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Orchidectomy sensitizes male rats to the action of diazepam on burying behavior latency: role of testosterone

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Abstract

The anxiolytic-like effect of diazepam (0.0, 0.5, 1.0 mg/kg) on the cumulative burying behavior and the burying behavior latency were compared between intact and castrated male rats. In both groups a clear reduction in cumulative burying, denoting an anxiolytic-like response, was observed. However, castrated males were more sensitive to diazepam in the burying behavior latency, a parameter indicating reactivity. Thus, orchidectomized males showed an increase in burying behavior latency after 1.0 mg/kg diazepam treatment. This dose has no effect on burying behavior latency in intact animals. The higher sensitivity to diazepam on reactivity seems to be androgen dependent because it was reversed by chronic treatment with testosterone propionate (TP, 0.0625 mg/rat for 2 weeks). TP (0.0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/rat) produced a dose-dependent reduction in burying behavior after chronic treatment (four injections separated 48 h). At no dose did TP affect burying behavior latency. These results indicate that some actions of diazepam vary in males depending upon the endocrine milieu. Results also support the idea of androgens possessing anxiolytic-like actions.
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Keywords: Intact versus castrated males; Testosterone propionate; Diazepam; Anxiolytic-like effects of androgens; Differential effects of benzodiazepines

1. Introduction

At present, there is controversy about putative sex difference in the anxiolytic action of benzodiazepines. Thus, while some authors have described sex-differential effects (Fernández-Guasti and Picazo, 1990, 1997; Bitran et al., 1991a), others (Wilson, 1992; Stock et al., 1999) have failed to find sex- or endocrine-related changes in anxiolysis produced by benzodiazepines. The reasons underlying such sex differences most likely rely on variations in the levels of progesterone and its derivatives in females that, as previously demonstrated (Majewska, 1992), may also interact with a recognition site within the $GABA_A$-benzodiazepine receptor (see below). Although some androgens also interact with this receptor (Gee et al., 1988; Turner et al., 1989), little has been explored in the male regarding putative alterations in the anxiolytic actions of benzodiazepines. Indeed, in many studies, intact males are frequently used as "controls" for comparing possible changes in females (Wilson, 1992; Pesce et al., 1994; Reddy and Kulkarni, 1999).

Data from the clinical literature shows enhanced effects of benzodiazepines and adverse reactions in the elderly regardless of the sex (Castleden et al., 1977; Giles et al., 1978). Such higher sensitivity may be caused by the loss of steroid secretions that characterize this period (Wise, 2000) and by an increased free drug concentration consequent to a decrease in serum albumin (McLeod et al., 1979). These data suggest that differences in the anxiolytic action in the male could be related to changes in the endocrine system.

In animal models, where the endocrine milieu can be experimentally modified, insufficient research regarding specifically the anxiolytic action of benzodiazepines has been studied in male subjects. However, it has been explored if tolerance development to the anticonvulsive or anxiolytic actions of diazepam depends on the endocrine status. The results showed that tolerance to the anticonvulsive actions of benzodiazepines develops in intact males (Wilson and Biscardi, 1992). Conversely, in orchidectomized animals, the seizure threshold for bicuculline—after

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chronic diazepam exposure—was elevated, suggesting a lack of tolerance in this group (Wilson and Biscardi, 1992). Regarding tolerance to the anxiolytic effects of diazepam in the plus maze test, Stock et al. (2000) have also shown that in intact males the chronic administration of diazepam produces an anxiolytic-like effect indicating a lack of tolerance. By contrast, in castrated males the chronic administration of diazepam does not reduce anxiety suggesting the development of tolerance to this benzodiazepine. These interesting results indicate that orchidectomy may modify the response to benzodiazepines and that endogenous androgens could play an important role in tolerance development to diazepam.

As aforementioned, it has been demonstrated that the reduced testosterone metabolites, 5-alpha-androstan-3-alpha, 17-beta-diol (androstanediol) and 5-alpha-androstan-3-alpha-ol-17-one (androsterone), potentiate the GABA-mediated function in a similar manner to that observed for the reduced metabolites of progesterone. Thus, androstanediol (Gee et al., 1988) and androsterone (Turner et al., 1989) have been shown to displace ^{35}S-*t*-butyl-bicyclophosphorothionate (TBPS) from the convulsant recognition site of the $GABA_A$ receptor with potency comparable to that of barbiturates. This finding opened the possibility that at least some androgens may exert an anxiolytic action. Following this idea, Bitran et al. (1993) and Melchior and Ritzman (1994) reported anxiolytic-like responses of various androgens in males in the plus maze test.

The present study has two main purposes: (1) to analyze if the anxiolytic actions of diazepam vary between intact and castrated male rats and whether these differences depended on testosterone and (2) to establish if TP produces anxiolytic-like actions. To fulfil these objectives male rats were tested in the burying behavior paradigm. A huge body of evidence indicates that this paradigm reflects anxiety-like changes after anxiolytic or anxiogenic compounds (Fernández-Guasti and Picazo, 1990, 1997; Treit, 1985; Treit et al., 1981). Importantly, the burying behavior paradigm also reveals variations in anxiety depending upon the endocrine status (Fernández-Guasti and Picazo, 1992; Picazo and Fernández-Guasti, 1993, 1995).

2. Methods

2.1. Animals

Male Wistar rats weighing 270–350 g were used in this study. All animals were housed in a room under inverted light/dark cycle conditions (lights on at 2200 h) with ad-libitum access to water and Purina Rat Chow throughout the experiments. Males were kept five to six per cage from weaning onwards and isolated in individual cages 72 h before the anxiety tests. Rats were divided into two main groups: orchidectomized and intact. Orchidectomy was performed through a ventral incision above the scrotum under pentobarbital (40 mg/kg ip) anesthesia. After orchidectomy animals were left for recovery for at least 3 weeks.

The general principles of laboratory animal care were followed (NIH publication 85-23, 1985). For these series of experiments the local ethical committee approved the protocol for animal use.

In the first experiment, diazepam (0.0, 0.5, 1.0 mg/kg ip, Hoffman-La Roche, Basel, Switzerland, −30 min) administration was common to both conditions—intact and orchidectomized animals (8–10 animals per group). Diazepam was dissolved in propylene glycol 40% and injected in a volume of 2.0 ml/kg. The diazepam doses and latencies were selected according to previous reports (Fernández-Guasti and Picazo, 1990, 1997). For all tests, observers were unaware of drug treatments. In a second experiment, orchidectomized males (9–10 rats per group), were treated with TP (0.0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/rat sc, Sigma Chemicals, St. Louis, MO, USA). Testosterone propionate (TP) was dissolved in sesame oil and chronically injected (in a volume of 0.1 ml/rat) giving a total of four injections separated by a 48-h interval. The behavioral tests after chronic TP were conducted 24 h after the last injection. Additionally, to explore the putative anxiolytic-like effect of acute TP, this androgen at the highest dose (1.0 mg/rat) was administered 4 h before the test. Finally, in a third experiment (8–11 males per group) a group treated with an effective dose of diazepam (1.0 mg/kg) combined with TP (0.0625 mg/rat) chronically administered was studied. This TP dose was selected because it did not produce any change in burying behavior per se. The respective control groups, vehicle, diazepam or TP alone were also included.

2.2. Anxiety test: burying behavior

The burying behavior test was conducted in a red, dimly lit room. For this test, a cage measuring $27 \times 16 \times 23$ cm (identical to the animal home cage) with an electrified prod (7 cm long) emerging from one of its walls 2 cm above the bedding material (fine sawdust) was used. Thus, when the rat touched the prod it received an electric shock of 0.3 mA (the electric source was a constant current shocker, model 5806, LaFayette Instruments). After the animal was placed in the test cage, its behavior was observed for 10 min. Once the animal received a shock it displayed a behavior characterized by pushing the sawdust ahead with rapid alternating movements of the forepaws oriented to cover the electrified prod. The parameters scored in this test were burying behavior latency, i.e., time from the first shock to the burying behavior display and cumulative burying behavior, i.e., cumulative time that the rat spends burying the prod during a 10-min period. The cumulative burying behavior has been directly related with the experimental anxiety levels (Picazo and Fernández-Guasti, 1995; Pinel and Treit, 1978; Treit et al., 1981), while the burying behavior latency may inversely reflect the animal's reactivity (Rodríguez-Manzo et al., 1999). Interestingly, treatment with anxiolytics

reduces the cumulative burying indicating that this parameter directly reflects an anxiolytic-like behavior. However, for some compounds such as benzodiazepines, but not for serotonergic agonists, higher doses produce an increase in burying behavior latency denoting that this parameter reveals changes in reactivity.

2.3. Activity test

To assess potential effects of those treatments that reduced burying behavior on the motor activity, male rats were tested in an actimeter measuring $43 \times 36 \times 19$ cm placed over a 38×40-cm sensitive plaque (Stoelting, Chicago, IL, USA). The chamber used to perform this test was similar to that used to keep the animals before being isolated in individual cages. The number of counts over a 10-min session was recorded and expressed as percent change from the control value considered as 100%. The activity test was performed immediately after the burying behavior test. A new testing cage was used for each rat.

3. Results

Fig. 1, panel A, shows the effect of various doses of diazepam on burying behavior of intact and castrated male rats. This benzodiazepine at the doses of 0.5 and 1.0 mg/kg produced a similar reduction of cumulative burying in both groups. Thus, the two-way ANOVA revealed statistically significant differences considering treatment [$F(2,49) = 7.42$, $P < .002$] but not considering the animals condition [$F(1,49) = 2.03$, NS] or the interaction between these factors [$F(2,49) = 0.41$, NS]. The action of diazepam on burying behavior latency, which is inversely related to the animal's reactivity, in intact and castrated male rats is shown in Fig. 1, panel B. These doses of diazepam did not affect the burying behavior latency in intact males. However, in castrated male rats the dose of 1.0 mg/kg produced a clear increase in this parameter. These data indicate that castrated male rats are more sensitive to the effects of diazepam on burying behavior latency. Thus, the two-way ANOVA revealed statistical significance for treatment [$F(2,49) = 5.49$, $P < .007$], animal's condition [$F(1,49) = 7.90$, $P < .007$] and the interaction between these factors [$F(2,49) = 4.42$, $P < .017$].

The action of various doses of chronic treatment with TP on cumulative burying behavior and burying behavior latency is shown in Fig. 2, panels A and B, respectively. TP at 0.125 and 0.25 mg/rat produced a mild reduction in the cumulative burying behavior; however, at 0.5 and 1.0 mg/rat, this steroid drastically reduced cumulative burying. The one-way ANOVA for the TP chronic treatment on cumulative burying showed statistically significant differences, $F(5,52) = 4.35$, $P < .002$. Panel B shows the burying behavior latency, a parameter that was unmodified by TP chronically administered [one-way ANOVA, $F(5,52) = 1.63$, $P < .16$]. Moreover, acute treatment with TP at a dose as high as 1.0 mg/rat was ineffective in altering the cumulative burying (mean ± S.E., control value 161.30 ± 21.75 vs. TP value

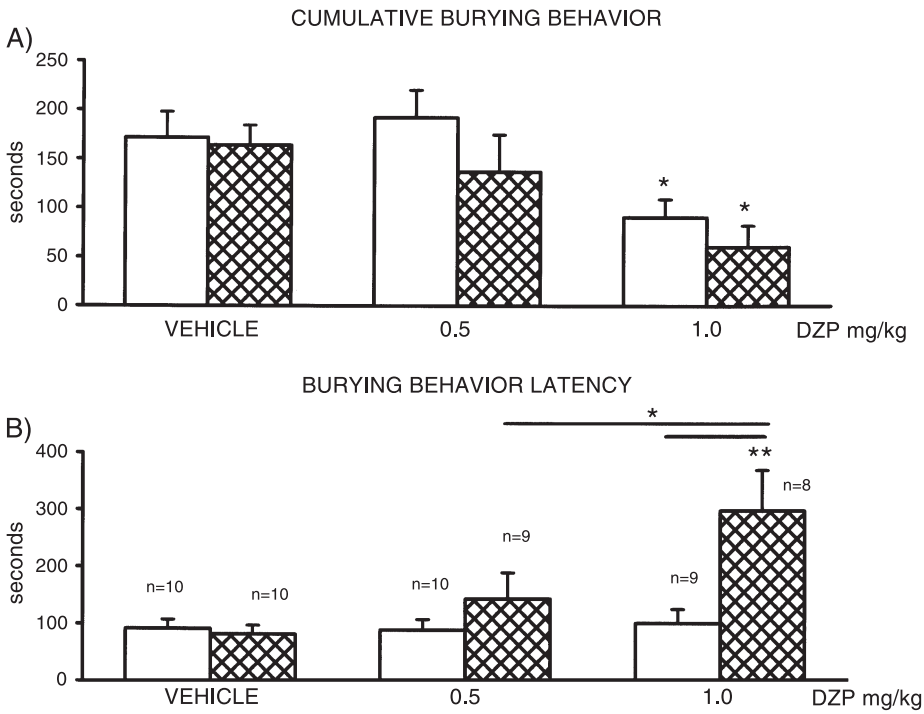

Fig. 1. Effect of diazepam (DZP) in intact (empty bars) and orchidectomized males (double dashed bars) on the cumulative burying behavior (panel A) and the burying behavior latency (panel B). Asterisks over columns represent comparisons vs. the control vehicle-treated group. Brackets show other comparisons. The results of the two-way ANOVA are described in the text. Tukey test, $*P < .05$; $**P < .001$.

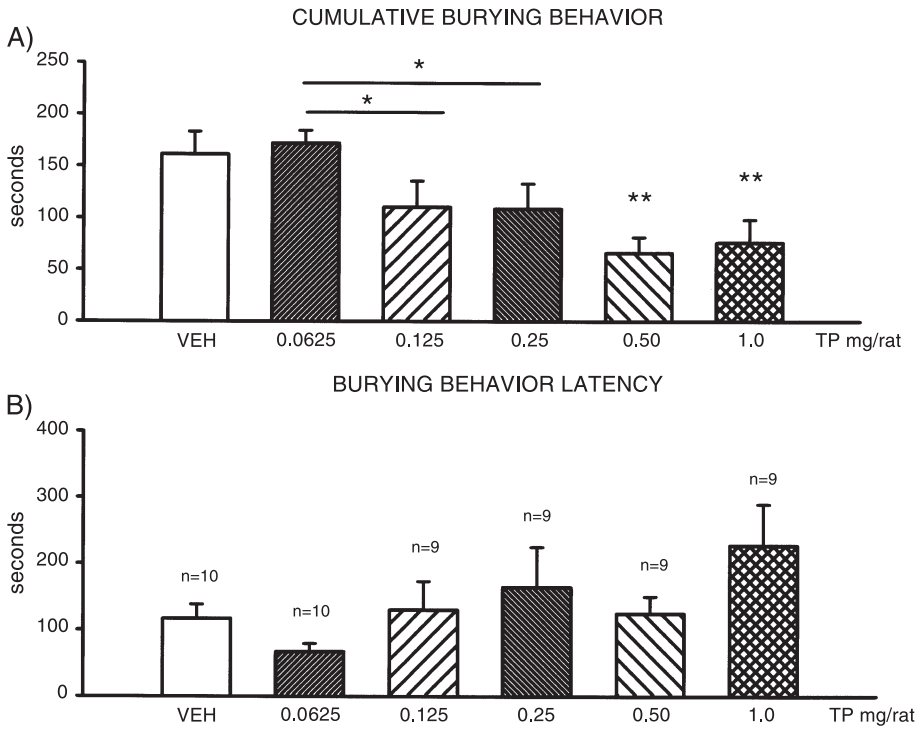

Fig. 2. Effect of chronic administration of various doses of TP on the cumulative burying behavior (panel A) and the burying behavior latency (panel B). Asterisks over columns represent comparisons vs. the control vehicle-treated group. Brackets show other comparisons. The results of the one-way ANOVA are described in the text. Dunnett test, $*P<.05$; $**P<.001$.

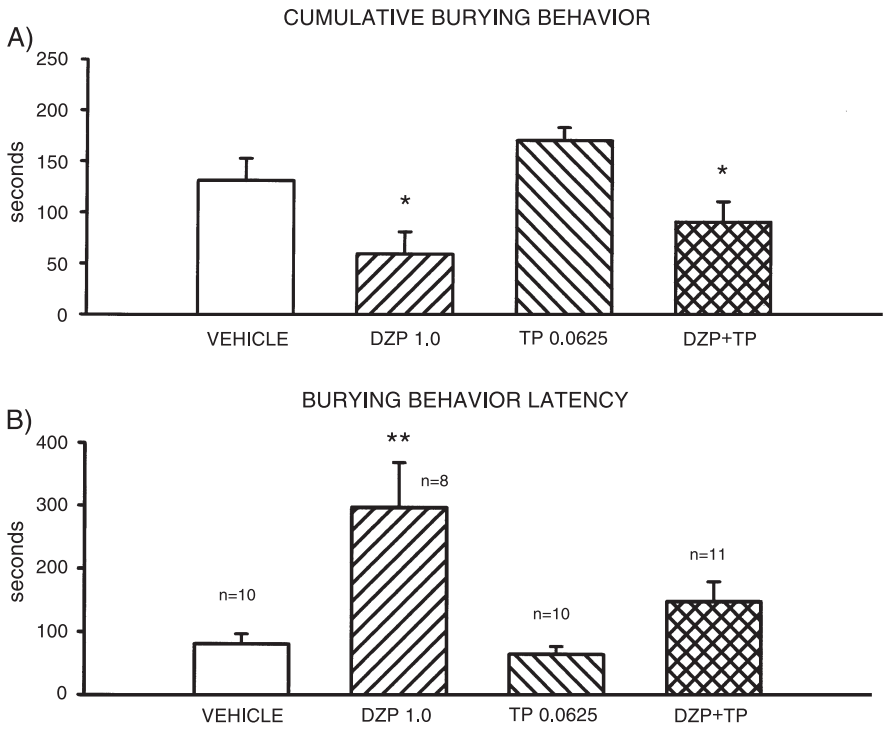

Fig. 3. Effect of the combination of an effective dose of diazepam (DZP) with a suboptimal dose of TP on the cumulative burying behavior (panel A) and the burying behavior latency (panel B). Asterisks over columns represent comparisons vs. the control vehicle-treated group. The results of the one-way ANOVA are described in the text. Dunnett test, $*P<.05$; $**P<.001$.

Table 1
Ambulatory activity of intact and castrated males after treatment with diazepam (DZP), testosterone propionate (TP) and the combination of DZP and TP

Groups	Mean ± S.E.	N
Effect of diazepam on castrated and intact males		
Vehicle intact	100 ± 7.60	10
DZP 1.0 mg/kg intact	83.6 ± 10.4	9
Vehicle castrated	100 ± 9.5	9
DZP 1.0 mg/kg castrated	77.1 ± 12.8	8
Two-way ANOVA: hormonal condition $F(1,32) = 2.75$, NS; treatment $F(1,32) = 1.60$, NS; interaction $F(1,32) = 0.23$, NS.		
Effect of TP		
Vehicle	100 ± 9.5	10
TP 0.5	107.1 ± 8.2	9
TP 1.0	124.0 ± 14.5	9
One-way ANOVA: $F(2,28) = 1.45$, NS		
Effect of the combination of DZP plus TP		
Vehicle	100 ± 9.5	10
DZP 1.0 mg/kg	77.1 ± 12.8	8
DZP 1.0/TP 0.0625	81.7 ± 10.3	11
One-way ANOVA: $F(2,28) = 1.63$, NS		

NS, non-significant.

167.12 ± 17.20; *t* test, $P < .45$) as well as the burying behavior latency (mean ± S.E., control value 116.30 ± 21.54 vs. TP value 117.50 ± 45.78; *t* test, $P < .90$).

Fig. 3 shows the results of the combined administration of diazepam (1.0 mg/kg) and TP (0.0625 mg/rat) into orchidectomized rats. Note that this experiment was performed to explore whether the higher diazepam sensitivity of castrated males depends on the absence of testosterone. As previously shown, in castrated males, diazepam (1.0 mg/kg) produced a prolongation in burying behavior latency and a reduction in cumulative burying. Chronic treatment with a low dose of TP (0.0625 mg/rat) did not modify the reduced cumulative burying produced by diazepam in castrated males, the one-way ANOVA was $F(3,37) = 7.31$, $P < .001$. However, this androgen blocked such drastic increase in burying behavior latency produced by diazepam. Results of one-way ANOVA were $F(3,37) = 6.69$, $P < .001$.

Table 1 shows the effect of diazepam, TP and their combination on ambulatory behavior. Clearly, no treatment affected this parameter excluding that the reduced cumulative burying or the increased burying behavior latency was due to unspecific motor actions.

4. Discussion

Three main conclusions can be generated from the present study. First, orchidectomized males were more sensitive to the actions of diazepam on burying behavior latency than intact males. Second, TP, chronically administered, reduced cumulative burying behavior and did not affect burying behavior latency. Finally, the higher sensitivity of castrated males to the actions of diazepam was reversed by TP.

Present results confirm that TP produces anxiolytic-like actions. As previously mentioned, Bitran et al. (1993) reported that TP reduces anxiety in the plus maze test. Some interesting methodological differences between Bitran et al.'s report and the present data deserve attention. In Bitran et al.'s study intact males were used, while in the present study orchidectomized animals were selected to control for endogenous testicular secretions. Additionally, Bitran et al. implanted their animals with silastic capsules filled with TP, producing an estimated release rate of 3.61–4.05 mg/kg/day, which corresponds to an approximate dose of 1.5 mg/rat. Conversely, in the present study we followed an injection treatment that allows determination of a dose–response curve. Following this scheme, the present results show that TP already at a dose of 0.125 mg/rat tends to reduce burying behavior.

Interestingly, Bitran et al. (1991b) also reported that withdrawal from chronic TP treatment resulted in anxiogenic-like behaviors suggesting tolerance development. Such suggestion is further supported by the lack of anxiolytic-like action after a long-time implant of TP (14 days) (Bitran et al., 1993). Present data showing a reduced burying behavior after four injections do not support the development of tolerance, although the different treatment schedules with TP may influence such development as is true for other drugs (Kayan and Mitchell, 1972; Lê and Khanna, 1989). In a recent experiment it has been reported that an acute injection of testosterone or dihydrotestosterone produced anxiolytic-like behavior in male mice within 30 min (Domek et al., 1992). Unfortunately the lack of detailed information of this report precludes any possible data interpretation. In the present experiment the acute treatment with TP at 1.0 mg/rat did not affect burying behavior.

In 1994, Melchior and Ritzman found that two testosterone metabolites, dehydroepiandrosterone (DHEA) and its sulfate metabolite DHEAS, produced anxiolytic-like effects in the murine plus maze. Interestingly, these anxiolytic-like actions seem to be mediated by the $GABA_A$-benzodiazepine receptor since flumazenil, a benzodiazepine antagonist, and penthylenetetrazol, a chloride ionophore blocker, prevent their anxiolytic-like actions. These data suggest that the TP anxiolytic-like effect is mediated by its reduced metabolites and that they act by stimulating the $GABA_A$-benzodiazepine receptor. Future studies should be undertaken to explore these possibilities.

The present results comparing the actions of diazepam in intact and castrated male rats show that this anxiolytic produced a similar reduction in burying behavior (denoting an anxiolytic-like effect) but a differential action on the burying behavior latency (reflecting the animal's reactivity). Regarding the latter, orchidectomized males are more sensitive to the diazepam effects. In agreement with these findings, Stock et al. (2000) showed similar anxiolytic-like actions of diazepam in the plus maze in intact and orchid-

ectomized rats but differential effects on other parameters such as the number of entries to the close arms. Furthermore, in females we have found that in animals in metaestrus, with low levels of steroids, diazepam produced a drastic increase in burying behavior latency (Fernández-Guasti and Picazo, 1990). All these data, taken together, suggest an important role of gonadal hormones in modulating the actions of diazepam on reactivity. Recently, Stock et al. (2000) also showed that chronic treatment with diazepam only produced tolerance in orchidectomized males and not in intact rats. These data indicate that this benzodiazepine produces some differential actions in castrated and intact males. Present results further support this idea.

Besides these results regarding anxiety-related behaviors, others (Pesce et al., 1994; Wilson and Biscardi, 1992) have reported on the differences produced by castration in the convulsions produced by flumazenil or bicuculline in males chronically treated with diazepam. Thus they showed that after administration of flumazenil or bicuculline to castrated males, the incidence of seizures significantly increased, suggesting the lack of development of tolerance to the anticonvulsant diazepam actions. Interestingly, the administration of testosterone to males significantly reduced the increase in seizures produced by castration. This last result indicates that the castration-induced changes in the behavioral effects of diazepam depend on the absence of steroid hormones secreted by the testes. Present data showing the reversal after TP treatment of the increased sensitivity of orchidectomized males to the effects of diazepam fully agree with this idea.

The mechanisms through which testosterone affects anxiety-like behaviors and modifies the effects of benzodiazepines remain unknown. However, as aforementioned, the interaction of androgens with the $GABA_A$-benzodiazepine receptor has been proposed (Gee et al., 1988; Turner et al., 1989). Additionally, it has been shown that the long-term castration of rats causes a decrease in GABA concentration and GABA synthesis in several brain regions (Earley and Leonard, 1978; Yoo et al., 2000). Furthermore, testosterone substitution was effective in reversing the changes associated with castration (Earley and Leonard, 1978). Interestingly, the formation rates of diazepam metabolites are activated in the presence of increasing doses of testosterone suggesting that diazepam is better metabolized in intact than in orchidectomized animals (Kenworthy et al., 2001). Such changes may explain the different reactivity to diazepam observed in orchidectomized and intact males. However, Wilson and Biscardi (1992) measured the brain levels of benzodiazepines using a radioreceptor assay and found that brain diazepam levels were similar between intact and orchidectomized males. This result suggests that the differential behavior between these groups is not based on pharmacokinetic factors but rather depends either on differences in the receptor number or in the receptor sensitivity. Regarding the benzodiazepine receptor number, it has been reported that this parameter is unmodified by the chronic administration of testosterone or of the antiandrogen, cyproterone acetate (Amiri et al., 1991). Interestingly, Bitran et al. (1993) showed that the sensitivity to the $GABA_A$ receptor was significantly increased in animals exposed to testosterone. These data, taken together, suggest that the mechanism involved in the differential action of diazepam between intact and castrated males involves a different $GABA_A$ receptor sensitivity. Further studies, however, should be undertaken to confirm this idea.

Finally, clinical data reveal that the elderly show enhanced effects and adverse reactions after diazepam administration. Such higher sensitivity does not seem to be related with changes in pharmacokinetic factors but rather to alterations in the receptor sensitivity probably mediated by endocrine changes according to age (McLeod et al., 1979). These data together with present results suggest that the doses of benzodiazepines should be adapted to patients that physiologically (by age) or pathologically (by surgical or pharmacological castration) lose their endogenous source of testicular hormone secretion.

In closing, the present results show that orchidectomy increases the diazepam sensitivity on reactivity and that such higher sensitivity depends on the presence of testosterone. This steroid, chronically injected, produces a dose-dependent anxiolytic-like behavior. All data, taken together, indicate that male testicular steroids may influence the action of benzodiazepines.

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