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Increase in testosterone metabolism in the rat central nervous system by formalin-induced tonic pain

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Abstract

The effects of formalin-induced tonic pain (FITP) on testosterone (T) concentrations in the central nervous system (CNS) and serum were investigated in rats. T was nearly eliminated from the brain and spinal cord 1.5 and 24 h after a single subcutaneous injection (100 μl/rat, sc) of 5% formalin and its levels were similar to that seen following castration. In serum, T concentrations were decreased significantly 1.5 h following formalin injection, but after 24 h, the serum level of T was within normal range. T concentrations in the brain, spinal cord, and serum were not modified 20 min after formalin injection. Pretreatment of rats with finasteride, a 5α-reductase (5α-R) inhibitor (5 mg/kg, sc) blocked T elimination from the brain and spinal cord by FITP, but it failed to prevent decrease in serum T. However, 3 h after administration of exogenous T (5 mg/kg, sc), FITP did not cause a significant decrease in T levels in the CNS and serum. These results suggest that FITP eliminates endogenous T in the brain and spinal cord by increasing 5α-R activity in the CNS.
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Keywords: Formalin-induced tonic pain; Testosterone; 5α-Reductase; Brain; Spinal cord; Serum; Rat

1. Introduction

The formalin test is used as a model of acute tissue injury-induced pain in rodents (Abbott et al., 1995). Immediately after formalin injection into an animal's paw, there is a vigorous behavioral response, presumably due to direct stimulation of C-fibers by formalin. The initial response subsides after a few minutes, and 15–20 min later, shaking, mouthing, and protection of the limb reappear and continue for 30–60 min (Matthies and Franklin, 1992; Yamamoto and Yaksh, 1992). The second phase is thought to reflect tonic pain associated with inflammation and the brainstem plays a critical role in its generation (Matthies and Franklin, 1992).

Evidence from numerous studies suggests that sex steroids, such as testosterone (T), influence pain processing (Fillingim and Ness, 2000). In animal models of transient (phasic) pain, castration of males has been reported to slightly decrease analgesia (Forman et al., 1989; Kepler et al., 1989), increase it (Rao and Saifi, 1981), or to not alter nociceptive responses (Ali et al., 1995; Kepler et al., 1991). In the second phase of the formalin test, castrated rats experience analgesia (Nayebi and Ahmadiani, 1999). It is postulated that the long-lasting painful stimuli in the second phase of the formalin test may be better analogous to chronic pain in people (Aloisi et al., 1998).

Although the studies on the role of sex steroids in pain perception and pain inhibition have received great attention, the effects of pain on endogenous levels of various steroids have not been extensively studied. Acute stress has been shown to elicit a marked rapid increase in brain progesterone and its metabolites, allopregnanolone and allotetrahydrodeoxycorticosterone (Barbaccia et al., 1996a,b; Purdy et al., 1991). These two metabolites that are produced de novo in the brain (Pinna et al., 2000) are among the most potent known ligands of gamma aminobutyric acid type A ($GABA_A$) receptors in the central nervous system (CNS) (Concas et al., 1999). Changes in the brain concentrations of neuroactive steroids may play an important role in the homeostatic mechanism that counteract the neuronal overexcitation elicited by acute stress (Barbaccia et al., 2001).

It has been reported by numerous investigators that stress inhibits T production in male rats (Srivastava et al., 1993).

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In addition, chronic painful stress decreases plasma T (Aloisi et al., 1998; Klimek, 1983). Interest in this study was based on the notion that the effect of formalin-induced tonic pain (FITP) on T concentration in the CNS had not been studied.

The CNS is a target for peripheral T, which is lipid soluble and can cross the blood–brain barrier, become concentrated in many brain regions (Sar and Stumpf, 1977), and act on target tissues (Tsutsui et al., 2000). Local synthesis of T has been reported to occur in glial cells in rat (Zwain and Yen, 1999) and frog brain (Mensah-Nyagan et al., 2001), however, it is not clear how much of the T found in the rat brain is produced locally.

The objective of the present study was to measure T levels in the rat CNS and periphery following FITP and to investigate whether attenuating T metabolism would alter such effects.

2. Materials and methods

2.1. Drugs and chemicals

T (4-androsten-17β-ol-3-one) and finasteride (F) were from Sigma (St. Louis, MO, USA). Dichloromethane, *n*-heptane, petroleum benzine (boiling range of about 40 °C), formalin, and salts were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

All experiments were performed using adult male Sprague–Dawley rats (250–300 g), kept on a 12-h dark/light cycle (lights on at 05:00 h) with ad-libitum access to food (pellet from Pars) and water. Castration was performed under pentobarbital anesthesia (50 mg/kg ip) and the rats were killed 14 days after surgery. Experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985).

2.3. Drug treatment

For the time-course study, animals were subcutaneously injected with a single 100 μl of 5% formalin in the right hind paw, 20 min and 1.5 and 24 h before killing. In subsequent experiments, FITP was induced with a formalin injection 1.5 h before killing.

F (5 mg/kg, Sigma) or T (5 mg/kg, Sigma) were dissolved in ethanol–castor oil (20:80, v/v) and were injected subcutaneously in the neck. Rats were pretreated with two subcutaneous injections of F. The F injections were given 2 h apart, i.e., 2 and 4 h before killing. Control rats for F receiving groups received an equal volume of vehicle (1 ml/kg, sc). T was injected 3 h before killing.

2.4. T extraction and assay

All animals were killed by rapid decapitation between 10:30 and 11:30 h. The brains and spinal cords were rapidly (<4 min) removed and kept frozen at −20 °C until T extraction. Trunk blood was collected and centrifuged at 900 × *g* for 20 min, after 1 h room temperature. Following centrifugation, serum was frozen.

Brains (1.35–1.50 g) and spinal cords (0.28–0.33 g) were homogenized using a glass/Teflon homogenizer in 7.5 and 1.5 ml of distilled water, respectively. T was extracted from the homogenate with dichloromethane (1:4, v/v) and dried at 45 °C. The residue was dissolved in 1 ml of *n*-heptane and applied to Amprep C_2 cartridge (1 ml, Amersham, UK), previously conditioned with 5 ml of methanol and 5 ml of water. The column was washed with 10 ml of dichloromethane/*n*-heptane (30:70, v/v) and then T was eluted with 10 ml of dichloromethane/petrolbenzine (70:30, v/v). The eluent was collected and evaporated at 45 °C. In this step, no lipidal residue could be seen in tube. Then, the extract was reconstituted in 200 μl of phosphate buffer saline (0.1 M, pH 7.4). T assays were performed using commercially available RIA kit (Immunotech, Marseilles, France). The minimal measurable T levels were 50 pg/g and 0.1 ng/ml for the brain and serum, respectively.

2.5. Statistical analyses

Data are presented as mean ± S.E.M. The statistical significance of differences was assessed by analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons tests. Student *t* test was used when only two groups were compared. Statistical significance was accepted at level of $P < .05$.

3. Results

3.1. Effects of castration on T levels in serum and CNS

To determine whether T is actually eliminated from the rat brain and spinal cord following castration, we determined CNS and serum concentrations of T in rats 14 days following castration. The results showed that castration causes a marked reduction of T in the CNS and serum, and in most cases, T concentrations in the CNS and serum were below the detection limit of the assay method.

3.2. Effects of formalin injection on T levels in serum and CNS

T levels in the brain [$F(3,27) = 8.98$, $P < .0003$], spinal cord [$F(3,27) = 11.92$, $P < .0001$], and serum [$F(3,27) = 3.36$, $P < .03$] were significantly different at the various time points examined. T concentrations in the brain, spinal cord, and serum were not modified 20 min after formalin injection

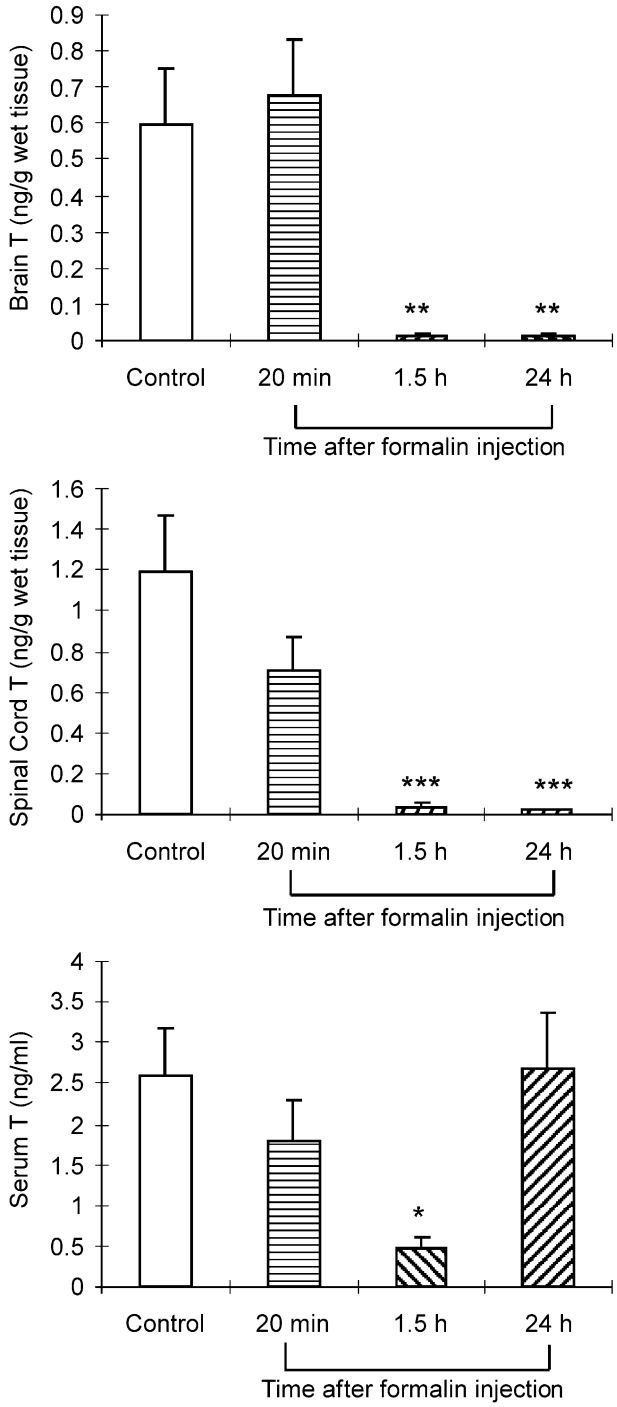

Fig. 1. Effects of formalin injection on the brain (upper panel), spinal cord (middle panel), and serum (lower panel) T concentrations. Formalin (5%) was injected (100 μl sc) to the hindpaw of male rats at the indicated times before killing, after which the concentrations of T were determined in the brain, spinal cord, and serum. Control animals were not injected. Data (mean ± S.E.M., 6–8 rats) are expressed as nanograms of T per gram wet tissue for the brain and spinal cord and nanograms per milliliter for serum. One-way ANOVA followed by Tukey–Kramer multiple comparison tests. * $P < .05$, ** $P < .01$, *** $P < .001$ vs. the control group.

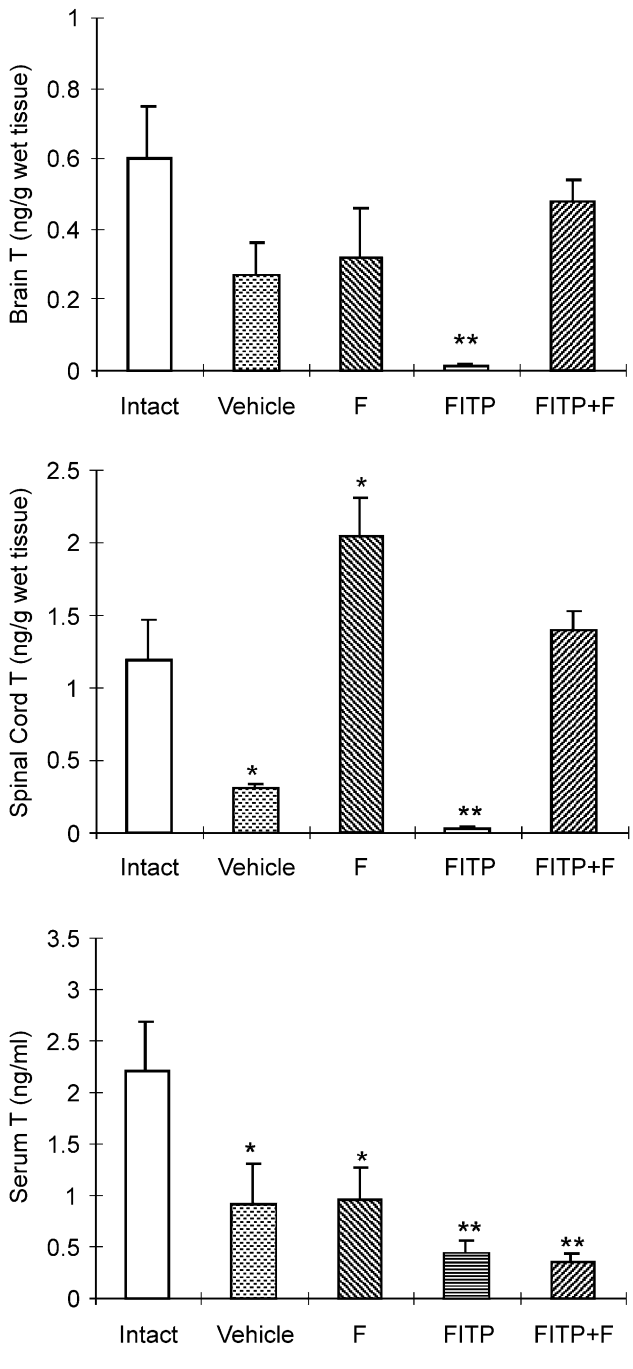

Fig. 2. Effects of finasteride (F) pretreatment on FITP (1.5 h after formalin injection into the right hind paw) decrease in the brain (upper panel), spinal cord (middle panel), and serum (lower panel) T concentrations in male rats. Control rats were no injected. The F injections (5 mg/kg sc) were given 2 and 4 h before killing. Vehicle group received two injections of an equal volume of vehicle and ethanol–castor oil (20:80, v/v) at the same times. Data (mean ± S.E.M., 6–8 rats) are expressed as nanograms of T per gram wet tissue for the brain and spinal cord and nanograms per milliliter for serum. One-way ANOVA followed by Tukey–Kramer multiple comparison tests. * $P < .05$, ** $P < .01$, *** $P < .001$ vs. the intact control group.

Table 1
Effects of FITP on T concentrations following exogenous T administration

Groups	T (ng/g wet tissue)		T (ng/ml)
	Brain	Spinal cord	Serum
T (5 mg/kg)	118.6 ± 40.25	16.1 ± 3.4	142.1 ± 41.1
T (5 mg/kg) + FITP	54.4 ± 33.9	7.7 ± 1.8	120.1 ± 23.8

T was administered subcutaneously 3 h before killing. Another group received formalin injection in addition to T 1.5 h before killing. Data are T concentrations and are expressed as mean ± S.E.M. values of six to eight rats. Two-sided Student *t* test was used.

(Fig. 1). However, T was nearly eliminated from the brain and spinal cord 1.5 and 24 h after formalin injection. In contrast, only 1.5 h after formalin injection, serum T concentration was reduced by more than 80% with respect to control.

3.3. Effects of F pretreatment on FITP-induced decrease in T levels

To determine whether FITP acts through induction of T metabolism, we used F to inhibit the brain 5α-reductase (5α-R). T levels in the brain [$F(4,32) = 3.97$, $P < .010$], spinal cord [$F(4,32) = 19.79$, $P < .0001$], and serum [$F(4,32) = 5.99$, $P < .001$] were significantly different between groups. As presented in Fig. 2, F pretreatment blocked T elimination from the brain and spinal cord by FITP, but it failed to prevent decrease in serum T. Surprisingly, vehicle injections decreased ($P < .05$) T concentrations in serum and spinal cord, which shows that it is effective on T concentration. F administration's decrease in serum T may be due to its vehicle but it increased T ($P < .05$) concentrations in spinal cord as compared to intact group.

3.4. Effects of FITP on T levels following exogenous T administration

The results of F study suggested that FITP induces T metabolism in the CNS. We injected T to see whether FITP has any effect on T concentrations following a large dose of T. As expected, the brain, spinal cord, and serum concentrations of T were markedly increased after exogenous T injection (Table 1). The results showed that FITP could not produce a statistical difference in T concentrations in comparison with the group that had not received formalin injection.

4. Discussion

In the present study, endogenous levels of T were determined in the brain and spinal cord. As expected (Baulieu and Robel, 1998), T was eliminated from the brain and serum following castration. More interestingly, T was also eliminated from the brain and spinal cord 1.5 and 24 h after formalin injection, while after 20 min, T levels were not modified. Serum T was decreased only 1.5 h after formalin injection but in the 24-h formalin-injected group, serum T comes back to its normal range. These results show that the brain and spinal cord are targets of FITP to decrease T, and serum T levels cannot predict the concentration of T in the CNS. Formalin-induced acute pain (the first phase of formalin test) did not change endogenous T concentration in the CNS, while the effects of FITP on the CNS T levels were long-lasting and continued at least for 24 h after formalin injection. This shows that the elimination of T from the CNS needs more time than 20 min. Therefore, other animal models of transient pain such as tail-flick or hot plate may also have no quick effect on the CNS T levels.

Rapid elimination of T from the CNS following tonic pain raised the question of whether FITP can increase T metabolism. We pretreated rats with F in doses that could inhibit the brain 5α-R (Celotti et al., 1997). F completely inhibited the elimination of T from the CNS following FITP and this indicated that FITP increases the activity of the 5α-R of the brain and spinal cord. However, F could not inhibit decrease in serum T. This may be explained by inadequate injected doses of F to inhibit the peripheral 5α-R, involvement of other metabolic pathways, or decreased synthesis of T in gonads instead of increasing metabolism by FITP. That vehicle administration decreased T concentration in the F study will require further investigation of its mechanism. The results also show that F and its vehicle, respectively, increase and decrease T levels in spinal cord. This may indicate that the concentration of T in the brain and spinal cord could be changed independently. However, further studies are needed to support this finding. Using exogenous T injection, we found that the brain and spinal cord 5α-R have a limited capacity to metabolize exogenous T. Since the FITP does not deplete T in the CNS when a large dose of T is injected peripherally, it would appear that T is being broken down in brain.

The results of this study demonstrate that FITP results in the depletion of T in the brain and spinal cord 1.5 h after formalin injection. The question arises, what does this mean for the animal? It is possible that the depletion in the brain T is a useful pain-modulating mechanism for the animal suffering from pain. This suggestion is supported by the previous behavioral findings that surgical castration or flutamide injection can produce analgesia in the second phase of formalin test, and administration of T to castrated rat eliminates castration-induced analgesia (Nayebi and Ahmadiani, 1999). Another possibility is that the depletion of T is a neuroendocrine disturbance effect of FITP and inhibits analgesia-producing mechanisms. Therefore, we need to compensate it with administrating exogenous T to induce analgesia. In our laboratory, we have administered T to formalin-injected male rats but we have not seen any analgesia in both phases of formalin test (unpublished data). Therefore, antihyperalgesic effect of T in FITP is not supported.

The results of the present study suggest that FITP can influence steroidal metabolism within the CNS, a suggestion that is supported by the finding that inhibition of 5α-R by peripheral injections of F prevents the depletion of T by FITP. Again, the question that remains is: what is the benefit of increase in the CNS 5α-R activity? Interestingly, it may be assumed that the increase in the 5α-R activity within the CNS produces analgesia. Both T and progesterone, which are substrates for this enzyme, may be involved in this action. 5α-R activity is widely distributed in the brain and spinal cord (Maclusky et al., 1987; Mensah-Nyagan et al., 1999). In the brain, T is readily metabolized by 5α-R to dihydrotestosterone (DHT), which is then reduced by 3α-hydroxysteroid dehydrogenase (3α-HSD) to 5α-androstane-3α,17β-diol (3α-diol) (Celotti et al., 1997; Frye et al., 2001; Kellogg and Frye, 1999). It has been reported that the activities of 5α-R and 3α-HSD are spatially and functionally coordinated (Celotti et al., 1997; Pinna et al., 2000). T and DHT have high affinities for intracellular androgen receptors but 3α-diol does not (Cunningham et al., 1979). 3α-diol is produced de novo in the brain (Martini et al., 1996) and is a very effective modulator of GABA/benzodiazepine receptor complexes (GBRs) (Frye et al., 1996b), while neither T nor DHT is particularly effective on modulating GBRs (Gee, 1988). Plasma 3α-diol is increased in response to stressful stimuli (Erskine and Kornberg, 1992; Frye et al., 1996a), while plasma T is decreased by stress via inhibition of activities of steroidogenic enzymes (Srivastava et al., 1993). Analgesic effects have been reported for 3α-diol (Frye et al., 1996b, 2001). Therefore, FITP may enhance levels of 5α-reduced metabolites of T specially 3α-diol and reduce feelings of pain. In addition to this metabolite, we have to consider other T metabolites such as 3β-androstanediol. Therefore, further studies are needed to determine DHT, 3α-diol, and 3β-androstanediol levels in the CNS following FITP.

The enzyme 5α-R is not selective for T and in fact, progesterone is a better substrate for it rather than T (Celotti et al., 1997). Therefore, we can expect that the levels of 5α-reduced metabolites of progesterone (allopregnanolone and allotetrahydrodeoxycorticosterone) are also increased following FITP. The effects of progesterone on nociception in transient pain models are still a matter of controversy, but it has recently reported that progesterone attenuate inflammatory hyperalgesia at the spinal cord level (Ren et al., 2000). It has been shown that 5α-reduced metabolites of progesterone induce analgesia via $GABA_A$ receptors (Frye and Duncan, 1994) or by enhancing the action of GABA agonists on $GABA_A$ receptors (Caba et al., 1994). It seems that to provide insight into the role of progesterone and its metabolites in FITP, it is necessary to measure their levels following FITP.

In conclusion, our data provide the first biochemical evidence for an eliminative effect of FITP on the CNS concentrations of T. This effect is related to an increase in the CNS 5α-R activity. These findings raise the need for more research on the effects of FITP on the other neuroactive steroids concentrations in the CNS to clarify the interaction between FITP and these steroids.

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