ORIGINAL RESEARCH

Effects of acute and chronic administration of neurosteroid dehydroepiandrosterone sulfate on neuronal excitability in mice

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Background: Neurosteroid dehydroepiandrosterone sulfate (DHEAS) has been associated with important brain functions, including neuronal survival, memory, and behavior, showing therapeutic potential in various neuropsychiatric and cognitive disorders. However, the antagonistic effects of DHEAS on γ-amino-butyric acid$_A$ receptors and its facilitatory action on glutamatergic neurotransmission might lead to enhanced brain excitability and seizures and thus limit DHEAS therapeutic applications. The aim of this study was to investigate possible age and sex differences in the neuronal excitability of the mice following acute and chronic DHEAS administration.

Methods: DHEAS was administered intraperitoneally in male and female adult and old mice either acutely or repeatedly once daily for 4 weeks in a 10 mg/kg dose. To investigate the potential proconvulsant properties of DHEAS, we studied the effects of acute and chronic DHEAS treatment on picrotoxin-, pentylentetrazole-, and N-methyl-D-aspartate-induced seizures in mice. The effects of acute and chronic DHEAS administration on the locomotor activity, motor coordination, and body weight of the mice were also studied. We also investigated the effects of DHEAS treatment on [³H]flunitrazepam binding to the mouse brain membranes.

Results: DHEAS did not modify the locomotor activity, motor coordination, body weight, and brain [³H]flunitrazepam binding of male and female mice. The results failed to demonstrate significant effects of single- and long-term DHEAS treatment on the convulsive susceptibility in both adult and aged mice of both sexes. However, small but significant changes regarding sex differences in the susceptibility to seizures were observed following DHEAS administration to mice.

Conclusion: Although our findings suggest that DHEAS treatment might be safe for various potential therapeutic applications in adult as well as in old age, they also support subtle interaction of DHEAS with male and female hormonal status, which may underline observed sex differences in the relationship between DHEAS and various health outcomes.

Keywords: dehydroepiandrosterone sulfate, mice, age and sex differences, seizure threshold, motor activity, [³H]flunitrazepam binding

Introduction

Dehydroepiandrosterone sulfate (DHEAS) is a neurosteroid associated with various important functions in the mammalian brain.[1–4] Namely, DHEAS has been reported to modulate neuronal plasticity,[5–9] cognition, and emotions,[10–15] thus showing therapeutic potential in a variety of neuropsychiatric and cognitive disorders.[2,16–20] It appears that the effects of DHEAS in the central nervous system are mediated through its action on multiple signaling pathways and neurotransmitter systems, which are also involved in regulating the balance between excitation and inhibition in the brain.[3,15,21–23] Perhaps, the

most documented has been the ability of DHEAS to bind and allosterically modulate the γ-amino-butyric acid$_A$ (GABA$_A$) receptor complex.[3,15,21,24] In addition to its antagonistic effects on GABA$_A$ receptors,[25–30] DHEAS has been shown to act as a positive modulator of N-methyl-D-aspartate (NMDA) receptors and facilitate NMDA-mediated glutamatergic neurotransmission through central sigma receptors.[31,32]

Some of these actions of DHEAS might lead to enhanced brain excitability and possible seizures[33] and thus limit its potential therapeutic applications. Increase in the motor activity and body weight reduction may also appear consistently with potential proconvulsive, anxiogenic, and provoking effects of DHEAS.[34,35] However, it is not yet fully elucidated to what extent DHEAS complex pharmacological profile contributes to its central actions, so the actual in vivo effects of DHEAS remain uncertain. Namely, in contrast to the reports suggesting that DHEAS can induce seizures when administered systemically or directly into the brain,[35,36] other studies failed to confirm proconvulsant effects of DHEAS.[37–41] Moreover, there is also a variety of data regarding the effects of DHEAS on the motor activity and body weight of the animals.[34,37,42–45]

Observed age-related decline in DHEAS levels suggests possible role of this neurosteroid in many disorders of aging.[2,46,47] Low DHEAS levels have been associated with age-related changes and decreased lifespan in various animal studies,[48] while exogenous DHEAS has been suggested to effectively ameliorate aging symptoms.[49] However, the findings of some studies are inconsistent or negative.

Although observed beneficial effects of DHEAS, such as proimmune, antidiabetic, antiobesity, antiatherosclerosis, antiosteoporosis, anticarcinogenic, antidementia, antiaging, and many other effects,[50,51] probably require rather long-term therapeutic strategy, so far most studies have investigated the effects of acute DHEAS treatment.[15] Moreover, despite the knowledge that susceptibility to various mental disorders, including the sensitivity to seizures,[52] as well as association between DHEAS and different health outcomes,[53] often differ by sex, most preclinical studies of DHEAS were performed only in males.

Hence, given the chronic and age-related nature of many conditions for which DHEAS could be prescribed, as well as potential interaction of DHEAS with male and female hormonal status,[42,54] we studied the effects of both acute and long-term DHEAS treatment in adult and aged mice of both sexes. Namely, in our study, we investigated possible age and sex differences in the susceptibility to seizures, as well as changes in locomotor activity, motor coordination, and

body weight in mice following acute and chronic DHEAS administration.

In addition, sex differences in the seizure sensitivity might be due to the different modulation of the neurotransmitter receptor activity by DHEAS-derived sexual hormones and their metabolites. Therefore, we also studied the potential changes in the action of DHEAS at GABA$_A$ receptors using [³H]flunitrazepam binding on the brain membranes obtained from male and female mice following DHEAS treatment.

Materials and methods
Animals
Adult (~3 months old, weighing 20–25 g) and aged (~18 months old, weighing 30–45 g) male and female CBA mice, bred in Rudjer Boskovic Institute, Zagreb, Croatia, were used. The animals were housed at a constant temperature (22°C) and under a light cycle of 12-hour light/12-hour darkness (lights on at 7 am). Food and water were freely available. Mice were caged in groups of ten. As the female mice were caged next to the male mice, their estrus cycle was presumably synchronized due to the Whitten effect.[55] All animal care and experimental procedures were carried out in accordance with the Directive 2010/63/EU of European Parliament and Council of the European Union on the protection of animals used for scientific purposes and the Croatian law on animal welfare. The ethical approval was obtained from Rudjer Boskovic Bioethics Committee.

Drugs
DHEAS (Aldrich Chemical Co, St Louis, Mo, USA), pentylenetetrazole (PTZ, Sigma-Aldrich Co., St Louis, MO, USA), picrotoxin (Sigma), and NMDA (Ascent Scientific, Bristol, UK) were dissolved in saline. Following the determination of animal body weight, DHEAS or saline was administered to mice intraperitoneally (ip) in a volume of 1 mL per 100 g body weight. In the acute experiments, 10 mg/kg DHEAS or saline was given 30 minutes prior to the animal testing. In the chronic experiments, mice were treated with DHEAS (10 mg/kg) or saline once daily for 4 consecutive weeks. The DHEAS dose was selected according to the literature data reporting that in mice, concentrations of dehydroepiandrosterone (DHEA/S) from 0.1 up to 1,200 mg/kg have been found to be active, while in the human studies, the administered doses range from 0.1 to 40 mg/kg/day.[56] The animal testing started 30 minutes following single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) DHEAS or saline administration. In addition to DHEAS, [³H]flunitrazepam (specific activity 96 Ci/mmol, Amersham Biosciences Ltd, Buckinghamshire, UK) and diazepam (Sigma) dissolved

in 0.1 N HCl were used in radioligand binding studies on brain membranes, isolated from mice 30 minutes following DHEAS or saline administration.

Locomotor activity measurement

The locomotor activity measurements were performed between 8 am and 1 pm in a quiet room under normal laboratory lighting. The horizontal ambulatory activity in individual mice was registered by Ugo-Basile Activity Cage. The activity was recorded for 10 minutes, starting after placing the animal into the test cage, 30 minutes following the single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) administration of DHEAS or saline. The observer left the room after placing the animal in the apparatus. The movements the animal makes inside the cage interrupted one or more infrared beam/s. The beam interruptions were counted, recorded by the electronic unit, and expressed as counts/10 minutes.

Rota-rod test

The motor coordination testing was also performed between 8 am and 1 pm in a quiet room under normal laboratory lighting. Motor coordination was evaluated using a rota-rod test. Prior the experiment, all mice were tested in two 2-minute period sessions per 2 consecutive days on the rotating bar at a constant speed of 15 rpm. Only the animals with stable performance on the rotating rod were used in the experiment and tested again 30 minutes following the single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) administration of DHEAS or saline. Each mouse was placed on the rota-rod (Ugo-Basile Rota-rod Treadmill for mice, Ugo-Basile, Monvalle VA, Italy; speed of rod 15 rpm) and the number of falls and latency to fall were measured for up to 2 minutes.

Seizure threshold determination

All experiments regarding determination of proconvulsive activity were carried out between 8 am and 1 pm, 30 minutes following the single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) administration of DHEAS or saline. The animal was taken from its home cage and placed in a glass cylinder (20×7 cm^2) with numerous holes for ventilation, while its tail was drawn through a hole of the plastic cover and warmed for 1 minute under a tensor lamp. A butterfly infusion needle (length 20 mm, gauge 27) was inserted into the tail vein, and correct placement was verified by the appearance of blood in the infusion tubing.

The convulsants acting on distinct ligand-gated ion channels (GABA$_A$ and NMDA glutamate receptors) – PTZ

(4 mg/mL), picrotoxin (0.75 mg/mL), and NMDA (8 mg/mL) – were administered by constant intravenous (iv) infusion. The infusion rates, controlled by a microinfusion pump, were 1.1 mL/min for PTZ and picrotoxin and 0.55 mL/min for NMDA. During the convulsant infusion, the mouse was held lightly by the tip of the tail and observed, and the time between the start of infusion and the onset of seizures (latency) was recorded. Timed tail vein infusion provides qualitative assessments of several different convulsive responses, which occurred in progression and are divided into two categories: clonus indicates rapid rhythmic movements due to alternating contraction and relaxation of muscles, whereas tonus indicates rigidity due to contraction of muscles.[57,58]

For PTZ, the first convulsive sign was myoclonus (a sudden involuntary muscle jerk, usually accompanied by a head twitch), followed by running and bouncing clonus (violent whole-body clonus, including running and explosive jumps) and tonic hindlimb extension (characterized by extreme rigidity, with forelimbs and hindlimbs extended caudally). As myoclonic twitch was not observed consistently in all of the animals that were challenged with picrotoxin and NMDA, only two convulsive signs, running and bouncing clonus and tonic hindlimb extension, which reliably characterize picrotoxin- and NMDA-induced seizures, were reported.[57] For each animal, the threshold dose of convulsant (mg/kg of body weight) required to elicit particular convulsant sign was calculated from the time of infusion (latency), infusion rate, concentration of convulsant, and body weight.

Preparation of the brain membranes

The animals were sacrificed 30 minutes following the administration of DHEAS or saline. The mice forebrains were quickly isolated on ice, washed with ice-cold saline, and stored at −20°C. Brain membranes were prepared mainly as previously described.[59] The frozen brains were homogenized in ice-cold 50 mM Tris-citrate buffer (pH 7.4) and homogenates centrifuged at 47,000 rpm for 20 minutes at 4°C. The pellet was washed in ice-cold 50 mM Tris-citrate buffer (pH 7.4), resuspended, and centrifuged three more times, than resuspended again and stored at −20°C. On the day of assay, the suspension was thawed, centrifuged once more at 20,000× g for 20 minutes at 4°C and used in [^{3}H]flunitrazepam binding studies.

[^{3}H]Flunitrazepam binding assay

For the [^{3}H]flunitrazepam binding assay, aliquots of the brain membrane preparation (~100 mg protein/mL) were incubated

Standard

with [³H]flunitrazepam in 50 mM Tris-citrate buffer (pH 7.4) containing 150 mM NaCl at 4°C for 90 minutes. Concentrations of DHEAS in the range from 1 μM to 1 mM were added to 1 nM of [³H]flunitrazepam in inhibition studies. Nonspecific binding was determined in the presence of 100 μM diazepam. In all binding assays, the total assay volume was 0.5 mL. Each assay tube was run in duplicate. Radioactivity bound to membranes after vacuum filtration on Whatman GF/C filters was measured using β-scintillation counter (Wallace 1409 DSA, PerkinElmer Inc., Waltham, MA, USA).

Protein concentration determination

Protein content was determined in a 10 μL sample, according to the method of Lowry et al[60] using bovine serum albumin as a standard.

Data analysis

All results are expressed as mean values ± standard error of the mean (SEM). The analysis and graphic presentation of data were performed using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Percentage change in [³H]flunitrazepam binding produced by DHEAS was defined as (specific binding in the presence of drug/specific binding in the absence of drug) ×100. The inhibition curves were analyzed using the sigmoidal equation and the values of half-maximum and maximum inhibition of [³H]flunitrazepam binding produced by DHEAS were determined. Statistical analysis of the results was conducted by Student's t-test (for the comparison of two mean values) or by one-way analysis of variance (ANOVA). ANOVA was followed by the Newman–Keuls multiple comparison test. In animal testing, there were seven to eight animals per group, while data in binding studies were from three independent experiments performed in duplicate. P-values <0.05 were considered significant.

Results

The effects of DHEAS treatment on the seizure threshold

To investigate the possible proconvulsant activity of DHEAS in mice, we examined the effects of acute and chronic DHEAS administration on seizures induced by iv infusions of three different convulsants: pentylentetrazole, picrotoxin, and NMDA.

As shown in Figure 1, 10 mg/kg DHEAS administered acutely 30 minutes prior to iv infusion of convulsants failed to significantly affect the doses of noncompetitive GABA$_A$ receptor antagonists pentylentetrazole (Figure 1A and B) and picrotoxin (Figure 1C and D), as well as the doses of NMDA, an agonist of NMDA glutamate receptors (Figure 1E and F), needed to produce convulsive signs in the male as well as in the female adult mice. Even considerably higher doses of DHEAS (25 and 50 mg/kg), administered acutely in animals of both sexes, have not produced any significant effects on the threshold of seizures induced by pentylentetrazole, picrotoxin, and NMDA (Table 1).

Because of the limited number of aged animals, we tested only the effects of acute DHEAS treatment on the picrotoxin-induced seizures. DHEAS applied acutely in the dose of 10 mg/kg has not significantly modified the seizure reactivity of aged male (Figure 2A) as well as aged female mice to picrotoxin (Figure 2B), when compared to control group administered with saline.

Moreover, following chronic DHEAS (10 mg/kg) treatment, we observed no significant differences compared to control group in the doses of pentylentetrazole (Figure 3A and B), picrotoxin (Figure 3C and D), and NMDA (Figure 3E and F) needed to induce clonic and tonic seizures in adult mice of both sexes.

Sex and age differences in the seizure susceptibility following DHEAS treatment

When control adult male and female mice were compared, significant differences were revealed in the doses of all three convulsants, required to elicit particular convulsant signs (Figure 4). Namely, in saline-treated animals, we observed significant sex differences in the clonic (P<0.04, Student's t-test) PTZ-induced seizures (Figure 4B), as well as in the tonic convulsions produced by picrotoxin (P<0.002, Student's t-test) (Figure 4E) and NMDA (P<0.001, Student's t-test) (Figure 4G).

However, following DHEAS administration, we determined some discrete but significant changes regarding sex differences in the susceptibility to clonic seizures in comparison to control groups (Figure 4). Namely, as opposed to the control group, significant sex differences in the clonic PTZ-induced seizures were not observed in the adult mice treated acutely or chronically with 10 mg/kg of DHEAS (Figure 4B). These sex differences were not observed in the myoclonus (Figure 4A) or tonic seizures (Figure 4C), produced by PTZ, in saline- as well as in DHEAS-treated mice. On the other hand, significant differences (P<0.04, Student's t-test) between adult male and female mice were found in the dose of NMDA needed to produce clonic convulsions (Figure 4F) following chronic DHEAS (10 mg/kg)
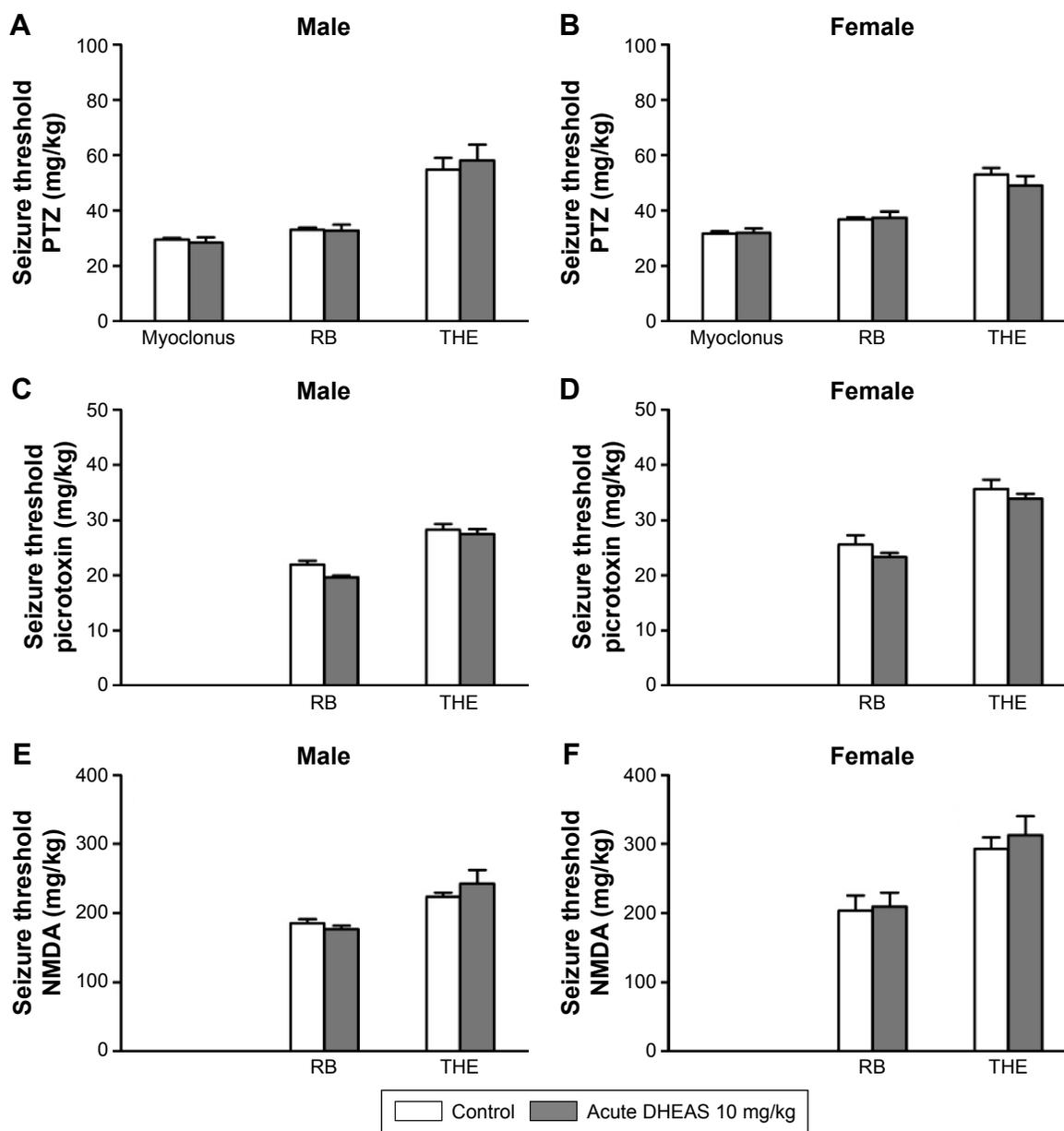
Figure 1 The effects of acute DHEAS treatment on the seizure thresholds for (**A** and **B**) PTZ, (**C** and **D**) picrotoxin, and (**E** and **F**) NMDA in adult male and female mice.
Notes: Saline (control group) or 10 mg/kg of DHEAS was administered intraperitoneally to mice 30 minutes prior to the intravenous infusion of convulsant. The observed convulsive signs – myoclonus, RB, and THE. The bars represent mean ± SEM from seven to eight animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; NMDA, N-methyl-D-aspartate; PTZ, pentylenetetrazole; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.

treatment, although these sex differences were not observed in control animals or mice treated with DHEAS acutely. In the case of threshold for tonic NMDA-induced seizures, significant differences between male and female mice were present in control, as well as in animals treated acutely and chronically with DHEAS (Figure 4G). In contrast to the saline-treated animals, after both acute ($P<0.002$, Student's t-test) and chronic ($P<0.05$, Student's t-test) DHEAS treatment, significantly lower clonic picrotoxin-induced seizure thresholds were also found in adult male when compared to adult female mice (Figure 4D). However, as shown in Figure 4E, significant sex differences in the threshold of tonic seizures produced by picrotoxin were observed in both saline- and DHEAS-treated groups (acute and chronic treatment).

In aged mice, the findings regarding sex differences in the picrotoxin-induced seizure thresholds were opposite form the results obtained on adult mice (Table 2). Namely, in contrast to adult animals, when control aged male and female mice were compared, significant differences were

Table 1 The effects of higher DHEAS doses (25 and 50 mg/kg) on the seizure thresholds for PTZ, picrotoxin, and NMDA in adult male and female mice

| Seizure threshold	Myoclonus	RB	THE
PTZ (mg/kg)			
Control male mice	29.59±0.58	33.03±0.89	52.90±3.41
Control female mice	31.67±0.79	36.93±0.80	53.06±2.40
DHEAS-treated male mice (25 mg/kg)	31.19±1.65	34.69±1.97	42.36±2.99
DHEAS-treated female mice (25 mg/kg)	29.50±2.60	34.12±2.82	46.33±4.29
DHEAS-treated male mice (50 mg/kg)	27.98±1.79	31.21±2.10	47.94±5.80
DHEAS-treated female mice (50 mg/kg)	31.04±1.81	34.71±1.90	43.53±1.60
Picrotoxin (mg/kg)			
Control male mice	–	21.17±0.34	28.28±0.98
Control female mice	–	25.62±1.62	35.63±1.69
DHEAS-treated male mice (25 mg/kg)	–	19.01±0.66	27.80±1.04
DHEAS-treated female mice (25 mg/kg)	–	23.66±0.64	32.81±0.78
DHEAS-treated male mice (50 mg/kg)	–	19.57±0.50	27.83±0.51
DHEAS-treated female mice (50 mg/kg)	–	23.52±0.30	36.17±1.66
NMDA (mg/kg)			
Control male mice	–	185.5±6.15	223.8±6.09
Control female mice	–	203.6±22.31	293.4±16.49
DHEAS-treated male mice (25 mg/kg)	–	194.9±8.26	236.0±7.28
DHEAS-treated female mice (25 mg/kg)	–	191.8±3.95	286.3±4.73
DHEAS-treated male mice (50 mg/kg)	–	171.6±5.91	213.9±7.18
DHEAS-treated female mice (50 mg/kg)	–	222.4±8.43	310.8±7.96

Notes: Saline (control group) and 25 and 50 mg/kg of DHEAS were administered intraperitoneally to mice 30 minutes prior to the intravenous infusion of convulsant. The observed convulsive signs – myoclonus, RB, and THE. The values represent mean ± SEM from seven animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; NMDA, *N*-methyl-D-aspartate; PTZ, pentylenetetrazole; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.

revealed in the doses of picrotoxin required to elicit clonic convulsive signs. On the other hand, in aged mice acutely treated with 10 mg/kg DHEAS, no significant sex differences were observed in clonic seizures produced by picrotoxin. In the case of picrotoxin-induced tonic convulsions, no sex differences were observed in aged mice treated acutely with saline or 10 mg/kg DHEAS, as opposed to the adult animals.

As shown in Table 2, the aged mice of both sexes demonstrated higher seizure sensitivity to picrotoxin when compared
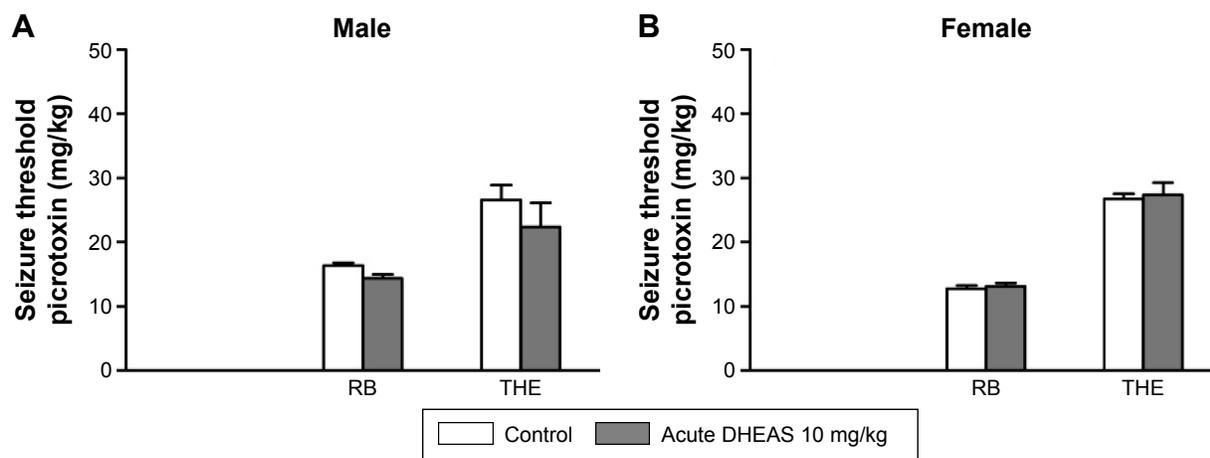
Figure 2 The effects of acute DHEAS treatment on the seizure thresholds for picrotoxin in aged (**A**) male and (**B**) female mice.
Notes: Saline (control group) or DHEAS (10 mg/kg) was administered intraperitoneally to mice 30 minutes prior to the intravenous infusion of picrotoxin. The observed convulsive signs – RB and THE. The bars represent mean ± SEM from seven animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.
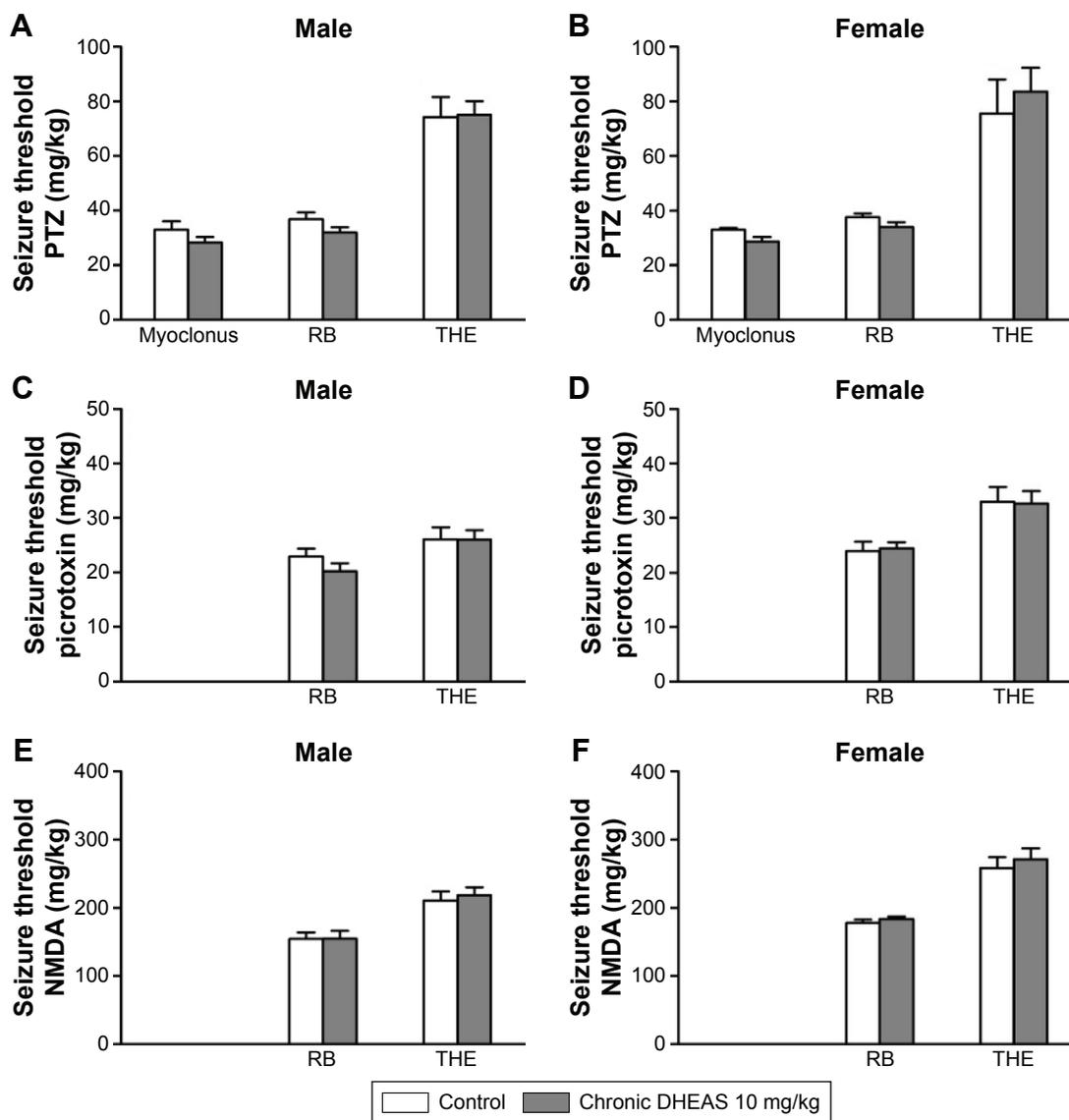
Figure 3 The effects of chronic DHEAS treatment on the seizure thresholds for (**A** and **B**) PTZ, (**C** and **D**) picrotoxin, and (**E** and **F**) NMDA in adult male and female mice.
Notes: Saline (control group) or DHEAS (10 mg/kg) was administered intraperitoneally to adult mice once daily for 4 consecutive weeks. On the last day of chronic treatment, intravenous infusion of convulsant started 30 minutes following last DHEAS or saline administration. The observed convulsive signs – myoclonus, RB, and THE. The bars represent mean ± SEM from seven to eight animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; NMDA, N-methyl-D-aspartate; PTZ, pentylenetetrazole; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.

to adult mice, regardless of whether they were acutely treated with 10 mg/kg DHEAS or not. However, in the case of threshold for tonic convulsions, the age-related differences in male mice have not reached a level of significance.

The effects of DHEAS treatment on the locomotor activity

The results of ANOVA demonstrated that acute as well as chronic administration of 10 mg/kg DHEAS has not affected the number of counts measuring the movements produced by adult male or female mice during 10 minutes stay in the activity cage, suggesting unchanged locomotor activity of the adult animals of both sexes (Table 3).

The effects of DHEAS treatment on the motor coordination

As shown in Table 4, in experiments addressing the effect of acute and chronic 10 mg/kg DHEAS treatment on motor coordination, we observed no significant differences (ANOVA) in the number of falls and the latency to fall from the rota-rod treadmill between control- and DHEAS-treated adult male and female mice.
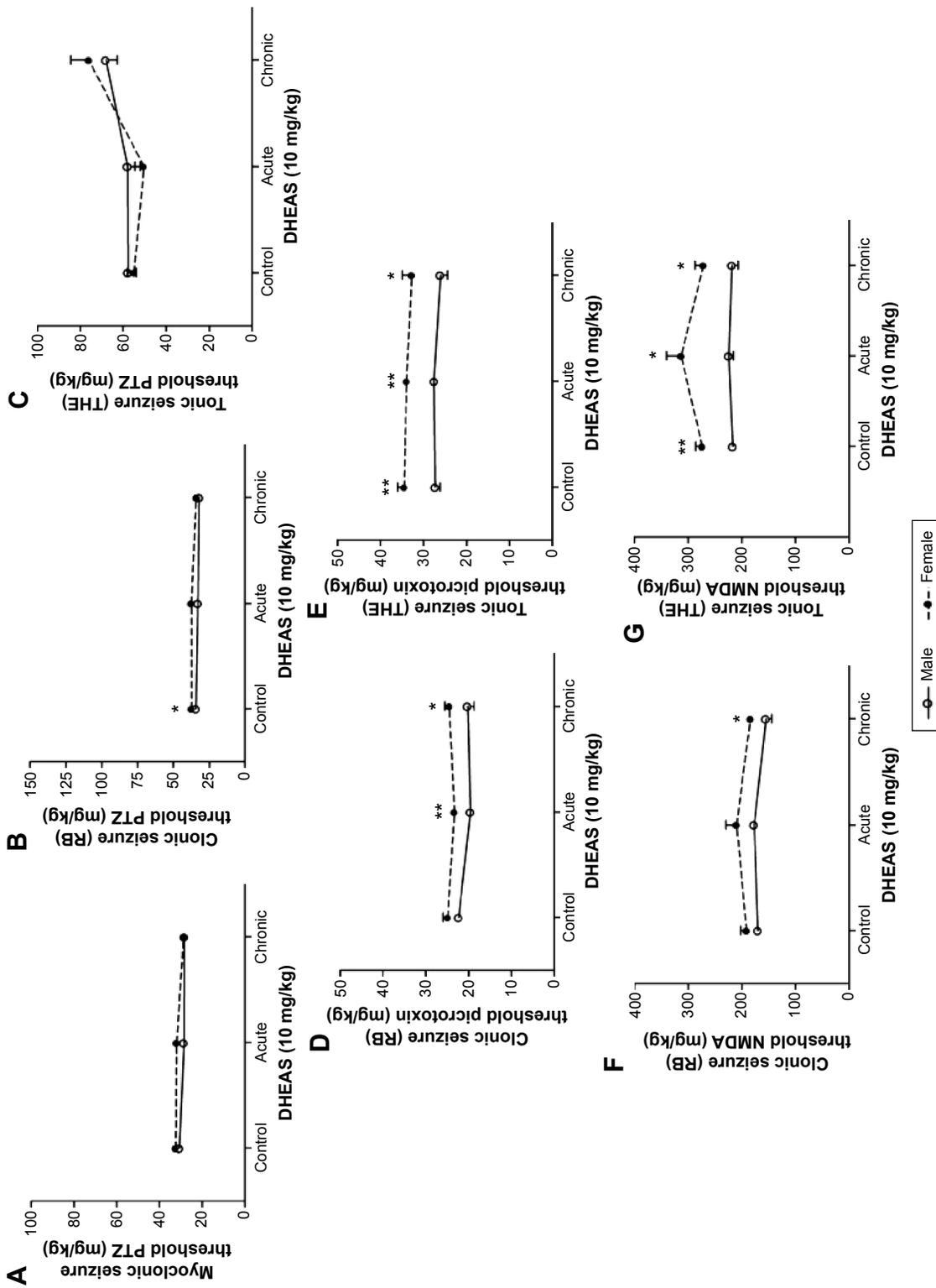
Figure 4 The sex differences in the effects of acute and chronic DHEAS treatment on the seizure thresholds for (**A** and **C**) PTZ, (**D** and **E**) picrotoxin, and (**F** and **G**) NMDA in adult mice.
Notes: Saline (control group) or DHEAS (10 mg/kg) was administered to adult male and female mice intraperitoneally 30 minutes prior to the intravenous infusion of convulsant (acute treatment) or once daily for 4 consecutive weeks (chronic treatment). The observed convulsive signs – myoclonus, RB, and THE. The dots represent mean ± SEM from seven to eight animals per group. *$P<0.05$; **$P<0.002$ versus male mice (Student's t-test).
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; NMDA, N-methyl-D-aspartate; PTZ, pentylenetetrazole; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.

Table 2 The sex and age differences in the effects of acute DHEAS treatment on the seizure thresholds for picrotoxin in mice

Seizure threshold picrotoxin (mg/kg)	RB	THE
Adult control male mice	21.88±0.76	28.28±0.98
Adult control female mice	25.62±1.62	35.63±1.69
Adult DHEAS-treated male mice (10 mg/kg)	19.59±0.41	27.50±0.91
Adult DHEAS-treated female mice (10 mg/kg)	23.32±0.79	33.89±0.94
Aged control male mice	16.33±0.47**	26.56±2.37
Aged control female mice	12.79±0.53$^{\psi,***}$	26.76±0.74***
Aged DHEAS-treated male mice (10 mg/kg)	14.38±0.58***	22.34±3.76
Aged DHEAS-treated female mice (10 mg/kg)	13.13±0.56***	27.40±1.88**

Notes: Saline (control group) or 10 mg/kg of DHEAS were administered intraperitoneally to adult and aged mice of both sexes, 30 minutes prior to the intravenous infusion of picrotoxin. The observed convulsive signs – RB and THE. The values represent mean ± SEM from seven animals per group. $^{\psi}P<0.02$ aged control female versus male mice; **$P<0.01$; ***$P<0.0004$ adult versus aged mice of the same sex and group (Student's t-test).
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; RB, running and bouncing clonus; SEM, standard error of the mean; THE, tonic hindlimb extension.

The effects of DHEAS treatment on the body weight

Following determination of animal body weight, saline or 10 mg/kg DHEAS was administered ip to male and female mice once daily for 4 consecutive weeks. As expected, the male mice had a higher body weight than female mice both in control- and DHEAS-treated groups ($P<0.001$, ANOVA followed by Newman–Keuls test), on the first day as well as on the last day of the chronic treatment (Table 5). Animals in all groups gained body weight after 4 weeks of treatment, but only the body weight of DHEAS-treated female mice was significantly higher on the last day when compared to the first day of the treatment ($P<0.05$, ANOVA followed by Newman–Keuls test). However, as shown in Table 5, there were no differences in the body weight between control- and DHEAS-treated groups of both male and female mice, on the first day as well as on the last day of treatment.

Table 3 The effects of acute and chronic DHEAS treatment on locomotor activity of adult male and female mice

Locomotor activity (counts/10 min)	Acute (10 mg/kg) DHEAS treatment	Chronic (10 mg/kg) DHEAS treatment
Control male mice	536.00±30.99	522.50±27.45
Control female mice	592.00±19.52	575.00±35.72
DHEAS-treated male mice	614.00±24.24	587.40±30.30
DHEAS-treated female mice	622.10±26.35	644.30±25.02

Notes: The locomotor activity of adult male and female mice was recorded for 10 minutes in the test cage, 30 minutes following single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) DHEAS (10 mg/kg) or saline intraperitoneal administration. The values represent mean ± SEM from seven to eight animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; min, minutes; SEM, standard error of the mean.

The effects of DHEAS treatment on the [³H]flunitrazepam binding to the brain membranes

DHEAS inhibited the binding of [³H]flunitrazepam to the brain membranes isolated from both saline- and DHEAS-treated adult male and female mice in a concentration-dependent manner (Figure 5). The inhibitory potency of DHEAS (the half-maximum inhibition value) was in the micromolar range, maximum inhibition obtained with DHEAS was approximately 70%, and slope factor was near unity in all investigated groups. As shown in Table 6, there were no significant differences in the maximum inhibition and half-maximum inhibition of [³H]flunitrazepam binding by DHEAS between saline- and DHEAS-treated adult male and female mice.

Discussion

This study demonstrated no differences in the body weight between control- and DHEAS-treated groups of both male and female mice. Our findings are in line with some previous animal studies reporting that acute and chronic administration of DHEAS has not affected body weight gain and food intake.[43,44] On the other hand, our results are in contrast with the decrease in the body weight of mice observed following chronic DHEAS treatment, which the authors found to be consistent with reported proconvulsive, anxiogenic, provoking, as well as hypophagic effects of DHEAS.[34,35,61,62] The same authors have also shown that DHEAS (1 and 2 mg/kg) increased the motor activity in mice.[34] However, in the present study and some other studies, locomotor activity, general ambulation, and motor coordination of animals were not affected by DHEAS treatment.[37,42,45] Hence, as the present study has not provided experimental evidence on the provoking and proconvulsant effects of long-term DHEAS

Table 4 The effects of acute and chronic DHEAS treatment on motor coordination of adult male and female CBA mice

Motor coordination	Acute (10 mg/kg) DHEAS treatment		Chronic (10 mg/kg) DHEAS treatment	
	The number of falls	The latency to fall (seconds)	The number of falls	The latency to fall (seconds)
Control male mice	0.22±0.15	106.70±8.82	0.56±0.29	95.56±12.37
Control female mice	0.50±0.50	108.80±11.25	0.78±0.22	77.78±10.77
DHEAS-treated male mice	0.50±0.27	95.00±12.39	0.33±0.24	104.40±10.42
DHEAS-treated female mice	0.33±0.17	93.33±10.54	0.25±0.25	102.20±11.76

Notes: The pretrained adult male and female mice were placed on the rota-rod treadmill (constant speed of 15 rpm) 30 minutes following single (acute treatment) or the last (after 4 consecutive weeks of chronic treatment) DHEAS (10 mg/kg) or saline intraperitoneal administration. The number of falls and latency to fall was measured for up to 2 minutes. The values represent mean ± SEM from seven to eight animals per group.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; SEM, standard error of the mean.

administration, perhaps it is not surprising that no changes in animal weight were observed as well.

Namely, our results demonstrated that both acute and chronic DHEAS treatments have not modified the seizure reactivity of mice to PTZ, picrotoxin, or NMDA, widely used convulsants in animal seizure models. These results are in agreement with studies in which DHEAS displayed neither proconvulsant nor anticonvulsant effects. For instance, DHEAS administered to mice in the doses from 1 to 40 mg/kg did not have any effects against acutely PTZ-induced or kindled convulsions or on the development of PTZ seizures.[39] DHEAS (25 and 50 mg/kg) also demonstrated no effect on kainate-induced convulsions, but elevated the kainate-induced lethality in mice.[41] In line with our results, ip administration of DHEAS in doses of 12.5, 50, and 100 mg/kg showed no effect on the NMDA-induced seizures.[37] On the other hand, 25 mg/kg of DHEAS significantly increased the dose of NMDA necessary to induce clonic convulsions in 50% of the tested mice, suggesting protective effect of DHEAS against NMDA-induced

Table 5 The effects of chronic DHEAS treatment on the body weight of adult male and female mice

Body weight (g)	First day of the treatment	Last day of the treatment
Control male mice	24.08±0.45*	24.90±0.76*
Control female mice	19.28±0.38	21.22±0.63
DHEAS-treated male mice	23.72±0.40*	25.45±0.30*
DHEAS-treated female mice	18.64±0.33	21.06±0.76$^{\psi}$

Notes: Saline or DHEAS (10 mg/kg) was administered intraperitoneally to adult mice once daily for 4 consecutive weeks. The body weight of adult male and female animals was determined on the first and the last days of treatment. The values represent mean ± SEM from seven to eight animals per group. *$P<0.001$, group of male mice versus corresponding group of female mice; $^{\psi}P<0.05$, DHEAS-treated female mice last day versus first day of the treatment (analysis of variance followed by Newman–Keuls test).
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; SEM, standard error of the mean.

seizures.[37] Previously, DHEAS was also shown to protect, both in vitro and in vivo, hippocampal neurons against excitatory amino acids-induced toxicity.[63] Moreover, DHEAS (20 mg/kg) administered daily with 45 mg/kg of cocaine for 12 days decreased the number of mice exhibiting cocaine-induced convulsions.[40]

In contrast to these results are the reports suggesting that DHEAS can induce seizures when administered systemically or directly into the brain.[35,36] Namely, it has been shown that intracerebroventricular injection of DHEAS-induced clonic-tonic convulsions in mice in a dose-dependent manner.[36] Moreover, Reddy and Kulkarni[35] reported that although acute DHEAS treatment did not demonstrate proconvulsant effects, mice treated for 4 weeks with 10 mg/kg DHEAS exhibited increased seizure sensitivity to PTZ. The results are also contradictory in human studies. While some studies demonstrated high levels of DHEAS in female patients with epilepsy,[64] other authors reported that women with more frequent seizures had an increase of cortisol and a decrease of DHEAS levels.[38]

The reasons for such discrepancies are not clear, but are usually explained by differences in the experimental approach or protocol, such as the solvent applied, seizure model, route of drug administration, administered dose, time of neurosteroid administration, as well as species or even animal strains used. For instance, unlike Reddy and Kulkarni[35] who induced PTZ seizures in mice by a single ip injection, in our study, convulsions were induced by iv infusion of PTZ. PTZ infusion test is described as a sensitive model that allows determination of drug effects on separate components of seizure behavior and has been suggested for laboratory evaluation of anticonvulsant drugs.[65,66] It is generally accepted that both PTZ and picrotoxin act as noncompetitive antagonists via picrotoxin site of the $GABA_A$ receptor,[67,68] although Huang et al[69] suggested that these two
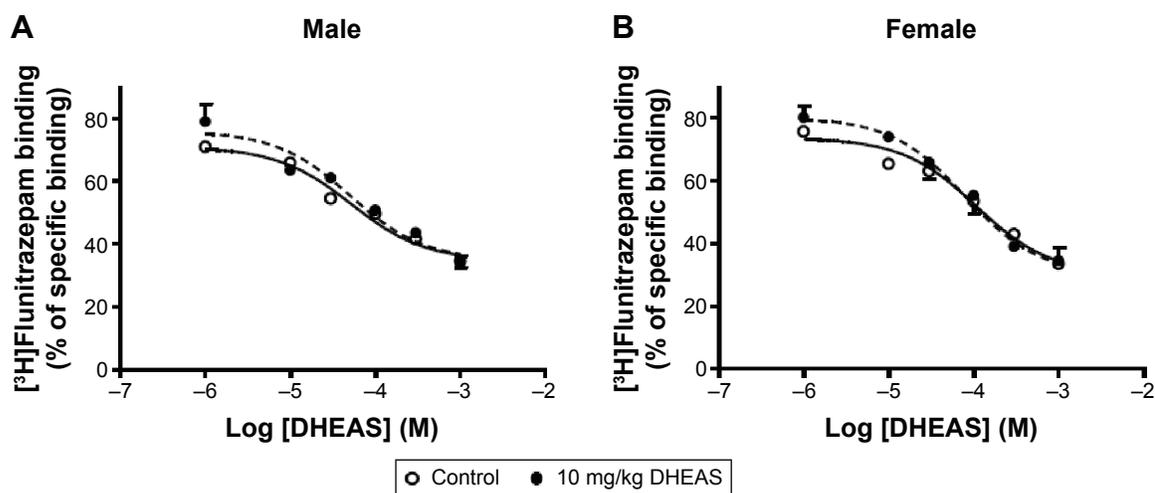
Figure 5 The effects of acute DHEAS treatment on DHEAS-produced inhibition of [³H]flunitrazepam binding to the brain membranes of adult male and female mice.
Notes: The animals were sacrificed 30 minutes following intraperitoneal administration of 10 mg/kg DHEAS or saline. Isolated brain membranes were incubated with increasing concentrations of DHEAS (1 µM to 1 mM) and fixed concentration (1 nM) of [³H]flunitrazepam. Radioactivity bound to membranes after vacuum filtration was measured using β-scintillation counter. Nonspecific binding was determined in the presence of 100 µM diazepam. The inhibition curves were analyzed, and values of half-maximum and maximum inhibition of [³H]flunitrazepam binding produced by DHEAS were determined. The dots represent mean ± SEM from three independent experiments performed in duplicate.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; SEM, standard error of the mean.

convulsants interact with overlapping but distinct domains of the GABA$_A$ receptor.

As DHEAS has been also reported as an allosteric antagonist of GABA$_A$ receptor,[26,27,30] which binds at or near the picrotoxin/t–butylbicyclophosphorothionate site on the GABA$_A$ receptor complex,[70] it is possible that DHEAS competes with PTZ and picrotoxin for the same binding site or displaces their binding by steric hindrance. Such an interaction would explain why proconvulsive effects of DHEAS were not observed in PTZ- and picrotoxin-induced seizures. The interaction of DHEAS with excitatory amino acid transmission also seems rather complex.[37] In line with

our results, demonstrating that NMDA-induced seizures were not affected by chronic DHEAS treatment, some authors failed to detect significant effects of DHEAS on glutamate binding sites and glutamate uptake.[71]

The presence of sex differences in the sensitivity to GABA-related convulsants also seems to depend on the convulsive drug and methodology used to inject it.[52] However, various results have been reported even when using convulsants that share the same receptor binding site. In contrast to the study demonstrating that PTZ, which acts via picrotoxin binding site, affects more female than male mice,[52] Pericic et al observed higher picrotoxin sensitivity in male than in female mice.[72] Even the same route of drug administration has not presented consistent results. For instance, intravenously injected bicuculline has been reported to affect more females[73] or males[72,74] or to have the same effect on both sexes.[75] Moreover, regarding intravenously administered PTZ, Kokka et al[76] demonstrated that male rats were more susceptible than females, while Finn and Gee[73] found no seizure differences between sexes.

The discrepancy in the results might also be due to the differences in the stages of the female estrous cycle. Although the estrus cycle in our study was presumably synchronized due to the Whitten effect, we have not controlled the stage of the estrous cycle between experiments. As the estrous cycle is short, a variation of the estrous stages between different experiments is possible and might influence the effect of DHEAS on neuronal excitability of female

Table 6 The effects of acute DHEAS treatment on the potency of DHEAS for inhibiting [³H]flunitrazepam binding to the brain membranes of adult male and female mice

DHEAS inhibition of [³H]flunitrazepam biding	IC$_{50}$ (µM)	I_{max} (%)
Control male mice	53.8±10.0	65.66±1.59
Control female mice	102.1±28.0	65.56±4.35
DHEAS-treated male mice	60.8±34.8	69.60±1.56
DHEAS-treated female mice	89.2±47.4	70.93±6.04

Notes: The animals were sacrificed 30 min following the intraperitoneal administration of 10 mg/kg DHEAS or saline. Isolated brain membranes were incubated with increasing concentrations of DHEAS (1 µM to 1 mM) and fixed concentration (1 nM) of [³H]flunitrazepam. Nonspecific binding was determined in the presence of 100 µM diazepam. The inhibition curves were analyzed, and the values of half-maximum (IC$_{50}$) and maximum (I_{max}) inhibition of [³H]flunitrazepam binding produced by DHEAS were determined. The values represent mean ± SEM from three independent experiments performed in duplicate.
Abbreviations: DHEAS, dehydroepiandrosterone sulfate; IC$_{50}$, half-maximum inhibition; min, minutes; SEM, standard error of the mean.

mice. Different stages in the estrus cycle might be the reason why in our study sex differences were not always significant. However, male adult mice generally demonstrated lower thresholds for seizures induced by all three convulsants, when compared to female adult mice, regardless of whether they were acutely or chronically treated with DHEAS or treated with saline.

In contrast, in aged mice, we observed significant sex differences only in the control group, with female mice showing lower threshold for clonic picrotoxin-induced seizures. In line with our results, Zhang et al[77] comparing adult and aged male and female mice following kainic acid treatment found that aged female mice demonstrated more severe seizure activity. On the other hand, our findings demonstrating sex differences in the susceptibility to tonic picrotoxin-induced convulsions only in adult but not in aged CBA mice are in line with a study reporting that unlike adult mice, 2-year-old CBA mice fail to display sex differences following picrotoxin administration.[78] Nevertheless, since aged mice were not submitted to all experimental procedures as adult mice, our limited results regarding differences in neuronal excitability of aged male and female mice should be taken with caution.

In our study, the aged mice of both sexes demonstrated higher seizure sensitivity to picrotoxin when compared to adult mice, regardless of whether they were acutely treated with DHEAS or not. These results are in line with various findings demonstrating that aging is associated with an increased risk of seizures/epilepsy.[79,80] It has been shown that aged rats exhibit altered EEG activity and clinical manifestations during kainate-induced status epilepticus.[81] In addition, increased hippocampal excitability has been found in aged mice suggesting its relevance to the increased seizure susceptibility observed in aged subjects.[82] However, different studies have reported both increased or decreased seizure susceptibility associated with advanced age, depending on the model and animal strain used.[83]

Following DHEAS administration, we determined some discrete but significant changes regarding sex differences in the susceptibility to clonic seizures in comparison to control groups. Because DHEA and its sulfate ester DHEAS serve as precursors for both androgenic and estrogenic steroids,[1,84,85] some effects of DHEAS may be attributable to its conversion into these sexual hormones.[53] Baulieu et al[86] reported that following long-term DHEA administration to elderly men and women, in addition to the higher concentration of DHEAS, a small increase of testosterone and estradiol was noted, particularly in women, which may be involved in the observed beneficial physiological–clinical manifestations. Moreover, it

has been suggested that both DHEA and DHEAS might have either estrogen- or androgen-like effects depending on the hormonal environment and thus influence the receptor type with which these neurosteroids interact.[87] The suggestion of such interaction between DHEAS and male or female hormonal milieu is in line with previously reported sex-related differences in the DHEAS serum/plasma levels.[54,88–90]

Hence, the small changes regarding sex differences in the seizure sensitivity, observed following DHEAS treatment, could be explained by different modulation of the neurotransmitter receptor activity by DHEAS-derived sexual hormones and their metabolites. However, our findings showing that acute DHEAS treatment has not modified the potency of DHEAS to inhibit [^{3}H]flunitrazepam binding on the brain membranes of adult male and female mice do not support this hypothesis, although they confirm previous results, which demonstrated that DHEAS behaves as an allosteric antagonist of the GABA$_A$ receptor.[25–30] Further studies are needed in order to elucidate the role of GABAergic and other neurotransmitter systems in the complex effects of DHEAS on neuronal excitability.

The limitations of this study are a lack of lower doses of DHEAS (1 and 2 mg/kg), which have been also shown to be active in mice, a lack of control for the stage of the mice estrous cycle between experiments, as well as the fact that aged mice were not submitted to all experimental procedures as adult mice.

Conclusion

Our findings demonstrating that DHEAS does not modify the locomotor activity, motor coordination, seizure susceptibility, brain [^{3}H]flunitrazepam binding, and body weight in the mice of both sexes suggest that DHEAS treatment might be safe for various potential therapeutic applications in both adult and aged population. The results of our study also suggest discrete interaction of DHEAS with male and female hormonal status, which may underline observed sex-related differences in the association of DHEAS with various indicators of health and morbidity, although the underlying molecular mechanisms are not clear.

Acknowledgments

Zagreb, Croatia, September 18–21, 2013, as a poster presentation with interim findings. The abstract of the poster was published in "Poster Abstracts" in *Periodicum Biologorum* Vol 115, Suppl 3. Vitale, Branko (ed.), Zagreb, Croatian Society for Natural Sciences, Rudjer Boskovic Institute, LASERplus, 2013. 96–96.

Disclosure

The authors report no conflicts of interest in this work.

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