Pharmacology Biochemistry and Behavior, Vol. 65, No. 1, pp. 35–38, 2000

0091-3057/00/$–see front matter

PII S0091-3057(99)00131-8

Hypoexpression of Benzodiazepine Receptors in the Amygdala of Neophobic BALB/c Mice Compared to C57BL/6 Mice

Y. HODE,* C. RATOMPONIRINA,* S. GOBAILLE,* M. MAITRE,* C. KOPP† AND R. MISSLIN†

*Institut de Chimie Biologique et UPR 416 CNRS, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France; and †Laboratoire d'Ethologie et de Neurobiologie, 7 rue de l'Université, 67000 Strasbourg, France

Received 26 February 1999; Revised 17 May 1999; Accepted 17 May 1999

HODE, Y., C. RATOMPONIRINA, S. GOBAILLE, M. MAITRE, C. KOPP AND R. MISSLIN. *Hypoexpression of benzodiazepine receptors in the amygdala of neophobic BALB/c mice compared to C57BL/6 mice.* PHARMACOL BIOCHEM BEHAV **65**(1) 35–38, 2000.—The distribution of benzodiazepine receptors in the brain of neophobic BALB/c mice was studied by autoradiographic analysis using [^{3}H]-diazepam and compared to that of the same receptors of the "nonemotional" C57BL/6 mice. This technique revealed no significant interstrain difference except for a lower density of diazepam binding sites in the amygdala of BALB/c mice. Therefore, the expression of benzodiazepine receptors in the amygdala of the two strains of mice were quantified by binding studies on brain membranes. The amygdala of BALB/c mice exhibited a fivefold decrease in the density of benzodiazepine receptors compared to C57BL/6 mice. These results suggest that the trait anxiety (neophobia) that characterizes BALB/c mice could be due, at least in part, to a genetic modulation of benzodiazepine receptor expression in the amygdala, a structure known to be strongly involved in fear behavior. © 1999 Elsevier Science Inc.

Benzodiazepine receptors Amygdala BALB/c and C57BL/6 mice Trait anxiety

HIGHLY inbred strains of mice were often used to investigate relations between genes and behavior. Strains were inbred from different stocks of mice and exhibited substantial phenotypic differences (10). C57BL/6 and BALB/c mice have repeatedly been found to differ strongly in several behavioral responses (3,15,16) as well as in neurodevelopmental and neurochemical parameters (14). For example, BALB/c mice exhibit more strongly anxious responses in the light/dark choice test (3), in the open-field paradigm (15), and in a runway traversal locomotor activity test (16). More recently, BALB/c mice have been reported to exhibit strong defensive responses toward unfamiliar places (neophobia), in a free-exploratory paradigm, when compared with C57BL/6 mice (9). BALB/c mouse neophobia has been proposed as exemplifying the so-called "trait" anxiety (4,11,19), which refers to an enduring feature and a stable characteristic of behavior (2,9). On the other hand, "state" anxiety can be considered as a form of anxiety that is triggered by stressful situations, as in many models of anxiety based on the forced exposure of rodents to unfamiliar enclosures (7). Moreover, it has been demonstrated (3), using a principal component analysis, that behavioral parameters recorded in a free-exploratory test, devoid of stressful components (13), and presented as an effective method for measuring neophobia in BALB/c mice (9), were not described by the same factors as the parameters recorded in test situations involving stressful components. These results suggest that trait and state anxieties must be considered as referring to different behavioral responses and probably to different neurochemical mechanisms. Interestingly, it has been shown that drugs that are agonists at benzodiazepine recognition sites were able to completely abolish neophobia in BALB/c (9). In contrast, other anxiolytic compounds such as the 5-HT_{1A} receptor agonist 8-OH-DPAT, the 5-HT_3 antagonist zacopride, the CCK-B receptor antagonist PD 135158, the α_2-adrenoreceptor antagonists yohimbine and idazoxan, as well as the D_2 dopaminergic antagonist sulpiride were ineffective in reducing neophobic responses of BALB/c mice (2,9). Furthermore, it has been demonstrated that

Requests for reprints should be addressed to Y. Hode, Institut de Chimie Biologique et UPR 416 CNRS, Faculté de Médicine, 11 rue Humann, 67085 Strasbourg, Cedex, France.

BALB/c mice exhibited higher susceptibility than C57BL/6 to convulsions induced by the inverse benzodiazepine receptor agonist, β-carboline-3-carboxylate (β-CCM) (6). A lower level of benzodiazepine binding sites, without changes in K_d, has been found in the whole brain of BALB/c mice compared to C57BL/6, using either [^{3}H]-diazepam or [^{3}H]-flunitrazepam as ligands (5,17). Finally, a diazepam potentiation of the GABA-mediated chloride influx into brain microsacs has been reported to be lower in BALB/c than in C57BL/6 mice, showing for the first time hypofunctional ability of diazepam to potentiate GABAergic mechanisms in BALB/c mice (12). Taken together, these data suggest a possible implication of brain benzodiazepine receptors in the differing reactivity of the two strains toward unfamiliarity as well as in their β-CCM sensitivity.

Therefore, the present study was designed to examine if the differences in benzodiazepine binding observed in the whole brain of BALB/c mice compared to C57BL/6 were, in fact, related to a more specific brain localization, such as limbic structures, involved in the control of emotional responses. An autoradiographic study with [^{3}H]-diazepam was first carried out on brain slices to detect potential regional differences in the density of benzodiazepine binding receptors. Second, because the autoradiography revealed significant variations only in the amygdalas, a saturation curve analysis on brain membranes obtained from this structure was performed to determine more precisely the kinetic parameters of diazepam binding.

METHOD

Animals

Male BALB/c and C57BL/6 mice from IFFA CREDO (France) Breeding Center were used, and were 10 weeks of age when sacrificed. Mice were housed by five in standard cages (26 × 20 × 14 cm) with food pellets and water available ad lib under controlled conditions of a light:dark cycle (12 L:12 D, lights on at 0100 h) and temperature (24 ± 1°C). Animals were sacrificed at 1000 h. The experimental procedures carried out in this study were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Receptor Autoradiography

The animals were killed by decapitation, and their brains were rapidly removed and frozen in isopentane maintained at −40°C with dry ice. Coronal section of the brains (20 μm thick) were cut with a cryostat and thaw mounted onto gelatin-coated slides. Sections were stored at −80°C until the assay. For binding experiments, slices of both strains were treated in the same set of experiments to ensure comparable conditions. Slices were incubated for 1 h in Tris-HCl buffer (50 mM, pH 7.4, 4°C) with [^{3}H]-diazepam (0.5 to 4 nM; 2.9 Tbq/mmol or 78 Ci/mmol, Dupont NEN) followed by washing in two baths of the same buffer (30 s each time) and rinsed briefly in distilled water. Then slices were dried in a stream of cold air. Autoradiograms were generated by exposing the labeled tissue sections to tritium-sensitive film (Hyperfilm, Amersham, UK) for a 3-week period. Optical densities of autoradiograms were determined using a computerized analyzer system (SAMBA software, Alcatel, France).

Binding Studies on Brain Membranes

After mice were killed by decapitation, brains were rapidly dissected on a cold glass plate. Amygdalas of each animal were extracted and kept frozen in liquid nitrogen over a period not exceeding 2 weeks. Samples were homogenized in 10 vol of Tris-HCl buffer (50 mM, pH 7.4, 4°C). The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged again at 50,000 × *g* for 10 min. Pellets were resuspended in the same volume of ice-cold buffer and washed three times under the same conditions. Finally, the washed pellets were resuspended in a volume of the same buffer that yielded a final protein concentration of about 500 μg/ml in the assay tube. This receptor preparation was then divided in tubes to which a series of dilutions of the radioligand ([^{3}H]-diazepam; specific activity 2.9 Tbq/mmol or 78 Ci/mmol Dupont NEN) were added in triplicate, to obtain a final volume of 0.5 ml/tube.

Nonspecific binding was estimated by the addition of 1 μM of clonazepam in the incubation medium. Incubations of 30 min at 4°C were followed by a rapid filtration on Whatman GF/B filters, and the membrane fragments on the filters were washed two times with 2 ml of cold incubation medium. The filters were placed in vials with 5 ml of scintillation fluid and left overnight before counting on a Beckman scintillation counter.

Protein concentrations were determined by the BCA method (Pierce, USA). *B*max and K_d were calculated by nonlinear regression analysis that provides more reliable estimations than Scatchard linearization (GraphPad Prism software, San Diego, CA). Deviation from the model was checked with the runs test. The significance of differences between BALB/c and C57BL/6 for both *B*max and *K*d was assessed by using Student's *t*-test.

RESULTS

Anatomical Localization of Differences in the Density of Benzodiazepine Sites Between the Two Strains of Mice

In preliminary experiments, several concentrations of [^{3}H]-diazepam (0.5 to 4 nM) were used to determine the optimal binding conditions for investigating [^{3}H]-diazepam binding in the brains of the two strains of mice. The best images were obtained using 1 nM of [^{3}H]-diazepam and an exposure time of 3 weeks. The washing and rinsing times were also varied. The conditions that provided the best ratio of total to nonspecific binding with enough signal were two washing periods of 30 s followed by a quick rinsing period. Under these conditions, the autoradiographic images of BALB/c and C57BL/6 brains showed a significant difference in [^{3}H]-diazepam binding sites only in the amygdala (central part mainly) (Fig. 1). Quantification of the optical density of the autoradiographs of this region confirmed the existence of a difference of about 11% ($p < 0.0049$, $n = 4$) between the two strains. The other regions (see Table 1) did not present any significant differences in optical densities.

Determination of the Kinetic Parameters of Benzodiazepine Binding in the Amygdala of the Two Strains

Based on the autoradiographic results, the measurement of the kinetic parameters of [^{3}H]-diazepam binding was carried out on brain membranes from the amygdalas of both strains of mice. Studies were performed on amygdalas obtained from 30 mice of each strain. Saturation curves for both strain were clearly different (Fig. 2). According to the runs test, the deviation from the model of one site binding was never significant, and the goodness of fit was always satisfactory (r^2 above 0.95). K_ds for diazepam binding showed no significant differences between the two strains (Table 2). However, the B_{max} was significantly lower in BALB/c than in C57BL/6, confirming the indication of the autoradiographic

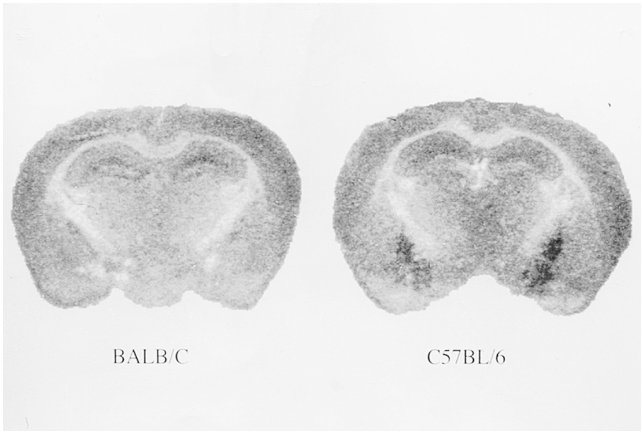

FIG. 1. Autoradiograms of diazepam binding. Central amygdala of BALB/c mice (left) is less labeled than central amygdala of C57BL/6 mice (right). No differences were observed in other brain structures.

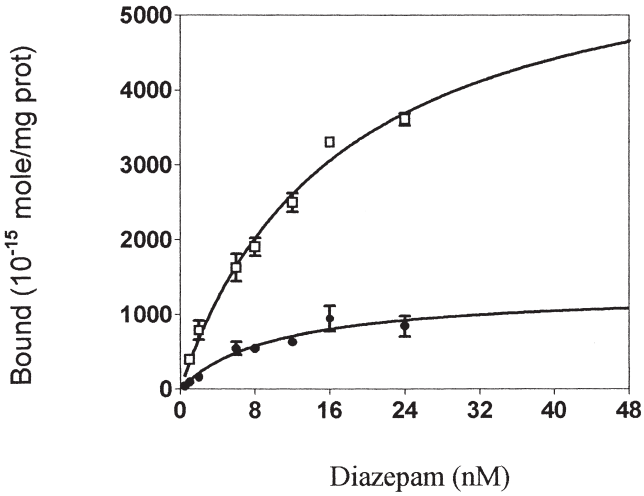

FIG. 2. Specific binding of [^{3}H]-diazepam as a function of free ligand concentration at equilibrium. The data were obtained from amygdala membranes of C57BL/6 (white squares) and BALB/c (black circles).

experiments (1.3 ± 0.24 pmol/mg protein for BALB/c vs. 6.3 ± 0.72 pmoles/mg protein for C57BL/6; $p < 0.0004$).

DISCUSSION

The aim of the present study was to carry out systematic comparisons of the density of benzodiazepine binding sites in several brain areas of mice originated from two inbred strains, known to be "emotional" (BALB/c) and "nonemotional" (C57BL/6) mice. Two different techniques were used: an autoradiographic approach with [^{3}H]-diazepam followed by a brain membrane binding study. The present results show that, among the examined brain areas, amygdala was the one that allowed to best discriminate BALB/c mice from C57BL/6 mice with regard to the density of benzodiazepine binding sites: BALB/c mice were found to present a significantly lower density than C57BL/6 mice in this structure. These data are in good agreement with previous studies performed on the whole brain (5,17). It must be emphasized that the differences between the two strains of mice were less striking in autoradiographic study than in binding study on membranes. These two techniques are known to provide data that are not strictly similar in regard to the differences in receptor availability and in specific/nonspecific binding ratio. In the present autoradiographic study, only total binding was measured, while in the study on membranes the specific binding was determined. Because the autoradiographic technique is unable to detect small changes, differences in the density of binding sites between the two strains cannot be entirely excluded in brain areas other than amygdala.

It has been well established that amygdala plays a critical role in many of the components of fear responses (18,20). As a consequence, it is not surprising that BALB/c mice, generally presented as an emotional strain, exhibited a lower expression of benzodiazepine receptors, particularly in amygdala. Interestingly, benzodiazepine receptor agonists were previously found to completely abolish the neophobia of BALB/c mice in the free-exploratory paradigm, whereas they were shown to be devoid of anxiolytic effect in C57BL/6 mice (9). In this context, it can be remembered that, in the rat, the beneficial effects of a benzodiazepine have also been shown to closely depend on the baseline conditions of the animals, re-

TABLE 1

COMPARISON OF OPTI CAL DENSITY MEASURED ON THE AUTORADIOGRAPHIC IMAGES OF [^{3}H]-DIAZEPAM BINDING IN DIFFERENT BRAIN REGIONS OF BALB/c AND C57BL/6 MICE

Brain Regions	BALB/c strain	C57BL/6 strain	Significance
Prefrontal cortex	156.2 ± 6.1	157.5 ± 3.9	ns
Frontal cortex	159.5 ± 2.1	160.5 ± 2.2	ns
Parietal cortex	155.0 ± 2.8	155.2 ± 2.8	ns
Temporal cortex	154.0 ± 2.2	156.2 ± 4.0	ns
Hippocampus	148.6 ± 2.0	150.3 ± 4.8	ns
Olfactori tubercles	143.5 ± 3.1	143.5 ± 2.1	ns
Caudate-putamen	149.5 ± 6.2	148.0 ± 2.2	ns
Globus pallidus	142.3 ± 2.5	138.7 ± 3.3	ns
Nucleus accumbens	149.3 ± 5.7	146.0 ± 2.7	ns
Thalamus	147.5 ± 3.4	147.2 ± 3.7	ns
Amygdala	146.2 ± 4.9	162.8 ± 5.8	$p < 0.005$

Data are expressed as the mean ± SD ($n = 4$) of the optical density. These values were obtained from digitized images of autoradiograms and analyzed with Student's *t*-test.

TABLE 2

KINETICS OF [^{3}H]-DIAZEPAM BINDING EXPERIMENTS OBTAINED WITH MEMBRANES PREPARED FROM THE AMYGDALA OF BALB/c AND C57BL/6 MICE (DETERMINATIONS PERFORMED IN TRIPLICATE ON 30 ANIMALS)

	BALB/c strain	C57BL/6 strain	Significance
B_{max} (10^{-12} mol/mg)	1.3 ± 0.24	6.3 ± 0.72	$p < 0.0004$
Kd (nM)	10.3 ± 4.2	16.8 ± 3.5	NS
r^2	0.95	0.99	

Values are means ± SEM. Data were analyzed by Student's *t*-test.

garding the density of benzodiazepine receptors or the behavioral responses in the elevated plus-maze test (1,8). Because the amygdala no longer appeared as a structural or a functional unit (20), it may be noted that the differences between BALB/c and C57BL/6 mice in the autoradiographic images of diazepam binding were mainly localized in the central nucleus of the amygdala, and to a lesser extent in a part of the medial nucleus. Thus, the central nucleus, which is known to be involved in fear-related behaviors (18), could be a good candidate for a key region implicated in the genetically determined predisposition to anxiety.

In conclusion, the present results, along with previous neurochemical and behavioral findings, suggest that the deficit in benzodiazepine receptors of amygdala could account, at least in part, for the high level of general emotional responsiveness of BALB/c mice (3,15,16), as well as for their neophobia, which can be considered as a characteristic feature of trait anxiety (2,9). However, it would be of great interest to assess whether flumazenil, a benzodiazepine receptor antagonist, injected in the central nucleus of amygdala could counteract the neophobia-reducing properties of intraperitoneally-administered chlordiazepoxide in BALB/c mice to further investigating the relationship between the deficit in benzodiazepine receptors in the amydgala of BALB/c mice and their neophobia.

ACKNOWLEDGEMENTS

Clonazepam was a generous gift from Hoffmann–La Roche, Switzerland.

REFERENCES

1. Andrews, N.; Zharkovsky, A.; File, S. E.: Acute handling stress downregulates benzodiazepine receptors: Reversal by diazepam. Eur. J. Pharmacol. 210:247–251; 1992.
2. Belzung, C.; Berton, F.: Further pharmacological validation of the BALB/c neophobia in the free exploratory paradigm as an animal model of trait anxiety. Behav. Pharmacol. 8:541–548; 1997.
3. Beuzen, A.; Belzung, C.: Link between emotional memory and anxiety states: A study by principal component analysis. Physiol. Behav. 58:111–118; 1995.
4. Cattell, R. B.: Personnality and mood by questionnaire. San Francisco: Jossey Bass; 1979.
5. Chapouthier, G.; Bondoux, D.; Martin, B.; Desforges, C.; Launay, J.-M.: Genetic difference in sensitivity to β-carboline: Evidence for the involvement of brain benzodiazepine receptors. Brain Res. 553:342–346; 1991.
6. Desforges, C.; Venault, P.; Dodd, R. H.; Chapouthier, G.; Roubertoux, P. L.: β-Carboline-induced seizure in mice: Genetic analysis. Pharmacol. Biochem. Behav. 34:733–737; 1989.
7. File, S. E.: Animal models of different anxiety states. Adv. Biochem. Psychopharmacol. 48:93–113; 1995.
8. File, S. E.; Andrews, N.; Wu, P. Y.; Zharkovsky, A.; Zangrossi, H.: Modification of chlordiazepoxide's behavioural and neurochemical effects by handling and elevated-plus maze experience. Eur. J. Pharmacol. 218:9–14; 1992.
9. Griebel, G.; Belzung, C.; Misslin, R.; Vogel, E.: The free-exploratory paradigm: An effective method for measuring neophobic behavior in mice and testing potential neophobia-reducing drugs. Behav. Pharmacol. 4:637–644; 1993.
10. Lindzey, G.; Thiessen, D., eds.: Contribution to behavior-genetic analysis. The mouse as a prototype. New York: Appleton-Century-Grafts, Educational Division/Meredith Corporation; 1970.
11. Lister, R. G.: Ethologically-based animal models of anxiety disorders. Pharmacol. Ther. 46:321–340; 1990.
12. Mihic, J.; Van Berckel, B.; O'Dowd, B.; Nguyen, T.; Wu, P.: Effects of sedatives on GABA-mediated chloride flux into cerebral cortical microsacs prepared from emotional and non-emotional mice. Eur. J. Pharmacol. 218:283–286; 1992.
13. Misslin, R.; Cigrang, M.: Does neophobia necessarily imply fear or anxiety? Behav. Process. 12:45–50; 1986.
14. Nowakowski, R. S.: The mode of inheritance of a defect in lamination in the hippocampus of BALB/c mice. J. Neurogenet. 1:249–258; 1984.
15. Oliverio, A.; Eleftheriou, R. S.; Bailey, D. W.: A gene influencing active avoidance performance in mice. Physiol. Behav. 11:497–501; 1973.
16. Peeler, D. F.; Nowakowski, R. S.: Genetic factors and the measurement of exploratory activity. Behav. Neural Biol. 48:90–103; 1987.
17. Robertson, H. A.: Benzodiazepine receptors in "emotional" and "non-emotional" mice: Comparison of four strains. Eur. J. Pharmacol. 56:163–166; 1979.
18. Rosen, J. B.; Schulkin, J.: From normal fear to pathological anxiety. Psychol. Rev. 105:325–350; 1998.
19. Spielberger, C. D.: Anxiety and behavior. New York: Academic Press; 1966.
20. Swanson, L. W.; Petrovitch, G. D.: What is the amygdala? Trends Neurosci. 21:323–331; 1998.