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Correlation between exploratory activity in an elevated plus-maze and number of central and peripheral benzodiazepine binding sites

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Summary. Two groups of rats were selected from a small animal population on the basis of their exploratory activity in an elevated plus-maze model of anxiety. One group had a considerably lower and the other one a higher exploratory activity than the average total population. These subgroups were termed "anxious" and "non-anxious", respectively. In both groups central benzodiazepine binding sites in various brain structures were labelled with ^{3}H-flunitrazepam. Peripheral benzodiazepine binding sites labelled in vitro with different tritiated ligands were also studied in several peripheral organs including blood platelets and lymphocytes. "Anxious" animals had a significantly lower number of ^{3}H-flunitrazepam binding sites in the cerebral cortex but not in the hippocampus and cerebellum. In this subgroup ^{3}H-Ro 5-4864 binding to peripheral benzodiazepine recognition sites was also lower than in the other one in adrenals, kidneys, platelets and lymphocytes. In the heart no differences of ^{3}H-Ro 5-4864 binding between subgroups studied were found. Although in "anxious" rats ^{3}H-diazepam and ^{3}H-PK 11195 binding was significantly lower only in lymphocytes, a somewhat decreased binding to these ligands was also present in platelets. No significant differences in the affinity were found between the two groups throughout the experiments described. The results indicate that behavioral anxiety in rats is correlated not only with the lower number of central benzodiazepine receptors but also with a lower density of peripheral benzodiazepine binding sites in several peripheral organs including platelets and lymphocytes.

Key words: Elevated plus-maze – Behavioral differences – Anxiety – Central and peripheral benzodiasepine binding sites

Introduction

On the basis of the selectivity for their ligands benzodiazepine (BD) binding sites may be divided into central and peripheral-type. While the central BD binding sites are localized mainly in neuronal tissue, peripheral BD binding sites occur in several peripheral organs like heart, lungs, liver, kidneys, adrenals etc. Although both the central and peripheral BD binding sites are found in the brain they seem to be different entities in regard to their distribution and functions (for review see Saano et al. 1989). The central BD binding site, mostly referred to as BD receptors, is an integral part of the $GABA_A$receptor-chloride channel complex in the mammalian CNS (Haefely 1987). The physiological role and function of peripheral BD recognition sites are still obscure. Several lines of evidence demonstrate that central BD binding sites, like other parts of $GABA_A$/benzodiazepine/Cl-ionophore receptor complex, are affected by stress (Medina et al. 1983; Miller et al. 1987; Corda and Biggio 1986; Concas et al. 1987). However, relatively little is known about the effect of stress and anxiety on the peripheral BD binding sites. Recently it has been demonstrated that some exogeneous stressful stimuli can increase not only central but also peripheral BD binding sites in rat kidney (Okun et al. 1988; Rägo et al. 1989a). However, in man the density of peripheral BD binding sites in platelets of anxious patients has been shown to be reduced in comparison to normal controls (Weizman et al. 1987).

To clarify this discrepancy and to find a more accessible model of "endogeneous" anxiety for studying possible changes of peripheral BD binding sites we decided to differentiate animals in potentially anxious and non-anxious according to their behavioral response and to use these subgroups for further studies. In most studies various environmental (exogeneous) factors have been used to elicit stress reactions in animal and man but up to now very little is known how more subtle factors, probably of more endogenous nature, like genetic differences or social interactions in animal population can modulate molecular mechanism involved in anxiety. Previously we have demonstrated that it is possible to differentiate mice according to their emotional response in an elevated plus-maze test into anxious and non-anxious

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Table 1. Selection experiment according to the behavioral response of rats in an elevated plus-maze. Animals with low exploratory activity were classified as anxious, those with high exploratory activity were termed as non-anxious. Results are from a typical experiment expressed as a mean ± SEM

Group	No of animals	Latency of 1.arm entry (s)	No of sections croseed in open arm	To time spent in open arm
Total group	46	49 ± 8	16 ± 3	32 ± 5
Non-anxious subgroup	7	15 ± 3**	30 ± 5*	87 ± 16*
Anxious subgroup	8	169 ± 13**	6 ± 2*	12 ± 4**

* $P < 0.05$ as compared to total group
** $P < 0.01$ as compared to total group

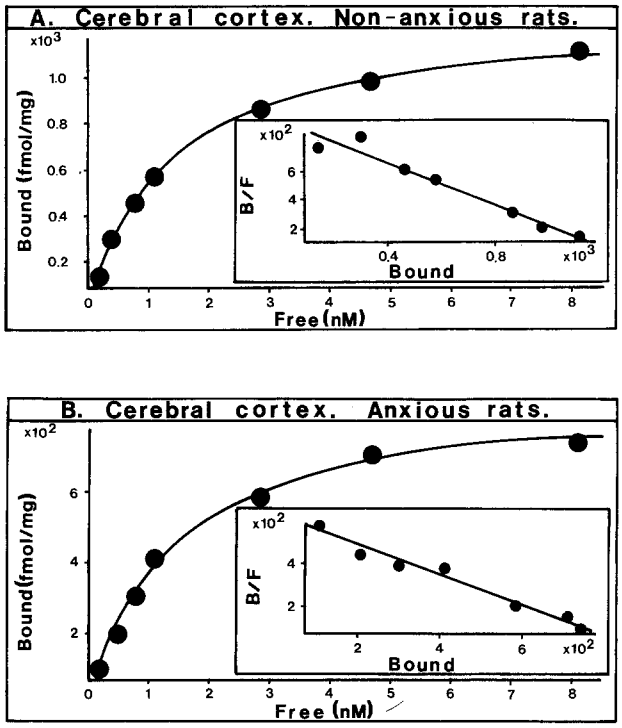

Fig. 1 A, B. Saturation studies of ^{3}H-flunitrazepam binding to cerebral cortex membranes in rats selected according to their exploratory response in an elevated plus-maze. The data presented are from a typical experiment out of 4 separate experiments each carried out in triplicate. **A** Non-anxious rats: $B_{max} = 1282$ fmol/kg; $K_D = 1.33$ nM. **B** Anxious rats: $B_{max} = 891$ fmol/mg; $K_D = 1.39$ nM. The mean ± SEM values of all experiments were: $B_{max} = 1286 \pm 85$ fmol/mg; $K_D = 1.42 \pm 0.13$ nM for non-anxious and $B_{max} = 1017 \pm 62$ fmol/mg; $K_D = 1.38 \pm 0.15$ nM for anxious rats respectively

animals. The anxious animals had significantly lower BD and $GABA_A$ receptor densities in the cerebral cortex (Rägo et al. 1988).

Due to the lack of sufficient amount of blood cells for binding studies with peripheral BD ligands from mice we decided to use rats selected in the elevated plus-maze to find out possible correlations between emotional status and peripheral BD binding sites in various organs and blood cells (platelets and lymphocytes). Here we report that rats with low exploratory activity in elevated plus-maze (termed "anxious" group) have a lower number of central BD binding sites in the cerebral cortex and of peripheral BD binding sites in adrenals, kidneys, lymphocytes and platelets than non-anxious rats.

Materials and methods

Animals. Male albino laboratory rats (local strain from Rappolovo Farm, Leningrad) weighing 220–260 g were used in this study. The animals were maintained on food and water ad libitum and were housed 20–25 per cage in a 12:12 h light/dark cycle (lights off from 2000–0800 h) at 20 ± 1°C. The experiments were carried out between 1700–2000 h.

Exploratory activity in an elevated plus-maze and animal selection. The elevated plus-maze used was the same as recently described by Pellow and File (1986) with minor modifications in recorded parameters. The plus-maze consisted of two open arms, 50 × 10 cm, and two enclosed arms, 50 × 10 × 25 cm with an open roof, arranged such that the two arms of the same kind were opposite to each other. The central compartment of the plus-maze was an open square, 10 × 10 cm. Each open arm was devided into 5 sectors (10 × 10 cm) by lines of water proof crayon. The maze was elevated to the height of 25 cm. During a 4-min test period the following measures were taken by an observer: (a) the latency period to first open arm entry, (b) the number of sectors crossed in open arms, (c) total time spent in open arms. At the start of the epxeriment rats were placed at the centre of the plus-maze.

For selection experiments animal populations consisting of 22–26 rats per cage were used. The animal populations used were kept together in the same home cages at least four weeks before the selection experiment. After testing in plus-maze every rat was marked and returned to the home cage till the beginning of binding experiments.

In vitro binding studies. Animals were killed by decapitation, trunk blood collected into plastic tubes containing 0.5 ml acid citrate dextrose anticoagulant and organs (brain, heart, kidneys and adrenals) rapidly removed on ice. Cerebral cortex (frontal part), hippocampus and cerebellum were rapidly dissected and homogenized in 30 volumes of ice-cold Tris-HCl (pH 7.4) using a Potter-S glass-teflon homogenizer (1000 rpm, 10 strokes). For peripheral organs a Brinkman Polytron homogenizer (setting 10, during 15 s) and the same buffer were used. The homogenates were centrifuged at 48000 g for 15 min and the resulting pellets stored at −20°C till subsequent binding studies. After melting the pellets were resuspended in Tris-HCl buffer and washed once by centrifugation before binding experiments. Binding of ^{3}H-flunitrazepam (0.125–8 nM, spc. act. 80 Ci/mmol, Amersham Radiochemicals) and ^{3}H-Ro 5-4864 (for peripheral organs except blood cells, 0.5–12 nM, spec. act. 81 Ci/mmol, New England Nuclear) were carried out in a total incubation volume of 500 µl in the presence or absence of 10 µM flunitrazepam and Ro 5-4864 (Hoffman-LaRoche, Basel, Switzerland) respectively. After 60 min incubation on ice the reaction was stopped by rapid filtration over Whatman GF/B filters.

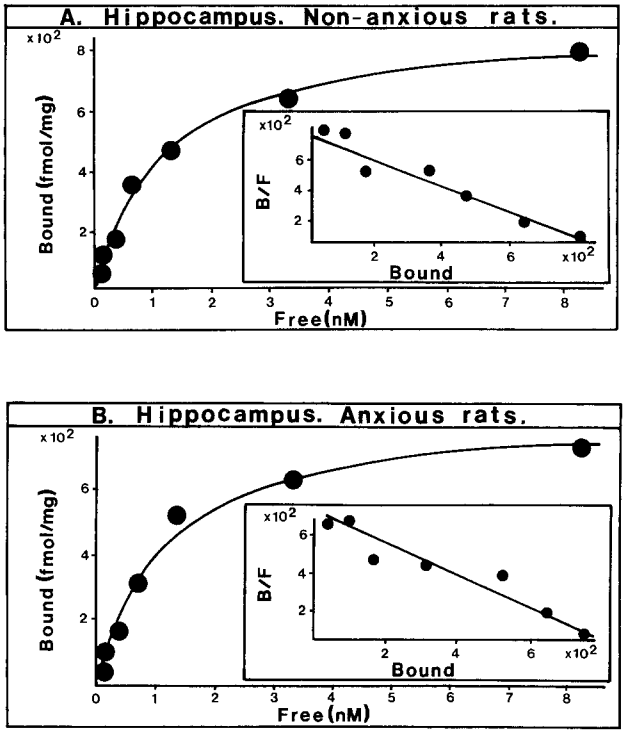

Fig. 2A, B. Saturation studies of ^{3}H-flunitrazepam binding to hippocampus membranes in rats selected according to their exploratory response in an elevated plus-maze. The data presented are from a typical experiment out of 3 separate experiments each carried out in triplicate. **A** Non-anxious rats: $B_{max} = 907$ fmol/mg; $K_D = 1.16$ nM. **B** Anxious rats: $B_{max} = 899$ fmol/mg; $K_D = 1.30$ nM. The mean ± SEM values of all experiments were: $B_{max} = 856 \pm 58$ fmol/mg; $K_D = 1.31$ nM for non-anxious and $B_{max} = 891 \pm 45$ fmol/mg; $K_D = 1.28 \pm 0.15$ nM for anxious rats respectively

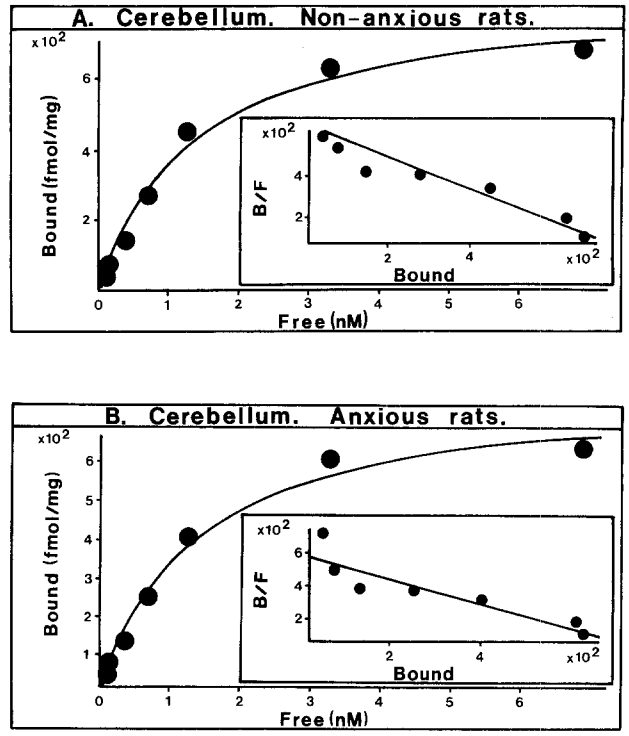

Fig. 3A, B. Saturation studies of ^{3}H-flunitrazepam to cerebellum membranes in rats selected according to their exploratory response in an elevated plus-maze. The data presented are from a typical experiment out of 3 separate experiments each carried out in triplicate. **A** Non-anxious rats: $B_{max} = 837$ fmol/mg; $K_D = 1.29$ nM. **B** Anxious rats: $B_{max} = 798$ fmol/mg; $K_D = 1.37$ nM. The mean ± SEM values of all experiments were: $B_{max} = 803 \pm 38$ fmol/mg; $K_D = 1.25 \pm 0.13$ nM for non-anxious and $B_{max} = 715 \pm 47$ fmol/mg; $K_D = 1.54 \pm 0.18$ nM for anxious rats respectively

The filters were washed with 4 × 3 ml of ice-cold Tris-HCl buffer. The blood samples were centrifugated at 190 g for 15 min at room temperature. The platelet rich plasma obtained was washed twice with phosphate buffered slaine (PBS-I) at 400 g for 15 min and the pellet resuspended in PBS containing 5 mM KCl and 1 mM $MgCl_2$ (PBS-II, pH 7.4) was used for binding studies. The blood cells remained after removing PRP fraction were used to obtain lymphocytes according to the method of Boyum (1968) using the Ficoll-Pague gradient. The lymphocytes isolated were washed twice with PBS-II before using in binding experiments. The intact cells (controlled under microscope, number of damaged cells usually less than 3%) were used in current studies. ^{3}H-PK 11195 (0.25–8 nM, spec. act. 91 Ci/mmol, New England Nuclear), ^{3}H-Ro 5-4864 (0.5–16 nM, other data see above) and ^{3}H-diazepam (0.5–24 nM, spec. act. 82 Ci/mmol, Amersham Radiochemicals) were used in 8 to 10 different concentrations to label BD binding sites on blood cells. Unlabelled Ro 5-4864 (10 µM) was used to determine nonspecific binding. The binding was performed in PBS-II in a total volume of 125 µl during 45 min on ice. The reaction was stopped by rapid filtration over Whatman GF/B filters followed by 3 × 1.5 ml washing with ice-cold PBS-II. Specific binding was calculated by subtracting the nonspecific from total binding at each given radioactivity concentration. Protein content was measured by Lowry et al. (1951) method.

Calculations and statistics. Maximum binding (B_{max}) and affinity constants (K_D) were calculated using Scatchard plot analysis. Scatchard plots were computed first using linear regression program. For brain structures only plots with correlation coefficent of 0.95 or more and for other studies with 0.85 or more were accepted. The final results presented were computed and curves fitted using a non-linear least squares regression analysis. Student's *t*-test for paired observations was used to determine statistical significance.

Results

Animal selection according to their exploratory response in an elevated plus-maze

Naive rats were tested in the elevated plus-maze test. The data of a typical experiment are presented in Table 1. The mean data obtained in the elevated plus-maze of the rats from both cages (containing 22 and 24 rats per cage) used in this experiment did not differ statistically (data not presented). From the cages where animal populations of 20–25 rats had been housed together for at least month it was always possible to find individuals with very different exploratory behavior in the elevated plus-maze. The differences seem to disappear when the selected rats are housed separately. However, retesting of animals in the elevated plus-maze is complicated. The test is largely based on the novelty of the situation (neophobia) for animals and therefore can be used successfully only once. Already during next testing overall considerably lower exploratory activity can be followed. This was confirmed

Table 2. Scatchard analysis of saturation data of 3H-Ro 5-4864 binding in rats selected according to their exploratory activity in an elevated plus-maze. For experiments pooled tissue of 6–8 animals was used. Each value is the mean ± SEM of at least four experiments each carried out in triplicate

Animal group	^{3}H-Ro 5-4864 binding	
	B_{max} (fmol/mg)	K_D (nM)
Adrenals		
Non-anxious	13894 ± 959	6.83 ± 0.71
Anxious	9128 ± 1121*	5.93 ± 0.64
Kidneys		
Non-anxious	8343 ± 619	4.94 ± 0.51
Anxious	6518 ± 438*	4.65 ± 0.71
Heart		
Non-anxious	4974 ± 534	7.03 ± 0.65
Anxious	5674 ± 695	6.19 ± 0.48

* $P < 0.05$ as compared to non-anxious group

Table 3. Scatchard analysis of saturation data of ^{3}H-Ro 5-4864, ^{3}H-diazepam and ^{3}H-PK 11195 in blood platelets of rats selected according to their exploratory activity in an elevated plus-maze. For each experiment the pooled trunk blood of 6–8 animals was used. The values represented are the mean ± SEM of at least four experiments each carried out in triplicate

Animal group	Ligand binding characteristics	
	B_{max} (fmol/10^8 cells)	K_D (nM)
^{3}H-Ro 5-4864		
Non-anxious	2117 ± 209	10.8 ± 0.9
Anxious	1329 ± 164*	9.3 ± 1.1
^{3}H-diazepam		
Non-anxious	451 ± 35	11.4 ± 1.2
Anxious	345 ± 46*	9.3 ± 0.8
^{3}H-PK 11195		
Non-anxious	1549 ± 243	7.0 ± 0.9
Anxious	1284 ± 138	6.8 ± 1.1

* $P < 0.05$ as compared to non-anxious animals

when after two weeks the previously tested rats were retested in an elevated plus-maze. However, the individuals belonging to both subgroups gained the tendency to display the same behavioral characteristics as during first trial (data not shown). To clarify the problem the plus-maze selected animals were also retested in the two-compartment test devised by Crawley and Goodwin (1980). The rats with lower exploratory activity in the plus-maze had a decreased number of visits into the brightly lit area (data not shown). However, probably due to big individual variations and a relatively small number of animals (6 per group) no statistically significant differences between the subgroups was found.

Previously it has been demonstrated that anxiolytic benzodiazepines enhance, while anxiogenic β-carbolines (like methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, DMCM) reduce the exploratory activity of rats and mice in elevated plus-maze (Pellow et al. 1985; Pellow and File 1986; Rägo et al. 1988). After testing the animals two subgroups of individuals with "anxiolytic" (diazepam-like) and "anxiogenic" (DMCM-like) behavioral characteristics were selected. Animals with diazepam and DMCM-like behavior were termed non-anxious and anxious respectively. The exploratory activity of these two newly formed subgroups differed significantly from the mean of the whole group (Table 1).

Comparative characterization of ^{3}H-flunitrazepam binding in various brain structures of non-anxious and anxious rats. In vitro experiments demonstrated that the number of ^{3}H-flunitrazepam binding sites was significantly lower in cerebral cortex but not in hippocampus or cerebellum of anxious animals (Figs. 1–3). No differences in the affinity for the ligand were found in the brain regions studied.

Comparative characterization of ^{3}H-Ro 5-4864 binding in adrenals, kidneys and heart of non-anxious and anxious rats. ^{3}H-Ro 5-4864 binding was considerably lower in adrenals and kidneys of anxious rats. No significant differences were found in heart. The affinity did not differ in all organs studied (Table 2).

Comparative characterization of peripheral BD binding sites in blood platelets and lymphocytes of non-anxious and anxious rats with different tritiated ligands. ^{3}H-Ro 5-4864 binding was significantly decreased in blood platelets of anxious rats. Although ^{3}H-diazepam and ^{3}H-PK 11195 binding was also lowered in anxious rats the differences between the groups studied were statistically insignificant. No clearcut changes in affinity for the ligands studied were found in platelets (Table 3). In lymphocytes the maximum binding of all ligands used was markedly lower in anxious rats. No affinity differences were seen in lymphocytes between the anxious and non-anxious groups (Table 4).

Discussion

The finding that the number of central BD receptors in cerebral cortex is decreased in rats demonstrating lowered exploratory activity in an elevated plus-maze test is in good correlation with our previous studies. Similar selection experiment carried out with mice resulted in a decreased number of cerebral cortex ^{3}H-flunitrazepam binding sites in anxious animals (Rägo et al. 1988). After the selection of mice according to their behavioral response to baclofen the subgroup of animals termed baclofen responders had also a lower number of central BD binding sites (Rägo et al. 1986) and decreased exploratory activity in an elevated plus-maze (Rägo et al. 1989a). These data altogether seem to support the idea that the behavioral differences observed in individuals from more numerous animal populations are possibly due to neurochemical changes caused by social interactions (social hierarchy in home cages) and/or genetic differences. However, the behavioral differences registered seem to reflect more the individual trait for the induction

Table 4. Scatchard analysis of saturation data of ^{3}H-Ro 5-4864, ^{3}H-diazepam and ^{3}H-PK 11195 binding in lymphocytes of rats selected according to their exploratory activity in an elevated plus-maze. For each experiment the pooled trunk blood of 6–8 animals was used. The data represented are mean ± SEM of at least four experiments each carried out in triplicate

Animal group	Ligand binding characteristics	
	B_{max} (fmol/10^6 cells)	K_D (nM)
^{3}H-Ro 5-4864		
Non-anxious	828 ± 62	7.6 ± 0.8
Anxious	632 ± 43*	8.5 ± 0.5
^{3}H-diazepam		
Non-anxious	127 ± 16	10.6 ± 0.8
Anxious	81 ± 13*	11.1 ± 1.7
^{3}H-PK 11195		
Non-anxious	1031 ± 83	4.8 ± 0.5
Anxious	819 ± 47*	5.3 ± 0.3

* $P < 0.05$ as compared to non-anxious group

of fear by novel situations. The present results demonstrating that peripheral BD binding sites in anxious rats are decreased in various peripheral organs like adrenals and kidneys are in line wiht some previous data. It has been reported that an inescapable tail shock produces a reduction of ^{3}H-Ro 5-4864 binding sites in rat kidney and heart (Drugan et al. 1986). Decrease of ^{3}H-flunitrazepam binding after foot shock was also found in adrenals and kidneys (Kiivet et al. 1988). However, other models of stress (laparatomy, swimming stress) have been shown to icnrease the number of peripheral BD binding sites in kidneys (Okun et al. 1988; Rägo et al. 1989a). It appares that increase or decrease in the density of peripheral BD recognition sites depends on the nature, intensity and duration of stressful stimuli. Data obtained in this study and our previous results (Rägo et al. 1989a) confirm that BD binding sites in the heart are less sensitive to anxiety or stress. Our data demonstrating that in anxious animals the number of peripheral BD binding sites is reduced both in blood platelets and lymphocytes are supported by the data obtained in anxiety patients. Recently it has been shown in several studies that the binding capacity of the BD binding sites on platelets and lymphocytes of anxious patients is reduced in comparison to normal controls (Weizman et al. 1987; Ferrarese et al. 1989; Ferrero et al. 1989). In present series of experiments with blood cells we used several ligands (^{3}H-Ro 5-4864, ^{3}H-diazepam and ^{3}H-PK 11195) to label peripheral BD recognition sites. No principal differences occurred although changes in binding of some ligands were not statistically significant. Probably more numerous experiments could demonstrate the significance of the changes observed. Ro 5-4864 is believed to be an agonist while PK 11195 is considered an antagonist of peripheral BD binding sites (Mestre et al. 1985). Our results demonstrate that the differences in binding of presumed peripheral BD recognition sites agonists and an antagonist in anxious rats are similar. One of the main findings of the present study is that in anxious animals the central BD receptors in cerebral cortex are downregulated similarly to peripheral BD binding sites in several peripheral tissues including blood platelets and lymphocytes. The existence of correlation between central BD receptor changes in cerebral cortex and peripheral binding sites in kidneys (an increase in both tissues) has been demonstrated also in earlier studies (Okun et al. 1988; Rägo et al. 1989a). This may indicate the possibility to use peripheral BD binding sites on platelets and/or lymphocytes as readily available markers of the functional activity of central BD receptors.

In spite of the considerable recent interest and success in peripheral BD binding site research the mechanisms responsible for the up- and downregulation of these binding sites remain unclear. Acute stress in rats (Rägo et al. 1989b) and humans (Karp et al. 1989) causes rapid increase of peripheral BD binding sites in platelets. This may indicate the presence of some endogeneous modulator (ligand?) in peripheral blood. So far several substances of known (porphyrins, DBI) and unknown chemical structure have been reported to interfere with peripheral BD binding sites (for review see Saano et al. 1989). However, further studies are necessary to assertain the role of possible endogeneous modulators in regulation of peripheral BD recognition sites.

In conclusion, the data presented here are evidence that central BD receptors in cerebral cortex and peripheral-type benzodiazepine binding sites in many peripheral tissues including platelets and lymphocytes are lower in endogeneously anxious rats than in non-anxious rats. The mechanisms involved in this phenomenon remain to be elucidated.

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