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# INDEPENDENT BENZODIAZEPINE AND BETA-CARBOLINE BINDING SITES IN THE BRAIN OF AGGRESSIVE AND TIMID-DEFENSIVE MICE

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The view has now been formulated that specific ligands of benzodiazepine binding sites constitute a continuum: from complete agonists through antagonists to complete inverting agonists, the beta-carbolines [4]. However, binding sites with increased affinity for beta-carbolines only may perhaps exist.

The aim of this investigation was to study the distribution of specific binding sites of labeled benzodiazepine and beta-carboline derivatives in parts of the brain of intact aggressive and timid-defensive mice, and also of animals subjected to subchronic administration of diazepam.

## EXPERIMENTAL METHOD

Experiments were carried out on 90 male albino mice weighing 28-30 g — aggressive (AG) and timid-defensive (TD) animals, kept in isolation for 6 weeks. The TD mice were kept in the same cage as an AG partner, which was changed daily, and was subjected to painful electrical stimulation of threshold strength (5 min).

Intact animals and also mice receiving subchronic treatment with diazepam (14 days, 5 mg/kg intraperitoneally), were decapitated 24 h after the last injection, the brain was removed and the cerebral hemispheres, diencephalon, and brain stem were separated in the cold [8] and frozen in liquid nitrogen. The tissue was then thawed and homogenized in isolation medium (0.32 M sucrose, 0.05 M phosphate buffer, pH 7.4, at 25°C) in a glass homogenizer with Teflon pestle and for 10 sec on a RT-2 mechanical tissue microblender. The homogenate (10%) was centrifuged for 10 min at 1000g and the supernatant for 20 min at 12,000g. The residue of the fraction of unpurified synaptosomes thus obtained was washed 5 times in 0.05 M phosphate buffer (pH 7.4), poured into polyethylene flasks and kept at −20°C for not more than 2 weeks. The incubation mixture for binding of $^3$H-flunitrazepam ($^3$H-flu) and $^3$H-beta-carboline-3-carboxylate ethyl ester ($^3$H-BCCE; both substances were obtained from Amersham Inrernational, England) contained $^3$H-flu in concentrations of 0.5, 1.1, 1.8, 2.4, 3.1, 3.7, 4.4, and 5 nM or $^3$H-BCCE in concentrations of 0.6, 1.4, 2.1, 2.9, 3.7, 4.4, 5.2, and 6 nM, nonradioactive diazepam ($10^{-7}$ M), phosphate buffer, and 0.4 ml of homogenate of synaptic membranes. Incubation continued for 60 min at 0°C when the reaction was stopped by addition of 4 ml of cold buffer to each sample, followed by filtration of the material in vacuo through glass fiber

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TABLE 1. Relative Duration (in relative units) of Manifestation of Individual Behavioral Actions and Postures for Isolated AG and TD Mice (M ± m)

| Group of mice | Aggression/defense | Intraspecific sociability | Motor activity | |
|---|---|---|---|---|
| | | | horizontal | vertical |
| Intact: | | | | |
| AG | 12,6±2,26* | 18,7±5,89* | 14,6±1,46 | 11,45±2,24 |
| TD | 34,8±4,89* | 9,9±2,03* | 5,0±1,18 | 3,3±1,84* |
| Receiving diazepam | | | | |
| AG | 0,8±0,31 | 50,8±3,72 | 14,3±1,94 | 9,2±1,45 |
| TD | 7,4±3,60 | 32,6±4,74 | 6,8±0,90 | 11,4±2,18 |

Legend. *$p < 0.01$ for comparison of group of intact mice with group of mice receiving diazepam.

CTB filters (Whatman, England). The filters were washed twice with 4 ml of the same buffer and placed in flasks containing 10 ml of Bray's scintillator. After incubation for 12 h at room temperature their radioactivity was determined with an SL-4000 counter (Contron, France). Adsorption isotherms were analyzed by Scatchard plot. The protein content in the samples was determined by the method in [6]. The behavioral parameters were assessed by a computerized ethologic method [1, 2] in tests in which AG and TD mice, kept in isolation, were paired with other males kept in a group. The relative duration of exhibition of behavioral actions and postures was determined. The statistical significance of differences was assessed by Student's t-test.

## EXPERIMENTAL RESULTS

The AG behavior of male mice interacting with a partner was characterized by predominance of attacking and threatening behavior, accompanied by little attempt to investigate the partner. The defensive behavior of the mice was characterized, not only by the paucity of locomotor and investigative activity, but also by dominance of sideways and vertical defensive stances, standing still, and avoiding the partner (Table 1).

Analysis of specific binding of $^3$H-flu and $^3$H-BCCE with brain membranes of animals not receiving diazepam (Table 2) showed that the concentration of specific binding sites for both ligands in both AG and TD mice was significantly higher in the cortex ($p \leq 0.01$) than in other brain regions. Differences between groups of AG and TD mice were discovered only by analysis of brain-stem structures: the concentration of specific binding sites for $^3$H-flu in AG animals in these regions was significantly lower, whereas that for $^3$H-BCCE was significantly higher (in both cases $p \leq 0.01$) than in TD mice. It is important to note that the concentration of specific binding sites for $^3$H-BCCE in the cortex of AG and TD animals and in the brain-stem structures of AG mice was significantly higher than the concentration of benzodiazepine receptors measured with the aid of $^3$H-flu.

Subchronic administration of diazepam (5 mg/kg) to AD mice led to a significant decrease in aggression and an increase in investigation of the partner and the surroundings (Table 1). In TD mice the defensive type of reaction was reduced, and social investigation or passive interaction with the partner predominated. Analysis of receptor changes during subchronic administration of diazepam (Table 2) showed that the drug did not change the affinity of the $^3$H-flu binding sites but significantly reduced $K_d$ or the $^3$H-BCCE binding sites. Under the influence of diazepam the concentration of $^3$H-flu binding sites changed variously depending on the group of animals and the brain region. For instance, in AG mice it was reduced in the diencephalic structures, whereas in TD animals it was increased in the brain stem but reduced in the diencephalon and cortex. Administration of diazepam did not change the concentration of $^3$H-BCCE binding sites in the brain of AG mice. Changes in this characteristic in TD animals were in the same direction as with $^3$H-flu: an increase in the concentration of $^3$H-BCCE binding sites in the brain stem but a decrease in the cortex compared with animals not receiving diazepam.

The presence of similar affinity and similar concentration of binding sites for $^3$H-flu in the cortex and diencephalon of AG and TD animals correlates with data obtained by the writers on whole mouse brain homogenate [3]. These data show that mice kept in isolation have similar values of $K_d$ and $B_{max}$ for $^3$H-flu binding, which differ significantly from animals kept in a group. These changes in benzodiazepine receptors are linked with the general reaction to the stress of isolation and interaction between agonists. The differences between the brain-stem

TABLE 2. Values of $K_d$ and $B_{max}$ for $^3$H-flu and $^3$H-BCCE Obtained for Synaptic Membranes from Different Parts of the Brain of AG and TD Mice (M ± m)

| Group of mice | Brain structure | $^3$H-flu | | $^3$H-BCCE | |
|---|---|---|---|---|---|
| | | $K_d$, nmoles | $B_{max}$, fmoles/mg protein | $K_d$, nmoles | $B_{max}$, fmoles/mg protein |
| Intact: | | | | | |
| AG | A | 1,54±0,13 | 443±18* | 1,08±0,14 | 714±37 |
| AG | B | 1,79±0,12 | 892±33 | 0,68±0,11 | 777±45 |
| AG | C | 1,57±0,13 | 1198±56* | 1,35±0,08 | 2197±73 |
| TG | A | 1,45±0,11 | 675±26* | 1,17±0,07 | 597±17 |
| TG | B | 1,61±0,08 | 922±25 | 0,89±0,08 | 873±32 |
| TG | C | 1,52±0,12 | 1224±50* | 1,21±0,10 | 2203±96 |
| Receiving diazepam | | | | | |
| AG | A | 1,74±0,12 | 404±12* | 0,66±0,10* | 724±40 |
| AG | B | 1,86±0,15 | 422±16*,** | 0,62±0,08 | 775±38 |
| AG | C | 1,44±0,12 | 1220±52* | 1,07±0,6* | 2077±57 |
| TD | A | 1,61±0,73 | 874±18*,** | 0,75±0,04* | 1480±30* |
| TD | B | 1,60±0,08 | 802±21*,** | 0,59±0,06* | 1561±29* |
| TD | C | 1,40±0,08 | 978±30*,** | 0,65±0,03* | 1808±28* |

Legend. A) Brain stem structures, B) diencephalic structures, C) cerebral cortex. *Significant differences obtained when comparing binding characteristics of $^3$H-flu and $^3$H-BCCE; **) significant differences obtained when comparing group of intact mice with animals receiving diazepam.

structures of AG and TD mice revealed by a more detailed analysis may be connected with the specific nature of the adaptive response of animals of different groups to stress.

The marked predominance of $^3$H-BCCE binding sites in the cerebral cortex of all the experimental animals and in the brain-stem structures of AG mice relative to $^3$H-flu binding sites is evidence that specific binding sites exist for beta-carboline-3-carboxylate (BCC). These results may be confirmed by data published previously for the rat brain [5], showing incomplete identity of binding sites for $^3$H-flu and $^3$H-BCCE. The possibility cannot be ruled out that binding sites for BCC, which are independent, are partially coupled with benzodiazepine binding sites. Preliminary treatment of brain membranes in vitro with diazepam, for instance, led to increased binding affinity for BCC [7]. In our own experiments with subchronic administration of diazepam in vivo, $K_d$ for $^3$H-BCCE binding sites also fell in virtually all parts of the experimental animals' brain irrespective of the behavioral characteristics. Thus the animal brain contains BCC receptors which differ structurally and functionally from benzodiazepine receptors.

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