, sham Gx, and in testosterone-replaced Gx rats. In contrast, the simultaneous injection of μ- or κ- and δ-opioid receptor antagonists or the injection of each selective opioid receptor antagonist alone had no effect. Importantly, neither the nonselective opioid receptor antagonist naloxone nor the combination of selective antagonists for either μ- or κ-opioid receptors affected the behavioral response of Gx rats, indicating that these opioid-related effects occur only in the presence of testosterone.

Although the mechanism underlying the testosterone requirement for μ- and κ-opioid receptor coactivation to protect male rats from developing TMJ nociception is unknown, a μ/κ receptor heterodimerization has been previously demonstrated by estrogen-induced antinociception in the spinal cord.[20] However, whether testosterone involves an analogous process to protect males from developing TMJ pain remains to be investigated. Alternatively, μ- and κ-opioid receptors may be located in different neurons of the same pathway involved in the protective effect of testosterone, and activation of each one of them may be essential to activate this pathway.

Testosterone could activate the opioid system either by elevating the release of opioid peptides or opioid receptor expression. In both cases, blockade of opioid receptors would block the protective effect of testosterone, as demonstrated in the present study. Consistent with the view that testosterone could activate the opioid system by increasing opioid peptide release are previous findings that androgenic steroids increase the expression of beta-endorphin levels in the ventral tegmental area in the male rat brain.[21] In addition, gonadectomy reduces, while testosterone replacement significantly increases, opioid concentration in some brain regions as well as in the plasma.[19] However, it is not known whether testosterone affects opioid receptor expression.

In the present study, opioid antagonists were infused into the dorsal portion of the brainstem and adjacent to the caudal component (subnucleus caudalis) of the spinal trigeminal nucleus (also known as the medullary dorsal horn[22,23]). The subnucleus caudalis receives the majority of the orofacial nociceptive afferents and has an important role in modulating orofacial nociceptive information that is transmitted to higher brain levels.[22,23] Therefore, the protective effect of testosterone may be modulated by a neuronal circuit located in this region, which is consistent with the evidence that opioid receptors occur in the spinal trigeminal nucleus.[22–25] In this region, as well as in the spinal dorsal horn, opioid receptors are located either presynaptically, in the central terminal of the peripheral neurons, or postsynaptically, in second-order neurons. Although ligand-binding studies have demonstrated a substantial reduction in the number of binding sites for opioid receptors in rats treated neonatally with capsaicin,[26] suggesting that a large number of opioid receptors are presynaptic, immunohistochemical studies suggest that μ- and κ-opioid receptors are targeted more at postsynaptic sites.[27,28] Therefore, the location of the opioid receptors involved in the protective effect of testosterone remains to be elucidated. In this regard it is also important to point out that because the antagonists can diffuse through the cerebrospinal fluid to neighboring regions, the involvement of regions other than the spinal trigeminal nucleus cannot be excluded.

Conclusions

This study has shown that testosterone activates a central opioid mechanism mediated by the coactivation of μ- and κ-opioid receptors to protect male rats from developing TMJ nociception. Thus, understanding the mechanisms of the modulation of pain development by testosterone could potentially shed light on strategies for preventing chronic pain development as well as increase the understanding of the neural basis of pain development.

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