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Long-Lasting Effect of Early Handling on the Peripheral Benzodiazepine Receptor

RONIT WEIZMAN,* JULIA LEHMANN,† SVETLANA LESCHINER,‡ ISABELLE ALLMANN,† THOMAS STOEHR,† CHRISTIAN HEIDBREDER,† ANNETTE DOMENEY,† JORAM FELDON† AND MOSHE GAVISH‡§

*Tel Aviv Community Mental Health Center and Sackler Faculty of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel;
†Laboratory of Behavioural Biology, The Swiss Federal Institute of Technology, CH-8603 Schwerzenbach, Switzerland;
‡Department of Pharmacology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, 31096 Haifa, Israel; and
§Rappaport Family Institute for Research in the Medical Sciences, 31096 Haifa, Israel

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WEIZMAN, R., J. LEHMANN, S. LESCHINER, I. ALLMANN, T. STOEHR, C. HEIDBREDER, A. DOMENE Y,J. FELDON AND M. GAVISH. *Effect of early handling on the peripheral benzodiazepine receptor.* PHARMACOL BIOCHEM BEHAV **64**(4) 725–729, 1999.—The present study determined the impact of early handling (EH) in rats on behavioral response to environmental stress and on peripheral benzodiazepine receptor (PBR) binding characteristics (B_{max} and K_d) in various organs. The behavioral consequences of EH in rats were expressed as increased exploratory activity in an open-field paradigm, when compared with nonhandled control rats. These findings are interpreted in terms of decreased emotionality. The biochemical consequences of EH, in both male and female rats, were expressed as the upregulation of PBR in the adrenal and kidney and the downregulation of gonadal (testis and ovary) PBR. It is possible that the long-lasting adrenal and renal changes in PBR expression in EH rats may enable better regulation of the hypothalamic–pituitary–adrenal axis, renin–angiotensin system, and autonomic nervous system responses to stress in adulthood. The significance of the EH-induced reduction in gonadal PBR for gonadal activity in adulthood is as yet unclear. © 1999 Elsevier Science Inc.

Early handling Locomotor activity Peripheral-type benzodiazepine receptor Steroidogenesis Stress Rats

PERIPHERAL benzodiazepine receptors (PBR) are found in both central nervous system and peripheral organs, and are labeled by the radioligands [^{3}H]PK 11195 (an isoquinoline carboxamide derivative) and [^{3}H]Ro 5-4864 (4-chlorodiazepam). PBR are especially abundant in steroidogenic tissues like adrenals, ovaries, testes, and glial cells (10). In contrast to the central benzodiazepine receptor (CBR), which is localized on the postsynaptic neuronal membrane, PBR are located mainly on the outer mitochondrial membrane (7). PBR density in the endocrine organs is altered by changes in their corresponding trophic hormones (6,14,30). This receptor seems to play a role in steroidogenesis by regulating cholesterol translocation from the outer to the inner mitochondrial membrane, the rate-limiting step in steroid biosynthesis (26).

Early postnatal environmental manipulations in animals have been reported to have profound and long-lasting effects on both neurodevelopment and behavior (1). When mature, early-handled (EH) rats are reported to exhibit a number of characteristic behavioral features. For example, EH rats explore more and defecate less than nonhandled (NH) rats in an open field (22), show less reactivity in a reaction-to-handling test (1), and are also faster and more accurate performers in avoidance-learning paradigms (19). On the neuroendocrine level, EH rats show attenuated activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to stressful stimuli (3,22,25). This finding has led to the suggestion that EH in the rat leads to an enhanced ability to cope with stress (17,20). PBR are sensitive to stress, as has been demonstrated

Requests for reprints should be addressed to Moshe Gavish, Department of Pharmacology, The Bruce Rappaport Faculty of Medicine, Technion-Institute of Technology, P.O.B. 9649, 31096 Haifa, Israel.

in several animal models and in human studies. Although PBR are known to be sensitive to the immediate acute and chronic effects of stress, a phenomenon that may be related to modulations in HPA axis activity, little is known about the long-term effects of manipulations that lead to long-lasting behavioral and endocrinological effects. Further, the modulatory effect of stress on PBR expression is in an organ-specific fashion and bidirectional, i.e., upregulation after exposure to acute stress and downregulation following chronic stress (31). It is of note that the precise function of PBR is as yet unclear, and hence, the putative link with stress or stress-related disorders is very intriguing at present (11).

Because a shift in PBR response may subserve the pathophysiological demands associated with stress or stress-related disorders, the aims of the present study were to evaluate the impact of early postnatal (first 21 days of life) handling, a repeated stress paradigm in infancy associated in adulthood with decreased stress reactivity, on the expression of PBR in steroidogenic (gonad and adrenal) and nonsteroidogenic (kidney) organs in adult (6-month-old) male and female rats. However, it should be noted that the present work was not designed in the general context of "acute vs. chronic stress," but rather in the context of early developmental manipulations and their long-term biochemical and behavioral effects.

METHOD

Animals

Behavioral and biochemical experiments were carried out on two separate groups of adult male and female Wistar rats (Institute of Toxicology, Zürich, Switzerland) that were individually housed in a climatically controlled room (temperature 21 ± 1°C and humidity 55 ± 5%) under a reversed 12L:12D cycle, with the light phase commencing at 1900 h. Food (Nafag-9431, Eberle Nafag AG, Gossau, Switzerland) and water were available ad lib. All experiments were carried out in agreement with the Swiss Federal Legislation for Animal Experimentation.

Two experimental conditions were generated—early handling (EH), and nonhandling (NH) of animals.

Early Experience Procedures

The two experimental groups were bred in the same animal facility. All litters were born within a 4-day period. No culling of litters was carried out, to avoid a handling effect by the culling itself (8). The NH rats were left completely undisturbed with the mothers until weaning (day 21). To carry out the EH procedure on each day between birth and weaning, the mother was removed from her litter to a holding cage and the pups were placed individually into small plastic cups with wood shavings as bedding for 15 min. At the end of the 15 min the mother and the pups were returned to the maternity cage. Approximately 1 week after weaning, all rats were placed in grid-floor cages (macrolon, 48 × 27 × 20 cm), five animals per cage (same sex and condition, but from different litters), and were left undisturbed until adulthood.

Behavioral StudOpen Field

Apparatus. Locomotor activity was measured in four open-field (76.5 × 76.5 × 49 cm) arenas made of dark gray plastic. Behavior in the arenas was recorded by a video camera mounted on the ceiling and relayed to a monitor and a video tracking, motion-analysis, and behavior-recognition system (Etho Vision®, Noldus, Wageningen, The Netherlands). The test room was dimly illuminated with indirect lighting (three 60-W bulbs).

Animals and procedure. The experiment was performed on 20 male and 20 female 3-month-old Wistar rats (10 EH and 10 NH rats in each group) without any pretest handling in adulthood. Within the same experimental condition, no littermates were used. Rats were run in squads of four, and all animals were habituated to the testing room for 20 min before the start of each session. Spontaneous locomotion and habituation were assessed in five consecutive daily sessions. Animals were tested at the same time each day, and each rat was placed in the center of one of four arenas and allowed to explore it for 20 min.

Behavioral data collection and analysis. The computer software (Etho Vision) calculated the distance a rat traveled while in the arena, the number of entries into the center of the arena, and the time the animal spent in the center. All data were analyzed by a 2 × 2 × 5 analysis of variance (ANOVA), with the main factors gender (male, female), treatment (EH, NH), and a repeated-measures factor of days (1 to 5).

Biochemical Studies: Animals and Preparation of Membranes

Rats (20 male and 20 female) belonging to the same cohort (but to different litters within the cohort) as those which participated in the open-field study were sacrificed at the age of 6 months. Adrenals, ovaries, testes, and kidneys were removed and stored at −70°C until assayed for receptor binding.

The various organs were homogenized separately in 50 vol of 50 mM potassium phosphate, pH 7.4, using a Brinkmann Polytron (setting 10) for 15 s. The homogenates were centrifuged at 49,000 × *g* for 15 min. The pellets were resuspended in 50 vol of 50 mM ice-cold potassium phosphate buffer, pH 7.4. The homogenates were diluted 50- to 100-fold in the buffer and used for binding assays.

[^{3}H]PK 11195-Binding Assay

[^{3}H]PK 11195 binding was conducted as previously described (14). The binding assay in a final volume of 500 μl contained 400-μl membranes of organ (100–200 μg of protein) and 25 μl of [^{3}H]PK 11195 (final concentration 0.2–6 nM) in the absence (total binding) or presence (nonspecific binding) of 1 μM unlabeled PK 11195. After incubation for 60 min at 4°C, samples were filtered under vacuum over Whatman GF/C filters and washed three times with 3 ml of potassium phosphate buffer. Filters were placed in vials containing 4 ml Opti-Fluor (Packard, Groningen, The Netherlands) and counted for radioactivity after 12 h. The binding parameters [maximal binding capacity (B_{max}) and dissociation constant (K_d)] were analyzed for each animal individually, using Scatchard analysis of saturation curves of [^{3}H]PK 11195 binding to the membranes of the examined tissues.

Statistical Analysis

A 2 × 2 ANOVA with the main factors of gender (male, female) and treatment (EH, NH) was used for intergroup comparisons of B_{max} and K_d values of adrenal and kidney. Data of testis and ovary were analyzed separately for males and females by a one-way ANOVA with the main factor of treatment. All results are expressed as mean ± SEM.

RESULTS

Spontaneous Locomotor Activity in the Open Field

Total distance traveled. The 2 × 2 × 5 ANOVA yielded a significant effect of the repeated-measures factor of days, $F(4, 144) = 3.81$, $p < 0.01$, reflecting differences in locomotor activity over the 5 days of testing. A significant main effect of gender was also obtained, $F(1, 36) = 6.57$, $p < 0.05$ (see Fig. 1), indicating higher activity of females compared with males. EH animals demonstrated increased locomotor activity when compared with NH animals, as indicated by the significant main effect of treatment, $F(1, 36) = 5.35$, $p < 0.05$ (see Fig. 2). All other interactions did not reach significance.

Time in center. The 2 × 2 × 5 ANOVA revealed a significant effect of the repeated-measures factor of days, $F(4, 144) = 7.58$, $p < 0.0001$, indicating an increase in time spent in the center due to habituation over the 5 days of experiment. EH rats spent significantly more time in the center than NH rats, $F(1, 36) = 8.02$, $p < 0.01$ (see Fig. 3). All other interactions did not achieve significance.

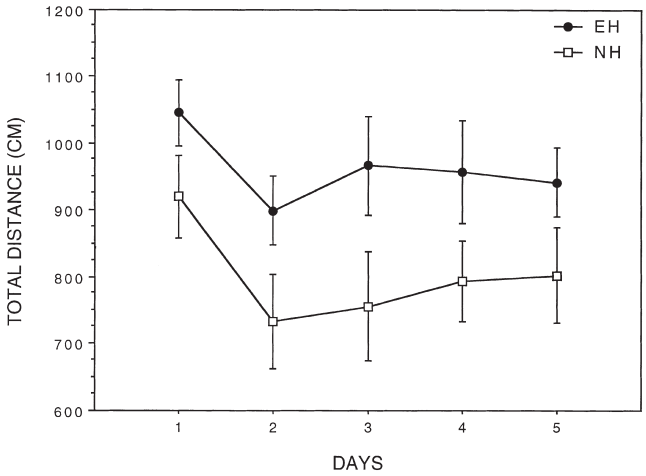

FIG. 2. Total distance traveled in the open-field arena over 5 days of testing for EH and NH rats. Results are means ± SEM. Effect of treatment: $p < 0.05$.

PBR Binding

Kidney. The ANOVA revealed a significant main effect of gender, demonstrating higher PBR binding in females vs. males, $F(1, 36) = 34.79$, $p < 0.0001$, and a significant main effect of treatment, $F(1, 36) = 45.97$, $p < 0.0001$, reflecting an elevation of PBR binding in EH vs. NH subjects. The significant interaction of gender × treatment, $F(1, 36) = 12.39$, $p < 0.002$, indicates that the difference between EH and NH animals was more pronounced in males than in females, due to an increased gender difference in NH rats (Table 1).

Adrenal. The ANOVA revealed only a significant main effect of treatment, demonstrating higher PBR binding in EH vs. NH animals, $F(1, 36) = 22.97$, $p < 0.001$ (see Table 1). No gender differences could be observed.

Ovary and testis. The two separate one-way ANOVAs of the ovary and the testis data each revealed a significant effect of treatment [ovary: $F(1, 18) = 53.33$, $p < 0.001$; testis: $F(1, 16) = 6.74$, $p < 0.02$], reflecting, in contrast to kidney and adrenal, a decrease in PBR binding in EH vs. NH rats (see Table 1). This effect was observed in both male and female rats, but seemed to be more pronounced in females.

Dissociation constants (K_d). The K_d values in all the tested peripheral organs were in the nanomolar range (Table 1). The K_d values in the testis, $F(1, 18) = 0.79$, $p = 0.39$, NS, and ovaries $F(1, 18) = 1.83$, $p = 0.19$, NS, did not differ between the NH and EH rats. However, a significant interaction of gender × treatment was observed in the kidney, $F(1, 36) = 13.20$, $p < 0.001$, and adrenal, $F(1, 36) = 20.10$, $p < 0.0002$, indicating that the increase in adrenal B_{max} values in male rats was also associated with increased affinity to the ligand, a phenomenon that was not obtained in the kidney.

DISCUSSION

In the present study, female rats displayed a higher level of activity compared with males. In addition, EH rats showed in-

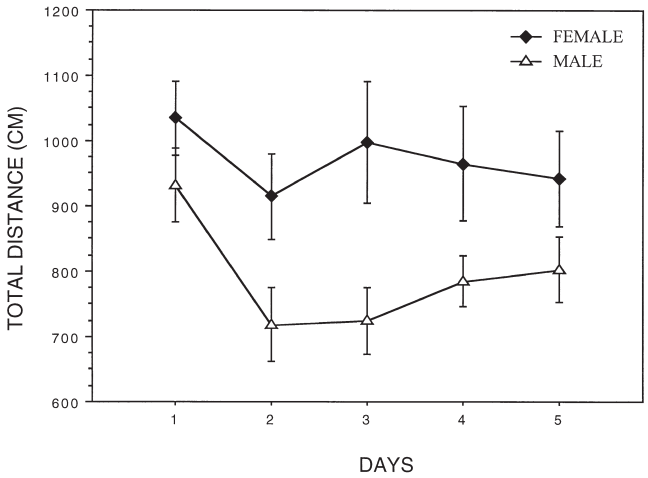

FIG. 1. Total distance traveled in the open-field arena over 5 days of testing for male and female rats. Results are means ± SEM. Effect of gender: $p < 0.05$.

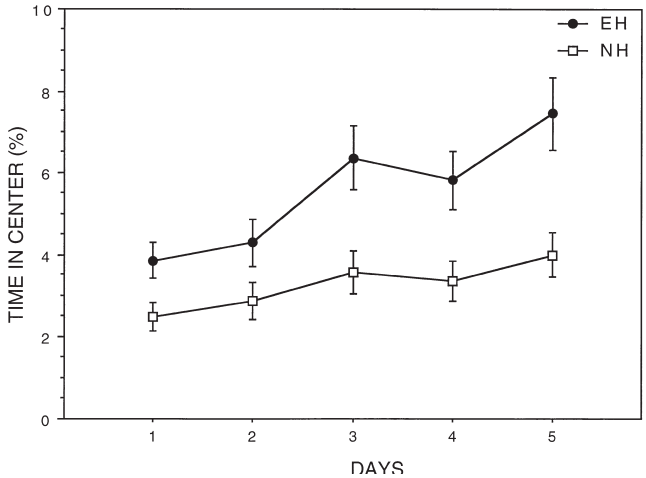

FIG. 3. Percentage of time spent in the center of the open-field arena over 5 days of testing for EH and NH rats. Results are means ± SEM. Effect of treatment: $p < 0.01$.

TABLE 1

EFFECT OF EARLY HANDLING ON B_{max} (fmol/mg PROTEIN) AND K_d (nM) VALUES OF [^{3}H]PK 11195 BINDING IN THE EXAMINED TISSUES IN MALE AND FEMALE RATS

	EH Male (n = 10)	EH Female (n = 10)	NH Male (n = 10)	NH Female (n = 10)
Adrenal				
B_{max}*	73,331.7 ± 4,346.2	76,110.5 ± 994.3	55,429.4 ± 6,341.8	56,283.8 ± 1,369.8
K_d‡	3.11 ± 0.19	4.23 ± 0.10	3.59 ± 0.32	2.99 ± 0.15
Kidney				
B_{max}*†‡	10,203.2 ± 233.5	10,680.4 ± 174.9	8,136.1 ± 715.4	10,026.1 ± 157.5
K_d‡	2.06 ± 0.17	1.73 ± 0.10	1.71 ± 0.12	2.24 ± 0.07
Testis/ovary				
B_{max}*	4,659.1 ± 143.1	7,486.0 ± 218.4	5,193.0 ± 147.7	10,082.7 ± 280.6
K_d‡	0.92 ± 0.11	2.35 ± 0.19	1.03 ± 0.09	2.64 ± 0.10

Values are means ± SEM. EH, early-handled; NH, nonhandled.
*Indicates a significant effect of treatment.
†Indicates a significant effect of gender.
‡Indicates a significant interaction of treatment × gender.

creased levels of exploratory activity in the open-field paradigm in comparison with their NH counterparts, as well as an increase of time spent in the center of the open-field arena. The radioligand binding studies revealed that EH of rats was associated with a long-lasting (of at least 6 months) increase in adrenal and renal PBR density, and a reduction in the expression of this receptor in the gonads. Moreover, the elevation in adrenal PBR density in male rats was also associated with increased affinity of the receptor to [^{3}H]PK 11195, indicating a rise in both number and sensitivity of PBR in this organ in males. The persistent upregulatory effect of EH on renal PBR density was more prominent in males than in females, while a downregulatory effect in the gonads was more pronounced in female rats. Sexual dimorphism in renal PBR response to stress has previously been ascribed to gender difference in the responsivity of the renin–angiotensin system to stress (12,13,15).

The behavioral results from the present study are consistent with previous reports in the literature, namely, that females in general are more active than males and that EH results in increased exploratory activity (5,22,29,32). Furthermore, the finding that EH animals, compared with NH controls (irrespective of gender), spent more time in the center of the open field suggests that EH animals show decreased emotionality in this paradigm, as is supported by the literature (2,25).

Animals exposed to neonatal handling show in adulthood altered HPA responsivity to stress (2,4,16,21,22). On the molecular level, it has been shown that early postnatal handling alters glucocorticoid receptor gene expression in the hippocampus and frontal cortex, regions involved in the negative feedback regulation of corticotropin-releasing hormone and arginine vasopressin (24). Thus, it seems that handling during the first 3 postnatal weeks induces a persistent increase in forebrain glucocorticoid receptor density, which in turn, amplifies the sensitivity of the HPA axis to the inhibitory effect of stress-induced elevation of circulating glucocorticoids (23,24). Acute exposure to stress is reported to be associated with an increase in HPA axis activity as well as with an increase in renal PBR expression. In contrast, repeated exposure to stress is reported to lead to a reduction in PBR density (31).

In the present study, the rats underwent a postnatal handling procedure that lasted for 3 weeks. This paradigm of repeated stress in infancy has been reported to be accompanied by reduced reactivity of the HPA axis to environmental stress in adulthood (24). Because PBR play a role in steroidogenesis (26), we hypothesized that PBR in the steroidogenic tissues would be reduced in EH rats. However, adrenal PBR, contrary to our expectations, were shown to be elevated in the EH male and female rats. Similar results were obtained in renal tissue. The decrease in gonadal PBR was the only finding that was in accord with our assumption.

Because EH is associated with enhanced efficacy of a negative-feedback regulatory mechanism of the HPA axis via increased forebrain glucocorticoid receptors (24), it is possible that supersensitized adrenal PBR are essential to counterbalance the enhanced inhibitory tone at the hippocampal–hypothalamic level in order to maintain adequate glucocorticoid activity.

It has been suggested that stress-induced regulation of renal PBR is coupled with the renin–angiotensin system (11), a phenomenon that is relevant to stress-induced hypertension. The increased renal PBR in EH rats may contribute to higher basal maintenance of the renin–angiotensin system, obviating the need for a further increase in the activity of this system and resulting in diminished activation of the autonomic nervous system response to stress in adulthood. Although dysfunction in the HPA, renin–angiotensin, and autonomic nervous systems could be important, the putative role of endocrine and homeostatic factors in regulating PBR response remains speculative, especially because no functional measurements were performed in the present study. Unknown factors or mechanisms, other than the mentioned neuroendocrine ones, may contribute in regulating PBR response.

The physiological significance of the EH-induced decrease in gonadal PBR in both male and female rats is unclear. Indeed, infantile stimulation by preweaning handling has previously been reported not to modify gonadal maturation (9). It should, however, be reiterated that the latter study used gonadal weights, vaginal opening, and first estrus as experimental indices. It is known that gonadal PBR play a role in the biosynthesis of sex steroids (18,27); thus, it is possible that the experience of EH is associated with interference with gonadal activity in adulthood. However, it should be noted that other proteins are also involved in the synthesis of sex steroids (28), and such proteins may compensate for the reduction in gonadal PBR expression. The impact of EH on the reproductive

activity of male and female rats exposed in infancy to this manipulation merits further investigation.

In summary, the behavioral consequences of EH may be associated with better coping with stress, while biochemical consequences seem to be associated with long-lasting organ-specific changes: an increase in adrenal and renal PBR and a decrease in gonadal PBR. The role of long-term changes in PBR expression for better adaptation to stressful situations in adulthood is as yet poorly understood.

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