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EFFECTS OF OVARIECTOMY AND STEROID REPLACEMENT ON $GABA_A$ RECEPTOR BINDING IN FEMALE RAT BRAIN

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Summary—The specific binding of tritiated muscimol to γ-aminobutyric acid (GABA) receptor sites was studied in distinct brain areas of female rats during different endocrine states. In diestrous rats with intact ovaries the highest receptor densities were found in the cortex (10.24 pmol/mg protein) and the lowest concentrations in the mediobasal hypothalamus (3.29 pmol/mg protein). Four weeks after removal of the ovaries, the number of binding sites was enhanced up to 2.4-fold in all brain areas investigated: the preoptic brain area, mediobasal hypothalamus, corticomedial amygdala, and cerebral cortex. The affinity of the binding sites remained unchanged. Substitution of estradiol and progesterone reduced the number of binding sites to values seen before ovariectomy. The induction of an afternoon surge of LH by estradiol that could be blocked by enhancing the GABAergic tone was accompanied by a distinct reduction in B_{max} in the preoptic area in the morning.

These results give evidence that ovarian hormones modulate GABAergic neurotransmission by regulation of $GABA_A$ receptor synthesis or degradation.

INTRODUCTION

Gonadal steroid hormones modulate central nervous functions such as sexual behaviour or gonadotropin release from the pituitary gland by alterations of the morphology of synapses, the release of neurotransmitters, or the properties of transmitter receptors (for reviews see Refs [1, 2]).

Within these neuroendocrine circuits the GABAergic system reportedly plays an important role. Its function, in turn, is modulated by sex steroids which interfere with the formation of γ-aminobutyric acid (GABA) in different brain areas [3, 4], the release of GABA from the medial preoptic area [5], the turnover of GABA in the preoptic hypothalamic brain area [6, 7], or the binding of radioligands to $GABA_A$ receptor sites in the CNS [8–11]. The results concerning GABA receptor binding studies, however, are inconsistent. While some investigators observed an **increase** in the total number of binding sites after estrogen administration [8, 9], others [10, 11] reported a **decrease** in muscimol binding induced by estrogen. Since the two groups used different binding techniques, the discrepancies might be due to procedural differences. We have recently established an improved binding assay which enables the *in vitro* characterization of [^{3}H]muscimol to $GABA_A$ receptor sites in distinct brain areas of individual animals [12]. This technique was used to study the effects of ovariectomy and steroid replacement on the *in vitro* binding of [^{3}H]muscimol in different brain areas of the female rat, the preoptic brain area (POA), the mediobasal hypothalamus (MBH), corticomedial amygdala (AMY) and the cerebral cortex (COR). In addition, we measured the *in vitro* binding of [^{3}H]muscimol to $GABA_A$ receptor sites in the four brain areas during different phases of reduced and enhanced LH secretion since alterations in GABAergic neurotransmission have been shown to be associated with negative and positive feedback actions of sex steroids on LH release [5, 6, 13–18].

EXPERIMENTAL

Chemicals

Tritiated muscimol (15.9–25.8 Ci/mmol) was obtained from DuPont de Nemours (Dreieich, F.R.G.). Non-labelled GABA and aminooxyacetic acid were purchased from Sigma (St

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Louis, Mo., U.S.A.). All other chemicals were bought in the highest quality from commercial sources.

Animals

Adult female rats of the strain Han: SPRD (Sprague–Dawley) were purchased from the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.) and kept under a cycle of 12 h light to 12 h darkness (lights on from 0600 to 1800 h). Food and water were available *ad libitum*. The study included three different groups of rats: (I) diestrous rats, (II) long-term ovariectomized, otherwise non-treated animals, and (III) long-term ovariectomized rats that received consecutive injections of estradiol and progesterone.

The sex hormone state of the diestrous rats with intact ovaries (group I) was controlled by inspection of vaginal smears for at least one complete ovarian cycle. The rats were killed at the age of 80 ± 10 days between 0900 and 0930 h in the morning. Ovariectomy (groups II and III) was performed under ketamine anaesthesia at the age of 50–60 days. Four weeks later, a subgroup of the ovariectomized animals (IIIa) received single s.c. injections of estradiol benzoate (EB, 20 μg) on day 0 and progesterone (P, 2.5 mg) on day 3 according to a previously published time schedule [19]. These rats were killed between 0900 and 0930 h in the morning on day 6. To stimulate LH secretion [19, 20], some ovariectomized rats (group IIIb) received 3 consecutive s.c. injections: EB (20 μg) on day 0 and 5 and P (2.5 mg) on day 3. These animals were killed either at 0900, 1200, or 1500 h on day 6.

Blood sampling

For the determination of plasma LH levels, blood was sampled from different animals. It was collected from a chronic cannula implanted under light ether anaesthesia into the external jugular vein [21]. Blood samples were withdrawn at 40 min intervals and the plasma was stored frozen at −20°C until assayed.

Determination of LH

The concentration of LH was measured using a specific radioimmunoassay for rat LH [22]. Materials and instructions were supplied by the NIDDK Rat Pituitary Hormone Distribution Program. Values are given in terms of the respective NIDDK-rat RP-3 preparation. All determinations were run in duplicate.

Preparation of membrane fractions

The brains were quickly removed after decapitation and chilled in physiological saline (0°C). The preoptic brain area (POA), mediobasal hypothalamus (MBH), corticomedial amygdala (AMY) and cerebral cortex (COR) were dissected out [23]. The brain areas were sampled from individual animals. Membrane fractions were prepared as described previously [12]. In brief, the tissue was homogenized in 50 mM Tris–citrate buffer in the presence of Triton X-100 (0.05%, v/v). The homogenate was then incubated at 37°C, followed by a centrifugation at 50,000 *g* for 10 min. The pellet was resuspended, incubated in a shaking waterbath (4°C, 15 min), and recentrifuged. The resulting membrane fraction was stored frozen at −80°C until assayed for the specific binding of [^{3}H]muscimol.

Binding assay

The binding assay was performed at room temperature (20°C) by incubation of the membrane fractions with [^{3}H]muscimol as described previously [12]. GABA (0.1 mmol/l) was included for determination of non-specific binding that accounted for 10–15% of total [^{3}H]-muscimol binding. The protein concentration was 50–60 μg/ml.

Incubation was terminated by rapid (< 3 s) filtration over Whatman GF/C filters. The filter bound radioactivity was eluted using 3 ml scintillation fluid (Aqualuma, Baker-Chemicals, Groß-Gerau, F.R.G.) and measured by liquid scintillation counting. All determinations were run in duplicate.

The data obtained from the binding experiments were submitted to Scatchard analysis [24]. The equilibrium dissociation constant (K_d) is expressed in terms of radioligand concentration (nM) and the total number of binding sites (B_{max}) in terms of protein concentration of the samples [25].

Statistical tests

Significant differences between mean K_d- or B_{max}-values were determined by Student's t-test. Significance of differences in sequential changes in LH plasma levels was determined by the paired t-test. The results were considered as significant for $P < 0.05$.

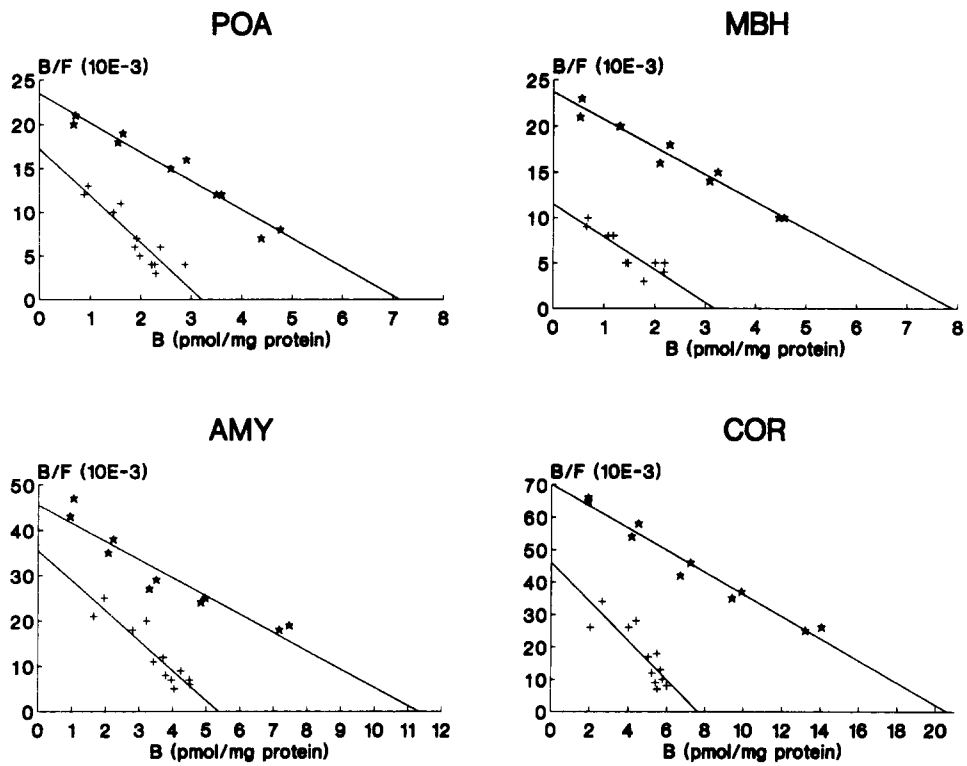

Fig. 1. Representative Scatchard plots of the specific binding of [^{3}H]muscimol to membrane fractions from distinct brain areas of long-term (4 weeks) ovariectomized (*) and diestrous (+), female rats at the age of 80 ± 10 days. POA = preoptic brain area; MBH = mediobasal hypothalamus; AMY = corticomedial amygdala; COR = cerebral cortex.

RESULTS

Effects of ovariectomy on the binding of [^{3}H]muscimol

Using the binding assay as described here, we were able to measure the specific binding of tritiated muscimol to crude membrane fractions obtained from **distinct** brain areas (15–30 mg wet wt) of individual animals and determine K_d- and B_{max}-values. Linear Scatchard plots revealed a single class of binding sites with high affinities for [^{3}H]muscimol in all brain areas investigated (Fig. 1).

The receptor densities ranged from 3.29 pmol/mg protein in the MBH to 10.24 pmol/mg protein in the COR of diestrous rats (Table 1). The K_d-value was lowest in the AMY (11.2 nmol/l) and highest in the MBH (23.3 nmol/l). The total number of binding sites was not significantly different in the POA and MBH but differed significantly from the values found in the AMY and COR (Table 1).

Ovariectomy of the rats resulted in a 2.5-fold increase in B_{max} in the POA and MBH ($P < 0.05$ and 0.01, respectively) and in an approximately 2-fold increase in the AMY and COR ($P = 0.051$ and $P < 0.05$, respectively; Table 1). The dissociation constants of GABA receptor sites for muscimol decreased from 11.2 to 18.6 nM in the AMY while they were not affected by ovariectomy in the other brain areas as indicated by unchanged K_d-values (Table 1).

Effects of steroid replacement on the binding of [^{3}H]muscimol

In the MBH and COR, the increase in the maximal number of binding sites induced by ovariectomy could be completely reversed by consecutive *in vivo* administration of estradiol benzoate and progesterone (Table 1). The effect of ovariectomy on GABA receptor densities in the POA and AMY was reversed to 50% under these conditions, yet not being significant. Moreover, estradiol benzoate plus progesterone reduced the K_d-values in the AMY to values seen before ovariectomy while no further changes were found in the other brain areas. The characterization of muscimol binding sites at 0900, 1200, and 1500 h did not reveal significant daytime dependent variations in the binding parameters of GABA receptors (Fig. 2). When the ovariectomized animals that had been

Table 1. Effects of ovariectomy and steroid hormone substitution on the maximal number of binding sites

Brain area	Diestrous rats	Ovariectomized rats	
		Non-treated	EB/P-treated
		B_{max}	
POA	3.64 ± 0.33	8.92 ± 1.24**	6.37 ± 0.56*
MBH	3.29 ± 0.06	7.72 ± 0.13**	4.36 ± 0.34
AMY	6.11 ± 0.55	11.87 ± 1.40	9.06 ± 0.92
COR	10.24 ± 2.15	19.34 ± 0.97*	11.35 ± 1.31
		K_d	
POA	17.2 ± 2.4	15.3 ± 0.8	17.4 ± 2.1
MBH	23.3 ± 1.0	23.7 ± 0.9	18.7 ± 4.4
AMY	11.2 ± 0.2	18.6 ± 2.4*	10.3 ± 1.4
COR	18.4 ± 5.4	16.3 ± 1.1	9.3 ± 1.9

Maximal number of binding sites (B_{max}, pmol/mg protein) and dissociation constants (K_d, nM) of [^{3}H]muscimol binding to membrane fractions from distinct brain areas of female rats. Steroid hormone substitution was performed by consecutive s.c. injections of estradiol benzoate (EB, 20 μg) on day 0 and progesterone (P, 2.5 mg) on day 3. Hormone-treated animals were killed between 0900 and 0930 h on day 6. B_{max}- and K_d-values were calculated from binding experiments submitted to Scatchard analysis. Values represent the mean ± SEM of independent experiments obtained from brain areas of 6 animals. POA = preoptic brain area; MBH = mediobasal hypothalamus; AMY = corticomedial amygdala; COR = cerebral cortex. *$P < 0.05$ vs diestrous rats; **$P < 0.01$ vs diestrous rats.

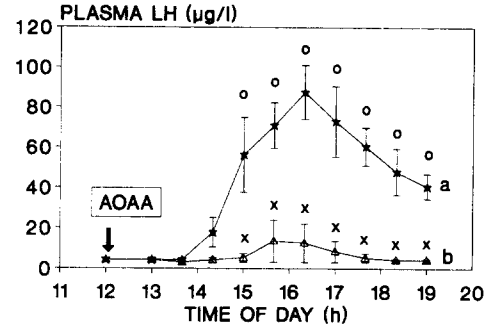

Fig. 3. Plasma concentrations of luteinizing hormone (LH) in long-term (4 weeks) ovariectomized rats after s.c. administration of estradiol benzoate (EB, 20 μg) on day 0 and 5 and of progesterone (P, 2.5 mg) on day 3. On day 6 the animals received i.v. injections of either vehicle (a, physiological saline) or aminooxyacetic acid (b, AOAA, 30 mg/kg) at 1200 h. Plasma was prepared from blood samples drawn from an external jugular vein cannula on day 6. Values represent the mean ± SEM of 6 animals $^{x}P < 0.01$ vs vehicle-treated control; $^{o}P < 0.01$ vs 12 h.

primed with estradiol on day 0 and progesterone on day 3 received a second injection of EB on day 5 the K_d- and B_{max}-values were similar to those of animals that had been injected with single doses of EB and progesterone (Fig. 2).

Muscimol binding during phases of enhanced LH secretion

Subcutaneous injections of estradiol benzoate (EB, 20 μg, s.c.) on day 0 and progesterone (P, 2.5 mg) on day 3 followed by another injection of EB on day 5 resulted in a surge of LH starting at 1430 h on day 6 of steroid treatment and

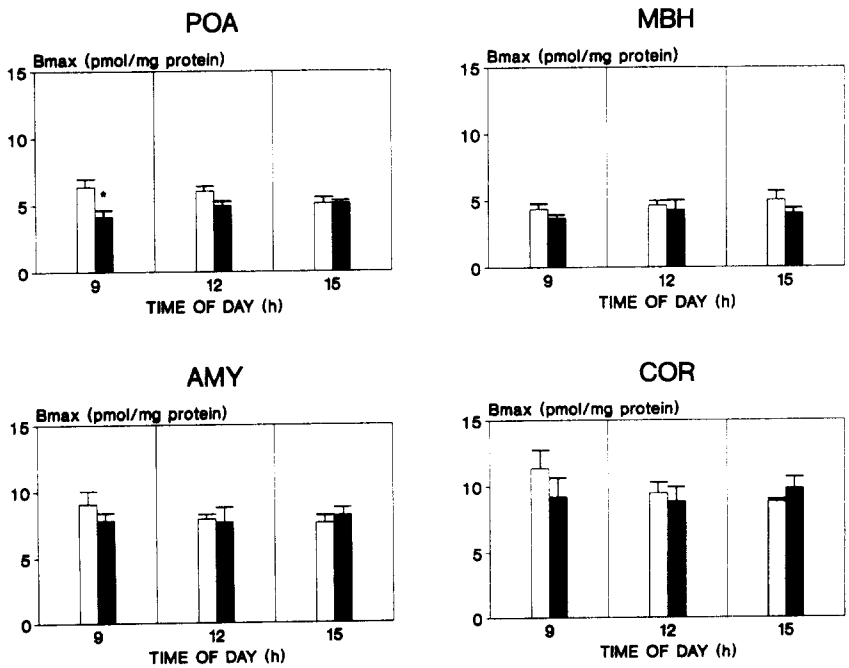

Fig. 2. Maximal number of binding sites (B_{max}, pmol/mg protein) of [^{3}H]muscimol binding to membrane fractions from distinct brain areas of rats after s.c. administration of estradiol benzoate (EB, 20 μg) on day 0 and progesterone (P, 2.5 mg) on day 3. The animals received a further injection of EB or sesame oil (VEH) on day 5 and were killed by decapitation at different times of day on day 6. The number of binding sites was calculated from binding experiments submitted to Scatchard analysis. Values represent the mean ± SEM of independent experiments obtained from brain areas of 6 animals. POA = preoptic brain area; MBH = mediobasal hypothalamus; AMY = corticomedial amygdala; COR = cerebral cortex.

reaching peak values at 1700 h (Fig. 3). The surge of LH could be completely blocked by aminooxyacetic acid which enhances the GABAergic tone by inhibition of GABA degradation [16]. As we have shown previously [19], administration of vehicle (sesame oil)—instead of EB—on day 5 did not bring about a surge of LH in the afternoon of day 6.

In the morning of day 6 (at 0900 h), when LH levels were still at baseline concentrations, the GABA receptor sites exhibited unchanged B_{max}- and K_d-values as compared to diestrous rats (Table 1). Within the following 6 h, the K_d-values did not change significantly in any of the four brain areas investigated. In the POA of EB-P-EB-treated rats, however, B_{max} was by 55% lower than in animals that had received sesame oil instead of EB (Fig. 2). The effect was restricted to the POA. No further changes in the amount of receptor sites were observed in the other brain areas.

DISCUSSION

The results of the present investigation support and extend the view that $GABA_A$ receptors in the central nervous system are under the modulatory control of ovarian sex steroid hormones. To our knowledge, investigations on the effects of ovariectomy on GABA receptor binding in the brain have not yet been reported in the literature whereas sex steroid induced alterations have been studied by several groups with inconsistent results. Estradiol has been found to increase [8, 9] or reduce [10, 11] the binding of [^{3}H]muscimol in rat brain. Since different methods have been used, it might be suggested that the discrepant findings are due to procedural differences. This suggestion is supported by findings showing that *in vitro* binding studies on the $GABA_A$ receptor demand special procedural precautions. Endogenous ligands, allosteric modulators or proteolytic enzymes may be present in the membrane preparations and interfere with the binding of the radioligand to the receptor sites [26–28]. The groups who described an estrogen induced **up-regulation** of GABA receptors [9, 29] prepared membrane fractions by using procedures that reportedly will not sufficiently remove endogenous GABA [26]. O'Connor *et al.* [10] and Schumacher *et al.* [11] who described an estrogen-induced **down-regulation** of [^{3}H]muscimol binding used brain tissue slices for quantitative autoradiography. Though interactions with endogenous ligands have not been studied for the latter technique it seems likely that slice preparations contain at least as much endogenous ligands as crude membrane fractions obtained by tissue homogenization and centrifugation [26] since removal of endogenous ligands is restricted to a 30 min preincubation of the slices in buffer solutions that do not contain detergents [10, 11]. The lack of detergent, however, will certainly not sufficiently remove endogenous ligands [26].

Taking these problems into consideration it can be supposed that hormone induced alterations in muscimol binding reported in the literature might not only be the result of changes in the content or properties of $GABA_A$ receptor sites but also in the formation or disappearance of endogenous ligands that interfere with the *in vitro* binding of [^{3}H]muscimol. This may dilute conclusions on the mechanisms of hormone actions.

Another methodological problem concerns the schedule of hormonal manipulation. Apud *et al.* [30] have demonstrated that single or repeated administrations of estradiol differentially modulate GABAergic neurotransmission: the activity of tuberoinfundibular GABA neurons is not affected by a single dose of estradiol while chronic treatment (for 2 months) does so. Schumacher *et al.* [11] showed that treatment of ovariectomized and adrenalectomized female rats with estradiol results in a decrease of muscimol binding. Progesterone has no effect when administered alone but affects muscimol binding after estradiol priming [11].

With regard to the present investigation special attention was therefore given to the use of sophisticated *in vitro* and *in vivo* methods. An assay procedure was included that efficiently removes endogenous contaminants [12] and reveals B_{max} and K_d of a single class of high affinity binding sites in distinct brain areas of individual animals. To investigate hormone effects some of the ovariectomized animals received consecutive injections of estradiol and progesterone to imitate the cyclic variations in the production of ovarian hormones in rats with intact ovaries. This hormone regimen induces both, negative and positive feedback actions on LH release [19].

Ovariectomy enhanced the specific binding of [^{3}H]muscimol to GABA receptor sites. It seemed that these changes were due to cessation of ovarian steroid production as they could be

reversed by substitution of estradiol and progesterone. With regard to the mechanisms that brought about the observed effects the following might be relevant: an increase in the release of GABA which in turn resulted in a down-regulation of receptor sites [6, 7, 31] or alterations in the synthesis or degradation of GABA receptor protein [8]. Moreover, the effects might have been mediated via classical intracellular hormone receptors and subsequent alterations in protein synthesis [11] or by direct steroid effects on membrane associated proteins as it has been shown for progesterone metabolites which bind to the GABA receptor ionophore complex and enhance [32] or decrease GABAergic neurotransmission [33]. It seemed likely that most of the effects shown in the present investigation were the result of genomic actions since the most prominent effects were observed in brain areas with high concentrations of intracellular receptors [34]. Moreover, the changes in B_{max} pointed to a steroid dependent modulation in the synthesis or degradation of GABA receptor protein because B_{max} is widely used as a measure for receptor concentration. The involvement of non-genomic mechanisms, however, must be considered, too, since the endocrine manipulations affected the cerebral cortex that is almost devoid of classical intracellular receptors for sex steroids [34]. Direct modulatory actions of steroids on the $GABA_A$–receptor complex might also explain the observed increase in K_d in the amygdala after ovariectomy and the steroid hormone induced reversion of the effect.

With regard to the physiological relevance of the observed effects on GABA receptor binding it is noteworthy that various reproductive functions are both, under feedback control of gonadal hormones and modulated by central GABAergic systems (for reviews see Refs [1, 2]). This includes the central nervous regulation of gonadotropin release where GABA plays an important inhibitory role [3, 5, 6, 13–17, 35–37]. Functional interactions between GABA and gonadotropin release systems were also found in the present investigation. The induction of a surge of LH by estradiol could be blocked by aminooxyacetic acid which enhances the GABAergic tone by inhibition of GABA degradation [16]. The distinct effect of reduced B_{max} in the preoptic area supported the view that the GABA receptor system in the preoptic area is a major target for central estrogen actions [35]. This brain area plays an important role for the control of LHRH secretion. It contains axosomatic contacts between LHRH producing perikarya and GABAergic nerve terminals [18] and concentrates estradiol in GABAergic nerve cell nuclei [35]. However, it remains to be established as to whether the observed distinct effect of estradiol on GABA receptor binding in the preoptic area is functionally related to the induction of LH surges during preovulatory phases.

The results of the present investigation taken together confirmed and extended the view that ovarian steroid hormones interfere with GABAergic neurotransmission by interaction with the $GABA_A$ receptor. *In vivo* manipulations of available sex steroid hormone concentrations in female rats were accompanied by marked alterations in the properties of [^{3}H]muscimol binding to carefully washed membrane fractions of distinct brain areas some of which seemed to be functionally related to the neuroendocrine control of gonadotropin secretion.

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