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Activational action of testosterone on androgen receptors protects males preventing temporomandibular joint pain

L.E. Fanton [a], C.G. Macedo [a], K.E. Torres-Chávez [a], L. Fischer [c], C.H. Tambeli [b,*]

[a] Department of Physiology, Piracicaba Dental School, State University of Campinas - UNICAMP, Piracicaba, São Paulo, Brazil
[b] Department of Functional and Structural Biology, Institute of Biology, State University of Campinas - UNICAMP, Campinas, São Paulo, Brazil
[c] Laboratory of Pain Physiology, Department of Physiology, Federal University of Parana, Curitiba, Parana, Brazil

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ABSTRACT

Background: Testosterone protects male rats from Temporomandibular Joint (TMJ) pain. This study investigated whether this protective effect is mediated by an organizational action of testosterone during nervous system development, by central estrogen and androgen receptors and by the 5α-reduced metabolite of testosterone, dihydrotestosterone.

Methods: A pharmacological approach was used to assess the ability of the androgen receptor antagonist flutamide, the estrogen receptor antagonist ICI 182 780 and the 5-α reductase inhibitor dutasteride to block the protective effect of testosterone, evaluated through the behavioral response induced by a TMJ injection of 0.5% formalin. Flutamide and ICI 182 780 were injected into the medullary subarachnoid space, and dutasteride and testosterone were systemically administered.

Results: The TMJ injection of 0.5% formalin induced a significant nociceptive behavioral response in gonadectomized male and naïve female, but not in sham gonadectomized male rats, confirming that endogenous testosterone prevents TMJ nociception in males. Testosterone administration prevented formalin-induced TMJ nociception in males gonadectomized either in the neonatal (at the day of birth) or adult period and in naïve female rats, suggesting that the protective effect of testosterone on TMJ nociception does not depend on its organizational actions during critical periods of development. The administration of flutamide and dutasteride but not of ICI 182 780 blocked the protective effect of testosterone.

Conclusions: We conclude that the protective effect of testosterone on TMJ nociception depends on activational actions of dihydrotestosterone on androgen receptors rather than on organizational androgenic actions during central nervous system development or estrogenic actions.

1. Introduction

Sex differences in pain result, at least in part, from the influence of gonadal hormones in pain (Craft et al., 2004; Evrard, 2006). In general, estrogens appear to increase pain and testosterone to decrease it (Craft et al., 2004), although their effect depends on the pain model. For example, orofacial pain induced by the injection of formalin into the upper lip was increased by adult gonadectomy in female, but not in male rats, suggesting that estrogen rather than testosterone decreases nociception in this model (Pajot et al., 2003).

Temporomandibular dysfunctions (TMD) are among the pain conditions modulated by gonadal hormones. TMD pain affects the masticatory muscles and temporomandibular joint (TMJ) with greater prevalence, severity and duration in women than in men (Anastassaki and Magnusson, 2004; LeResche, 1997). We have used the injection of formalin into the rat's TMJ as a model of pain associated with TMD (Roveroni et al., 2001). Recently, we have demonstrated that the injection of 0.5% formalin into the rat's TMJ induces a significant nociceptive behavior in gonadectomized (Gx) males and naive females but not in naive males, suggesting that testosterone protects naive males, preventing TMJ nociception (Fischer et al., 2007). While this finding may help to explain, at least in part, the lower prevalence of TMD in males, the nature of testosterone's protective mechanism remains unclear.

Testosterone is endogenously found in high levels in males in which it induces sexual differentiation of the nervous system and determines structural changes (organizational effects) essentials for its action during male adulthood (activational effects) (Kawata, 1995; Viger et al., 2005). Although testosterone is the main androgen synthesized in the testes and circulating in the plasma, in many cases it functions as a pro-hormone. The aromatization of testosterone by the action of the enzyme aromatase converts it into 17-β estradiol, which acts on estrogen receptors to mediate some of the effects attributed to testosterone (Evrard, 2006; Gillies and McArthur, 2010). In the central nervous

* Corresponding author at: R. Monteiro Lobato, 255 - Cidade Universitária, Campinas, - SP 13083-862, Brazil.
E-mail address: tambeli@unicamp.br (C.H. Tambeli).

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system, this conversion has been described as a new mechanism of pain modulation (Evrard, 2006). On the other hand, the action of the enzyme 5-α reductase converts testosterone into 5α-dihydrotestosterone (DHT). While the effects of both testosterone and DHT are mediated by androgen receptors, DHT has a greater androgenic potency (Pluchino et al., 2009) being the active androgen in many, but not all, androgen target tissues (Carson and Rittmaster, 2003).

The aim of this study was to investigate whether the protective effect of testosterone, preventing formalin-induced TMJ nociception, is mediated by its organizational action during nervous system development, by the activation of central estrogen and androgen receptors and by its 5α-reduced metabolite, DHT.

2. Materials and methods

2.1. Animals

This study was carried out in male and female albino Wistar rats obtained from the Multidisciplinary Center for Biological Research - University of Campinas. The animals were housed in plastic cages with soft bedding (five rats/cage) in a temperature-controlled room (23 ± 1 °C) on a 12:12 light cycle (lights on at 6:00 A.M.), with food and water available ad libitum. Experimental protocols were approved by the Committee on Animal Research of the State University of Campinas and conformed to the International Association for the Study of Pain (IASP) guidelines for the study of pain in conscious animals (Zimmermann, 1983).

2.2. Gonadectomy

Gonadectomy was performed on the day of birth (neonatal gonadectomized (Gx) males) or at six weeks of age (adult Gx males). Six-week-old male rats were Gx (Fischer et al., 2008) under anesthesia induced by an intramuscular injection of a mixture of ketamine (55 mg/kg) and xylazine (5.5 mg/kg). A single scrotal incision was performed, and the testicular bundles were ligated with 4–0 silk sutures, then the testes were removed, and the skin was closed with 5–0 silk sutures. Neonatal male rats were Gx, at the day of birth, under continuous anesthesia induced by inhalation of a mixture of oxygen and halothane (2%). A single small cutaneous incision was made in the lower abdomen, and the peritoneal cavity was entered to expose the testes. The vascular bundles were tied off with 4–0 silk suture, and the testes were removed. The cutaneous incision was closed with 5–0 silk suture (Cicero et al., 2002). Sham-operated animals underwent a surgical procedure similar to that of Gx animals, except that the testes were not removed. The efficacy of gonadectomy was confirmed by the decrease in testosterone levels in Gx animals.

2.3. Drug administration

2.3.1. Formalin

Formalin solution was prepared from commercially available stock formalin (aqueous solution of 37% of formaldehyde) diluted in 0.9% NaCl to the concentration of 0.5%. Animals were anesthetized by a brief inhalation of halothane, and a 30-gauge needle was introduced into the TMJ to allow the TMJ injection of 0.5% formalin or its vehicle (0.9% NaCl), as previously described (Roveroni et al., 2001). 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 s after discontinuing the anesthetic. Formalin was obtained from Sigma, São Paulo, SP, Brazil. At the conclusion of the experiment (45 min after TMJ formalin injection), animals were anesthetized by an intramuscular injection of a mixture of ketamine (55 mg/kg) and xylazine (5.5 mg/kg). The Evans blue dye (30 mg/kg) was injected systemically, and 10 min 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 *postmortem* observation of formalin-induced TMJ plasma extravasation (Roveroni et al., 2001).

2.3.2. Testosterone

Testosterone administration was performed in adult, three-month-old rats. Males Gx at neonatal or adult period and intact females received a daily subcutaneous administration of testosterone propionate (2 mg/kg) or its vehicle for three days (Banu et al., 2002; Liu et al., 2006). The systemic administration of testosterone was performed to replace it, reaching, in gonadectomized rats, a physiological level compatible with that observed in sham Gx ones. Nociceptive testing was performed on the next day. Testosterone (17β-Hydroxy-3-oxo-4-androstene) was obtained from Sigma, São Paulo, SP, Brazil and diluted in propylene glycol. Serum testosterone level was determined by radioimmunoassay using a specific kit (DSL-4100) from Diagnostic System Laboratories, Inc. (Webster, TX, USA).

2.3.3. Estrogen and androgen receptor antagonists

In order to deliver the estrogen receptor antagonist ICI 182 780 (60, 90 and 120 μg (Ji et al., 2011); or the androgen receptor antagonist flutamide (60 and 120 μg; (Kumar et al., 2015), in the dorsal portion of the brainstem and adjacent to medulla, a medullary subarachnoid injection was performed as previously described (Fischer et al., 2005). Rats were anesthetized by inhalation of a mixture of oxygen and halothane (2%) and a small area of skin overlying the high cervical region was shaved with an electric razor. With the animals dorsally positioned, a 30-gauge needle connected to a Hamilton syringe by a polyethylene cannula was inserted into the medullary subarachnoid space. Total volume injection was 10 μl. Flutamide (2-methyl-N-(4-nitro-3-[trifluoromethyl]phenyl)propanamide) was obtained from Sigma, São Paulo, SP, Brazil and ICI 182 780 (7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) was obtained from Tocris, St. Louis, MO, USA. Both were dissolved in dimethyl sulfoxide (DMSO). All injections were performed at a rate of 1 μl/s. Each animal regained consciousness approximately 30 s after discontinuing the anesthesia.

2.3.4. 5α-reductase inhibitor

Dutasteride was administered orally (1.0 mg/day) during eight consecutive days, a protocol that completely suppresses the conversion of testosterone into DHT without overt signs of toxicity, as previously described (Attardi et al., 2010). Nociceptive testing was performed on the next day. Dutasteride was obtained from Interprise, SP, Brazil and dissolved in sesame oil (250 μl).

2.4. Nociceptive assay

Behavioral testing was performed in adult rats (three months old) during the light phase (between 9:00 AM and 5:00 PM) in a quiet room maintained at 23 °C (Rosland, 1991). On the day of the experiment, each animal was individually placed in a test chamber (30 × 30 × 30 cm mirrored-wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress. Some of the animals received a medullary subarachnoid injection and were returned to the test chamber for a recovery period of 10 min. After the TMJ injection of 0.5% formalin or its vehicle, each animal was returned to the test chamber for counting two types of behaviors, rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw and flinching the head in an intermittent and reflexive way characterized by high-frequency shakes of the head. These behaviors were quantified in blocks of 5 min for 45 min. For each block of 5 min, a chronometer recorded the amount of time that the animal exhibited the rubbing behavior, and a hand tally counter recorded the occurrence of the flinching head behavior. Considering that the flinching head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The TMJ