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Involvement of the Spinal Serotonergic System in Analgesia Produced by Castration

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NAYEBI, A. R. AND A. AHMADIANI. *Involvement of the spinal serotonergic system in analgesia produced by castration.* PHARMACOL BIOCHEM BEHAV **64**(3) 467–471, 1999.—The involvement of spinal serotonergic system in testosterone influence on formalin-induced pain was studied in male rats. Four weeks after castration, there was an analgesia in the late phase of formalin test that was reversed by intraperitoneal injection of testosterone enanthate (1 mg/kg) for 3 days. Flutamide (testosterone antagonist) produce analgesia in the late phase on intraperitoneal (10 mg/kg, IP) and intrathecal (60 μg/rat, IT) injections, but not on intracerebroventricular (60 μg/rat, ICV) administration. The antinociceptive effect of castration and IP flutamide (10 mg/kg) was abolished by pretreatment with 5,7-dihydroxytryptamine (5,7-DHT, 100 μg/rat, IT). IT-administered 5-HT (100 μg/rat) produced analgesia in the early and late phase of formalin test. Microdialysis sampling was used to characterize the extracellular concentration of 5-hydroxytryptamine (5-HT, serotonin) in the dorsal horn of the lumbar spinal cord. This technique demonstrated that levels of 5-HT were increased in 4-week castrated and IP flutamide (10 mg/kg) injected rats. The results may indicate that the analgesia produced by castration and flutamide administration is mediated through functional alteration in spinal cord serotonergic system. © 1999 Elsevier Science Inc.

Castration Formalin test Serotonin Spinal cord Microdialysis

THE formalin test was introduced in 1977 (5), and has continued to gain popularity as a model of tonic inflammatory pain. The pain produced in the formalin tests differs from that of acute nociceptive tests. Specifically, formalin creates a tonic pain secondary to tissue injury, inflammation, and central sensitization. pain behavior in the formalin test has been postulated to better reflect the pain commonly experienced by humans.

Evidence from different studies suggests that testosterone has a role in nociception (7,14,16,28,29,32,37). It has been shown that in castrated rats there is an analgesia against thermal algesic tests (30). Leuprolide (a slow-release gonadotropin-releasing hormone analogue) can cause a significant improvement in the severity of the cluster headache and has been postulated that the benefit of it is related to lowering of serum levels of testosterone (23). Absence of testosterone in normal female mice has been found to be responsible for the development of the estrogen-dependent swim stress-induced analgesia (SSIA) (19,37). In contrast, a different study showed that castration reduces both opioid and nonopioid SSIA in rats, deficits reinstated by testosterone replacement (32). However, the effects of testosterone on nociception are still a matter of controversy. Interest in this study is based on the notion that the effect of testosterone on formalin-induced tonic pain has not been studied clearly.

The dorsal horn of the spinal cord is innervated by serotonergic neurons, which are involved in the modulation of nociceptive transmission (1,38,39). Hyperalgesia consecutive to the administration of 5-HT receptor antagonists or the lesion of the raphe-spinal 5-HT system has been reported in the tail-flick and hot-plate tests (6). Activation of descending bulbospinal neurons by electrical stimulation of the nucleus raphe magnus promotes the release of 5-HT and increase its turnover in the dorsal horn of the spinal cord (12,31). Serotonin 5-HT_3 and 5-HT_{1A} receptors have been identified in the dorsal horn (24). The localization of these receptors in this region subserving nociception and analgesia, together with the documented involvement of serotonin in these processes, suggests a role for serotonin in pain modulation (24,38).

There are many reports about testosterone interaction with serotonergic system. It has been reported that in men with secondary hypogonadism there is an increase in urinary

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5-hydroxyindoleacetic acid (5-HIAA) who respond to testosterone therapy (35). Others have suggested that castration increases the biosynthesis of 5-hydroxytryptophan in limbic forebrain and diencephalon of the rat (18). There is also evidence that androgens interact with serotonin to control sexual dimorphisms in the rat lumbosacral spinal cord (4). Whether serotonin mediates testosterone effects on nociception has not yet been understood. The present study aimed to investigate the involvement of spinal serotonergic system in influence of castration on formalin-induced pain.

METHOD

Subjects

The experiments were carried out on male Sprague–Dawley rats weighing 250–300 g. Animals were housed in standard polypropylene cages, six per cage, under a 12L:12D schedule at an ambient temperature of 23 ± 1°C, and were allowed food and water. Following surgical implantation of microdialysis probe or IT and ICV cannulas, animals were housed one per cage to avoid possible displacement or disruption of the probe or cannulas. Experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publ. No. 85-23, revised 1985.)

Drugs

All drugs were prepared on the days of experimentation. Testosterone enanthate (Sigma), flutamide (Sigma) as a testosterone antagonist and 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT, Sigma) were dissolved in sterile caster oil, ethanol–water (2/1, v/v), and 0.9% saline containing 0.2 mg/ml ascorbic acid, respectively. Desipramine hydrochloride (Sigma) and 5-hydroxytryptamine creatinine sulfate (5-HT, Sigma) were dissolved in 0.9% saline. The neurotoxin, 5,7-DHT (100 μg/rat), was administered intrathecally 5 days before the experiment. Desipramine (10 mg/kg) was administered intraperitoneally 30 min before the 5,7-DHT administration to prevent uptake of 5,7-DHT into catecholaminergic neurons and subsequent damages. Other drugs were administered in a volume of 10 μl on IT and ICV routes 5 min before formalin injection. In the IP administration the drugs were injected 30 min before the experiments.

Formalin Test

The tests were carried out in a quiet room during the light phase of the light/dark cycle. Before injection of formalin, the rats were placed individually in a transparent plastic cage (30 × 30 × 30 cm) and were left there for at least 30 min. After adaptation to the cage, 50 μl of diluted formalin 2.5% was subcutaneously injected into the plantar region of the hind paw for noxious stimulation. Pain rating was recorded according the following behavioral categories: 0 – weight is born evenly on both rear paws, 1 — limps during locomotion or rests with injected paw favored, 2 — injected paw is elevated with at most the nails touching the floor, 3 — injected paw is groomed or bitten, (5). Rats were observed for 60 min by an observer blind to treatment. The results of formalin test are shown as mean of pain scores during the first 5 min (early phase) and 20–60 min (late phase) after formalin injection.

Surgical Procedures

The animals were anesthetized with IP injection of sodium pentobarbital (50 mg/kg). Castration were performed by exposing the testes via bilateral midscrotal incisions and crushing the vas deferens with a hemostat, after which the testes and testicular fat were removed. The sham surgery consisted of exposing the gonads without removing them. In the IT administration, animals were cannulated intrathecally with a PE-10 catheter inserted caudally 8.5 cm from the Atlanto-occipital membrane (40). An indwelling ICV cannula were implanted with stereotaxic coordinates, AP: −0.8 mm L: 1.4 mm V: 3.3 mm, according to Paxinos and Watson (1982) (21,26).

To implant microdialysis probe, the animals were anesthetized with sodium pentobarbital (50 mg/kg, IP), and additional anesthetic (3 mg/kg, IP) was given when necessary. For dorsal surgery, the skin of the back was incised in the dorsolumbar region, and the superficial and deep dorsal lumbar fascia were cut along the midline. Paravertebral muscles were excised at their insertions to expose the laminae of dorsal vertebrae T11–L3, and spinous processes of vertebrae T12–L2 were exposed. The body fixed in a horizontal position by temporally holding lateral clamps that were attached to the pedicles of vertebrae T11 and L3. Two anchoring screws, positioned on the right dorsal side and lateral portion of the laminae of vertebrae T13 and L2, were cemented together to obtain a solid arthrodesis between the two vertebrae. A hole was then drilled through the left side of the lamina of vertebrae L1. The underlying dura mater was cut longitudinally and the pia mater was slit. The microdialysis probe (external diameter: 240 μm; dialysis membrane length: 2 mm; cutoff: 20 kDa) then was fixed on a microdescending apparatus and vertically implanted in the dorsal horn of the spinal column at level L5 (vertebral body L1) in Rexed layers 1 to 5. Stereotaxic coordinates were set with references to the apex of the spinous process of vertebrae T13 [AP: 4.0 mm; L: 0.5 ± 0.1 mm; H: 2 ± 0.1 mm (from the surface of the spinal cord and after opening the dura mater)]. The probe was cemented to the bone. Muscles and skin were sutured individually (9).

Collection of Dialysate Samples

Microdialysis was carried out 5 days after surgery. The dialysis membrane was perfused with sterile artificial cerebrospinal fluid (ACSF = in mM: 114 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 1 $CaCl_2$, and 10 glucose, PH adjusted to 7.3–7.4 using 1 N NaOH) at a constant flow rate (2.0 μl/min) for 2.5 h a washout period before collecting samples. Dialysate samples were collected every 25 min (three samples for each group) and stored at −50°C until analysis by high-performance liquid chromatography (HPLC).

Chromatography

5-HT was separated by an analytical C18-Sil-X-5 column. The mobile phase consisted of 0.1 M sodium acetate and 1mM ethylenediaminetetraacetic acid (EDTA)(9); the pH of this buffer was adjusted to 3.5 with acetic acid. The mobile phase was degassed under vacuum by filtration through a 0.22-μm membrane and delivered at a flow rate of 1.0 ml/min. An electrochemical detector with an Ag/AgCl reference (potential fixed at 574 mv) and a 0.5 nA sensitivity was used for detection of 5-HT in the injected (40-μl) samples. The minimum levels of detection for 5-HT were 10 pg/40 μl.

Histology

All the animals were sacrificed at the end of the procedure. The brain and spinal dissects were prepared for all animals to

confirm the exact implantation of ICV cannula and microdialysis probe, respectively.

Statistical Analysis

The data were expressed as mean ± SEM. The significant differences between treatment conditions in each phase were first analyzed by Kruskal–Wallis nonparametric ANOVA test. In the case of significant variation (if $p < 0.05$), the values were compared by Dunn's multiple comparisons test. Data from microdialysis experiments were analyzed by variance (one-way ANOVA) followed by Tukey–Kramer multiple comparisons test. Statistical significance was accepted at the level of $p < 0.05$.

RESULTS

The effects of castration on formalin-induced pain were examined in the present study. 5-HT concentration in dorsal horn of the lumbar spinal cord (lamina 1 to 5) was measured by microdialysis technique. Histological analysis confirmed that ICV cannulas were located in the cerebral ventricle. Furthermore, microdialysis probes were implanted in dorsal horn of the spinal column at level L5 (vertebral body L1) and Rexed layers 1 to 5.

Effects of Castration and Testosterone Replacement Therapy on Formalin Test

Kruskal–Wallis nonparametric ANOVA revealed significant interaction between groups in the late phase (Kruskal–Wallis, $p = 0.0012$). There was a significant ($p < 0.05$) difference between 4-week castrated and sham-operated rats, but no difference was observed in pain sensitivity between sham-operated and intact rats. On the other hand, castration produced analgesia in the late phase of formalin test. In 4-week castrated rats, testosterone enanthate (1 mg/kg, IP) was administered for 3 days. Castration-induced analgesia was reversed significantly ($p < 0.05$) by testosterone replacement therapy (Fig. 1).

Effect of Flutamide on Formalin Test

Flutamide injection (IP and IT) produced marked differences of pain sensitivity in the late phase of formalin test (Kruskal–Wallis, $p = 0.0003$). Further analysis showed that pain sensitivity was different ($p < 0.05$) between IP injected flutamide (10 mg/kg) and vehicle-treated rats. Flutamide produced analgesia in the late phase on this route of administration. Furthermore, IT administration of flutamide (60 μg/rat) produced significant ($p < 0.05$) analgesia in the late phase, but there was no difference between ICV administered flutamide (60 μg/rat) and vehicle-treated rats (Fig. 2).

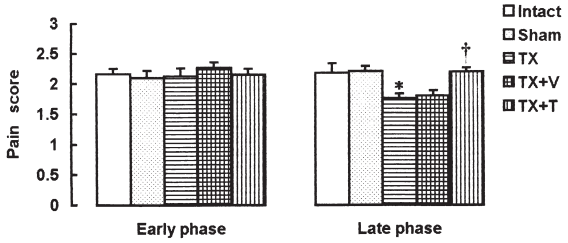

FIG. 1. The effect of castration and a hormonal replacement regimen of testosterone enanthate (1 mg/kg, IP) in castrated rats on formalin-induced pain. Each bar represents the mean ± SEM of pain score during the first 5 min (early phase) and 20–60 min (late phase) after formalin injection. $n = 6$ for each group. Kruskal–Wallis nonparametric ANOVA followed by Dunn's multiple comparisons test; *$p < 0.05$ when compared to sham-operated or intact rats, †$p < 0.05$, when compared to vehicle treatment in castrated rats. TX = castrated; V = vehicle; T = testosterone.

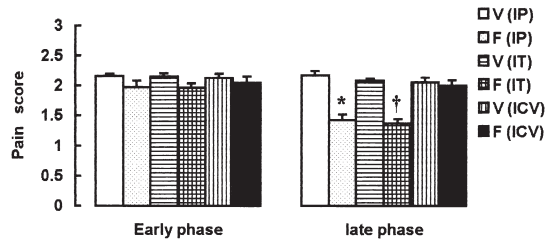

FIG. 2. The effect of IP (10 mg/kg), IT (60 μg/rat), and ICV (60 μg/rat) administered flutamide on formalin-induced pain. Each bar represents the mean ± SEM of pain score during the first 5 min (early phase) and 20–60 min (late phase) after formalin injection. $n = 6$ for each group. Kruskal–Wallis nonparametric ANOVA followed by Dunn's multiple comparisons test; *$p < 0.05$, when compared to IP vehicle-treated rats; †$p < 0.05$, when compared to IT vehicle-treated rats. F = flutamide; V = vehicle.

Effects of 5,7-DHT on Castration or Flutamide-Induced Analgesia

There was significant difference between groups in early phase (Kruskal–Wallis, $p < 0.0001$) and late phase (Kruskal–Wallis, $p < 0.0001$) of formalin-induced pain. In 4-week castrated rats, analgesia was reversed significantly ($p < 0.05$) by administration of 5,7-DHT (100 μg/rat, IT). In addition, analgesic effect of IP administered flutamide (10 mg/kg) was reversed significantly ($p < 0.05$) by 5,7-DHT (100 μg/rat, IT) pretreatment. The IT administration of 5-HT (100 μg/rat) produced significant ($p < 0.05$) analgesia in early and late phase of formalin test (Fig. 3).

5-HT Assay in Dialysate Samples

Results of 5-HT assay in the dorsal horn of the lumbar spinal cord showed statistically significant difference, $F(3, 20) =$

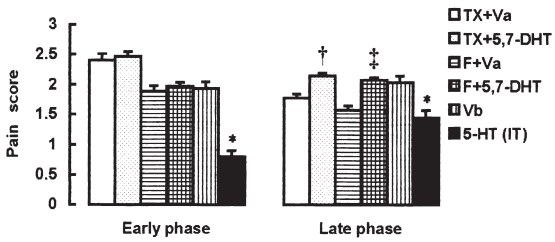

FIG. 3. The effect of 5,7-DHT (100 μg/rat, IT) in castrated rats, flutamide (10 mg/kg, IP) in 5,7-DHT (100 μg/rat, IT) pretreated rats and 5-HT (100 μg/rat, IT) on formalin-induced pain. Each bar represents the mean ± SEM of pain score during the first 5 min (early phase) and 20–60 min (late phase) after formalin injection. $n = 6$ for each group. Kruskal–Wallis nonparametric ANOVA followed by Dunn's multiple comparisons test; *$p < 0.05$, when compared to IT vehicle-treated rats; †$p < 0.05$ when compared to vehicle treatment in castrated rats; ‡$p < 0.05$, when compared to flutamide administration in vehicle-treated rats. TX = castrated; Va = vehicle of 5,7-DHT; F = flutamide; Vb = vehicle of 5-HT.

TABLE 1
EFFECT OF CASTRATION AND FLUTAMIDE ON 5-HT CONCENTRATION IN THE DORSAL HORN OF THE SPINAL CORD

Group	5-HT Concentration (pg/40 μl)
Intact	26.07 ± 1.16
Four-week castrated	40.42 ± 1.41*
Vehicle	25.55 ± 1.52
Flutamide	50.26 ± 3.23†

Spinal concentration of 5-HT was measured in intact, 4-week castrated, flutamide (10 mg/kg, IP) and vehicle-treated rats. Data are presented as mean ± SEM. $n = 6$ for each group. One way ANOVA followed by Tukey–Kramer multiple comparisons test.

* $p < 0.001$, when compared to intact rats.

† $p < 0.001$, when compared to vehicle-treated rats.

6.79, $p < 0.002$. The mean of 5-HT concentration in dialysate samples of 4-week castrated rats (40.04 ± 1.41 pg/40 μl) was higher ($p < 0.001$) than intact (26.07 ± 1.61 pg/40 μl) rats. Furthermore, 5-HT concentration in dialysate samples of flutamide (10 mg/kg, IP) injected rats (50.26 ± 3.23 pg/40 μl) was significantly ($p < 0.001$) higher than vehicle-treated rats (25.55 ± 1.52 pg/40 μl) (Table 1).

DISCUSSION

It has been reported that the aversive responses elicited by intraplantar injection of formalin are composed of two phases (5); the early phase seems to be caused predominantly by direct activation of C-fibers, while the late phase is dependent on the combination of an inflammatory reaction in the peripheral tissues and changes in the dorsal horn functions of the spinal cord (36). The late phase is considered to be a more closely relevant model to clinical inflammatory pain than the early phase. Therefore, the late phase is more interesting to us than the early phase. Results obtained in the present study show that the castration of male rats after 4 weeks diminishes pain-related behavior that was expressed 20 min after formalin injection and lasts for 40 min (late phase). These results confirm the previous report that showed castration induces analgesia against thermal and mechanical algesic tests (30). Some investigators have shown that castration results in significant reduction of pain threshold against electrical stimulation (27). However, our results failed to support the view that castration decreases pain threshold. Although, it should be noted that the difference may be due to the species of animals (15) and pain assay method (17) that we used in our experiment.

The IP and IT injection of flutamide, as a testosterone antagonist, produced analgesia in the late phase of formalin test, but on ICV administration did not cause analgesia. This may indicate that testosterone exerts its effects on pain nociception through spinal cord mechanisms. It has been shown that testosterone can directly regulate development and morphology of the lumbar genitofemoral nucleus (3). Other study indicates that structural alteration plus a reduced level of testosterone receptor in the spinal cord are involved in the pathogenesis of spinal and bulbar muscular atrophy, resulting in degeneration of motor neurons (22), but no studies have assessed the effect of testosterone on sensory neurons of dorsal horn. Previous studies also showed that plasma concentration of testosterone decreases significantly in 2-week castrated rats (18). In the present work, the analgesia induced by castration was reversed by IP-administered testosterone enanthate. Thus, one can assume that castration-induced analgesia is related to decrease of serum testosterone levels. Several lines of evidence support the hypothesis that aromatization of androgens to estrogens is a requisite step in the central action of sex steroids in males of several mammalian species (34). Other studies show that castration significantly reduces aromatase activity in the hypothalamus-preoptic area of adult rats (33), so it should be noted that estrogens may be involved in castration-induced analgesia. Testosterone has also been found to modify endogenous opioid peptides (EOP) levels in various sites in the hypothalamus and the secretion of β-endorphin in the hypophysial portal system (8). Therefore, the roles of EOP in castration-induced analgesia are not negligible.

Considerable amount of data implicate that descending serotonergic system has a pain modulatory effect in the dorsal horn of spinal cord (1,10,12,24,38). Analgesia produced after IT injection of 5-HT confirms the spinal role of 5-HT in modulation of nociception. The possibility has also been raised that some of testosterone effects are exerted through its interaction with spinal serotonin (4). Furthermore, according to many reports about testosterone interaction with serotonergic system (2,11,13,18,20,25,35), it seems that castration-induced analgesia may be exerted through the serotonergic system. The present data demonstrate that lesion of serotonergic neurons by IT administration of 5,7-DHT, as a neurotoxin, reverses castration and flutamide-induced analgesia. This may suggest the involvement of spinal serotonergic system in analgesia induced by castration and flutamide administration.

5-HT assay in the dorsal horn of the lumbar spinal cord shows that its concentration in 4-week castrated and flutamide (IP)-administered rats is significantly higher than control groups. These results are in agreement with present experiments that show castration- and IP flutamide-induced analgesia are reversed by 5,7-DHT. On the other hand, our data may indicate that 5-HT concentration in the dorsal horn of the spinal cord is increased in an analgesic state that can be produced by a decrease of testosterone levels (i.e., castration) or by blocking testosterone receptors.

We conclude that castration after 4 weeks and flutamide induce analgesia in the late phase of formalin test. This analgesic effect is related to increase of 5-HT levels in the dorsal horn of the lumbar spinal cord. The mechanism of testosterone interaction with spinal serotonin is not clear and remains to be elucidated.

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