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Effects of early-life stress on behavior and neurosteroid levels in the rat hypothalamus and entorhinal cortex

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Abstract

Recent evidence support the hypothesis that exposure to stress or trauma during early childhood may disturb the formation of functional brain pathways, in particular, of the limbic circuits. We examined the effects of exposure to early life trauma (juvenile stress) on emotional and cognitive aspects of behavior in adulthood as well as on dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) levels in relevant brain regions. Quantitative assessment of the effects of exposure to juvenile stress was made 1 month post-stress, and obtained by measuring: emotional (utilizing an open field and a startle response tests) and cognitive (Morris water-maze task) functions, as well as neurosteroids concentration (DHEA and its sulfate ester, DHEAS) in the hypothalamus and entorhinal cortex. We report here that an exposure to juvenile stress led to elevated levels of anxiety 1 month post-stress. Moreover, in a spatial learning task, the juvenile stress group performed poorer than the control group. Finally, an exposure to juvenile stress increased DHEAS but not DHEA concentrations both in the hypothalamus and the entorhinal cortex. These findings indicate that an exposure to juvenile stress has long-lasting effects on behavior and DHEAS levels in the hypothalamus and the entorhinal cortex. These effects may be of relevance to our understanding of early life stress-related disorders such as PTSD and major depression.

Keywords: Early life; Stress; Behavior; Neurosteroids

1. Introduction

As adults, victims of child abuse are at considerably greater risk for mental illness, as well as for obesity, diabetes, and heart disease [5,18,34].

Specifically, children that were exposed to an emotional trauma, such as sexual abuse [21,36] show higher tendency in adulthood to develop post-traumatic stress disorder (PTSD), major depression or generalized anxiety [2,6,17,20,23,42,48].

Previous findings from our lab indicate that an exposure of rats to a relatively brief stressful experience during their 4th week of life (juvenile stress) has profound and long-lasting behavioral effects [3]. The activation of the stress response system by exposure to stress in early life could lead to abnormalities in this system in adulthood [28]. Neuroactive steroids are suggested to be part of the stress response system [51] and thus could potentially be affected by the exposure to juvenile stress.

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are endogenous hormones secreted by the adrenal cortex in response to adrenocorticotrophin hormone (ACTH) [38]. They are also classified among the group of steroids known as neurosteroids, so named because they can be synthesized de novo in the central nervous system [32]. Moreover, their concentration was found to be considerably higher in the brain than in other organs [12]. DHEA and DHEAS have been also shown to behave as negative modulators of the gamma-aminobutyric acid type A ($GABA_A$) receptor [7,31] with possible anxiogenic properties. The GABAergic properties of neurosteroids are similar to those of anxiolytic or anxiogenic compounds acting at the $GABA_A$ receptor like benzodiazepine (BZD) or β-carbolines. Thus, a possible role of neurosteroids in the neuro physiological regulation of adaptive responses to stress has been hypothesized. In adult mice, DHEAS release has been shown to exhibit memory-enhancing, antidepressant, anxiolytic, and antiaggression properties [10,35,40,41]. Neuronal and glial sur-

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vival and differentiation have also been shown to be enhanced by DHEAS in dissociated cultures of mouse embryo brain and intact rats [26,27]. In addition, it has been proposed that DHEA and DHEAS may play a role in neurodevelopment, due to a transient expression of the steroidogenic enzyme P450 17-alphahydroxylase (P450c 17) [11] and the potential ability of DHEA and DHEAS to aid in neuronal pathway formation [9]. Taken together, DHEA and DHEAS seem to play a role in emotional- and cognitive-related processes in adulthood. However, Their involvement during developmental trajectory is still obscure. We hypothesize that they exert important effects during early life, and therefore an exposure to stressful event at that age will have long-lasting effects on behavior. Thus, here we focused on the effects of exposure to juvenile stress on behavior and its relation to neurosteroid concentrations in adulthood. This post-weaning pre-puberty age in the rat is suggested to physiologically resemble childhood pre-puberty, which is an age associated in humans with increased risk of child abuse-related mood disorders later in life [3]. At this age, high associative areas in the brain are not yet fully developed and they continue to develop until the rat is sexually mature [33]. Thus, it is reasonable to hypothesize that these late-developing regions and their emerging synaptic connections may be particularly vulnerable to the effects of an exposure to juvenile stress [25].

A variety of key regions in the young and adult brain are suggested to be involved in the emotional response to stress and to potentially be affected by the exposure to stress. One region is the hypothalamus, which is the core part of the stress response system, i.e., hypothalamic–pituitary–adrenal (HPA) axis [45]. Another relevant region is the entorhinal cortex (EC), which represents a hierarchically high level of associativity, where a network of intrinsic associational connections further increases the level of integration of multimodal polysensory information and serves as a major trajectory into the hippocampus and the amygdala [29].

Thus, we set out to examine whether the exposure to juvenile stress would have long-lasting effects on behavior and DHEA and DHEAS concentrations in the hypothalamus and the EC.

2. Materials and methods

2.1. Animals

Twenty-three male Wistar rats (15 controls and 8 in the juvenile stress group) weighing between 45 and 49 g (age 22 days) were purchased from Harlan (Jerusalem, Israel) and were given 4 days of acclimation. Rats were housed four per cage in 75.0 cm × 55.0 cm × 15.0 cm plexiglas cages in temperature-controlled (23 ± 1 °C) animal quarters on a 12-h light:12-h dark cycle (lights on 07:00–19:00 h). They had ad lib access to standard Purina Rat Chow pellets and water. All rats were weighed once a week.

All the behavioral tests were conducted between 11:00 and 17:00 h by the same experimenter.

2.2. Behavior

2.2.1. Elevated-platform stress

Following 4 days of habituation to the housing conditions, individual rats (age 26 days) in the juvenile stress group are placed for 30 min on an elevated black platform (12.0 cm × 12.0 cm) whose top lies 10.0 cm above the water surface. The platform is located in the middle of a water pool. Rats are subjected to this stress three times, separated by 1h in a resting cage, for 3 consecutive days [3].

2.2.2. Open field test

Four weeks after the exposure to juvenile stress (at the age of 8 weeks), all rats were tested in the open field according to methods described previously [8,30]. Briefly, the open field consists of a wooden box 90.0 cm × 90.0 cm × 38.0 cm, positioned in a dimly lighted room. The walls are painted black, the floor is white and divided by 1 cm wide black lines into 25 squares 17.0 cm × 17.0 cm. Rats are placed at the corner of the open field. For the following 3 min, the number of line crossings and the time spent in the central and the peripheral areas are manually recorded. The total line crossings represent the activity level of the rat. The ratio between line crossings in the peripheral area and the total line crossings, and the relative time spent, are considered a measure of anxiety [4].

2.2.3. The water-maze task

The water-maze [37] consists of a circular pool of water (1.7 m in diameter with a rim 0.5 m high), painted black. Water depth is 30.0 cm, and temperature is maintained at 23 ± 1 °C. The only means of escape from the maze is a hidden black platform (12.0 cm × 12.0 cm) whose top lies 3.0 cm beneath the water surface. The maze itself is featureless and the only obvious landmarks are those outside the water-maze, in the surrounding environment. Rats are placed in the maze and allowed to swim freely for a maximum of 1 min or until they reach the hidden platform. At the end of each trial, rats are either led to or left on the platform for 15 s. Each trial begins from a different starting point in a random order. The experimenter measures the escape latency with a stopwatch.

2.2.4. Massed spatial training

In a massed spatial learning task that was carried out 1 day after the open field test, rats were given a total of 12 trials with inter-trial intervals of 1 and 4 min, alternately [1,3].

2.2.5. The startle response test

The startle response [39] was measured using an automated JR. Startle box (Hamilton-Kinder, USA) that was positioned in a dimly lighted room. Immediately after exposure to the water-maze massed spatial learning task, rats are habituated for 30 min to the startle test room before placing them in the chamber. Rats are subjected to eight tones (40.0 ms 115 dB noise stimulus), separated by 1 min. The maximum startle response for each tone is measured in arbitrary units.

The average of the eight responses of each animal is taken as an index of the intensity of its startle reflex response.

2.3. Brain tissue preparations

Immediately after the startle response test, rats were decapitated. The hypothalamus and EC were dissected, rinsed of blood and immediately frozen on dry ice and stored at −80 °C until further use. Each specimen included two weighed hypothalami or EC's. Tissues were homogenized in 3 ml absolute ethanol with polytron (PCU, Lucerne, Switzerland) and stared for 24 h at −20 °C for protein precipitation and steroid extraction. Ethanol was separated by centrifugation (15,000 × *g*) for 30 min at 4 °C, evaporated till dryness, dissolved again in 0.6 ml absolute ethanol and stored at −20 °C till used. Recovery of ethanol extraction was determined using labeled radioisotopes and was found to be 88–91% for both DHEA and DHEAS (homogenization in buffer and extraction of DHEA using diethylether or hexane resulted in a lower recovery rate). The volume of brain extracts taken for evaluation was determined so it obtained a final concentration fitting the sensitivity and range of the RIA kit. All ethanol-extracted steroids were evaporated to dryness and dissolved in buffer steroid (DHEAS) or 0 ng/ml standard of the RIA kit (DHEA) containing the appropriate milieu needed for the assay, but with 0 ng/ml of the tested steroid. In addition to testing the recovery of ethanol extraction, recovery and linearity of dilution were tested using the different RIA assays. Recovery was tested by adding known quantities of steroids to the brain tissue extract and was found to be 84–110%. Linearity of dilution was tested using 0 ng/ml steroid standard and the observed values matched the expected ones by more than 88%.

2.4. Evaluation of steroids

2.4.1. DHEA

DHEA was determined using the DHEA-DSL 9000 Active TM DHEA coated tube radio immunoassay (RIA) kit (Diagnostic Systems Laboratories, Webster, TX, USA). The detection limit of the assay is 0.07 nmol/l (0.02 ng/ml); assay variability is 10.2% between runs and 5.6–10.6% within runs according to the level of DHEA in the sample; cross-reactivity with other steroids is <0.2%. 0.2 ml out of the 0.6 ml absolute ethanol extract of EC or hypothalamus (see Section 2.3) were evaporated to dryness and then dissolved in 120 μl standard 0 of the RIA kit. One hundred microliters were taken for determination.

2.4.2. DHEAS

DHEAS levels were measured using ICN ImmuChem TM Double Antibody RIA kit (Costa Mesa, CA, USA). The detection limit of the kit is 0.5 ng/ml; assay variability is 3.8–15% between runs, 8–9.4% within runs, cross reactivity with DHEA is about 58%, androstenedione 40%, androsterone 30% and all other <0.7%. This is the reason why free steroids were extracted and removed before assessment of DHEAS level. 0.15 ml out of the 0.6 ml absolute ethanol extractof EC or hypothalamus (see Section 2.3) were evaporated to dryness and reconstituted in 0.15 ml assay buffer. Free steroids were extracted using 1.5 ml of diethyl ether and removed from the sample. 0.10 ml was used for the RIA assay.

2.5. Statistical analysis

Results are presented as means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) for repeated measures, Student's *t*-test for independent samples and Pearson/Spearman correlations. All tests were two-tailed and a *P*-value of less than 0.05 was considered statistically significant. Partial η^2 computed in all measurements that are presented revealed non-significant effect of group size.

2.6. Approval

The experiments were approved by the institutional Animal Care and Use Committee, and adequate measures were taken to minimize pain or discomfort, in accordance with the guidelines laid down by the European Communities Council Directive and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3. Results

3.1. Effects of juvenile stress on behavior

All behavioral tests were conducted in early adulthood period, i.e., 4 weeks after the exposure to juvenile stress (Table 1).

The activity level was measured as the total line crossings in an open field test. A *t*-test revealed a significant decrease (∼30%) in activity of the juvenile stress group compared to the control group [$t(21) = 8.21$, $P < 0.0001$].

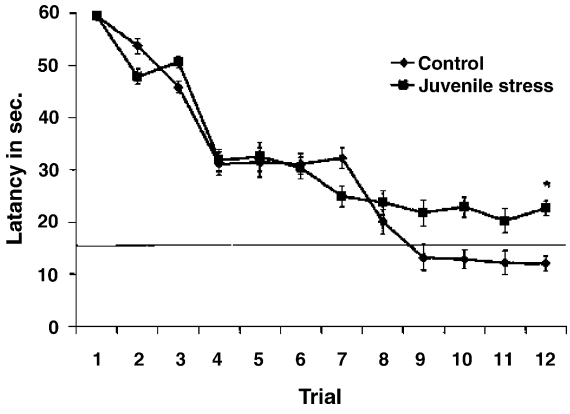

Fig. 1. Rats that were exposed to juvenile stress showed decremental performance in the acquisition of spatial learning task as they have reached to a longer latency asymptote of the learning curve ($^*P < 0.01$).

The ratio between the number of line crossings in the peripheral part of the open field and the total line crossings is considered a measure of anxiety.

A *t*-test revealed that exposure to juvenile stress significantly increased (∼18%) anxiety level compared to the control group [$t(10) = 7.18$, $P < 0.0001$]. The ratio between the time spent in the peripheral part of the open field and the total time in the maze also reflects anxiety levels. A *t*-test revealed that juvenile stress significantly increased (∼4%) anxiety level compared to the control group [$t(21) = 5.13$, $P < 0.0001$]. Twenty-four hours after testing anxiety level in the open field, both groups were trained for a spatial learning task in the Morris water-maze (Fig. 1).

During the last four trials, considered to most directly reflect spatial learning, the juvenile stress group performed poorly (longer latency) compared with control rats [$F(1,21) = 6.76$, $P < 0.017$].

Rats were then tested for their startle response (Table 1). A *t*-test revealed that exposure to juvenile stress significantly increased the startle response compared to the control group [$t(14) = 2.33$, $P < 0.035$].

3.2. Effects of juvenile stress on neurosteroids concentration

DHEA concentration in the hypothalamus and in the EC was not affected by exposure to juvenile stress compared to the con-

Table 1
Measured 1 month after the exposure to juvenile stress, rats that were exposed to juvenile stress showed a decrease in locomotor activity, as well as an increase in anxiety-like behaviors, compared with their counterpart controls

	Locomotor activity (total crossings)	Anxiety index (% of crossings)	Anxiety index (% of time)	Startle response (arbitrary units)
Control $n = 15$				
Average	111.46	63.71	88	723.46
S.E.M.	2.52	0.79	0.35	41.1
Juvenile stress $n = 8$				
Average	77.5	75.25	91.4	885.06
S.E.M.	3.56	1.79	0.54	61.71
Significant (*P*)	<0.0001	<0.0001	<0.0001	<0.035

Table 2
Whereas the exposure to juvenile stress did not affect DHEA levels in adulthood in the hypothalamus and the entorhinal cortex, it has increased the level of DHEAS by 34% in the hypothalamus and striking increase of 371% in the entorhinal cortex

	DHEA (pmol/g tissue)		DHEAS (pmol/g tissue)	
	Hypothalamus	Entorhinal cortex	Hypothalamus	Etorhinal cortex
Control $n = 15$				
Average	46.86	44.34	41.95	47.85
S.E.M.	3.88	3.8	2.59	3.13
Juvenile stress $n = 8$				
Average	32.94	35.35	56.09	225.1
S.E.M.	4.86	2.77	3.8	7.09
Significant (P)	n.s.	n.s.	<0.007	<0.0001

trols. However, exposure to juvenile stress induced a significant increase in the hypothalamic (∼34%; $t(13) = 3.19$, $P < 0.007$) and EC (∼371%; $t(18) = 28.26$, $P < 0.0001$) concentrations of DHEAS, as was measured in adulthood (Table 2).

3.3. Correlations between DHEA/DHEAS concentrations and behavior

DHEA concentration in the hypothalamus and in the EC showed no significant correlation with the behavioral measures (Table 3).

However, in the hypothalamus, DHEAS concentration showed a significant negative correlation with the level of activity ($r_s = -0.53$, $P < 0.04$), and a significant positive correlation with anxiety levels, based on the percentage of crossings the rat made in the peripheral part of the open field ($r_p = 0.71$, $P < 0.002$). In addition, DHEAS concentration in the hypothalamus showed a significant positive correlation with the escape latency on the 12th trial of the spatial learning task ($r_p = 0.64$, $P < 0.009$). Similar to the hypothalamus, DHEAS concentration in the EC showed a significant negative correlation with the level of total crossings ($r_s = -0.82$, $P < 0.0001$). Moreover, DHEAS concentration in the EC showed a significant positive correlation with the anxiety index based on the percentage of crossings ($r_p = 0.83$, $P < 0.0001$), as well as with anxiety index based on percentage of time ($r_p = 0.704$, $P < 0.001$). Similarity, DHEAS concentration showed a significant positive correlation with the startle reflex response ($r_p = 0.56$, $P < 0.029$). Finally, DHEAS concentration in the EC showed a significant positive correlation with escape latency on the 12th trial of the spatial learning task ($r_p = 0.74$, $P < 0.0001$).

Table 3
DHEA concentration in the hypothalamus and in the EC showed no significant corrrelation with the behavioral measures

	DHEA (pmol/g tissue)		DHEAS (pmol/g tissue)	
	Hypothalamus	Entorhinal cortex	Hypothalamus	Etorhinal cortex
Locomotor activity (total crossings)				
Correlation	0.32	−0.51	−0.53	−0.82
Significant (P)	n.s.	n.s.	<0.04	<0.0001
Anxiety index (% of crossings)				
Corelation	−0.42	0.31	0.71	0.83
Significant (P)	n.s.	n.s.	<0.002	<0.0001
Anxiety index (% of time)				
Correlation	−0.47	0.25	0.48	0.7
Significant (P)	n.s.	n.s.	n.s.	<0.0005
Startle response (arbitraty units)				
Correlation	0.03	0.44	0.39	0.56
Significant (P)	n.s.	n.s.	n.s.	<0.029
Spatial learning (12th trial latency)				
Correlation	−0.37	0.2	0.64	0.74
Significant (P)	n.s.	n.s.	<0.009	<0.0001

However, correlation between DHEAS levels in the hypothalamus and entorhinal cortex showed similar positive correlations with anxiety measures, as well as with the performance on the 12th trial in the spatial task.

4. Discussion

The results demonstrate that an exposure to juvenile stress has enduring effects on behavior and DHEAS but not on DHEAS brain concentration, as was observed 1-month post-juvenile stress.

The emotional consequences of an exposure to juvenile stress were examined both in the open field and the startle response tests. Rats that were exposed to juvenile stress showed in adulthood a decreased level of activity and their anxiety level were significantly increased compared to their control counterparts. Likewise, an exposure to juvenile stress increased the startle reflex response. In addition to the emotional tests, we have tested the effects of an exposure to juvenile stress on the performance of rats in a spatial learning task in the Morris water-maze. Rats that were exposed to juvenile stress performed poorly in the spatial task, compared with the controls. We also examined the long-lasting effects of exposure to juvenile stress on DHEA and DHEAS concentration in the hypothalamus and entorhinal cortex (EC). Measured 1-month post-juvenile stress, there were no significant differences in hypothalamic or EC DHEA levels between the juvenile stress and the control groups. In contrast, both hypothalamic and EC DHEAS levels were increased in the juvenile stress group and showed good correlations with the behavioral consequences. Supporting our findings, in previous study [43] a significant elevation was found both in plasma DHEA and DHEAS levels in human PTSD patients compared with control subjects.

DHEAS was shown to increase long-term potentiation (LTP) in the rat dentate gyrus (DG), in a dose-related manner [50]. Later, it was revealed that DHEAS could counteract the decremental effects of corticosterone on DG LTP when corticosterone is injected in doses that occupy type II glucocorticoids receptors (GR) [13,15,24].

These data led to the proposal [16] that DHEAS can counteract the putative depressogenic effects of GR hormones [13,14]. Support to this suggestion was found in studies on the effects of DHEA and DHEAS in rats, in which enhancement of cognitive functioning was attributed to modulation of hormones that affect the emotional state of the animals [19].

These findings are in accordance with the robust elevation in DHEAS that we found in the EC and the positive correlation of this increase with increased anxiety level. The EC is not often thought about in relation to stress responses and long-term consequences of stress. A role for the EC and its connections with the amygdala in coping with stress has been suggested by Henke and Ray [22] and was more recently demonstrated by Umegaki et al. [46]. They found a significant activation of c-fos following immobilization stress. Furthermore, lesion of the EC produced by ibotenic acid significantly attenuated the adrenocorticotropic hormone (ACTH) release evoked by immobilization. We have recently found that EC stimulation could induce LTP simultaneously in the dentate gyrus and the amygdala [49]. An exposure of rats to an elevated platform stress suppressed LTP in the dentate gyrus and enhanced it in the amygdala, suggesting that the EC plays an important role in mediating responses to stress [47].

Kimonides et al. [26] have found that DHEAS can prevent or reduce the neurotoxic actions of the glutamate agonist NMD A acid in the hippocampus both in vivo and in vitro, as well as that of two other glutamate receptor agonists, AMPA and kainic acid, in vitro. An increase in DHEAS level may thus have a protective action aimed to reduce the damage induced by the exposure to juvenile stress. Interestingly, and related to the apparent significant role of the EC in this respect, Sunanda et al. [44] found that lesioning the EC could protect hippocampal CA3 neurons from a 21-day restraint stress-induced damage. These results suggest that the neuronal vulnerability in CA3 to chronic stress is modulated at least in part by the EC projection into the hippocampal formation.

In summary, these results indicate that an exposure to a relatively brief stressful experience during early life has profound and long-lasting effects on both behavior and neurosteroid concentration in the hypothalamus and EC. Clearly, alterations in the concentration of DHEAS are not likely to be the sole basis of the behavioral alterations found 4 weeks after the exposure to the juevnile stress. Nevertheless, it is possible that these changes in DHEAS concentration in the hypothalamus and particularly in the EC may be indicative of a potential target of pharmacological intervention in stress related disorders.

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