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Dehydroepiandrosterone increases synaptosomal glutamate release and improves the performance in inhibitory avoidance task

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Abstract

Dehydroepiandrosterone (DHEA) exerts multiple effects in the rodent central nervous system (CNS), mediated through its nongenomic actions on several neurotransmitter systems, increasing neuronal excitability, modulating neuronal plasticity and presenting neuroprotective properties. It has been demonstrated that DHEA is a potent modulator of $GABA_A$, NMDA and Sigma receptors.

In the present study, we investigated the effect of DHEA on (i) basal- and K^+-stimulated L-[^{3}H]glutamate release from synaptosomes (both in vitro and ex vivo), (ii) synaptosomal L-[^{3}H]glutamate uptake (in vitro), and (iii) an inhibitory avoidance task (in vivo).

The results indicated that DHEA in vitro increased glutamate release by 57%, and its intracerebroventricular infusion increased the basal-[^{3}H]glutamate release by 15%. After 30 min of intraperitoneal administration, DHEA levels in the serum or CSF increased 33 and 21 times, respectively. Additionally, DHEA, intraperitoneally administrated 30 min before training, improved memory for inhibitory avoidance task. Concluding, DHEA could improve memory on an inhibitory avoidance task, perhaps due to its ability to physiologically strength the glutamatergic tonus by increasing glutamate release.

Keywords: Dehydroepiandrosterone; Synaptosomal glutamate release; L-[^{3}H]Glutamate uptake

1. Introduction

Dehydroepiandrosterone (DHEA) exerts multiple effects in rodent central nervous system (CNS), mediated through its nongenomic actions on several neurotransmitter systems. DHEA increases neuronal excitability, modulates neuronal plasticity and presents neuroprotective properties (Wolf and Kirschbaum, 1999).

Glutamate, the main excitatory neurotransmitter in the mammalian brain, participates in several physiological and pathological events. Glutamate receptors are involved in plastic processes related to ontogeny and ageing, memory and learning and formation of neuronal networks. Glutamate receptors have been implicated in memory formation (Izquierdo and Medina, 1997; Ozawa et al., 1998; Segovia et al., 2001).

Several studies has shown that NMDA type of glutamate receptor channel has been implicated in the mechanisms underlying memory formation, and NMDA receptor antagonists impair retention of many tasks (De-Paris et al., 2000). Inhibitory avoidance training induced a 29% increase in glutamate binding in hippocampus membranes obtained from rats sacrificed 5 min after training (Schroder et al., 2000). Intrahippocampal infusion of the NMDA receptor antagonist AP5 (aminophosphonopentanoic acid) impairs retention of inhibitory avoidance task (Vianna et al., 2000). Infusion of CNQX (6-cyano-7 nitroquinoxoline, 2,3-dione), a non-NMDA receptor antagonist, was amnesic when given 90 min after training (Mello e Souza et al., 2000). All these results point that the glutamatergic tonus modulates learning and memory processes (Izquierdo and Medina, 1997).

However, overstimulation of the glutamatergic system potentially leads to excitotoxicity, as observed in various brain disorders (Meldrum and Gaarthwaite, 1990; Danbolt, 2001; Maragakis and Rothstein, 2001; Haberny et al., 2002). To avoid the excessive increase in extracellular glutamate

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levels and, consequently, its excitotoxicity, glutamate must be taken up, mainly by astrocytes, from the synaptic cleft to the cytosol (Ozkan and Ueda, 1998; Anderson and Swanson, 2000; Danbolt, 2001), and further stored into synaptic vesicles located on presynaptic terminals. Glutamate stored in synaptic vesicles is available for release when presynaptic membrane is depolarized (Nicholls, 1998).

Baulieu (1998) found that steroids, such as pregnenolone, DHEA and their sulfates and lipoidal esters, are present in the nervous system (brain and peripheral nerves) and in plasma. The presence of these compounds in the CNS could be either due to peripheral synthesis and sequestration to the brain (Baulieu, 1998) or local production (Mellon and Griffin, 2002). DHEA and DHEA sulfate (DHEAS) can interact with various neurotransmitter systems, promoting neuronal remodeling (Campagnone and Mellon, 1998; Mao and Barger, 1998). It has been demonstrated that some neurosteroids are potent modulators of neurotransmitter receptors, such as $GABA_A$, NMDA and Sigma receptors (Baulieu and Robel, 1998; Foy et al., 1999; Rupprecht and Holsboer, 1999; Racchi et al., 2001). Moreover, DHEA and DHEAS are multifunctional, exhibiting a variety of actions on the CNS, including the modulation of memory consolidation, neuroprotection, and reduction of neurodegeneration (Majewska, 1992, 1995; Lapchak and Araujo, 2001; Lapchak et al., 2000).

Considering the DHEA effect on various parameters of the glutamatergic system, and also the involvement of glutamate in learning and memory processes, in the present study, we investigated in rats the effect of (i) DHEA in vitro (100 nM to 1 mM) on basal and stimulated L-[^{3}H]glutamate release from synaptosomes, (ii) acute unilateral intracerebroventricular (icv) administration of DHEA (4 μl/100 μmol) on L-[^{3}H]glutamate release from and uptake by synaptosomes and (iii) acute intraperitoneal (ip) administration of DHEA (100 μM) on inhibitory avoidance task.

2. Material and methods

2.1. Subjects and reagents

Two-month old male Wistar rats (250–300 g) were maintained on a 12:12-h light/dark cycle (lights on at 06:00 h), with tap water and standard lab chow (Guabi, Santa Maria, RS, Brazil) ad libitum. They were housed in plastic cages (five per cage). Animals were killed by decapitation without anesthesia, the brains rapidly removed, and the cerebral cortices dissected. The experimental protocol was approved by the Ethics Committee for animal research of Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

L-[^{3}H]Glutamate (49 Ci/mmol) was from Amersham International, UK. DHEA and dimethyl sulfoxide (DMSO) were from Reagent and other chemicals were from Sigma, St. Louis, MO, USA. The anesthetic sodium thiopental was obtained from Cristália (Itapira, SP, Brazil). DHEA was measured by a specific Radioimmunoassay (Kit from ICN Pharmaceuticals, Diagnostic Division, Costa Mesa, CA, USA).

2.2. Surgical procedure and intracerebroventricular Infusion techniques

The animals were anesthetized with sodium thiopental (25 mg/kg, 2 ml/kg, ip). In a stereotaxic apparatus, the skin of the skull was removed, and a 27-gauge guide cannula was placed 0.9 mm posterior to bregma, 1.5 mm right from the midline, and 1 mm above the lateral brain ventricle. The cannula was implanted through a 2-mm hole made at the cranial bone, and fixed with jeweler acrylic cement. Experiments were performed 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a 10-μl Hamilton syringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula, aiming the lateral brain ventricle. An intracerebroventricular infusion of either 4 μl of vehicle or DHEA (0.4 nM) in vehicle was performed, and rats were behaviorally observed for 30 min in Plexiglas chambers. After infusion, no significant alterations in behavior were observed.

2.3. Inhibitory avoidance

Animals were trained and tested for step-down inhibitory avoidance task in a 50 × 25 × 25 cm Plexiglas box, whose floor consisted of a grid of 1 mm caliber parallel bronze bars spaced 10 cm apart. The left side of the grid was covered by a 5 cm high, 7 × 25 cm wood platform. The rat was placed on the platform, and the latency to step down, placing the four paws on the grid, was measured. Immediately after stepping down on the grid, animals received a 0.4-mA, 2.0-s footshock. Retention test session was carried out 1.5 h (short-term memory—STM) and 24 h (long-term memory—LTM) after training and was procedurally identical to training, except that no footshock was presented. The same rats were used in STM and LTM test sessions. This protocol has been largely used (Izquierdo et al., 2000) and is adequate for studying both STM and LTM. Difference between the latency (maximum of 180 s) in training and test session was taken as a measure of memory. Animals received an intraperitoneal injection of saline, 1% DMSO, or 500 μl of DHEA 28 mg/kg dissolved in 1% DMSO (experimental group) 30 min before training.

2.4. Synaptosomal preparations

The animals were decapitated, and the forebrain was used to prepare synaptosomal preparations on a discontinuous Percoll gradient according to Dunkley et al. (1998). The preparations were used in the same day of preparations.

2.5. L-[^{3}H]Glutamate release from synaptosomal preparations

L-[^{3}H]Glutamate release was measured according to Migues et al. (1999), with minor modifications (Tavares et al., 2002). Synaptosomal preparations were incubated in HBSS, pH 7.4, for 15 min at 37 °C, in the presence of 0.1 μCi/ml L-[^{3}H]glutamate (final concentration, 500 nM). Then, aliquots of labeled synaptosomes (1.3 mg protein) were centrifuged at 13,000 × *g* for 1 min at 4 °C. Supernatants were discarded, and the pellets were washed four times in HBSS, by centrifugation at 13,000 × *g* for 1 min at 4 °C. To assess the basal release of L-[^{3}H]glutamate, the final pellet was resuspended in HBSS and incubated for 1 min at 37 °C in 1% DMSO (control group) or DHEA (0.0001–1 mM) in 1% DMSO. Incubation was terminated by immediate centrifugation (16,000 × *g* for 1 min at 4 °C). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. The released L-[^{3}H]glutamate was calculated as a percentage of the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period. K^+-stimulated L-[^{3}H]glutamate release was assessed as described for basal release, except that the incubation medium contained 40 mM KCl (NaCl decreased accordingly) to induce synaptosomal depolarization.

In further experiments, synaptosomes were prepared from rats previously injected with intracerebroventricular DHEA, to determine basal and stimulated L-[^{3}H]glutamate release, performed according to described above.

These synaptosomal preparations contained 5% contamination with inner and outer mitochondrial membrane fragments, microsomes, myelin, as well as neural and glial plasma membranes (Nagi et al., 1986).

2.6. [^{3}H] glutamate uptake by synaptosomes

The synaptosomal preparation from rats, previously intracerebroventricularly DHEA injected, was washed twice by suspending in 3 volumes 0.3 M sucrose and in 15 mM Tris/acetate buffer (pH 7.4) and centrifuged at 13,000 × *g* for 15 min. The final pellet was resuspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS (composition in mM: HEPES 24, NaCl 119, KCl 2.1, $MgSO_4$ 1.08, KH_2PO_4 1.08, glucose 10.8, $CaCl_2$ 0.9), pH 7.4 (adjusted with HCl), in the presence of 1 μCi/ml L-[^{3}H]glutamate (final concentration 100 nM), for 1 min at 37 °C. The reaction was stopped by filtration through GF/B filters. The filters were washed three times with 3 ml ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in Wallac scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptakes obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (nonspecific uptake).

2.7. Measurement of lactate dehydrogenase (LDH) activity

To evaluate the integrity of the synaptosomal preparation, lactate dehydrogenase (LDH; E 1.11.27) release was monitored by incubating preparations with DHEA (1 mM) or 1% DMSO (control) for 15 min. The LDH activity in the incubation medium and the total LDH content, which was determined by the disruption of the synaptosomal preparation using 1.5% Triton X-100, was assayed spectrophotometrically using an assay kit (Labtest reagents, Brazil), which measures the amount of a colored complex derived from the NADH formed by the enzymatic reaction, using a spectrophotometric method (510 nm).

2.8. Measurement of DHEA concentration

The measurement of DHEA concentration was determined by Radioimmunoassay, using a sensitive and specific antibody preparation (commercial kit from ICN Pharmaceuticals, Diagnostic Division, Costa Mesa), with low cross-reactivity to other physiologic compounds, including DHEA-S, and that does not require prior sample (serum and cerebrospinal fluid) extraction. Sensitivity is >20.0 pg/ml.

2.9. Measurement of protein content

The measurement for protein content was determined by the method of Lowry et al. (1951), using bovine albumin as standard.

2.10. Statistics

All experiments were performed in triplicates, and the mean was used for the calculations. Statistical significance was assessed by analysis of variance (ANOVA), followed by Komolgorov–Smirnov test, when the *Z* was significant, or Student *t* test for comparing control group and DHEA administration. A value of $P < .05$ was considered statistically significant. The values are expressed mean ± standard deviation. Short- and long-term retention of a step-down inhibitory avoidance task in rats previously treated was evaluated and represented with the median (interquartile ranges) of latencies to step-down. Due to the 180-s ceiling used for test session latencies, these data were analyzed by nonparametric procedures by Mann–Whitney test followed by Wilcoxon *W*, when the *Z* was significant in all two sessions.

3. Results

Synaptosomal preparations did not show any significant difference in the permeability of plasma membranes in our assay conditions, measured by the cytosolic marker LDH activity release. Groups with DMSO, or DMSO, plus 1 mM

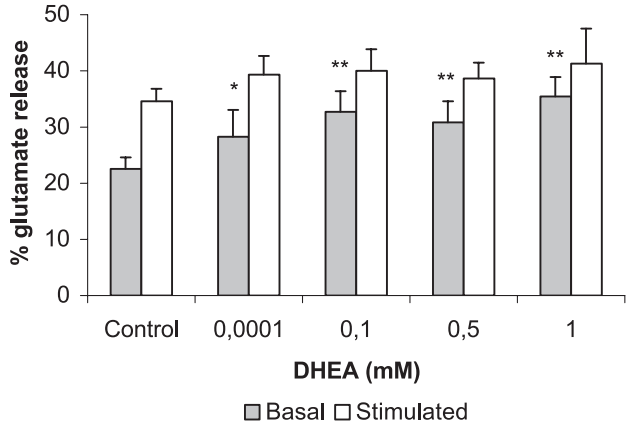

Fig. 1. DHEA (100 nM to 1 mM) in vitro effect on basal and K^+-stimulated L-[^{3}H]glutamate release from rat brain synaptosomes. [^{3}H]Glutamate released was calculated as a percentage of the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period. Data are mean ± S.D. from five independent experiments performed in triplicates. * And ** represent values significantly different from the control group at $P<.05$ or $P<.01$, respectively, by ANOVA, followed by Student–Newman–Keuls test.

DHEA presented (mean ± S.D.) 20.6 ± 5.1 and 21.3 ± 6.5, $n=5$, respectively (expressed as percentage of total disruption caused by 0.1% of SDS and NaOH 1 M), indicating that these compounds did not affect plasma membrane permeability in a significant way.

The in vitro and in vivo effects of DHEA on L-[^{3}H]glutamate release in basal (physiological extracellular K^+ concentration) or depolarized (40 mM KCl) conditions were evaluated. In vitro, DHEA (100 nM to 1 mM) increased up to 57% of the basal [^{3}H]glutamate release [$F(4,20)=8967$; $P<.01$], without affecting the K^+-stimulated release (Fig. 1). Additionally, intracerebroventricular infusion of 0.4 nmol DHEA also increased only the basal [^{3}H]glutamate release (by 15%; Student's t test, $P<.01$), without affecting the K^+-stimulated release (Fig. 2).

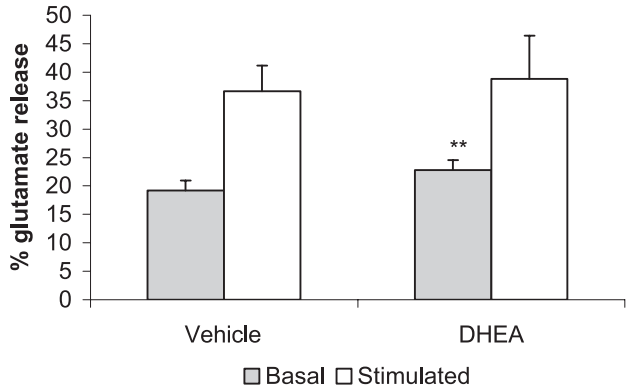

Fig. 2. Effect of intracerebroventricular 0.4 nM DHEA administration on L-[^{3}H]glutamate release from brain synaptosomes of rats. [^{3}H]Glutamate released was calculated as a percentage of the total amount of radiolabel in the synaptosomal preparation at the start of the incubation period. Data are mean ± S.D. from five independent experiments performed in triplicates. ** Represents values significantly different from vehicle basal group, at $P<.01$, by Student t tests.

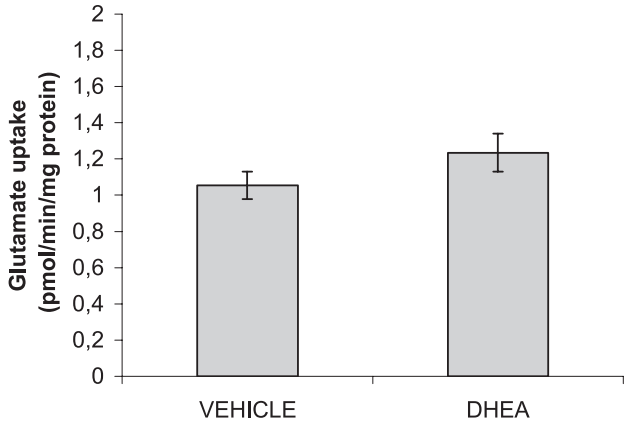

Fig. 3. Effect of intracerebroventricular 0.4 nM DHEA administration on [^{3}H]glutamate uptake into brain synaptosomes of rats. [^{3}H]Glutamate uptake is expressed as pmol mg protein^{-1} min^{-1}. Data are mean ± S.D. from five independent experiments performed in triplicates. No statistically significant difference was observed between vehicle and DHEA groups.

Fig. 3 shows that intracerebroventricular DHEA infusion had no effect on glutamate uptake by synaptosomes, when compared with vehicle (saline, 1% DMSO).

Considering the effect of DHEA on the glutamatergic system, and based upon the established modulatory role of this system on learning and memory, we investigated the effect of DHEA intraperitoneal administration on memory of inhibitory avoidance task (Fig. 4). Preliminary, we investigated the DHEA levels in serum and CSF after intraperitoneal DHEA administration (28 mg/kg). After 30 min of injection, DHEA levels in serum increased from

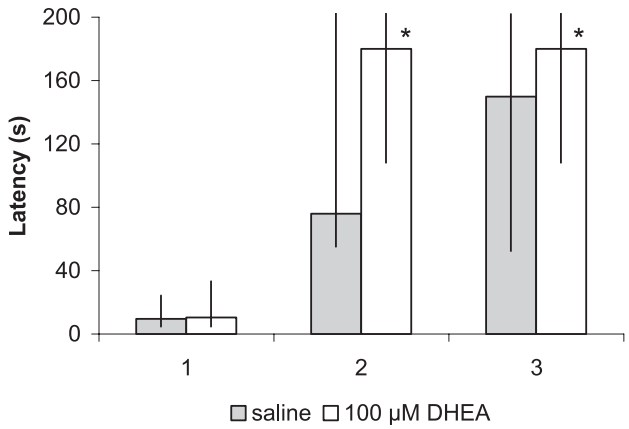

Fig. 4. Effect of DHEA (28 mg/kg ip) administration 30 min before training session on inhibitory avoidance STM and LTM. Latency to step-down (s) is expressed as medians (interquartile range). Statistical comparison among sessions was performed by Mann–Whitney U test, followed by Kolmogorov–Smirnov, two-tailed ($n=12$ per group). * Represents significant differences between saline and DHEA (in STM and LTM sessions) at $P<.05$, by Mann–Whitney U test, Komolgorov–Smirnov, two-tailed; ($n=12$ per group).

0.85 ± 0.07 (basal) to 28.00 ± 4.90 ng/ml, $n = 5$, and in CSF from 0.98 ± 0.08 to 20.80 ± 3.85 ng/ml, $n = 5$. Both differences were statistically different at $P < .001$. The results are expressed as mean ± S.D. Furthermore, we investigated the behavioral effect of intraperitoneal DHEA administration (28 mg/kg), 30 min before a training session for step-down inhibitory avoidance task. The latency of step-down in test sessions both for STM and LTM was significantly increased by intraperitoneal DHEA administration, at $P < .05$.

4. Discussion

In the 1980s, Baulieu and collaborators (Baulieu, 1998) made the important discovery that certain steroids are synthesized in the central and peripheral nervous systems. These compounds, known as neurosteroids, are produced locally in glial and neuronal cells and can exert important modulatory actions in the nervous system (Meyer et al., 2002).

Neurotransmitter release represents a target of a staggering number of studies on synaptic activity (Danbolt, 2001; Garcia-Sanz et al., 2001). We previously demonstrated (Lhullier et al., 2004) that the in vitro DHEA effect on synaptosomal glutamate release depends on the age of rats: it decreases the basal glutamate release from synaptosomes of old rats (12 months old), with no effect on young rats (17 days old). The K^+-stimulated release is not affected in both ages. Accordingly, here, we observed, in 2-month-old rats, that DHEA increased, also specifically, the basal release of glutamate. The relevance of age influence on this variable DHEA effect (increasing, decreasing or no effect) on basal glutamate release deserves further investigations.

There have been reports of DHEA and DHEAS actions on NMDA receptors. DHEA is a positive allosteric modulator of NMDA receptors (Mellon and Griffin, 2002), increasing various NMDA responses (Bergeron et al., 1996; Baulieu and Robel, 1998). As NMDA receptor activity has been shown to stimulate presynaptic glutamate release (Breukel et al., 1998), the NMDA receptors modulation by DHEA could be involved in its effects, reported here, on glutamate release. However, it is noteworthy that DHEA and DHEAS can prevent/reduce the neurotoxicity of NMDA, both in vitro and in vivo models (Kimonides et al., 1998). At molecular levels, the two hypotheses are either that DHEA and DHEAS reduce NMDA-stimulated Ca^{2+} entry into cells, a critical step in excitotoxic cell death, or that they act as antagonists of glucocorticoids (Kimonides et al., 1998), compounds that accentuate Ca^{2+} entry into neurons via voltage-gated channels (Ffrench-Mullen and Spence, 1991; Kimonides et al., 1998; Kurata et al., 2001). Hence, we could postulate that DHEA the effects on the glutamatergic system could either strengthen the physiological tonus of glutamate or act as neuroprotector against glutamate excitotoxicity.

Based upon DHEA effect on glutamate release, and considering the facilitatory effect of the glutamatergic system on the memory of various tasks, we investigated if DHEA could affect the performance of rats in inhibitory avoidance task. DHEA administered 30 min before training had a facilitator effect on both short-term (STM) long-term (LTM) avoidance memories, pointing that DHEA in vivo seems to strengthen the physiological tonus of glutamate. As the same protocol increased the DHEA levels both in the serum and CSF, it may be suggested that there is a central effect of DHEA on this behavioral task. This result is in agreement to reported behavioral roles of neurosteroids. It has been hypothesized that they exhibit a broad spectrum of biological actions, from developmental modulation of CNS to complex processes such as learning and memory (Frye and Sturgis, 1995; Flood and Roberts, 1998; Frey and Lacey, 1999; Wolf and Kirschbaum, 1999; Vallée et al., 2001; Mellon and Griffin, 2002). Several studies have reported facilitatory effects of the acute administration of DHEA, pregnenolone, or their sulfates on the memory of rats or mice (Flood and Roberts, 1998; Ladurelle et al., 2000; Racchi et al., 2001), in particular, on avoidance task. Regarding the reversal of amnesic procedures, pregnenolone and its metabolic derivatives antagonize the amnesic effect of the NMDA antagonist dizocilpine in rodents (Mathis et al., 1994), and pregnenolone sulfate blocks learning deficits induced by scopolamine, with an additive memory-enhancing effect, apparently through interaction with NMDA and/or $GABA_A$ receptors (Meziane et al., 1996).

In conclusion, based upon data from the literature pointing to a modulatory effect of glutamate on behavioral performances, the results presented here could indicate that by increasing glutamate release, DHEA could strengthen the physiological glutamatergic tonus, consequently improving the memory of inhibitory avoidance task.

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