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Changes in GABA–Benzodiazepine Receptor Complex and in Peripheral Benzodiazepine Receptors in Male Mice After Copulation

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SAANO, V., L. RÄGO, E. TUPALA AND M. M. AIRAKSINEN. *Changes in GABA-benzodiazepine receptor complex and in peripheral benzodiazepine receptors in male mice after copulation.* PHARMACOL BIOCHEM BEHAV 51(2/3) 529–533, 1995.—We studied the effect of copulation on GABA and benzodiazepine (BZD) receptors in the male mouse. After copulation, there was an 18% increase in the in vitro number of [^{3}H]muscimol binding sites in frontal cortex. No changes were observed in central BZD binding sites labelled either in vivo by [^{3}H]flunitrazepam or in vitro (in olfactory bulbs and in frontal cortex) by [^{3}H]flumazenil, but further in vitro studies demonstrated that the GABA-stimulated [^{3}H]flunitrazepam binding was reduced in both frontal cortex and olfactory bulbs. Copulation increased the number of peripheral BZD binding sites labelled by ^{3}H-Ro 5-4864 in olfactory bulbs by 22% and in heart by 36%, but not in frontal cortex or in testes. The changes of GABA/BZD and peripheral BZD receptors in mouse suggest that the GABAergic system may be affected by copulation.

Central- and peripheral benzodiazepine receptors GABA receptors Mouse brain Sexual behaviour

SLIGHTLY increased levels of anxiety may result in premature ejaculation, whereas high levels of anxiety completely inhibit copulatory behavior (25). The involvement of GABA$_A$/benzodiazepine-Cl-ionophore receptor complex in anxiety, alertness, and responses to stress is well established (2,7,21). Anxiolytic and anxiogenic ligands of benzodiazepine (BZD) receptors inhibit or facilitate some measures of sexual behaviour (9,17). Therefore, BZD receptors may be of importance not only in the control of anxiety but also in sexual behaviour. However, the role of GABA/BZD receptors in the neurochemical mechanisms of male sexual behaviour has been sparsely studied.

BZD tranquilizers bind with varying affinities to central and peripheral-type binding sites. The first type of these receptors is found only in the CNS, whereas the second is found in a variety of peripheral organs and tissues as well as in the CNS (23). The central BZD receptor is a part of the macromolecular GABA$_A$ receptor complex that includes binding sites for the inhibitory neurotransmitter GABA, BZDs, and chloride channel. In contrast to central BZD receptors, the peripheral-type BZD binding site is not modulated by GABA in vitro, nor does it contain a binding site for this neurotransmitter (16). GABAergic transmission influences the peripheral BZD receptors, albeit indirectly. They can be modulated by various types of stress and anxiety (7), such as forced swimming, which increases the density of BZD receptors in rat kidneys (22). Muscimol (GABA$_A$ agonist) increases the affinity of peripheral BZD binding sites in kidney and brain, and baclofen (GABA$_B$ agonist) also increases the number of these binding sites in rat kidney (20).

In the present study we investigated the effects of copulation on GABA and central BZD receptors in the mouse brain. We also studied the peripheral BZD binding sites in both the CNS and the periphery.

METHOD

Animals

Male and female NMRI mice (National Laboratory Animal Centre, University of Kuopio, Finland) weighing 32–41 and

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28–34 g, respectively, were used. Pelleted food (Astra-Ewos, Vadstena, Sweden) and tapwater were available ad lib. Animals were maintained on a 14-h light period (lights on 0700–2100 h). The temperature of the animals' room was 22 ± 1°C, and the relative humidity was 50–60%. Before the experiments (3 days) male mice were housed individually and females with two animals per cage. The reversed lighting cycle (lights on 2100–0700 h) was used during this period. Female mice were brought into estrus with 0.1 mg/kg SC stilbestrol (Honvan® 50 mg/ml; ASTA Pharma, Frankfurt, Germany) 24 h before the experiment. Observations of sexual behaviour began 3 h after the onset of darkness.

Experiment 1

[^{3}H]Flunitrazepam in vivo. Male mice were placed in a cage with two female mice brought into oestrus. The control male mouse was placed alone in a cage. Each mouse from this group was a control to one of the copulating mice, and the time kept in the cage was matched.

Immediately after the first intromission, or after the matched stay alone in the cage (controls), the central BZD receptors were labelled by injecting 0.46 μg/kg [^{3}H]flunitrazepam (total volume 12.5 ml/kg; spec. act. 77.4 Ci/mmol; New England Nuclear, Boston, MA) into the tail vein of the male mouse. Two minutes after the termination of the injection, the mouse was decapitated and the brain was rapidly removed. To minimize delays in tissue processing, only two tissue blocks were used: the cerebellum was processed as one block and the cerebrum as another. Tissue was homogenized into 32 vol. of 50 mM Tris-HCl (pH 7.4). Two minutes after the decapitation, two 500-μl aliquots of the homogenate were filtered onto Whatman GF/B filters (Whatman, Ltd., Maidstone, UK). To determine nonspecific binding, half of the homogenate was incubated for 60 min in the presence of 10 μM concentration of unlabelled flunitrazepam.

Experiment 2

Benzodiazepine and GABA binding in vitro. A male mouse was placed into a cage with two receptive female mice to start the experiment. After the first intromission the male mouse was immediately decapitated. The brain, heart, and testes were rapidly removed and put on dry ice. From the brain, the olfactory bulbs and ventral forebrain below the rhinal fissure containing the anterior olfactory nucleus, tuberculum olfactorium, and primary olfactory cortex were separated. Because of the small amount of tissue obtained from each animal, explants had to be pooled. Binding was determined using three to four separate tissue pools, each from tissue explants from four to six mice. The total number of animals was 15–20 per group. Control animals were matched with regard to body weight and the time spent in an empty cage which was kept in the lighting conditions of the laboratory where the experiment was performed. The frozen brains were stored on dry ice until the end of the experimental session.

Scatchard analyses were made from the binding data obtained using [^{3}H]flumazenil (Ro 15-1788; seven concentrations, range 0.25–10 nM; spec. act. 79 Ci/mmol, New England Nuclear) to label central-type BZD receptors in the brain, [^{3}H]muscimol (seven concentrations, range 2.5–70 nM; spec. act. 22 Ci/mmol, Amersham International, Plc, Chalfont, UK) to label $GABA_A$ receptors in the brain, and ^{3}H-Ro 5-4864 (seven concentrations, range 0.5–16 nM; spec. act. 89 Ci/mmol, New England Nuclear) to label peripheral-type BZD receptors in the brain, heart, and testis. Binding tests were carried out as described earlier (20,22). The concentration of unlabelled ligands (flumazenil, GABA, and Ro 5-4864, respectively) used for determining the nonspecific binding was 10 μM.

Experiment 3

GABA-stimulated [^{3}H]flunitrazepam binding. The brains of the copulated and control mice were homogenized in 30 vol. 25 mM K_2HPO_4/KH_2PO_4 containing 50 mM KCl (pH 7.4) with Ultraturrax® homogenizer (setting 6) for 5 s and washed twice in the same buffer after centrifugation (48,000 × *g* for 20 min). The final pellets were stored overnight at −20°C. After thawing they were rehomogenized and washed four more times in cold buffer. Binding of [^{3}H]flunitrazepam (1 nM; spec. act. 78 Ci/mmol, Amersham Radiochemicals) was carried out in the presence of 0 (controls), 0.01, 0.1, 1, and 10 μM concentrations of nonlabelled GABA, using a total incubation volume of 250 μl containing 0.1–0.15 mg of protein. For determining the nonspecific binding, a 10-μM concentration of unlabelled flunitrazepam was added. After 60 min of incubation on ice, the reaction was stopped by rapid filtration over Whatman GF/B filters. The filters were washed with 3 × 3 ml of ice-cold Tris-HCl (pH 7.4). The radioactivity was counted by liquid scintillation spectrometry. Specific binding was calculated by subtracting the nonspecific from the total binding. Protein content was measured as described elsewhere (13).

Calculations and Statistics

Maximum binding (B_{max}) and affinity constant (K_d) values were calculated using Scatchard analysis. The Scatchard plots

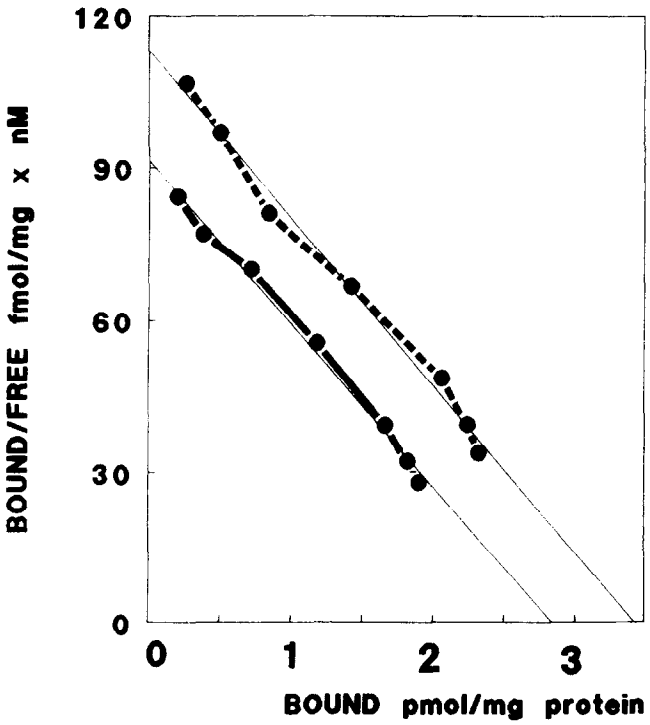

FIG. 1. Scatchard plot of the in vitro binding of [^{3}H]muscimol on GABA receptors in the frontal cortex of male mouse brain showing an increase in the receptor number without changes in binding affinity after copulation (dashed line). Solid line represents a control mouse.

TABLE 1

IN VITRO BINDING OF ^{3}H-RO 5-4864 TO PERIPHERAL BENZODIAZEPINE RECEPTORS IN MALE MOUSE OLFACTORY BULB, CEREBRAL CORTEX, HEART AND TESTIS AFTER COPULATION

Tissue	No. Tissue Pools	B_{max} (fmol/mg)	K_D (nM)
Olfactory bulb			
Control	4	1712 ± 84	3.68 ± 0.15
Copulation	4	2081 ± 139*	3.20 ± 0.48
Frontal cortex			
Control	4	441 ± 85	3.49 ± 0.66
Copulation	4	426 ± 82	3.86 ± 0.59
Heart			
Control	4	3453 ± 260	5.04 ± 0.94
Copulation	4	4680 ± 368*	5.71 ± 0.97
Testis			
Control	4	3816 ± 389	5.87 ± 0.88
Copulation	4	3303 ± 595	6.19 ± 0.86

The data are mean ± SEM. Each tissue pool was homogenized from tissues of four to six mice.

*$p < 0.05$ as compared to controls; analysis of variance and Student's t-test.

were computed with the help of Enzfit program (Elsevier Biosoft, Elsevier Science Publishers, Amsterdam, the Netherlands). The differences between control and treated mice were assessed by two-tailed Student's t test. $p < 0.05$ was considered statistically different. The number of samples was the number of tissue pools.

RESULTS

Experiment 1

[^{3}H]flunitrazepam in vivo. Copulation had no measurable effect on in vivo binding of [^{3}H]flunitrazepam to mouse cerebral or cerebellar central-type BZD receptors as compared to isolated controls (data not shown).

Experiment 2

Benzodiazepine and GABA binding in vitro. The number of $GABA_A$ binding sites, labeled in vitro by [^{3}H]muscimol, in the frontal cortex was higher (17.9%; $p < 0.05$) after copulation than in control mice (B_{max}, means ± SEM, copulation: 3662 ± 223 fmol/mg protein, four tissue pools, each from four to six animals; control: 3107 ± 271 fmol/mg; four tissue pools, each from four to six animals). Affinity was not changed (Fig. 1): K_d values were 31.21 ± 2.97 nM (copulated mice) and 27.02 ± 1.90 nM (controls). In other brain areas studied no statistically significant changes were detected in [^{3}H]muscimol binding (data not shown).

Copulation increased the number of ^{3}H-Ro 5-4864 peripheral BZD binding sites studied in vitro in olfactory bulbs and heart, but not in frontal cortex and testes. The affinity of the binding was not altered (Table 1 and Fig. 2).

After copulation, [^{3}H]flumazenil binding in vitro to mouse brain central BZD receptors in olfactory bulbs or in frontal cortex remained unaltered (means ± SEM: B_{max} 1981 ± 164 and 2009 ± 241 fmol/mg protein, and K_d 2.20 ± 0.21 and 2.14 ± 0.56 nM, respectively; data from three tissue pools, each from four to six animals) as compared to values from control mice (B_{max} 1859 ± 41 and 1981 ± 223 fmol/mg, K_d 2.92 ± 0.08 and 2.22 ± 0.63 nM, respectively; data from three tissue pools, each from four to six animals).

Experiment 3

GABA-stimulated [^{3}H]flunitrazepam binding. GABA-stimulated [^{3}H]flunitrazepam binding to central BZD sites decreased after copulation in all the structures studied in vitro (olfactory bulbs, frontal cortex, and ventral forebrain); the effect was most prominent in the olfactory bulbs and frontal cortex (Fig. 3).

DISCUSSION

Sex hormones modulate the GABAergic neurotransmission, for example, by increasing the number of $GABA_A$ receptors (14); in addition, the synthesis of GABA is altered (26). Progesterone increased the binding of flunitrazepam to BZD receptors whereas the estradiol increased or decreased it, depending on the brain area (5). In vivo, the response to benzodiazepines was altered according to phases of oestrus cycle (6).

GABAergic regulation of anxiety and anxiolysis seems to be important in male sexual behaviour, although little is known about the role of the GABA-BZD receptor complex in these functions. The role of GABA receptors is still unclear and complicated by the fact that in the CNS there are at least two pharmacologically and functionally distinct GABA receptor populations designated as $GABA_A$ and $GABA_B$ (4). The administration of a $GABA_A$ agonist, muscimol, into the

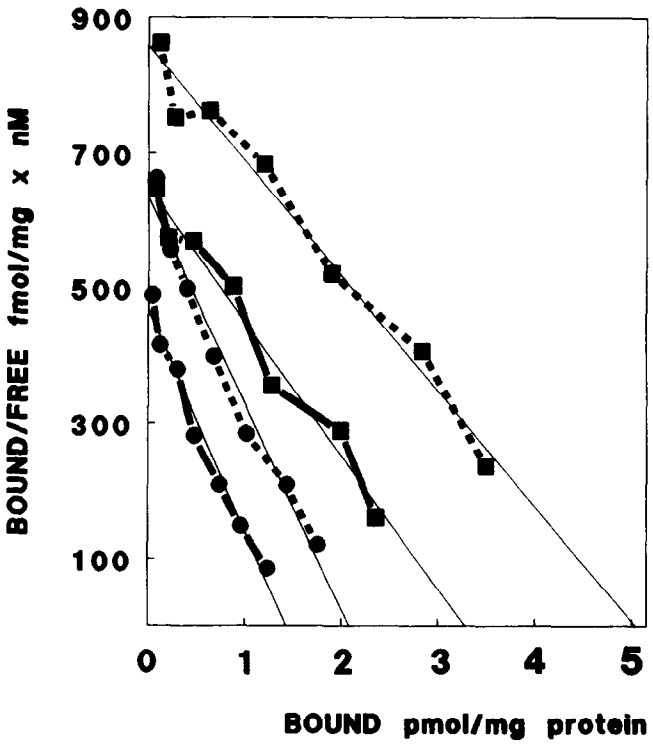

FIG. 2. Examples of Scatchard plots of in vitro binding of ^{3}H-Ro 5-4864 to peripheral-type benzodiazepine binding sites in heart (■) and in olfactory bulb (●). Solid lines represent the control mice, dashed lines the copulating mice.

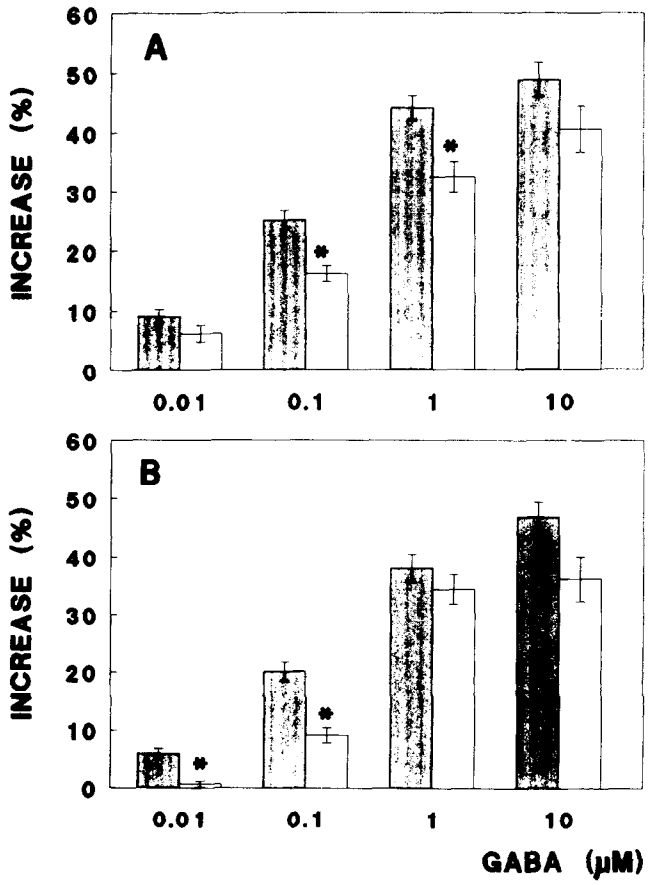

FIG. 3. Effect of copulation on the GABA-induced stimulation of in vitro [^{3}H]flunitrazepam binding in male mouse olfactory bulbs (A) and frontal cortex (B). Shaded bars are controls; white bars represent copulating mice. The data are mean ± SEM of three tissue pools, each from tissues of four to six mice. *$p < 0.05$ compared to control animals, Student's t-test.

medial preoptic area inhibited copulatory activity (9). In contrast, systemic administration of THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) or bicuculline, agonist, and antagonist of $GABA_A$ receptors did not affect penile responses in male rats (12). Interestingly, stimulation of $GABA_B$ receptors by the $GABA_B$ agonist baclofen inhibited the erectile response (3) and disrupted copulatory behaviour (1).

Our in vitro results indicate that in male mice, copulation increases the number of [^{3}H]muscimol binding sites on GABA receptors in frontal cortex, whereas their affinity remains unaltered. For in vivo binding, we had to use large blocks of brain; this is probably why no changes were seen. The concentration of GABA in cerebrospinal fluid of male rats has been shown to increase dramatically during the postejaculatory interval (19). Recently, a complex pattern of changes in [^{3}H]flunitrazepam binding was observed in male rats following copulation and various postejaculatory intervals (24). Also acutely after convulsions, the number of central-type BZD binding sites increased (11,18). Rapid changes such as those observed in this study may represent an opening of spare receptors induced by convulsions or by copulation, rather than synthesis of new receptors.

The receptors increased by copulation may have a different coupling from that normally seen between GABA and BZD receptors, as the effect of GABA stimulation on BZD receptor binding decreased after copulation. This may be an important indicator of the changes in receptor function that are linked to copulatory behaviour. Modulation of GABA-stimulated BZD receptor binding has been suggested to be the most accurate biochemical indicator of the anxiolytic efficacy of BZD compounds (15).

Anxiolytic compounds inhibit GABA-induced stimulation of BZD binding (15). In this study a similar change was seen after copulation. In some tissues, increased numbers of peripheral BZD receptors were also seen after copulation. In states of increased anxiety, as in anxious patients, lowered densities of peripheral BZD receptors have been observed. Anxiolytic treatment with diazepam increased the receptor number (27). We speculate that after copulation, animals experience a short period of tranquility, possibly euphoria, which is seen as a decreased motility and lowered level of exploratory behaviour. The increased number of GABA receptors in the frontal cortex may mediate an abnormally potent, pleasure-inducing GABAergic effect.

The possible involvement of central GABA–BZD receptor complex in sexual behaviour is also indirectly supported by data that anxiogenic BZD receptor ligands (β-carbolines) and stress can cause similar changes of GABA receptors (2). Anxiogenic β-carbolines in low doses stimulated sexual behaviour, whereas higher doses inhibited it (9).

In addition to affecting the central GABA–BZD receptor complex, sexual activation and copulation increased ^{3}H-Ro 5-4864 binding sites in olfactory bulbs and in the heart. Olfactory bulbs contain the highest density of peripheral BZD receptors in the CNS (10) and represent brain regions that are especially important for rodent sexual behavior. Also, other findings suggest a functional link between peripheral-type BZD receptors and GABAergic brain functions: Parallel regulation of GABA receptors in the CNS and peripheral BZD receptors in the heart (downregulation in both cases) has been reported after chronic postnatal phenobarbital treatment (8). Moreover, the models of stress that are known to affect GABA receptors also affect BZD receptors in many peripheral tissues (20,22).

In conclusion, our data show that both the densities of the peripheral BZD receptors in olfactory bulbs and in the heart, and the $GABA_A$/Cl-channel receptor complex in frontal cortex are increased in copulating male mice. These findings support the hypothesis that the peripheral BZD receptors and GABAergic transmission are functionally linked to each other: In addition to reacting in concert to stress, they also are influenced by copulation.

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