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Peripheral benzodiazepine receptors reflect trait (early handling) but not state (avoidance learning)

Julia Lehmann[a,1], Ronit Weizman[b], Svetlana Leschiner[c], Joram Feldon[a], Moshe Gavish[c,*]

[a]*Behavioral Neurobiology Laboratory, The Swiss Federal Institute of Technology, Zürich 8603, Schwerzenbach, Switzerland*
[b]*Tel Aviv Community Mental Health Center and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*
[c]*Department of Pharmacology, The Bruce Rappaport Faculty of Medicine, and Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, P.O. Box 9649, Haifa 31096, Israel*

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Abstract

Behavioral animal paradigms and experimental neuroendocrinological and neurochemical studies have shown that early environmental manipulations have profound effects on the late response to stress. The aim of the present study was to investigate the interactive effects of environmental manipulation (early handling) and experimentally induced behavioral differences on the peripheral benzodiazepine receptor (PBR) system, which is known to be involved in the response to stressors. Adult early-handled (EH) and nonhandled (NH; control) Wistar rats were placed in a two-way active avoidance/latent inhibition (LI) paradigm, and PBR densities in the adrenal glands, kidneys, and gonads were assessed. In line with previous studies, overall avoidance learning improved in the EH group, and LI was disrupted in the NH group (primarily in males). PBR densities were up-regulated in EH subjects, and more so in females than males. However, PBR densities did not correlate with any of the behavioral measures. These findings strengthen the hypothesis that differences in PBR densities between EH and NH rats are a reflection of trait rather than state, and they suggest that the PBR system is characterized by a highly stressor-specific response.

Keywords: Rats; Early handling; Peripheral benzodiazepine receptor; Two-way active avoidance; Latent inhibition

1. Introduction

Early environmental manipulations in rats have been reported to have profound effects on both neurodevelopment and behavior (Ader, 1965, 1968). One of the major types of manipulations used is brief (3–30 min) daily handling throughout the weaning period (Days 1–21). Compared with nonhandled (NH) animals, who are left undisturbed with the mother until weaning is completed, early-handled (EH) rats display decreased emotionality, as indicated by reduced anxiety, increased exploratory behavior, and decreased defecation (Levine et al., 1967). They also have better learning performance in the two-way active avoidance (2WAA) paradigm (Levine, 1956) and enhanced latent inhibition (LI) (i.e., retarded conditioning to a stimulus as the consequence of repeated preexposure to it) in other paradigms (Weiner et al., 1987; Shalev et al., 1998). In terms of neuroendocrine parameters, early handling of rats leads to a reduction in corticosterone release in response to acute stress during adolescence and adulthood (Levine et al., 1967; Ader, 1970; Núñez et al., 1996). Furthermore, since the handling is carried out largely during the quiescent period of the hypothalamic–pituitary–adrenal (HPA) axis, it has marked effects on HPA axis development, with EH animals demonstrating an increased number of hippocampal glucocorticoid receptors in adulthood (Meaney and Aitken, 1985). Indeed, this factor may account both for their reduced adrenocorticotropic hormone (ACTH) and corticosterone release following acute stress (Meaney et al., 1996) and for their reduced behavioral expression of emotionality (Levine, 1959; Hilakivi-Clarke et al., 1991). Thus, it seems that improved learning performance in an active avoidance paradigm as well as enhanced LI may be a consequence of the development of an improved stress-coping mechanism following early handling.

* Corresponding author. Tel.: +972-4-829-5275; fax: +972-4-829-5271.
E-mail address: mgavish@tx.technion.ac.il (M. Gavish).
[1] Current address: Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany.

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Besides the HPA, the peripheral benzodiazepine receptor (PBR) system is also involved in the stress response. There is evidence that PBR behaves bidirectionally, i.e., up-regulation in response to acute stress and down-regulation in response to repeated or chronic stress (Weizman and Gavish, 1993; Gavish et al., 1999). In a recent paper, we demonstrated that in Wistar rats, early handling increases basal PBR density in the adrenal glands and kidneys, and decreases PBR density in the gonads (Weizman et al., 1999). This change may be attributable to a compensatory shift in receptor density to accommodate the demands of coping with stress (Drugan, 1996; Drugan et al., 1993; Weizman and Gavish, 1993; Gavish et al., 1999).

One of the best investigated paradigms for studying behavioral changes after early handling is the 2WAA paradigm, wherein animals learn to avoid a weak foot shock by crossing a barrier in the middle of the box when the conditioned stimulus is presented (avoidance response). Animals who fail to avoid the shock usually display an escape response, i.e., they cross the barrier in response to the shock. This paradigm presents a stressful experience for the subjects, as can be shown by a rapid increase of corticosterone release during avoidance learning (Lehmann et al., 1999). Using an LI paradigm, i.e., preexposing half of the subjects to the conditioned stimulus prior to the test, it has been consistently reported that (i) EH subjects perform better in the 2WAA paradigm than their NH counterparts, and (ii) male NH subjects do not display LI, while there is normal LI in EH and female NH subjects (Weiner et al., 1985).

We recently demonstrated that the 2WAA paradigm itself does not affect PBR densities in the testis, adrenal glands, kidneys, or cortex in normally reared Wistar rats (Lehmann et al., 1999). In the present study, using the same paradigm, we sought to determine whether early manipulation changes the response to stress and whether experimentally induced behavioral differences (by preexposure) are reflected in PBR binding capacity. We hypothesized that we would find differences in PBR densities as a function of preexposure in the EH but not the NH subjects.

2. Methods

2.1. Animals

Male and female Wistar rats (Zur:WIST[HanIbm]; Research Unit, Schwerzenbach, Switzerland) were used in the experiments. 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 (Barbazanges et al., 1996). Only litters with 9–13 pups were used.

Animals were housed under reversed cycle lighting (lights on 1900–0700 h) at a temperature of 21 ± 1 °C and humidity of $55 \pm 5\%$ in a climatically controlled animal facility. Food (Nafag-9431; Eberle Nafag, Gossau, Switzerland) and water were available ad libitum in the home cages. All experiments were carried out in agreement with the Swiss Federal Legislation for Animal Experimentation; the experimental protocol was approved by the Cantonal Review Committee for the Use of Animal Subjects.

2.2. Early manipulation

Animals were divided into two groups, EH and NH, of 10 males and 10 females each. Within each treatment group all subjects were derived from different litters, and between treatment groups each subject had a maximum of one opposite sex littermate.

For the EH group, on each day between birth and weaning (Day 21), the mother was removed from its litter to a holding cage, and the pups were placed individually into small plastic cups lined with a layer of wood shavings for a 15-min period. Thereafter, the pups and the mother were returned to the home cage. The NH rats were left completely undisturbed with the mothers until weaning, except for the provision of food and water. Approximately 1 week after weaning, the rats were placed in grid floor cages ($48 \times 27 \times 20$ cm, five rats per cage; Macrolon) and were left undisturbed until the onset of the experiment at 6 months of age.

2.3. 2WAA

2.3.1. Apparatus

The apparatus consisted of four identical shuttle boxes (model E10-16TC; Coulbourn Instruments, Allentown, PA, USA), each set in a ventilated sound- and light-attenuating shell (model E10-20). The internal dimensions of each experimental chamber, as measured from the raised grid floor, were $35 \times 17 \times 21.5$ cm. The box was divided by an aluminum hurdle 17 cm long $\times$ 4 cm high; its thickness was only 1 mm, to prevent rats from balancing on top of the hurdle and avoid receiving a shock. Scrambled shocks were delivered from a constant current shock generator (model E13-14; Coulbourn Instruments) and scanner (model E13-13) set at 0.5 mA. The conditioned stimulus was an 85-dB tone produced by a 2.9-kHz tone module (model E12-02 (Coulbourn Instruments) placed behind the shuttle box on the floor of the shell. The chambers were illuminated during the experimental session with two diffuse light sources (house lights) mounted 19 cm above the grid floor in the middle of the sidewalls.

2.3.2. Procedure

Twenty male and 20 female 6-month-old adult Wistar rats were tested. One week prior to the start of the experiment, all animals were transferred to individual cages ($48 \times 27 \times 20$ cm; Macrolon) and were handled daily for 5 min. The testing procedure was divided into three stages 24 h apart.

2.3.2.1. Familiarization. Each animal was placed in the shuttle box with the house light on for 60 min.

2.3.2.2. Preexposure. Each rat was placed in the experimental chamber. Half the rats in each group received 50 presentations of the conditioned stimulus, with a variable interstimulus interval of 50 s (preexposed animals). The remainder were confined to the chamber for the same period of time without receiving the conditioned stimulus.

2.3.2.3. Testing. Each animal was placed in the shuttle box and underwent 100 avoidance trials at a variable interval of 50 s. Each avoidance trial began with a 12-s tone (conditioned stimulus), the last 2 s of which were concurrent with a 0.5-mA shock. If the animal crossed the barrier to the opposite compartment during the tone, the stimulus was terminated and no shock was delivered (avoidance response). If the animal crossed the barrier during the shock, the tone and the shock were terminated (escape response). If the animal failed to cross the barrier during the entire tone shock trial, the tone and the shock were terminated after 12 s from the onset of the tone. The number of avoidance and escape responses was recorded for 100 trials.

LI was considered present if the preexposed rats displayed a lower number of avoidance responses than the nonpreexposed rats.

2.3.3. Data collection and analysis

The 100 avoidance trials were divided into 10 blocks of 10 trials each. The number of avoidance responses per block was calculated and expressed as the percentage of total avoidance responses. These data were analyzed by a $2 \times 2 \times 2 \times 10$ ANOVA with main factors of early experience (EH, NH), gender (male, female), and preexposure (0, 50) and a repeated measurement factor of blocks (1–10) each consisting of 10 trials.

2.4. Benzodiazepine receptor binding analysis

2.4.1. Tissue preparation

The 2WAA subjects were decapitated, and adrenal glands, testes, kidneys, and brain were removed and stored at −70 °C until PBR and central benzodiazepine receptor binding assay. Briefly, the organs were homogenized separately in 50 vol of 50 mM potassium phosphate buffer, pH 7.4, using a Brinkmann Polytron (setting 10) for 15 s. The homogenates were centrifuged at $49{,}000 \times g$ for 15 min. The pellets were resuspended in 50–100 vol of 50 mM ice-cold buffer and assayed.

2.4.2. [^{3}H]PK 11195 binding

[^{3}H]PK 11195 binding was conducted as previously described (Gavish and Weizman, 1989). The binding assay in a final volume of 500 μl contained 400 μl of 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 of scintillation cocktail (Opti-Fluor; Packard, Groningen, the Netherlands) and counted in a scintillation counter following a 12-h equilibration period.

2.5. Statistical analysis

PK 11195 binding per organ was analyzed for each animal individually. A $2 \times 2 \times 2$ ANOVA with main factors of treatment (EH/NH), preexposure (PE/NPE), and gender (male/female) was carried out for each organ separately. Since the factor preexposure did not yield a significant effect or interaction in all the analyses, a 2×2 ANOVA with main factors of treatment (EH/NH) and gender (male/female) was carried out for intergroup comparisons. All results are expressed as mean ± S.E.M. To determine whether PBR density corresponded with the number of

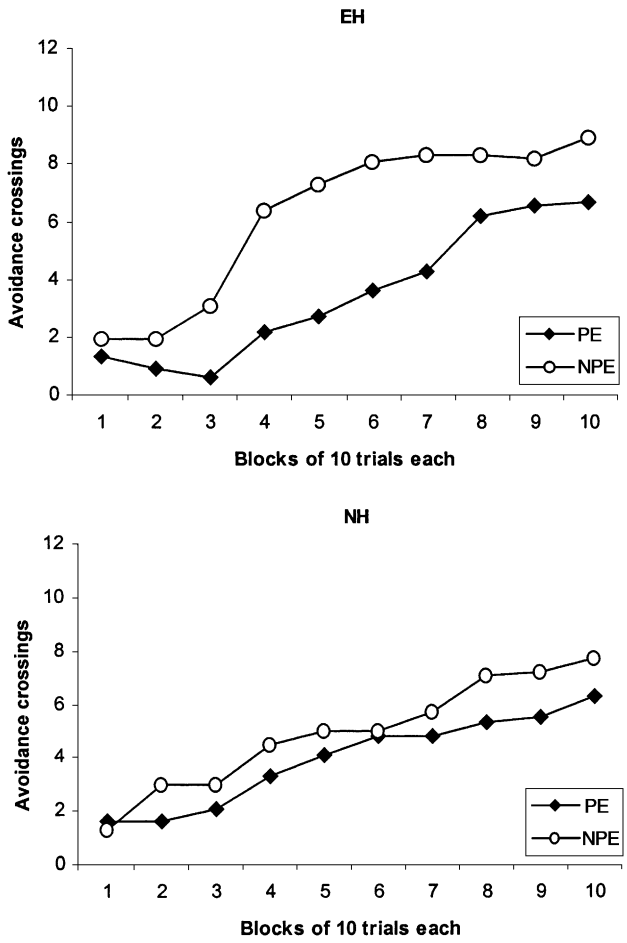

Fig. 1. Mean number of avoidance responses over 10 blocks of 10 acquisition trials in the test stage in EH and NH, preexposed (PE) and nonpreexposed (NPE) adult rats ($n = 10$ per condition). LI was observed in the EH but not in the NH condition ($P < .05$, ANOVA).

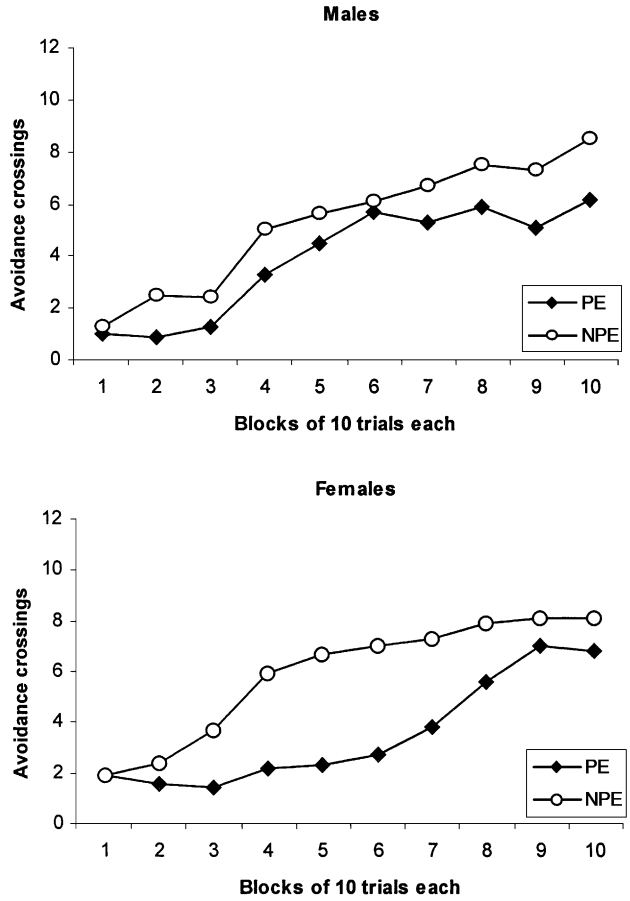

Fig. 2. Mean number of avoidance responses over 10 blocks of 10 acquisition trials in the test stage in male and female preexposed (PE) and nonpreexposed (NPE) adult rats ($n = 10$ per sex/treatment condition). While a significant sexual difference was observed in the PE condition (males better than females), no such difference was found in the NPE condition ($P < .03$, ANOVA).

shocks in individual rats, regression analysis was performed for all subjects together and for each treatment (EH/NH) separately for each organ, with PBR density as the dependent variable, and the total number of shocks throughout the experimental session and the number of shocks during the last 10 min of the experiment as the independent variables.

3. Results

3.1. Active avoidance and LI

The $2 \times 2 \times 2 \times 10$ ANOVA revealed a significant main effect of Blocks [$F(9,288) = 51.42$, $P < .0001$], indicating an overall increase in avoidance responses as a function of progressive training. There was a significant main effect of Preexposure [$F(1,32) = 7.1$, $P < .02$], with nonpreexposed rats acquiring the avoidance response faster and to a higher level compared with preexposed rats, and a significant interaction of Preexposure × Treatment × Blocks [$F(9,288) = 1.92$, $P < .05$]. This reflected that LI was present only in the EH group, as can be seen in Fig. 1. The absence of a preexposure effect in the NH group was primarily due to the reduced avoidance in the NH nonpreexposed subjects as compared with the EH nonpreexposed subjects. Thus, early handling, as reported before, facilitates avoidance learning. The disruption of LI in the NH condition as compared with the EH condition was supported statistically by two post hoc 2×10 ANOVAs comparing the preexposed and nonpreexposed subgroups within each main group. For EH

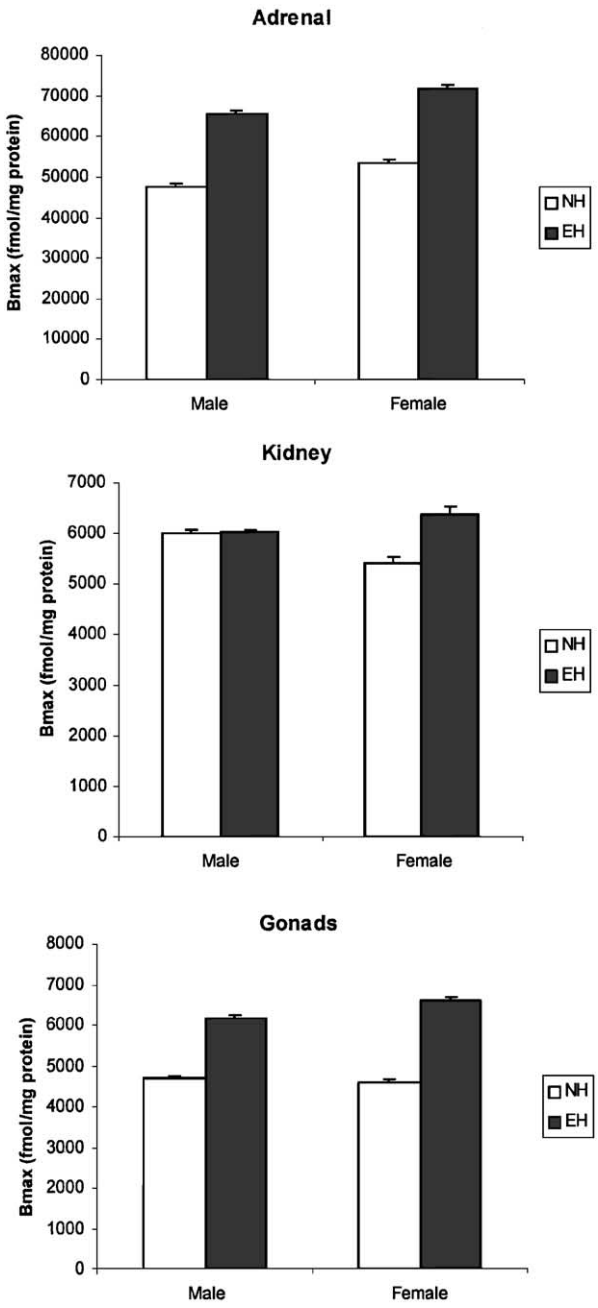

Fig. 3. B_{max} values (fmol/mg protein) in EH and NH male and female rat adrenal gland, kidney, and gonads following active avoidance testing. Values are mean ± S.E.M. ($n = 10$). See Results section for significance values.

subjects, the main effect of Preexposure and the interaction of Preexposure × Blocks were significant [$F(1,16) = 9.69$, $P < .007$; $F(9,144) = 3.09$, $P < .0025$, respectively], whereas for the NH subjects, neither effect was significant ($F = 0.85$, $P > .3$; $F = 0.57$, $P > 0.8$, respectively). The additional significant interaction of Preexposure × Gender × Blocks in the overall analysis [$F(6,288) = 2.22$, $P < .03$] demonstrated that preexposed males acquired the avoidance response faster than preexposed females (i.e., females showed enhanced LI compared with males); there was no such gender difference among the nonpreexposed rats (Fig. 2).

3.2. Peripheral-type benzodiazepine receptor

3.2.1. Adrenals

The 2 × 2 ANOVA revealed a significant effect of Treatment [$F(1,36) = 384.44$, $P < .0001$], with higher PBR density in the EH than the NH group, and a significant effect of Gender [$F(1,36) = 39.66$, $P < .0001$], with higher PBR density in females than males (Fig. 3).

3.2.2. Gonads

The 2 × 2 ANOVA revealed a significant effect of Treatment [$F(1,36) = 390.62$, $P < .0001$], with higher PBR density in the EH than the NH group. In addition, the interaction of Treatment × Gender was significant [$F(1,36) = 9.93$, $P < .005$], with females showing higher PBR density than males, and no gender difference in the NH group (Fig. 3).

3.2.3. Kidneys

The 2 × 2 ANOVA revealed a significant effect of Treatment [$F(1,36) = 23.99$, $P < .0001$], with higher PBR density in the EH group than the NH group. In addition, the interaction of Treatment × Gender was highly significant [$F(1,36) = 22.38$, $P < .0001$], indicating that the differences apparent in the EH group were attributable to changes that occurred in the females; there was no such treatment effect in males (Fig. 3).

Table 1
Correlation coefficients based on regression analysis between PBR densities and number of shocks received during the active avoidance paradigm [$n = 40$ (overall) and 20 (NH/EH)]

PBR	Total number of shocks (r)	Shocks during last 10 min (r)
Overall		
Adrenal	−.114	.151
Kidney	.037	.098
Gonads	−.020	.064
NH		
Adrenal	−.121	.216
Kidney	.186	−.158
Gonads	.382	−.351
EH		
Adrenal	−.023	−.063
Kidney	.003	.224
Gonads	.118	−.133

3.3. Behavior and PBR density

Overall regression analysis as well as the analysis for each of the treatment groups (EH, NH) and the organs separately indicated no significant relationship. The same finding was obtained when the independent variable was limited to the total number of shocks during the last 10 min of the experiment (as a more direct measurement of acute stress). Correlation coefficients are presented in Table 1.

4. Discussion

In the present study, we replicated the previously reported finding that in the 2WAA paradigm, early handling facilitates LI and active avoidance learning in general (Weiner et al., 1985) Additionally, we found that females displayed enhanced LI as compared with males (mainly due to the EH condition). In addition, there was an increase in PBR density in EH subjects in all organs investigated. However, PBR density was not correlated either with the number of foot shocks received or with learning performance in the paradigm. This finding implies that the alterations in PBR densities were not due to the experience in the 2WAA paradigm, but rather reflected a permanent change as a result of the early handling procedure (Weizman et al., 1999). This assumption is further supported by the finding that the PBR levels in the present study were very similar to those reported for naive EH/NH rats (Weizman et al., 1999).

It has previously been demonstrated that PBR densities are not affected by active avoidance testing in normally reared Wistar rats (Lehmann et al., 1999). The aim of the present study was to investigate whether NH animals, which are known to demonstrate increased emotionality and reduced learning capability in stressful situations, would be more sensitive, and therefore demonstrate more acute alterations in PBR densities, than EH animals, which have reduced emotionality and enhanced learning. Using an LI paradigm, we experimentally induced retarded learning in the preexposed subjects, expecting it to enhance stress and to challenge the PBR system. However, neither preexposure nor the differences in stress-coping mechanisms led to an acute PBR response. These results, together with those of previous studies carried out in our laboratory (Lehmann et al., 1999), indicate that PBR densities do not increase following 2WAA testing, remaining in a range similar to that in naive EH and NH subjects, and that the behavioral differences induced by preexposing the animals to the conditioned stimulus are not reflected in alterations in PBR densities.

In the present study, the number of foot shocks subjects experienced varied between 14 and 98. Despite this wide range, there was no evidence of a correlation of PBR

density with cumulative shocks received. Therefore, we conclude that the 2WAA paradigm, which involves a coping response, is not an appropriate stressor to induce alterations in PBR expression, not even when the behavioral response is experimentally manipulated. Although significant effects on PBR densities have been reported previously following a lower number of shocks than that used here, in most of these studies the shocks lasted 5–10 s, and shock levels ranged between 1 and 2 mA (Drugan and Holmes, 1991; Drugan et al., 1993). Therefore, one explanation for our finding that active avoidance testing does not affect PBR densities may be that the aversive component of the experimental paradigm was too short and the shock levels were too low. However, the weakness of the stressor cannot by itself account for the lack of PBR alteration because measures of corticosterone release after avoidance testing in other studies have indicated that the 2WAA paradigm is indeed a stressful experience (Lehmann et al., 1999). Furthermore, in addition to the present correlational studies, a direct pharmacological intervention is needed to clarify the role of PBR in the various experimentally produced behavioral alterations.

Another suggested explanation for the nonresponse of the PBR system is the avoidability of the stressor (Lehmann et al., 1999). Animals that show a deficit of avoidance learning usually display a high percentage of escape responses, regardless of whether the deficit was induced experimentally by preexposing the animals to the conditioned stimulus or by early environmental manipulations altering the stress-coping mechanisms (Lehmann et al., 1999). Holmes et al. (1992) reported that alterations in renal PBR densities are observed irrespective of the controllability or predictability of the stressor. The 2WAA paradigm, however, comprises both factors: the shocks are predictable and are controllable by the subjects. This probably underlies the lack of the noxious component that has been claimed to be necessary for the PBR system to respond (Lehmann et al., 1999). Also, our use of subjects that differed in avoidance learning ability and in responsiveness to stressful events (early handling has been shown to reduce and shorten the HPA response to stress; Levine et al., 1967; Ader, 1970; Núñez et al., 1996), and our additionally increasing the deficits in avoidance learning between the groups by preexposing some of the animals did not lead to differences in PBR densities between subjects other than those that have already been described (Weizman et al., 1999). This indicates that the responsiveness of the PBR system may occur independently of the HPA axis response (Holmes and Drugan, 1994).

The present study extends our previous finding that 2WAA exposure fails to produce an effect on the PBR system. The fact that the high number of shocks received by some of the subjects still failed to exert an effect on PBR density suggests that the lack of an effect is inherent to the experimental design, which includes predictability and controllability of the stressor. It has already been argued that uncontrollable stressors are more anxiety-producing than controllable events (Drugan and Holmes, 1991). However, in a study by Maier et al. (1986), levels of ACTH and corticosterone did not show such an effect of controllability, a finding in line with our previous observation that, following 2WAA, corticosterone levels, but not PBR densities, are increased (Lehmann et al., 1999). Unfortunately, in the present study, we did not assess blood corticosterone levels. Nevertheless, considering the results of our previous study (Lehmann et al., 1999), it is still possible that PBR densities display a much more stressor-specific response, in contrast to the nonspecific HPA axis response. In addition, nonhandling leads to a decrease in basal PBR densities, but not to a change in basal corticosterone levels (Lehmann et al., 1999), indicating a dissociation between the PBR and HPA systems. Some researchers have suggested that the response of renal PBR occurs independently of the HPA and the sympathetic autonomic nervous systems, but may be regulated by the renin–angiotensin system (Holmes and Drugan, 1994).

In conclusion, the present results support the hypothesis that an alteration in the PBR response to stress can occur independently of the HPA response, and another system that is not activated in 2WAA may be more dominant. The present study also supports the hypothesis that differences in PBR densities observed in EH/NH subjects are a reflection of trait rather than state.

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