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Stress Through Handling for Vaginal Screening, Serotonin, and ACTH Response to Ether

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SFIKAKIS, A., P. GALANOPOULOU, M. KONSTANDI AND D. TSAKAYANNIS. *Stress through handling for vaginal screening, serotonin, and ACTH response to ether.* PHARMACOL BIOCHEM BEHAV **53**(4) 965-970, 1996.—The effect of duration of handling for vaginal smear screening on the adrenal weight and acute ACTH response to ether were examined in 4-day-cycling female rats, sacrificed at 97-103 days of age on diestrus-2 after evaluation of resistance to handling, thymus weight, and hypothalamic serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). Prolonged handling paralleled increased resistance (behavioral response) to handling and adrenal weight but was inversely related to thymus weight. The hypothalamic 5-HT, 5-HIAA, and 5-HIAA/5-HT ratio, compared to controls with similar conditions of handling, were not modified after 2.5 min of ether despite the ACTH rise. In ether-stressed rats, the ACTH response to ether was lower after prolonged handling compared to short handling paralleling decreased thymus weight. In contrast, 5-HT, 5-HIAA, and the 5-HIAA/5-HT ratio were higher, paralleling increased resistance and adrenal weight. The results suggest chronic activation of the hypothalamo-pituitary-adrenal axis with positive serotonergic involvement after prolonged handling and resistance during vaginal screening and a negative implication of this activation on the acute ACTH response to ether.

Handling for vaginal smear screening Duration Resistance Adrenal weight ACTH Ether stress Diestrus-2 Hypothalamic 5-HT 5-HIAA

AS FAR AS WE KNOW, there is no information concerning the stress-inducing capacity of daily handling for vaginal smear screening (VSS), despite the importance of this screening in studies using female rats as experimental model. On the other hand, it has been reported that transfering rats from their home cage in a jar for 3 min results in a continuous rise of ACTH for 15 min, but at any time, ACTH concentrations are greater in females than in males (25). Furthermore, female rats have a higher adrenal weight than males (28), fail to adapt to a repeated stress procedure unlike male rats (24), and show a different corticosterone response to stress (19,25). In a recent study from our laboratory (30), increased adrenal weight and decreased thymus weight were found after prolonged handling for VSS. These findings on diestrus-2 paralleled an increase in hypothalamic 5-hydroxyindoleacetic acid (5-HIAA) and in the 5-HIAA/serotonin ratio.

In the present study, the individual reaction to handling for VSS was recorded at each handling and a resistance score was evaluated to examine the relationship of this resistance to the adrenal weight and duration of handling. We considered the weight of the adrenals to be a useful index of chronic activation of the hypothalamo-pituitary-adrenal (HPA) axis, because in the early bioassay for ACTH it has been used as an integrated measure of circulating ACTH (32), and because an increase in adrenal weight has been found in male and female rats after chronic stress (2,4) and after chronic administration of ACTH (1). Another purpose of the present study was to examine the effect of 2.5 min of ether stress on the hypothalamic serotonergic system compared with control rats after similar conditions of handling for VSS. On the other hand, we were interested to see whether differences in duration of handling for VSS in ether stressed rats might affect the acute

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ACTH response to ether and the serotonergic system, and to examine the relationship of the serotonergic system to adrenal weight, resistance to handling, and thymus weight. The weight of thymus was used as an approximate index of the amount of experienced stress after chronic handling for VSS because of the thymolytic effect of glucocorticoids (11,22). To limit the interference of age-dependent thymic involution (unpublished observations), the evaluation of thymus weight was done only in rats restricted in age at the time of sacrifice.

The results of the present study refer to a distinct stage of the estrous cycle, namely, diestrus-2, because differences in the hormonal milieu inevitable during the estrous cycle (9,31) might interfere with the pituitary adrenocortical system (7) and influence central 5-HT binding sites (6,36) as well as the concentrations of 5-HT and 5-HIAA (35) and the adrenal weight (30). Diestrus-2 was chosen because of the advantage of being separated by 3 days from the high estradiol levels of proestrus, and hence from its genomic effect (26).

METHODS

Animals: General Procedure

Virgin female rats of the Wistar strain, born and raised in our laboratory, living under an automatically controlled light cycle (lights on from 0700–2100 h, 21–23°C), were studied at 3–4 mo of age. The stage of the estrous cycle was determined by vaginal smears, which were obtained between 0830 and 1000 h. Rat chow and drinking fluid were available ad lib. The handling for VSS consisted of grasping the rat gently but firmly by the skin of the neck and back to be immobilized in the upright position. During this time, a cotton swab (sterilized before use in aluminum foil for 1 h at 160°C) humidified in drops of saline was inserted with caution in the vagina to obtain vaginal cytology by hemicircular movement. Vaginal smears were placed in absolute methyl alcohol for 5 s. After being dried, they were coloured by Giemsa solution 1 : 20 for 30 min and examined under microscope (31). All handled rats were autopsied at the end of each experiment on diestrus-2. After being removed and trimmed from surrounding fat and connective tissue on a filter paper saturated with saline, the two adrenals, uterus and thymus, were weighed to the nearest 0.01 mg on an electric balance.

Experimental Design

At each handling for VSS, the individual's reaction to this handling was recorded. The resistance score to handling for VSS was expressed mainly by three behaviors (unpublished observation): 1) uneasiness or restlessness during touching of the vagina, which imposed repeated grasping for immobilization; 2) vocal reaction; 3) attempts to hide during the removal of roommates from the cage, or attempts to escape from the cage or box during the waiting time.

In 12 rats 3–4 mo of age, handling for VSS started without habituation to simple handling for weighing. In these rats, after variable duration of handling for VSS, the resistance score was evaluated in relation to the adrenal weight.

In another group, habituation to simple handling for weight recording preceeded handling for VSS. Only 4-day cycling rats from this group were used. In nine of these rats, the duration of handling for VSS extended from 7–10 days, and in nine other rats, from 12–18 days. In these numbers, the final handling for vaginal sampling and weight recording on the day of sacrifice is not included. In both groups, the length of time for evaluating body weight gain was kept equal. The age of the 18 rats at trunk blood collection by decapitation, for the measurement of ACTH, extended from 97–103 days. A total of 12 rats were decapitated after 2.5 min of ether inhalation in a ventilated Plexiglas chamber between 0930 and 1130 in parallel with six rats which were sacrificed without ether about 10 min after handling for VSS and weighing. The duration of handling was similar in control and ether-stressed rats, as half of the rats in each group had prolonged duration of handling. Care was taken that rats with resistance be equally distributed in the control and ether-stressed groups. In 10 rats of comparable age not handled for VSS, sacrificed immediately after removal from their home cage, trunk blood was collected and pooled plasma used for the recovery control of ACTH, with the radioimmunoassay (RIA) used. These 10 rats were habituated to handling for simple weighing. In six rats that had at autopsy vaginal cytology and uterine weight excluding proestrus or estrus, ACTH by RIA was measured in individual blood samples to compare with controls handled for VSS.

In ether-stressed rats and the six controls, after the rats were decapitated, their brains were rapidly removed and the hypothalamus preoptic area immediately dissected from the base of the brain. The concentrations of 5-HT and 5-HIAA were determined fluorophotometrically following acid-butanol extraction according to the method of Curzon and Green (12), and the 5-HIAA/5-HT ratio (as an indirect index of 5-HT turnover) was calculated.

Plasma ACTH Determination

Blood was collected in plastic tubes with EDTA (1 mg/ml) kept on ice and centrifuged as soon as possible at 2500 rpm in a refrigerated centrifuge. Separated plasma was kept in a polystyrene tube at −20°C until assayed. After thawing the plasma before assay, the samples were centrifuged to eliminate fibrin. Plasma ACTH was measured by direct RIA with a kit provided by CIS Sorin. The antiserum was obtained in rabbits immunized with porcine ACTH coupled with bovine serum albumin. The ACTH hormone used for labelling, [^{125}I]-labelled ACTH, was from porcine origin. Human ACTH 1–39 served as standard. The percentage of cross-reaction of the antiserum was 100% for ACTH 1–39 human, 100% for β 1–

TABLE 1

EVALUATION OF RESISTANCE SCORE TO CHRONIC HANDLING FOR VAGINAL SMEAR SCREENING IN RELATION TO ADRENAL WEIGHT

	Low Adrenal Weight Range	High Adrenal Weight Range
Range (mg/100 g BW)	22.2–28.8	30.0–34.7
Adrenal weight (mg/100 g BW)	26.56 ± 1.00	31.58 ± 0.74
Adrenal weight (mg)	42.7 ± 1.45	55.26 ± 2.60*
Uneasiness or restlessness during handling‡	3.16 ± 0.87	6.66 ± 0.84†
Vocal reaction‡	2.83 ± 0.70	6.83 ± 0.70*
Attempts to escape‡	0.66 ± 0.42	3.16 ± 0.70†
Days of handling	8.16 ± 0.79	12.66 ± 2.15

Results are mean ± SEM (n = 6).
*$p < 0.005$.
†$p < 0.01$.
‡The results show the number of times corresponding behavior was expressed by rats during handling.

24 (synachtene), 3% for fragment 1-16, 1.5% for fragment 11-24, and 1% for β-endorphin. Cross-reactivity was negligible for β-LPH, α-melanocyte-stimulating hormone (MSH), β-MSH, fragment 1-10, and fragment 25-39.

Plasma samples for controls and stressed rats were always determined in duplicate or triplicate. The assay always included determinations in triplicate of the total activity, the nonspecific binding ability of the zero standard, the binding ability of the zero standard, and the five points of the standard curve at 24, 75, 190, 450, and 920 pg. Only the blank determinations of each plasma sample were not done in triplicate.

The within-assay coefficient of variation for two serum pools, with different ACTH concentrations and nine determinations for each pool, were 6.5% for 460 pg/ml and 6.2% for 720 pg/ml. The addition of 920 pg ACTH standard to pooled plasma gave recovery values of 99, 78.5, and 98.7% for serial dilutions of 1 : 1, 1 : 2, and 1 : 4, respectively. The interassay coefficient of variation was 12%. The minimal detectable dose was 10 pg/ml ACTH.

Statistical differences were evaluated by Student's unpaired t-test. $p > 0.05$ was considered significant. Logarithmic conversion of the data for statistical evaluation was done whenever needed.

RESULTS

Table 1 gives the mean score of each behavior expressing resistance to handling, in relation to the adrenal weight. Rats were separated into two groups of equal number according to their relative adrenal weight, and the mean score of resistance and days of handling were evaluated correspondingly. The three particular behaviors were significantly higher in rats belonging to the high adrenal weight range, whereas the duration of handling was longer, but the difference did not reach significance (Table 1).

Table 2 demonstrates the immediate effect of 2.5 min ether stress on plasma ACTH, hypothalamic 5-HT, 5-HIAA, and other studied parameters, compared to control rats with similar conditions of handling. The only significant difference was for ACTH plasma levels, which increased about six times compared to controls.

TABLE 2

HYPOTHALAMIC-PREOPTIC AREA CONCENTRATIONS OF 5-HT, 5-HIAA, AND 5-HIAA/5-HT RATIO AND THE ACTH RESPONSE TO 2.5 min ETHER-STRESS COMPARED TO RATS WITH SIMILAR CONDITIONS OF HANDLING

	Controls (6)	Ether-Stressed (12)
Plasma ACTH pg/ml	97.8 ± 12.84	604.1 ± 46.24*
Adrenals mg/100 g BW	28.9 ± 2.24	26.9 ± 1.36
Thymus mg/100 g BW	163.1 ± 9.52	154.8 ± 7.08
Uterus mg/100 g BW	150.0 ± 11.41	162.9 ± 7.97
5-HT μg/g tissue	1.32 ± 0.146	1.42 ± 0.135
5-HIAA μg/g tissue	0.827 ± 0.083	1.09 ± 0.136
5-HIAA/5-HT	0.64 ± 0.044	0.746 ± 0.031
Duration of handling	12.16 ± 0.47	12.58 ± 0.75
Restlessness	3.66 ± 1.28	3.0 ± 0.71
Vocal reaction	2.16 ± 0.47	2.58 ± 0.75
Attempts to escape	1.66 ± 0.55	1.33 ± 0.33
Body weight (g)	199.4 ± 6.53	190.6 ± 4.19
Body weight gain	19.7 ± 2.37	18.4 ± 2.07

Results are mean ± SEM. Number of rats is shown in parentheses.

*Significant difference from controls, $p < 0.005$ (after logarithmic conversion of value).

TABLE 3

EFFECT OF DURATION OF HANDLING FOR VSS ON BEHAVIORAL PARAMETERS, ORGAN WEIGHTS, AND BODY WEIGHT GAIN

Duration of Handling (days)	7-10 days (8.6 ± 0.33)	12-18 days (16.2 ± 0.82)
Uneasiness restlessness	1.44 ± 0.24	4.88 ± 0.96*
Vocal reaction	1.44 ± 0.47	3.44 ± 0.81†
Attempts to escape	0.55 ± 0.29	2.33 ± 0.23‡
Body weight (g)	190.0 ± 5.32	196.9 ± 4.80
Body weight gain for 16-18 days (g)	23.8 ± 1.60	14.0 ± 1.35‡
Adrenal weight (mg)	45.3 ± 1.71	59.9 ± 2.66‡
Adrenal weight (mg/100 g BW)	23.8 ± 0.85	30.8 ± 1.32‡
Thymus weight (mg)	330.9 ± 16.34	266.9 ± 16.30§
Thymus weight (mg/100 g BW)	173.3 ± 4.61	135.7 ± 8.10‖
Uterine weight (mg/100 g BW)	163.5 ± 10.35	153.8 ± 8.18

Mean ± SEM, $n = 9$. Each group was composed of three controls and six rats sacrificed after 2.5 min ether inhalation.

*$p < 0.001$ after logarithmic conversion; †$p < 0.05$; ‡$p < 0.001$; §$p < 0.02$; ‖$p < 0.005$.

The ACTH plasma levels in the six handled controls (Table 2) were higher ($p < 0.01$) compared to the ACTH levels (47.0 ± 8.52 pg/ml) found in six rats not handled for VSS and sacrificed within 20 s of being taken from their home cage. The effects of the duration of handling for VSS in control and ether-stressed rats are given in Table 3. The results revealed an increase in behavioral parameters expressing resistance after prolonged handling. This increase paralleled the increase in adrenal weight and the decrease in body weight gain and thymus weight. There was no difference in uterine weight.

These results are regrouped according to low and high relative adrenal weights in Table 4. There were significant differences in all parameters studied, except for body and uterine weight. The duration of handling and resistance score increased in the high adrenal weight range compared with body weight gain and thymus weight, which decreased (Table 4).

The acute ACTH response to 2.5 min ether inhalation, the corresponding hypothalamic serotonergic system, and other parameters studied were evaluated in relation to the duration of handling for VSS (Table 5). This subdivision of the rats was identical to one according to their adrenal weights (short duration of handling, adrenal weight range 20.5-26.6 mg/100 g body wt.; long duration of handling, adrenal weight range 27.2-36.3 mg/100 g body wt.). Thus, there was a positive correlation between handling and adrenal weight, and for both of them, between behavioral parameters expressing resistance to handling and concentrations of 5-HT and 5-HIAA and their ratio. In contrast, prolonged handling and higher adrenal weights were inversely related to acute ACTH response to ether, thymus weight, and body weight gain.

DISCUSSION

The results of our study demonstrate that handling for VSS represents a mild stress, as an increase in ACTH was found a few minutes after this handling. On the other hand, they demonstrate that resistance to handling for VSS, as expressed

TABLE 4

EVALUATION OF BODY WEIGHT GAIN, DURATION OF HANDLING, BEHAVIORAL PARAMETERS, AND ORGAN WEIGHTS IN CONTROL AND ETHER-STRESSED RATS IN RELATION TO ADRENAL WEIGHT

	Low Adrenal Weight	High Adrenal Weight
Range	20.56–26.62	27.2–37.79
Adrenal weight (mg/100 g BW)	23.6 ± 0.76	30.98 ± 1.25
Body weight (g)	193.5 ± 5.93	193.6 ± 4.38
Body weight gain (g)	23.2 ± 1.75	14.59 ± 1.61*
Duration of handling days	8.88 ± 0.48	16.0 ± 0.98†
Uneasiness or restlessness	1.55 ± 0.17	4.88 ± 0.96‡
Vocal reaction	1.33 ± 0.44	3.55 ± 0.80§
Attempts to escape	0.55 ± 0.29	2.33 ± 0.23‡
Thymus weight (mg/100 g BW)	170.59 ± 5.15	144.5 ± 9.15‖
Uterine weight (mg/100 g BW)	160.2 ± 11.26	157.0 ± 7.23

Results are mean ± SEM (n = 9). Each group included three control and six ether-stressed rats.

*$p < 0.005$.
†$p < 0.0005$.
‡$p < 0.001$.
§$p < 0.05$.
‖$p < 0.025$.

by three particular behaviors, is closely related to the activation of the HPA axis, as shown by the presence of a significant increase of these behaviors in the high adrenal weight range. This was also demonstrated by the increase of adrenal weight and decrease of thymus weight in rats after prolonged handling for VSS, paralleled by increased resistance to handling. The overall results favour the assumption that the combination of prolonged handling and resistance, and not the increased duration of handling per se, is the decisive factor for activation of the HPA axis. We cannot exclude the possibility that rats with resistance to handling for VSS were oversensitive to environmental stimuli and that they had already increased adrenal weight and decreased thymus weight at the beginning of the experiment, with the differences accentuated by the imposed stress of handling for VSS. The main conclusion is that female rats with resistance to handling for VSS should either be excluded from experimental studies related to stress and serotonin or be equally distributed between groups with similar durations of handling.

Evaluation of the hypothalamic serotonergic system after 2.5 min ether inhalation, compared to control rats after similar conditions of chronic handling for VSS, revealed no difference in the serotonergic system despite a sixfold increase in ACTH. These results do not favour a role for 5-HT in the stimulus-induced release of ACTH after ether, in agreement with previous studies in male rats (18,21,33). Separation of ether stressed rats into two groups according to the duration of handling revealed that prolonged handling was paralleled by increased resistance, increased adrenal weight, and decreased thymus weight. These results are consistent with previous findings showing that female rats fail to adapt to a repeated stress procedure (24). On the other hand, prolonged handling with resistance in ether-stressed rats was paralleled by increased adrenal weight and increased 5-HT, 5-HIAA, and 5-HIAA/5-HT ratio, but decreased thymus weight and body weight gain. The results concerning the increase in the concentration of 5-HIAA and 5-HIAA/5-HT ratio, the increase in adrenal, and the decrease in thymus weight were similar to those found after prolonged handling in rats sacrificed without ether anaesthesia in a previous study (30). The present findings, considered together with our previous results (30), suggest that chronic stress due to prolonged handling for VSS activates the hypothalamic serotonergic system in parallel with the HPA axis. In view of studies in vitro (8,16,27) and in vivo [for review, see (14)], and recent pharmacologic studies (3,10), all supporting activation of the HPA axis by 5-HT, it is tempting to suggest that the chronic activation of the HPA axis after prolonged handling for VSS and increased resistance may be causally related to the chronic activation of the hypothalamic serotonergic system.

Because the subdivision of rats according to their duration of handling for VSS or their adrenal weights was the same, these two parameters therefore had the same inverse relationship with the ACTH response to acute ether exposure. Longer duration of handling and higher adrenal weight also correlated with raised levels of 5-HT, 5-HIAA, and the 5-HIAA/5HT ratio, with reduced thymus weight and reduced body weight gain.

These findings of lower ACTH response to ether stress in the presence of a greater amount of experienced chronic stress suggest the possibility of a downregulation of corticotropin-releasing hormone (CRH) receptors (15), because of a repeated chronic release of CRH as a result of the chronic stress of prolonged handling and resistance. The inverse relationship between 5-HT, 5-HIAA, the 5-HIAA/5-HT ratio, and the acute ACTH response to ether found in the present study is in line with early pharmacologic studies in rats (5,34).

In view of the thymolytic effect of corticosteroids (11,22), our findings of a greater thymic involution in the high adrenal weight range, in parallel with decreased ACTH response to ether, compared to the low adrenal range, suggest that negative corticosteroid feedback (13,23) derived from the enlarged adrenals might be another reason for the diminished ACTH response to stress. The results of a recent study suggest that the nocturnal circadian rise of corticosterone might provide

TABLE 5

ACUTE ACTH RESPONSE TO ETHER AND HYPOTHALAMIC SEROTONERGIC SYSTEM ON DIESTRUS-2 IN RELATION TO DURATION OF HANDLING FOR VAGINAL SMEAR SCREENING

Duration of Handling (days)	7–10 days (8.5 ± 0.42)	12–18 days (16.6 ± 0.95)
Uneasiness and restlessness	1.5 ± 0.22	4.5 ± 1.14*
Vocal reaction	1.33 ± 0.61	3.8 ± 1.22†
Attempts to escape	0.55 ± 0.29	2.16 ± 0.307*
Plasma ACTH (pg/ml)	705.0 ± 42.7	503.3 ± 59.2‡
5-HT (μg/g tissue)	1.078 ± 0.0874	1.772 ± 0.1541*
5-HIAA (μg/g tissue)	0.741 ± 0.0672	1.450 ± 0.1614*
5-HIAA/5-HT ratio	0.686 ± 0.0347	0.806 ± 0.0395‡
Adrenals (mg)	42.6 ± 1.67	60.1 ± 2.61§
Adrenals (mg/100 g BW)	23.2 ± 1.07	30.5 ± 1.36§
Thymus (mg)	310.9 ± 16.6	259.9 ± 20.27†
Thymus (mg/100 g BW)	168.7 ± 5.43	140.8 ± 10.60‡
Body weight (g)	183.7 ± 5.23	197.4 ± 5.59
Body weight gain (g)	23.8 ± 1.89	13.0 ± 1.92*

Mean ± SEM, n = 6.
*$p < 0.005$; †$p < 0.05$; ‡$p < 0.025$; §$p < 0.001$.

delayed negative feedback (17), whereas attempts to demonstrate that endogenous corticosterone secreted after a first stress inhibits the response of ACTH to subsequent stress have proven unsuccessful (13,20,23,25).

Corticosterone, which rises to stress levels at the circadian maximum (13), might correlate at this time with adrenal weight, according to previous findings showing that at the end of 30 min of avoidable foot-shocks, corticosterone levels in female rats did not correlate with the amount of experienced stress but with the adrenal weight (29). The possibility that differences in delayed feedback as a result of differences in the circadian rise of corticosterone might be expressed by differences in the adrenal weight after chronic stress should be further investigated.

In conclusion, the overall results of the present study suggest a positive serotonergic involvement contributing to the activation of the HPA axis, after the psychogenic stress of prolonged handling for VSS combined with resistance to this handling. They suggest also a negative involvement of this activated HPA system on the acute ACTH response to ether stress, with possible participation of the hypothalamic serotonergic system.

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