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Brain Research 838 (1999) 151–157

BRAIN RESEARCH

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Research report

Testosterone and its metabolites affect afterdischarge thresholds and the development of amygdala kindled seizures

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Accepted 11 May 1999

Abstract

In boys with epilepsy, pubertal increases in seizure frequency may be associated with rising androgen levels. The present study tested the hypothesis that testosterone (T) and/or its metabolites might affect amygdala seizure thresholds and the development of secondary generalization from amygdala foci (kindling). Afterdischarge thresholds and kindling rate were measured in gonadectomized (GDX) male rats, with or without T replacement therapy. Drugs that block either androgen or estradiol (E_2) receptor-mediated responses were also tested. *Methods*: Kindling electrodes were implanted in the basolateral amygdala of adult male Wistar rats. In Experiment 1, subjects were GDX and implanted with a silastic capsule containing either: cholesterol (control); T; 5% E_2 in cholesterol; or 5α-dihydrotestosterone (DHT). In Experiment 2, intact subjects were treated with daily injections of vehicle (control); daily injections of flutamide (an androgen receptor antagonist); or Silastic implants containing 1,4,9-androstatriene 3,17-dione (ATD; an aromatase inhibitor). *Results*: In Experiment 1, initial afterdischarge (AD) thresholds were significantly lowered by E_2 treatment, as compared to cholesterol controls, and remained low throughout the kindling paradigm. In T replaced males, AD threshold significantly decreased over the kindling period, a response that was not observed in DHT treated rats. Rates of kindling were significantly faster as a result of T, E_2 and DHT treatment, as compared to cholesterol controls. E_2 treated males kindled the fastest of all 3 groups. In Experiment 2, initial AD thresholds were significantly lowered by flutamide treatment, as compared to cholesterol controls, and remained low throughout the kindling paradigm. AD threshold significantly decreased over the kindling period in intact males, a response that was blocked by ATD treatment. Both flutamide and ATD significantly slowed the rate of kindling, as compared to intact controls. ATD had the most dramatic inhibitory effect on kindling rate. *Conclusions*: In males, T and its two metabolites, E_2 and DHT, all appear to enhance the development of amygdala-kindled seizures. E_2 has the most potent epileptogenic effect. Antagonism of E_2 mediated effects in the brain may have potential therapeutic value for males with epilepsy. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Testosterone; Estradiol; Dihydrotestosterone; Flutamide; Aromatase inhibitor; Kindling model

1. Introduction

Androgen secretion may exacerbate seizure activity. Male epileptic patients sometimes exhibit an increase in frequency and severity of epileptic seizures with puberty [24,26]. Such responses are more common in patients with complex partial seizures than with generalized, tonic–clonic seizures [1,21,24,28], implying a specific role for steroid-receptor interactions in the temporal lobe, which contains the limbic structures. Limbic structures such as the amygdala might be particularly sensitive to the modulatory influences of gonadal hormones, as a result of their high concentrations of both steroid receptors (androgen and estrogen (E_2) receptors) and the aromatase enzyme which converts androgen into E_2 [3,25,30].

Past studies have not produced a clear picture of the effects of androgens on seizure susceptibility. Chronically administered testosterone (T) significantly lowers the threshold for electroshock seizures in intact male rats [32], suggesting a proconvulsant effect. This effect might be mediated by T itself or by T's two active metabolites, E_2 and 5α-dihydrotestosterone (DHT) [8,19]. E_2 has been shown to lower seizure thresholds and facilitate seizure development in the female rat [9,13,32], but neither E_2 nor DHT has been studied in the male. There is clinical evidence that E_2 enhances seizure activity in males, since seizure activity decreases in hypogonadal males receiving

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PII: S0006-8993(99)01620-0

T treatment combined with either an antiestrogen or blockade of E_2 biosynthesis using an aromatase inhibitor [12].

The kindling model is the best studied animal model of partial complex seizures [15,29]. Kindling involves the progressive increase in electrographic and behavioral seizures following repeated, often daily, stimulations of chronic indwelling electrodes placed in forebrain structures, usually limbic sites [29]. At first, epileptiform afterdischarges are localized to the site of stimulation and do not propagate to other parts of the brain [29]. With repeated stimulation, focal seizure threshold drops and seizures secondarily generalize to other brain regions, eventually leading to the onset of behavioral convulsions [15,29].

If the actions of T involve E_2 and/or DHT, then it might be possible to selectively isolate the seizure-potentiating effects of each steroid by specifically blocking one or other response pathways. This is the hypothesis that we have tested in the present study. Using either an androgen receptor antagonist or an inhibitor of E_2 biosynthesis, we have determined the relative contribution of E_2 and androgen receptor-mediated responses to the effects of T on limbic seizure thresholds and seizure development during amygdala kindling in male rats. Our data show that T potentiates the development of kindled seizures and that this response involves both androgen and E_2-mediated mechanisms.

2. General methods

2.1. Subjects

Adult male Wistar rats (225–275 g; Charles River, Canada) were housed individually in 24 cm × 24 cm × 45 cm transparent, plastic cages. Food (Purina Rat chow) and water were provided ad libitum. The colony was maintained at 18°C on a 12 h light:12 h dark schedule (lights on at 0700 h). Subjects were transported in their home cages to the experimental room and allowed to acclimatize for 30 min before experimentation.

2.2. Procedure for implantation of electrodes

Rats were stereotaxically implanted with a chronic indwelling bipolar electrode in the right basolateral amygdala exactly as described in the preceding paper [9].

2.2.1. Experimental group 1

2.2.1.1. Procedure for gonadectomy and hormone replacement. Seven days after electrode implantation, 44 rats were rapidly anesthetized with 1.5% halothane and bilaterally gonadectomized (GDX) via a single mid-line incision in the lower abdomen just above the bladder.

2.2.1.2. Procedure for hormone replacement. Eleven days after electrode implantation (4 days after gonadectomy), subjects were rapidly anesthetized with 1.5% halothane and implanted s.c. dorsomedial to the scapulae with a single Silastic® capsule (0.058 in. i.d., ×0.077 in. o.d.; Dow Corning, Midland, MI) containing either: cholesterol (control; capsule length 1.0 cm); T (capsule length 1.0 cm); 5% wt/wt 17β-estradiol in cholesterol (E_2 group; capsule length 1.0 cm), or 5α-dihydrotestosterone (DHT; capsule length 2.0 cm). It has been shown previously that treatment with 5% E_2 or 100% T capsules produces serum hormone levels of approximately 30–50 pg/ml E_2 and 2–5 ng/ml T, respectively [4,16]. The 2.0 cm capsules containing DHT maintain prostate weight in castrate male rats at close to normal intact male levels [unpublished observations]. Prior to implantation, capsules were equilibrated by incubation at 37°C for 24 h in phosphate-buffered saline containing 1% albumin [4].

2.2.2. Experimental group 2

2.2.2.1. Procedure for drug administration. Seven days after electrode implantation, 37 intact male rats were randomly assigned to one of three groups receiving either: daily injections of flutamide (an androgen receptor antagonist [16]); daily injections of vehicle (saline/propylene glycol (1:1)); or subcutaneous Silastic® implants (0.058 in. i.d., ×0.077 in. o.d.; Dow Corning, Midland, MI) of 1,4,9-androstratriene 3,17-dione (ATD, an aromatase enzyme inhibitor [16]; capsule length 4.0 cm; 3 capsules/subject). Flutamide was dissolved in the vehicle at a concentration of 25 mg/ml and was administered by s.c. injection. Capsules were implanted s.c. dorsomedial to the scapulae. The aromatase inhibitor, ATD, was chosen for this experiment because it has been previously shown to selectively block neuroendocrine and behavioural E_2 receptor-mediated responses in male rats [16]. All experimental protocols were approved by the relevant Institutional Animal Care Committees.

2.3. Threshold determination and kindling procedure

Afterdischarge (AD) thresholds were determined as previously described [9]. Twenty-four hours after AD threshold determination, kindling was begun. Daily kindling stimuli were administered, seizure intensities were recorded and the location of the kindling electrodes was verified using the procedures reported in the preceding paper [9].

2.4. Tissue collection

At the time of sacrifice, the pituitary, adrenals, prostate and seminal vesicles were collected. Fresh weights were recorded to the nearest 0.1 mg. As the prostate and seminal vesicles are critically dependent on androgen receptor me-

diated growth responses for tissue maintenance and function [18], their weight served as a bioassay for the effectiveness of hormone (T and DHT) and drug (flutamide) treatments. Any males that did not show the expected changes in organ weight were eliminated from subsequent analyses.

2.5. Statistical analyses

Kindling was terminated at criterion (five stage–five seizures) or after 25 ADs had been evoked. Subjects that failed to reach criterion were arbitrarily assigned a score of 25. Measurements including AD threshold, number of ADs to kindle to criterion during kindling and tissue and body weight were approximately normally distributed and were therefore analyzed by parametric techniques. Inequality of variance was corrected where necessary by log transformation of the data. Multiple group comparisons used the One-way Analyses of Variance (ANOVA). Provided that the overall ANOVA was significant, post-hoc analyses used Duncan's Multiple Range test. All tests were two-tailed with a critical significance level of $p < 0.05$.

3. Results

3.1. Tissue weights

Table 1 shows the effect of experimental treatment on prostate weight, seminal vesicle weight, adrenal weight and pituitary weight. Both prostate and seminal vesicle weight were significantly affected by experimental treatment. Removal of androgen responses in GDX + cholesterol, GDX + E_2, and intact + flutamide treated males significantly decreased prostate and seminal vesicle weights, as compared to all other groups. Prostate weight significantly increased in males receiving ATD, as compared to intact + vehicle injected controls. Adrenal weight and pituitary weight were also significantly increased in GDX males receiving E_2 replacement, relative to cholesterol controls.

3.2. Initial afterdischarge thresholds

Fig. 1 shows the mean (±S.E.M.) initial AD threshold in each experimental group. There was a significant overall effect of treatment on initial AD threshold (One-way ANOVA; $F_{6,73} = 2.9$, $p = 0.01$). Animals in which E_2 responses predominated (i.e., GDX + E_2, intact + flutamide) had significantly lower AD thresholds than cholesterol-treated controls or intact controls. Animals in which androgen responses predominated (i.e., GDX + T, GDX + DHT, intact + ATD) had initial AD thresholds that were statistically indistinguishable from GDX-cholesterol controls or intact controls. There was a trend for both DHT and ATD treatment to lower the initial AD threshold, as compared to their respective controls, but this did not reach statistical significance.

3.3. Reduction in afterdischarge thresholds

Fig. 1 also shows the AD thresholds recorded at the end of the kindling period. Experimental treatment had a significant overall effect on final AD threshold (One-way ANOVA; $F_{6,73} = 2.16$, $p = 0.05$). Final AD threshold was 25% lower than initial threshold in males with circulating T (i.e., GDX + T rats and intact control rats), being statistically lower than final AD threshold in either males without T (i.e., GDX + cholesterol controls) or males in which E_2 responses were ablated (i.e., GDX + DHT or intact + ATD rats). In the latter two cases, AD threshold did not change significantly during kindling and remained high. In males with only E_2 responses (GDX + E_2; intact + flutamide), AD thresholds were low at the start of the experiment and did not change significantly as a result of kindling (Fig. 1B).

Table 1
Effect of experimental treatment on tissue weights. Data are expressed as Mean ± S.E.M.

Group	Fresh weight (mg)			
	Prostate	Seminal vesicles	Adrenals	Pituitary
GDX + cholesterol	21.4 ± 6.1	108.1 ± 6.0	77.4 ± 6.8	12.7 ± 0.7
GDX + T	1013.4 ± 69.3*	1256.8 ± 61.7*	92.0 ± 4.8	13.1 ± 1.0
GDX + E_2	11.0 ± 4.3	129.6 ± 11.6	109.6 ± 6.1*	25.5 ± 3.4*
GDX + DHT	985.7 ± 63.1	886.8 ± 87.0*	63.1 ± 2.5	9.9 ± 1.0
Intact + vehicle	928.5 ± 46.7*	1168.3 ± 90.9	90.3 ± 5.3	16.3 ± 2.2
Intact + flutamide	53.6 ± 20.3	189.6 ± 32.3	72.8 ± 9.0	12.1 ± 1.3
Intact + ATD	1242.6 ± 57.4*†	1030.5 ± 59.81*	79.6 ± 2.8	13.4 ± 0.7

*Significantly different from GDX + Cholesterol control group (Duncan's test, $p < 0.05$).
†Significantly different from Intact + Vehicle group (Duncan's test, $p < 0.05$).

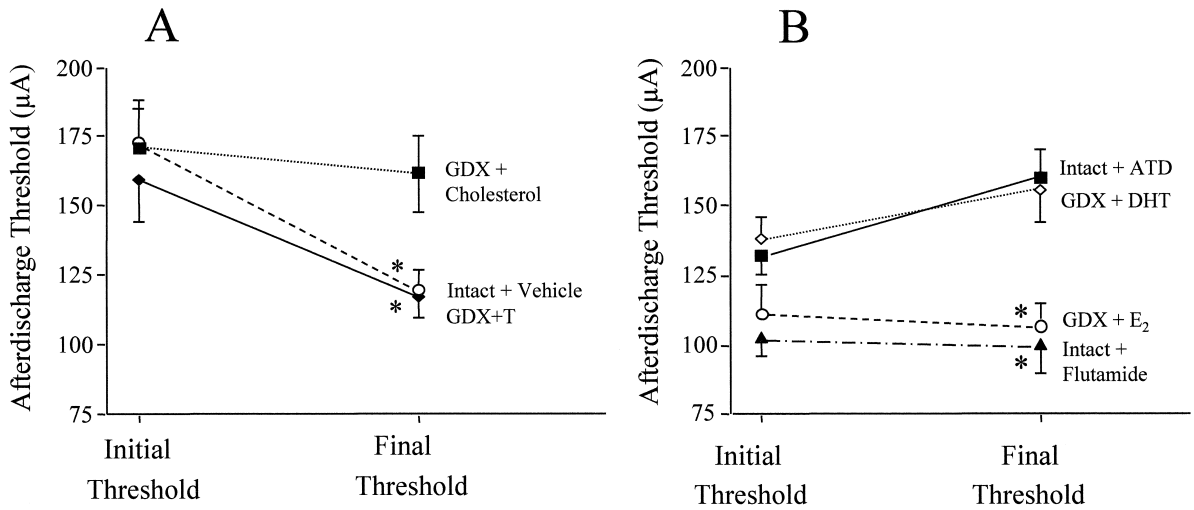

Fig. 1. Mean ± S.E.M. for initial and final AD thresholds (μA) over the kindling period in GDX + cholesterol, GDX + T treated and intact males (A), and GDX + E_2, GDX + DHT treated and intact males receiving ADT or flutamide treatment (B). Asterisks (*) represent significant differences in final afterdischarge threshold compared to GDX + cholesterol controls (Duncan's test, $p < 0.05$). Thresholds were measured peak-to-peak.

3.4. Rates of acquisition of kindled seizures

Fig. 2 shows the mean (±S.E.M.) number of ADs required to kindle to five stage–five motor seizures. There was a significant overall effect of experimental treatment on kindling rate (One-way ANOVA; $F_{6,73} = 8.1$, $p < 0.01$). Seizures developed significantly faster in males receiving T, E_2 and DHT, as compared to GDX + cholesterol controls, with E_2 males kindling the fastest of all treatment groups. Seizures developed significantly slower in males receiving flutamide or ATD, as compared to vehicle injected controls. Kindling rates were statistically comparable between GDX + cholesterol controls and males receiving either flutamide or ATD.

3.5. Afterdischarges in each seizure stage

Considering each motor stage separately, a One-way ANOVA showed that hormone replacement in GDX males had a significant overall effect on the number of ADs in stage-0 ($F_{3,41} = 5.13$, $p < 0.01$), but not any other stage. Males receiving T, E_2 and DHT spent significantly less time (i.e., fewer ADs) in motor stage-0 than cholesterol controls (Duncan's test, $p < 0.05$; data not shown).

In intact males, drug treatment had a significant overall effect on the number of ADs in stages 1 and 3 (One-way ANOVA; $F_{2,31} = 4.23$, 5.3, respectively; $p < 0.02$ for both stages). Males receiving ATD spent significantly more time (i.e., more ADs) in motor stages 1 and 3, as compared

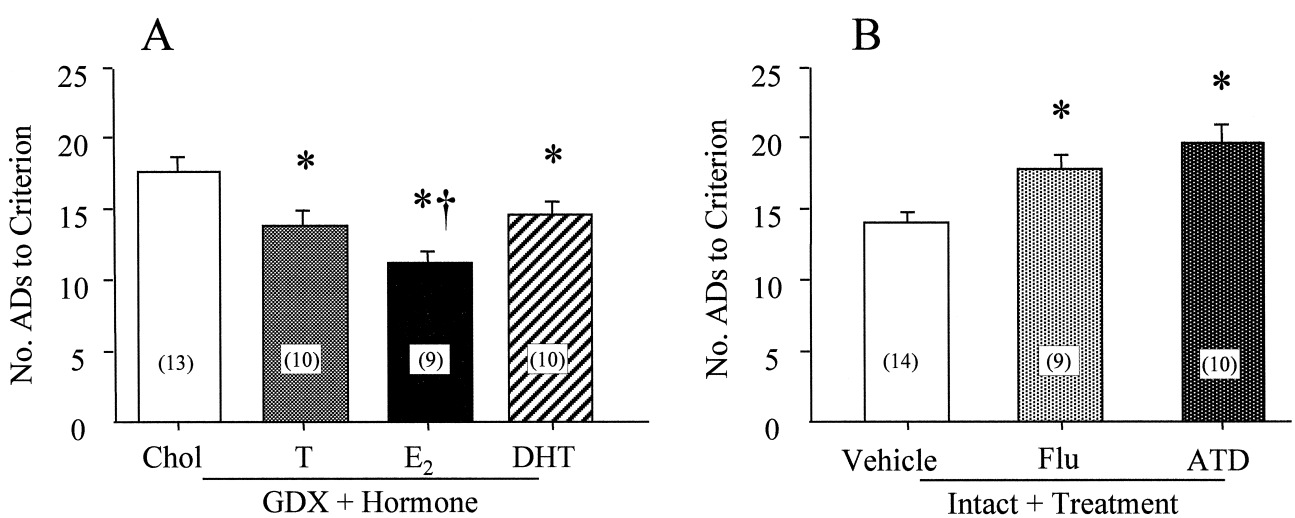

Fig. 2. Mean ± S.E.M. number of ADs required to evoke five stage–five motor seizures in GDX males receiving hormone replacement (A), and intact males receiving drug treatment (B). The number of animals per group is shown in each bar. Asterisks (*) represent significant differences from cholesterol controls (A), or intact + vehicle injected controls (B). The dagger (†) in panel (A) indicates that the data for this group (GDX + E_2) are also significantly different from those for the DHT treated and T treated males (Duncan's test, $p < 0.05$).

to vehicle injected controls (Duncan's test, $p < 0.05$; data not shown).

4. Discussion

The results of this study show that physiological levels of T in male rats hasten the secondary generalization of focal seizures initiated via the basolateral amygdala. T's effects appear to involve both E_2 and androgen receptors, with E_2 mediated effects being most pronounced. E_2 also had significant effects on initial afterdischarge thresholds and on the reduction in AD threshold induced by kindling.

4.1. Initial afterdischarge threshold

Chronic low seizure thresholds in patients with epilepsy are thought to underlie the initiation of spontaneous attacks [6]. In the present study, we found that initial AD thresholds were significantly lowered by E_2 treatment, suggesting that the neuronal circuitry close to the stimulation site is sensitive to the effects of E_2. Consistent with this hypothesis, treatment of intact males with flutamide also reduced seizure thresholds, to levels comparable to those observed in GDX males receiving E_2 replacement. Since flutamide blocks androgen receptors, T's effects in flutamide-exposed animals are presumably mediated largely if not entirely via local E_2 biosynthesis [16].

The threshold lowering effect of E_2 in the amygdala is consistent with previous data showing a significant reduction in amygdala threshold in ovariectomized female rats receiving E_2 replacement [9,13,31]. In intact cycling female rats, both amygdala thresholds and electroconvulsive shock seizure thresholds are lowest at proestrus — the day preceding ovulation when follicular E_2 production is maximal [9]. The amygdala has a high concentration of both E_2 receptors [3,30] and aromatase [25] and might therefore be expected to respond to T via local E_2 biosynthesis. Taken together, these results suggest that E_2 receptor-mediated effects reduce amygdala AD threshold, in both sexes.

Although not statistically significant, there was a trend for DHT, but not T, to lower initial AD thresholds in GDX animals. This suggests that the effects of T and DHT on seizure thresholds may be different. In the presence of T (i.e., GDX + T and intact males), the contribution of DHT is probably quantitatively smaller than that of T itself since T, not DHT, is the major androgen-receptor ligand in the male rat brain [19].

T may in fact oppose the threshold-lowering effects of both E_2 and DHT. This would explain why T itself did not decrease amygdala seizure thresholds, as compared to cholesterol controls. This hypothesis is consistent with studies showing that androgens antagonize E_2 receptor responses and down-regulate E_2 receptor levels in the brain (reviewed in Ref. [2]). The present data also agree with results of a previous study in which T treatment failed to lower electroshock threshold in intact male rats [32]. Only when androgen receptors are blocked by flutamide administration does the threshold-lowering effect of locally synthesized E_2 on the amygdala become apparent (Fig. 1B).

Since E_2 lowers amygdala thresholds, blockade of E_2 biosynthesis with ATD might be predicted to have the opposite effect, of raising AD thresholds. We found, however, that ATD had no significant effect on initial AD thresholds — indeed, there is a trend towards reduced thresholds in the ATD treated animals. This trend may be explained by the fact that ATD also has weak androgenic activity [5], as demonstrated by the increase in prostate weight of ATD treated males relative to intact, vehicle-injected controls. At the high doses required to achieve complete inhibition of aromatase [16], the androgenic effects of ATD may mimic those of DHT.

4.2. Kindling-associated changes in afterdischarge threshold

In epileptic patients, initially low seizure thresholds tend to drop even further as repeated attacks occur [23,29]. Thus, repeated episodes of neural excitation may contribute to chronic low seizure thresholds [6]. In the kindling animal model, AD thresholds are similarly lowered in the adult, male rat as a result of electrical stimulation, with reductions being greater in limbic structures than in the cortex [7,27].

In the present study, AD thresholds were lowered as a result of kindling in both intact and GDX + T replaced males. In both groups, thresholds were high at the outset and lowered to values comparable to those of males in which E_2 receptor responses predominated (i.e., GDX + E_2 males and intact + flutamide males). In contrast, threshold reductions were not seen in the GDX subjects treated with cholesterol or males in which androgen responses predominated (GDX + DHT and intact + ATD).

These observations suggest that reduced AD thresholds in the amygdala may be the result of E_2 action. Previous studies have shown that E_2 receptor occupation in the intact male rat amygdala is comparable to that observed in females at either the proestrus stage of the estrous cycle or following treatment with 5% E_2 Silastic capsules [17,19,22]. Nevertheless, AD thresholds are high in intact males. As indicated above, we postulate that this may be due to androgen antagonism of the effects of locally-synthesized E_2. One of the consequences of repeated electrical stimulation of the brain may be to increase the activity of the aromatase enzyme, either within the brain itself or elsewhere in the body, thereby shifting the balance of T metabolism towards increased E_2 biosynthesis. Consistent with this hypothesis, we have reported elsewhere that amygdala kindling in male rats is associated with a dramatic increase in circulating estradiol levels — to within the range observed in animals implanted with s.c.

5% E_2 capsules [10]. Given that E_2 treatment reduces AD thresholds even in non-kindled GDX males (Fig. 1), it seems entirely possible that the decline in AD threshold observed in intact animals may not in fact represent an effect of kindling per se, but may instead represent an indirect response to the kindling-induced increase in $T \rightarrow E_2$ conversion.

4.3. Rates of acquisition of kindled seizures

The facilitatory effect of E_2 during amygdala kindling suggests that the neural pathways projecting from the amygdala to the motor system(s) responsible for the onset of motor convulsions are E_2 sensitive. In GDX males, E_2 replacement was most effective in facilitating epileptiform spread in the brain, as compared to other hormonal treatments. Blockade of E_2 biosynthesis in intact males with ATD significantly prolonged the development of kindled seizures, as compared to intact controls.

T treatment of GDX males also significantly increased the rate of development of kindled seizures, as measured by the number of ADs required to kindle. Since treatment with the non-aromatizable androgen DHT also increased kindling rate, the most reasonable interpretation of the data is that androgen and estrogen receptor-mediated responses *both* contribute to the effects of T. The fact that the T treated males did not kindle as fast as those receiving E_2 can probably be ascribed to the fact that local E_2 biosynthesis occurs in only a few discrete region of the rat brain. At least initially, only target structures containing high concentrations of aromatase and E_2 receptors such as the amygdala, lateral septum and the stria terminalis [3,25,30] would be expected to respond to circulating T via aromatization. In contrast, in males receiving E_2 treatment, estrogen responses would occur throughout the brain.

A corollary of the hypothesis that androgen receptor-mediated responses accelerate kindling is that blockade of these responses should significantly slow the development of seizures. Flutamide treatment did significantly increase the number of ADs to kindle in intact male rats, albeit not to quite the same extent as was observed with ATD. The effects of flutamide cannot be considered solely in terms of inhibition of androgen action, however. They may also include a contribution from reduced local estrogen biosynthesis, since androgens induce aromatase activity in some regions of the brain [14].

In summary, the actions of T and its metabolites, E_2 and DHT, in the male brain all appear to facilitate the acquisition of kindled seizures originating from the basolateral amygdala. The epileptogenic actions of E_2 appear to be stronger than those of DHT. These results have important implications for the hormonal management of epilepsy. Since the great majority of the effects of T on reproductive function in men do not require E_2 biosynthesis, it seems possible that, in patients with seizures originating in androgen-sensitive regions of the limbic system, reduction in seizure activity might be possible through therapeutic use of aromatase inhibitors or anti-estrogens. This hypothesis is consistent with preliminary clinical data using an aromatase inhibitor in male epileptic patients, reported by Herzog [12]. Since the central effects of T on libido in men appear to be mediated to a large extent through androgen as opposed to E_2 receptor-dependent mechanisms [11,20], such treatments could conceivably reduce seizure activity with minimal adverse side effects on the patient's reproductive function.

Acknowledgements

The authors would like to thank Antonio Mendonca for his invaluable assistance in stereotaxic surgery. Partial financial support was provided by a grant from the Bloorview Childrens Hospital Foundation to Dr. W.M. Burnham and a Natural Sciences and Engineering Research Council doctoral scholarship to H.E. Edwards.

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