STUDIES OF THE EFFECTS OF HORMONES ON THE DEVELOPMENTAL PROFILE OF THE GABA-ERGIC SYSTEM IN THE RAT BRAIN

by

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To my wife.

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ABSTRACT

The present study addressed to the factors influencing the differentiation of the GABA-ergic system in mammals using a rat model. Male and female rat hypothalamus, cerebellum, and cerebral cortex were assayed for three parameters: glutamic acid decarboxylase (GAD) activity, and binding of the GABA and benzodiazepine (BDZ) receptors.

Levels of all three parameters were different for each brain region studied. The patterns of development of each parameter were similar in male and female rats, but there appeared to be a sex difference at discrete ages.

Treatment with dexamethasone for three weeks during early development stages decreased BDZ binding in the cerebellum of female rats. Treatment with diethylstilbestrol for the same time period reduced GAD activity in female hypothalamus and cerebellum and reduced GABA binding in the cerebellum and cortex of males. Treatment with dihydrotestosterone for the same duration reduced GABA binding in female cortex.

In conclusion, these data suggest that development of the GABA-ergic neuronal system is a complex process, and the early hormonal environment plays an important part in its ontogenesis. In addition, sex is an important determinant in the developmental profile of the GABA- ergic system.

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ABBREVIATIONS

Units of Measurement

wt	weight
g	grams
mg	milligrams
ug	micrograms
nmole	nanomole
pmole	picomole
fmole	femtomole
uM	micromolar
nM	nanomolar
uCi	microCurie
mosm	milliosmolar
R.T.	room temperature
min	minutes
Hr	hours
cpm	counts per minute

General Terms

CNS	central nervous system
SDN-POA	sexually dimorphic nucleus of the pre-optic area
ChAT	choline acetyl transferase
TH	tyrosine hydroxylase
GAD	glutamic acid decarboxylase
GABA	gamma- amino butyric acid
BDZ	benzodiazepine
FNZ	flunitrazepam
DA	dopamine

NE	norepinephrine
PRL	prolactin
E	estrogen
E2	estradiol
P	progesterone
LH	luteinizing hormone
T3	triiodothyronine
LH-RH	luteinizing hormone releasing hormone
ACTH	adenocorticotrophic hormone
TP	testosterone propionate
DEX	dexamethasone
DES	diethylstilbestrol
DHT	dihydrotestosterone
AOAA	aminooxyacetic acid
PLP	pyridoxal-5'-phosphate
HEPES	(4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
PBS	phosphate buffered saline
EtOH	ethanol
ddH_2O	distilled deionized water
DB	diluting buffer
SCG	superior cervical ganglion
DRG	dorsal root ganglion
NP 40	Nonidet-P 40
NaCl	sodium chloride
KPO_4	potassium phosphate

INTRODUCTION

I STUDIES ON THE DEVELOPMENT OF THE BRAIN

The development of the mammalian central nervous system is an extremely complex process. The control of endocrine function and behavior is achieved only after the incorporation of many different influences, both intrinsic and extrinsic, on the developing neurons. Among the most important of these influences is that of the hormones secreted by the gonads (1).

1) Sexual Differentiation of the CNS.

The original research leading to the current concepts of sex differences in the brain was done 50 years ago by Pfeiffer (2). He showed that factors released from the testes during early postnatal life caused the expression of masculine patterns of pituitary gonadotropin secretion in adulthood. When it was subsequently demonstrated that the functions of the pituitary are regulated by the hypothalamus, the influence of the testes on development of centers within the brain became well established (3).

In mammals, the intrinsic pattern of the central nervous system development is female, with differentiation toward masculine patterns of gonadotropin secretion and behavior occuring in the male as a result of exposure to testicular hormones during development (4). MacLusky and Naftolin (1)

state: "This mechanism is not the sole determining factor in sexual differentiation of the central nervous system. In many cases, however, there is good evidence that early hormonal experience makes at least some contribution." For a good review see (reference 1).

2) "Critical" Period of Brain Development

There is a time period in the development of the CNS when there is increased sensitivity to the organizational effects of gonadal hormones. This is the so-called 'critical period'. The endpoints used to define this period are those associated with the control of reproductive function and sexual behaviour (1).

Receptors for gonadal hormones in the brain have been widely demonstrated (5,6). These receptors have been shown to exist during the 'Critical Period' of brain development. Vito and Fox (6) showed estrogen receptors to be present in cytosol extracts of embryonic mouse hypothalamus, thus establishing a potential early beginning for the 'Critical Period' of the brain's sexual differentiation. Likewise, androgen receptors were demonstrated in embryonic and neonatal mouse and rat hypothalami (5).

The behavioral effects of exogenously administered gonadal hormones in utero have been well documented (7,8). Female rats exposed in utero to androgens are more masculinized than normal females (9). In addition, the

ability of the exogenously applied androgens to masculinize the females decreases with increasing age. Similarly, male rats exposed in utero to anti-androgens (that is, synthetic analogs of androgens), exhibit less "male-like" sexual behavior and more "female-like" sexual behavior as adults than normal males (7,10).

There is also evidence that the hormonal environment affects the cytoarchitectonics of the brain. The gross morphological sex differences in the sexually dimorphic nucleus of the pre-optic area of the brain (SDN-POA) can be controlled by the hormonal environment during the 'Critical Period' of brain sexual differentiation (11). This nucleus is an intensely staining component of the pre-optic area and is several fold larger in males than in females. It has been shown to accumulate and retain steroids in adulthood, but is not influenced by the adult hormone environment. The development of this nucleus is determined, in part, by testicular hormones during the postnatal period.

3) In Vitro Studies on the Developing Central Nervous System

The cell culture technique has been widely used to separate growth and differentiation of different types of neurons located within the mammalian brain and spinal cord. These studies have the advantage of being able to monitor directly the effects of exogenously applied drugs or

hormones, and also applying techniques adapted from *in vivo* studies to quantify the net changes in biochemical systems within these neurons.

In mouse or rat fetuses, previous studies have shown that most of the neurons in hypothalamus are formed between day 11 and day 14. For the cortex, this time period is between day 14 and day 16 (12). Between day 14 and day 16 of gestation, the neuronal perikarya begin to differentiate (13). Three neurotransmitter-synthesizing enzymes: ChAT, TH, and GAD, are present on day 14 of gestation (14). During prenatal and postnatal periods, the levels of these markers of neuronal differentiation increase at different rates. TH activity increases rapidly in the hypothalamus during the last days of gestation, almost reaching adult levels by day 10 postnatally. In the cerebral hemispheres, TH activity remains very low during fetal life and increases most rapidly only after 15 days of postnatal life. In contrast, GAD and ChAT activities represent only 2 to 3% of adult levels by the fourteenth fetal day in the hypothalamus and cerebral cortex and remain low until birth. After birth, ChAT and GAD rapidly increase between day 5 and day 20 and reach adult levels earlier in the hypothalamus than in the cerebral hemispheres (14). Thus, by culturing premature neurons, obtained before birth, neuronal maturation *in vitro* can be followed (13).

Puymirat (13) found the addition of 10^{-12} M 17β -estradiol to be an absolute requirement for cell attachment to culture plates, quality of culture and neuronal survival. T3 was found to greatly improve hypothalamic neuronal survival and neurite extension for up to one month when added on day 5. This hormone also reduced ChAT activity at a concentration of 10^{-12} M after 8 days in culture. In the cerebellum and hypothalamus cell cultures, 17β -estradiol increased the ratio of neuron like cells to basal glial-like cells (15).

4) Functional Specificity in the Central Nervous System

One of the primary choices the developing neuron makes is which neurotransmitter to produce, thereby determining the effect its synapses will have on its target cells. Different regions of the central nervous system have been shown to have higher amounts of synthesizing enzymes and receptors for particular neurotransmitters than others. The organization of these different regions has been shown to be influenced by several different factors. To date, the most concretely established influencing factors are the trophic factors. Trophic influences have been well-established in the peripheral nervous system for the effect of muscle on the development of the corresponding motoneurons (16 ,17).

Trophic factors are less clearly defined in the central nervous system. In particular, the effects of neuronotrophic

factors and the influence of hormones has not been widely studied.

II THE GABA-ERGIC SYSTEM

Gamma-aminobutyric acid (GABA) has been characterized as one of the most important putative neurotransmitters of the central nervous system (see references 18 and 19 for reviews). Its synthesis, storage, release, action, inactivation, and metabolism have been well documented (20). Studies examining the neuronal uptake of GABA and the localization of GAD (L-glutamate decarboxylase), the synthetic enzyme for GABA, indicate that presynaptic GABA terminals constitute between 30 and 45% of all presynaptic nerve terminals in all brain regions studied thus far (21). Most GABA neurons appear to be, in general, interneurons localized to particular brain structures, although some project elsewhere. For example, Purkinje cells project from the cerebellar cortex to Dieter's nucleus and GABA neurons project to the substantia nigra from the striatum and globus pallidus (22).

1) Metabolism of Gamma-aminobutyric Acid

a) Synthesis GAD catalyzes the conversion of L-Glutamic acid to form GABA. This enzyme is believed to be the rate-limiting enzyme that normally determines the steady-state levels of GABA in the mammalian nervous system

(23). GAD is a better marker for GABA-ergic neurons than is GABA, which may be redistributed or metabolized during tissue preparations (24).

b) Degradation GABA is catabolized by GABA-Transaminase (GABA-T) to form succinic semialdehyde. Further oxidation of this compound is catalyzed by the enzyme succinic semialdehyde dehydrogenase (SS.ADH) to form succinic acid and γ-hydroxybutyrate. GABA-T inhibition is most often accomplished by using aminooxyacetic acid (AOAA)(20). While this compound is a potent competetive inhibitor of GABA-T, it also inhibits other pyridoxal-dependent enzyme systems, including GAD (25).

2) Functional Aspects of Gamma-aminobutyric Acid

a) Localization of GABA-ergic Neurons Cortical GABA appears to be contained entirely in local circuit neurons, with no corticofugal projections. The same applies for the hippocampus, hypothalamus, olfactory bulb, retina, and spinal cord. The most well-defined GABA-using projection neurons in the central nervous system are associated with the basal ganglia and cerebellum (for review see reference 19).

b) Involvement of GABA-ergic Neurons in Development. Pharmacological manipulations of GABA-ergic transmission were reported to alter the secretion of most adenohypophysial hormones and of some related hypothalamic

hypophysiotropic hormones. GABA also decreased the electrical activity of the hypothalamo-hypophysial axons in vitro (for review see reference 26).

Direct and indirect relationships between GABA and several hormones have been established. Hyperprolactinemia induced by adenopituitary transplantation under the kidney capsule in rats caused a slight increase in GAD activity in the substantia nigra and medial basal hypothalamus and a more significant increase at the striatal level (27). The strong inhibitory link between prolactin (PRL) levels and DA in the hypothalamus has been well-documented and a functional link between DA transmission and the GABA-ergic system has been demonstrated in various brain regions (28). The parallel changes in PRL levels and GAD activity observed in acute treatments with apomorphine in rats and the return to normal of GAD and high PRL levels induced by haloperidol and sulpiride suggested a PRL-GABA subsidiary feedback loop (29).

The effect of estrogen in the GABA-ergic system has also been studied. Estradiol treatment reduces whole brain GAD activity in adult female rats, whereas ovariectomy increases GABA content in the cerebral hemispheres (30). Oophorectomized rats treated for 3 days with either estradiol benzoate (E) 1 ug/ day or progesterone (P) 500 ug/day or 2 days E and one day P, inhibited GAD activity. These findings suggest a role for LH in controlling GAD

levels, since conditions that inhibit LH secretion also reduce GAD activity (31).

Norepinephrine (NE) turnover in the medial preoptic area correlates with plasma LH levels. GABA turnover correlates inversely with LH and preoptic NE turnover (32). These findings seem to indicate that GABA-ergic neurons mediate the negative feedback action of estradiol to LH-RH perikarya located in the medial preoptic area. GABA may be the neurotransmitter of the E2-receptive neurons, and these neurons specify NE turnover locally.

GABA binding is increased in the midbrain and corpus striatum following adrenalectomy. This receptor change is reversed by administration of corticosterone. Hypophysectomy causes a significant reduction of GABA receptor binding that is not reversed by corticosterone. In addition, systemic administration of either ACTH 1-39 or ACTH 4-10 in unoperated animals causes an increase in midbrain and striatal GABA binding (33). These findings indicate that GABA receptor binding in rat brain can be modified by changes in the circulating levels of ACTH.

3) The Development of the GABA-ergic System

a) GAD Activity. Tests for GAD activity in different brain regions have been carried out in cat and rat models. Areas of highest activity include the midbrain, thalamus-hypothalamus (35) of rat, the substantia nigra and

subthalamic nuclei in cat (36), with cerebellum in both animals having relatively high levels of this enzyme.

The development of GAD activity and GABA concentrations have been studied in mouse. GAD activity showed a marked increase between postnatal day 8 and day 18 with a further increase up to day 43. GABA content increased two fold over the same time period (37).

b) γ-aminobutyric Acid and Benzodiazepine Receptors

Examination of GABA binding has revealed its association to benzodiazepine binding (20,38). Benzodiazepine recognition sites are present at birth in the cerebellum and cerebral cortex as well as in the rest of the brain (38). The changes in binding with age are due to an increase in the total number of binding sites. The developmental profile of the BDZ receptor in culture mimics its development *in vivo*, reaching maximum values between 35 and 42 days post-conception (39). This time-course parallels the developmental profile reported for GABA neurons.

The increase in the total number of GABA receptor sites in the cerebellum occurs at a relatively late ontogenetic stage (40). The best computer estimated fits of GABA binding in cerebellar membranes involved two receptor sites. The development of the two receptor sites is distinctly different with the high affinity site reaching its maximum density at about 2 weeks postnatally, while the low affinity site reaches its maximum development after 60 days.

AIMS

The aim of this research is to establish a method of monitoring the GABA-ergic system *in vivo* in order to examine the developmental pattern in male and female rats and effects of hormonal manipulation in the early stages of brain differentiation. The development of the GABA-ergic system is monitored for GAD activity, GABA receptor binding and BDZ receptor binding in the hypothalamus, cerebellum and cortex which have been shown to have high GABA content. These studies on the development of the GABA-ergic system will gain further information on the role of the hormonal environment in brain development and differentiation.

MATERIALS AND METHODS

I MATERIALS

1. Biological Materials

All laboratory animals, including pregnant rats and adult females with litters were obtained through the animal breeding facilities at the University of Manitoba.

2. Hormones

Dihydrotestosterone, dexamethasone, and diethylstilbestrol were all obtained from Sigma Chemicals, St. Louis, USA.

3. Chemical Reagents

[^{3}H]-glutamate, [^{3}H]-muscimol, and [^{3}H]-flunitrazepam were all obtained from New England Nuclear, Boston, USA. L-glutamate, aminooxyacetic acid (AOAA), pyridoxal phosphate, aminoethylisothiouronium bromide (AET), +Na-+K tartrate and HEPES were obtained from Sigma. AG1-X4 (formate form) (200 mesh) Resin, and Coomassie brilliant blue G-250 were obtained from Bio- Rad, California. RO5-2807 was a gift from NIH in Bethesda, Md. All other chemicals were obtained from Fisher Scientific, Winnipeg.

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II METHODS

1. Preparation of Brain Tissue Samples for Assay.

a) Dissection Rats were killed by decapitation and the brains were immediately removed from the skull and placed on ice cold Petri dish covered with filter paper soaked with PBS. The brain was weighed and the hypothalamus, cerebellum and cortex were immediately dissected as follows:

(i) The hypothalamus was delineated by the optic chiasm rostrally, the hypothalamic sulci laterally and the mamillary bodies caudally. The depth was determined by a line connecting the anterior and posterior commisures.

(ii)The cerebellum was removed from the brain stem and pons by transsection of the cerebellar peduncles.

(iii) The cortex was removed completely from the remainder of the brain tissue and dissected free of all white matter, then cut into several small blocks and a heterogeneous sample was made by combining several dissected blocks of tissue.

b) Membrane Isolation. The regions were weighed and then homogenized in 1 ml Potter-Elvejhem or 5 ml Ten-Broeck homogenizers with 15 volumes of 0.32 M sucrose according to the procedure of Zukin et al. (46) in Dilution Buffer (1.0 mM K_2HPO_4 with 1.0 mM +Na-+K-Tartrate and 1.0 mM HEPES pH 7.4) using ten firm but gradual strokes. Two samples of 50 ul each were used to assay the GAD activity using the two

column procedure of Swalman and Wu (48). The remainder of the homogenate (approximately 1.0 ml) was centrifuged at 1000Xg for 10 minutes. The supernatant was further centrifuged at 50,000Xg for 15 minutes. The supernatant from this spin was discarded and the pellet was then resuspended in 100 volumes of cold distilled deionized water and homogenized with a Brinkmann Polytron at setting 6 for 20 seconds. This homogenate was then centrifuged at 10,000xg for 20 minutes. The soft buffy uppercoat of the pellet was carefully resuspended with the supernatant from this spin without resuspending the rest of the pellet. The combined supernatant and buffy coat were centrifuged at 48,000Xg for 20 minutes. The supernatant was discarded and the pellet was frozen at -20° C for up to 1 month for assay of GABA and benzodiazepine receptor.

2. Measurement of Glutamic Acid Decarboxylase Activity.

50 ul samples of crude homogenate were tested in 60 mM phosphate buffer pH 6.5-6.8 (containing 1.2 mM PLP, 0.6 mM AET, and 0.36 mM HEPES and 0.2% Triton X-100). The samples were incubated at 37° C for 15 minutes in 12mmx75mm disposable glass culture tubes. After incubation, 25 ul samples were removed for protein assay. Aliquots of [^{3}H]-Glu cocktail containing 0.16nM [^{3}H]-glutamate (0.12 uCi/reaction mixture) and 10 mM l-glutamate were added to the assay mixture and the reaction was continued for 30 minutes. The

reaction was stopped by adding 0.5 ml of 50% EtOH. Each sample was then passed through a small column of prewashed AG1-X4, followed by 2 washings of 1.0 ml H_2O directly into an empty scintillation vial. Controls contained the protein sample and the GAD inhibitor AOAA. Samples were counted in a liquid scintillation counter after the addition of 10 ml Scinti Verse II.

3. Measurement of Specific Binding

Specific binding is calculated on each sample, using the total amount of bound radioactively labelled tracer in the absence or presence of 1000 fold excess inhibitor.

The frozen membrane samples were resuspended in 0.5 ml ddH_2O at R.T. for 20 minutes. The remainder of the procedures were performed at 4° C. Equal aliquots of this membrane preparation were centrifuged in a Fisher microcentrifuge for 15 minutes. After centrifugation the supernatant was removed. The pellet from one membrane aliquot was resuspended in buffer for GABA binding, containing 0.05% NP40 and warmed to 37°C for 20 minutes. The pellet from the other aliquot was resuspended in PBS at 4° C for benzodiazepine binding. Following centrifugation of the NP40-washed membrane sample in the microcentrifuge for 15 minutes at 4°C, the supernatant was discarded and the pellet was resuspended in buffer for GABA binding. The protein

content of all samples were measured and binding was assayed on the basis of equal amounts of protein.

a) Gamma-aminobutyric Acid Binding. Binding studies were performed using 1.5 ml plastic microcentrifuge tubes. Either 20 ul of 1 mM GABA in buffer or 20 ul of 50 mM potassium phosphate buffer pH 7.1, was added to 160 ul of membrane preparation in the same buffer After 10 minutes of incubation at 0° C, 20 ul of [^{3}H]-muscimol (final concentration of 54 nM, approximately 100,000 cpm) was added and incubated for 15 minutes at 0°C. The reaction was stopped by centrifuging in a microcentrifuge for 12-15 minutes. The supernatant was removed and the pellet was washed once quickly and superficially with 1.0 ml ice cold H_2O. The pellet was dissolved in 1.0 ml of 1% TX-100 and counted with a liquid scintillation counter.

b) Benzodiazepine Binding Either 20 ul of 0.1 mM RO5-2807 for specific displacement or 20 ul PBS for total binding was added to 160 ul of membrane sample in PBS to be assayed. The mixture was incubated for 30 minutes at 0° C, followed by addition of [^{3}H]-flunitrazepam (final concentration 5 nM, approximately 50,000 cpm) in PBS. After incubating for another 30 minutes at 0°C, the reaction was stopped, centrifuged and the supernatant then removed. After washing once quickly and superficially with ice cold H_2O, the pellet was left to dissolve in TX-100 for up to 24 hours before counting in a liquid scintillation counter.

4. Measurement of Protein

a) Glutamic Acid Decarboxylase Assay. Samples of 25 ul from each tube were assayed for protein content by the method of Bradford (53).

b). Binding Assays Aliquots of 20 ul from each sample were assayed for protein content by the method of Lowry (54). All samples were assayed for binding on equal amounts of protein in each tube, based on fmoles bound/mg protein.

5. Hormone Treatments

Pregnant animals were given either 20 ug DES, 20 ug DHT, or 20 ug DEX in sesame oil vehicle or vehicle alone on day 16 of gestation. After birth, the pups were given either 1.0 ug DHT or DES in sesame oil vehicle every day for 21 days. For DEX treatments, pups were given 1.0 ug DEX for the first 3 days, followed by 0.4 ug DEX on alternate days up to day 21. For controls, sesame oil vehicle alone was used for the same periods. Treated male and female pups and respective controls (groups of 5) were sacrificed between days 35 and 45.

6. Animals Used in Age and Sex Studies

a) Sex Differences in Glutamic Acid Decarboxylase Activity , Gamma-aminobutyric Acid and Benzodiazepine Binding. Rats from the same litters were sacrificed in groups of three animals each of males and females. In the studies of the hypothalamus of younger pups (day 2), three

separate tissue samples were pooled together in order to obtain enough membranes for the binding assays.

b) Development of GAD Activity, GABA and BDZ Binding.

Rats of ages 18 to 45 days were studied to include prepubescent stages to adult. Male or female animals were sacrificed in groups of three animals each on day 20±2, day 25±3, day 30±2, or day 45±2.

7. Statistical Analysis

For all the histograms in this study, statistical analysis was performed on the data using the student's 2-tailed t-test. $p<.05$ was taken as statistically significant. Values are expressed as means plus or minus standard deviations, where indicated.

RESULTS

I Conditions for Testing the GABA-ergic System: Effect of Protein Concentration.

1.Glutamic Acid Decarboxylase Activity

GAD activity was assayed according to the procedure of Swaiman and Wu (48) with slight modifications. In adapting the procedure, we had to determine whether the increase in activity was proportional to the amount of protein added.

The brain regions of hypothalamus, cerebellum, and cortex were tested (Fig. 1). The activities measured were proportional to the amount of protein. With a four fold increase in protein, there was a parellel increase in activity in each brain region studied.

2.Gamma-aminobutyric acid and Benzodiazepine Receptor

Membrane preparations and binding assays were performed according to the procedure for determining GABA binding by Zukin et al (46) with slight modifications. Assays of GABA receptor were performed using [^{3}H]-muscimol as ligand and [^{3}H]-flunitrazepam was used in assays of benzodiazepine receptor (Fig. 2).

In the assay of BDZ receptor, the total binding between the three brain regions was different and a linear increase of binding was observed with the amount of protein added.

Figure 1

Calibration of GAD activity assay in three discrete brain regions. Two samples each of male hypothalamus, cerebellum, and cerebral cortex were homogenized in 15 vols. of 0.32 M sucrose in DB. Appropriate sample sizes were then tested in duplicate for GAD activity to give final protein amounts of 83 ug and 334 ug in each of the brain regions.

Fig. 1

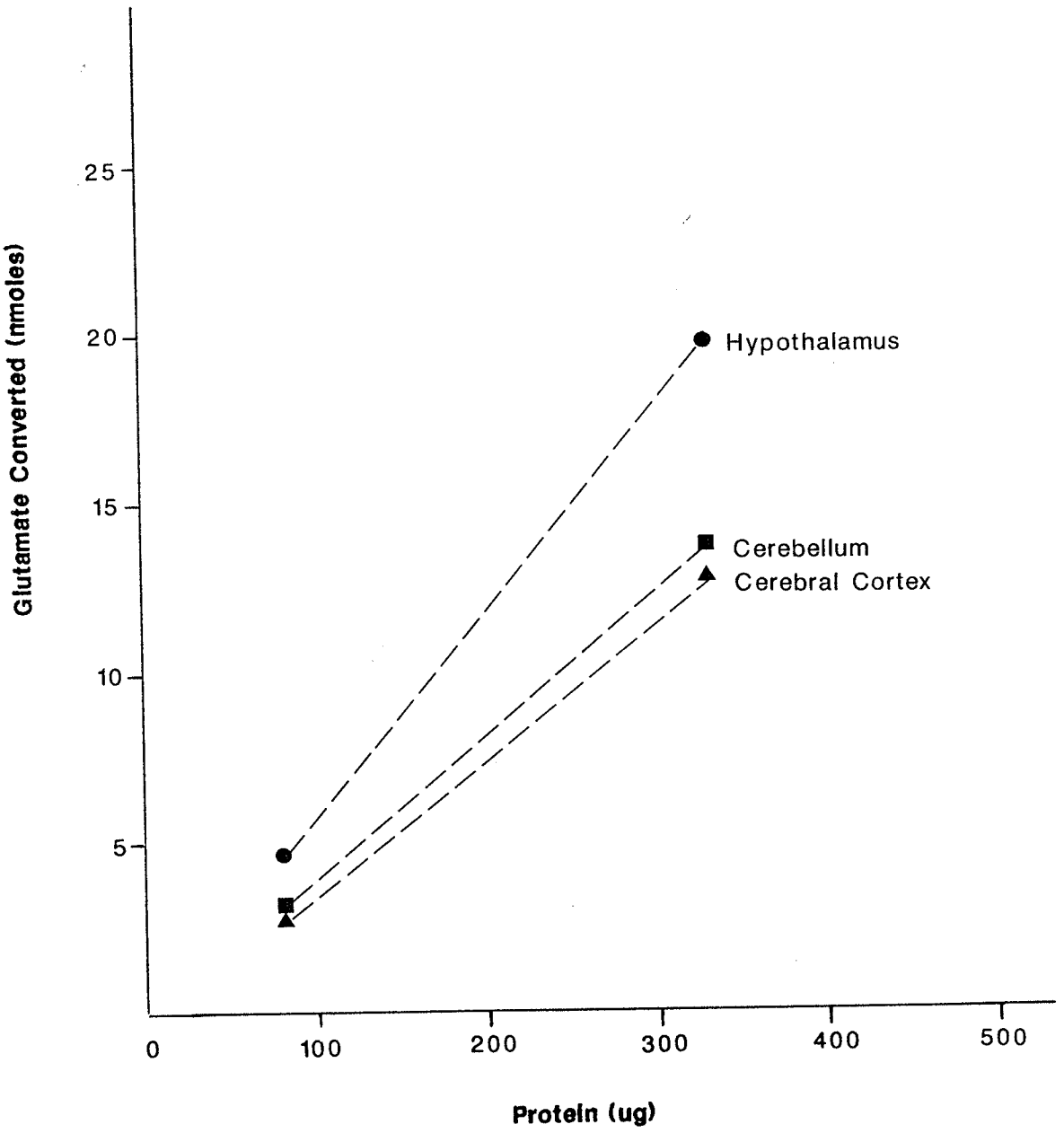
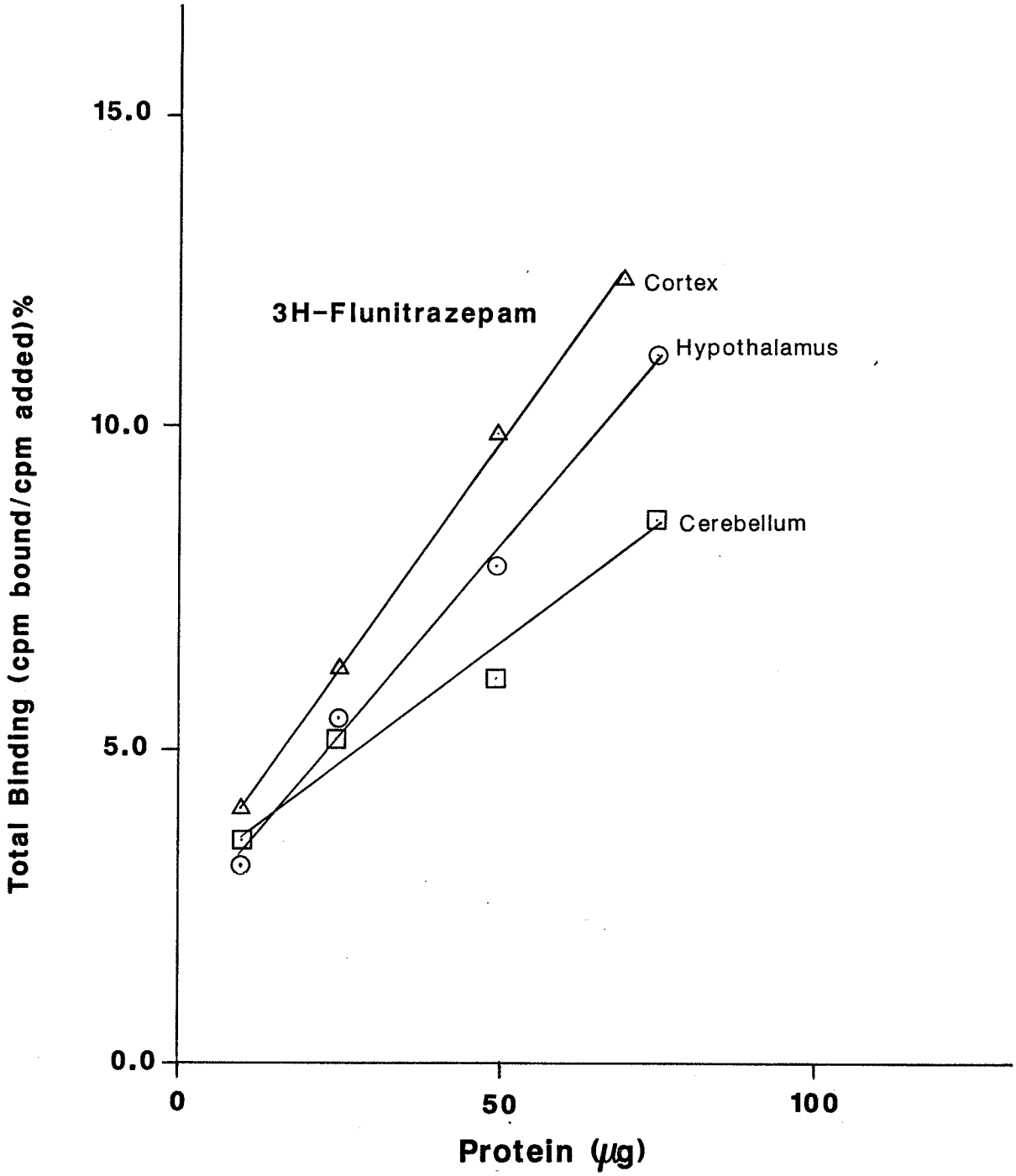

Glutamate Converted (nmoles)
25
20
15
10
5
0
100
200
300
400
500
Protein (ug)
Hypothalamus
Cerebellum
Cerebral Cortex

Figure 2

Titration curves for total binding of different ligands in discrete brain regions. Homogeneous samples of hypothalamus, cortex and cerebellum from four male rat brains were made by combining the various brain regions and homogenizing in .32 M sucrose in DB. Total [^{3}H]-flunitrazepam binding was tested in duplicate sample sizes of 10, 25, 50, and 75 ug of protein for each brain region. Each point is the average of duplicates from samples stored at -20° C for four months. Total [^{3}H]-flunitrazepam added was approximately 50,000 cpm in each case.

Fig. 2


15.0
10.0
5.0
0.0
0
50
100
Total Binding (cpm bound/cpm added)%
Protein (μg)
3H-Flunitrazepam
Cortex
Hypothalamus
Cerebellum

Similar results were obtained in GABA receptor (not shown), but showing lower specific binding.

With these procedures established we then examined the developmental pattern of the GABA-ergic system in male and female rats.

II Studies on the Development of the GABA-ergic System.

1. Development of Glutamic Acid Decarboxylase Activity With Age

GAD activity was measured in homogenates of the hypothalamus, cerebellum and cortex from rats in the early neonatal stages up to and including adult. At day 2, GAD levels were: 0.12 nmoles Glu converted/mg protein/min. in hypothalamus, 0.46 nmoles/mg/min. in the cortex, and 0.77 nmoles/mg/min. in the cerebellum (Table 1), for both males and females. The activity increased gradually until around 30 days of age (Fig. 3), when it appeared to level off.

The developmental patterns appear to be similar in each of the brain regions studied, despite differences in levels of this enzyme activity present. There appears to be a difference in enzyme activity between males and females at various developmental stages.

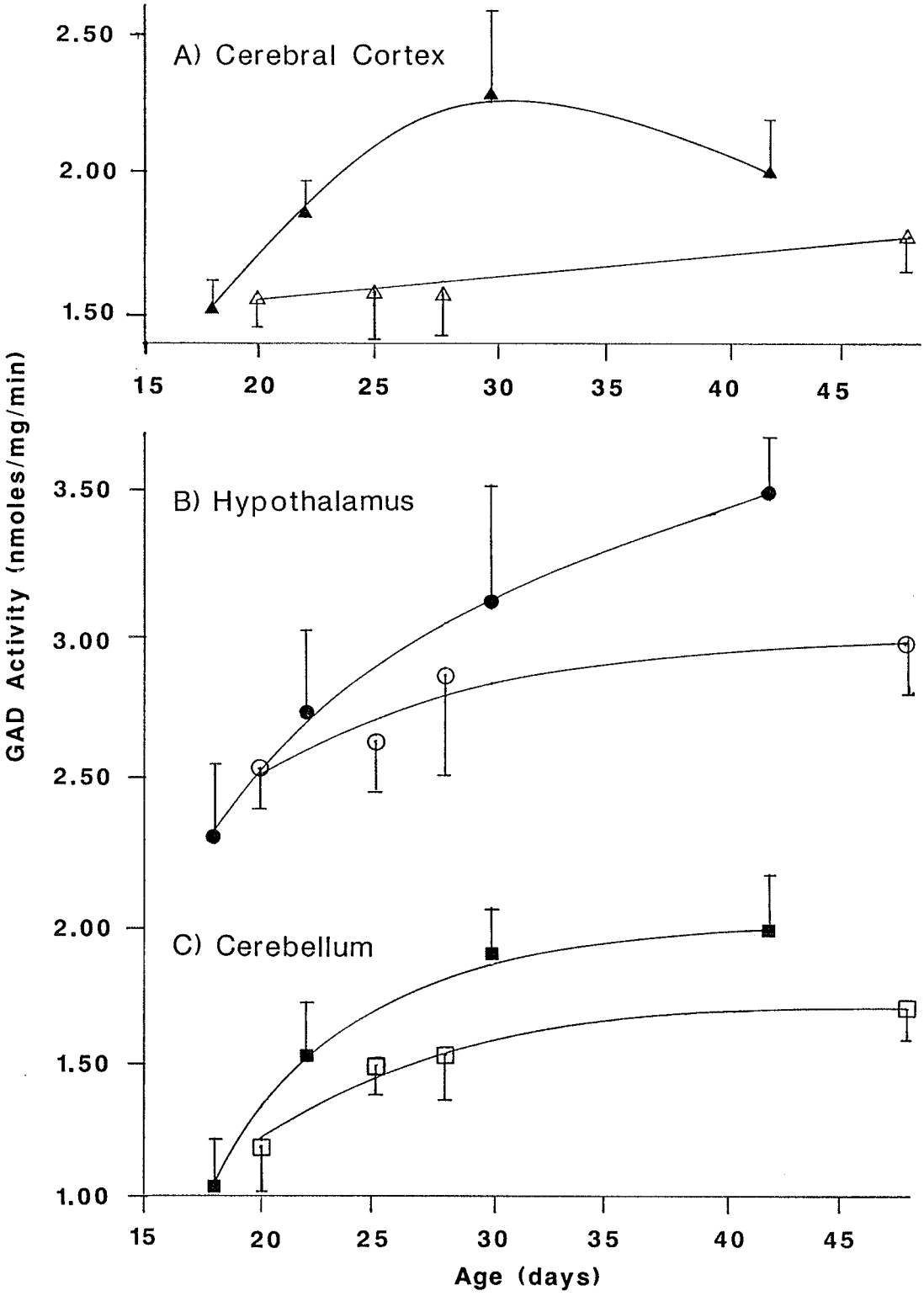
Table 1: GABA-ergic system in early development.

-	-	GAD ACTIVITY (nmoles/mg/min)		
AGE (days)	BRAIN REGIONS	Male	Mixed	Female
2	Hypothalamus	-	0.123	-
2	Cerebellum	-	0.77	-
2	Cerebral Cortex	0.56	-	0.35
12	Hypothalamus	1.73	-	1.83
12	Cerebellum	0.93	-	0.99
12	Cerebral Cortex	0.89	-	0.85
AGE	-	GABA BINDING		(fmoles ^{3}H-musc/mg
2	Hypothalamus	-	1250	-
2	Cerebellum	-	2000	-
2	Cerebral Cortex	1750	-	2100
12	Hypothalamus	1450	-	2750
12	Cerebellum	1200	-	720
12	Cerebral Cortex	2600	-	2050
AGE	-	BDZ BINDING		(fmoles ^{3}H-Flu/mg
2	Hypothalamus	-	660	-
2	Cerebellum	-	530	-
2	Cerebral Cortex	830	-	560
12	Hypothalamus	1220	-	1250
12	Cerebellum	-	351	-
12	Cerebral Cortex	1260	-	1450

Figure 3

Study of the development of GAD activity. The hypothalamus, cerebellum, and cerebral cortex were dissected from male and female rat brains and assayed for GAD activity in each of the four different age groups. Each point is the average of three animals with bars representing standard deviations. All animals from male or female groups were assayed at the same time. Male hypothalamus O, cerebellum □, and cortex △. Female hypothalamus ●, cerebellum ■, and cortex ▲.

Fig.3


A) Cerebral Cortex
B) Hypothalamus
C) Cerebellum
GAD Activity (nmoles/mg/min)
Age (days)
2.50
2.00
1.50
3.50
3.00
2.50
2.00
1.50
1.00
15
20
25
30
35
40
45

2. Sex Differences in Glutamic Acid Decarboxylase Activity.

To examine the differences at age 20 and 35 days, males and females of the same litter were used. In the hypothalamus, but not the cerebellum or cortex, GAD activity was higher in the male at 20 days of age. At age 35 days, the females had higher GAD activity in all brain regions studied, with the cerebellum being significantly higher (Fig. 4).

3. Development of Gamma-aminobutyric Acid Receptor.

The binding of [H]-muscimol to the GABA receptor was low in 2-day old rats. The average values for both males and females were: 1250 fmoles/mg protein in the hypothalamus, 2000 fmoles/mg in the cerebellum, and 1940 fmoles/mg in the cerebral cortex (Table 1).

The levels of GABA binding increased with age and levelled off at the age of 30 days (Figs. 5 and 6) in the cerebellum and cortex. The developmental profiles between the two brain regions as well as those between males and females were similar.

4. Sex Differences in the GABA Receptor.

GABA binding was assayed in male and female litter-mates at day 20 and day 35. At the age of 20 days, the binding of

Figure 4

Sex differences in GAD activity at two developmental stages. Male and female litter mates, ages 20 and 35 days, were sacrificed and their brains dissected to remove hypothalamus, cerebellum, and cerebral cortex. Each brain region was tested for GAD activity as previously described, and the mean of triplicate determinations is shown with lines representing standard deviations. In day 35 cortex, $p<.06$. * $p<.01$

Fig.4

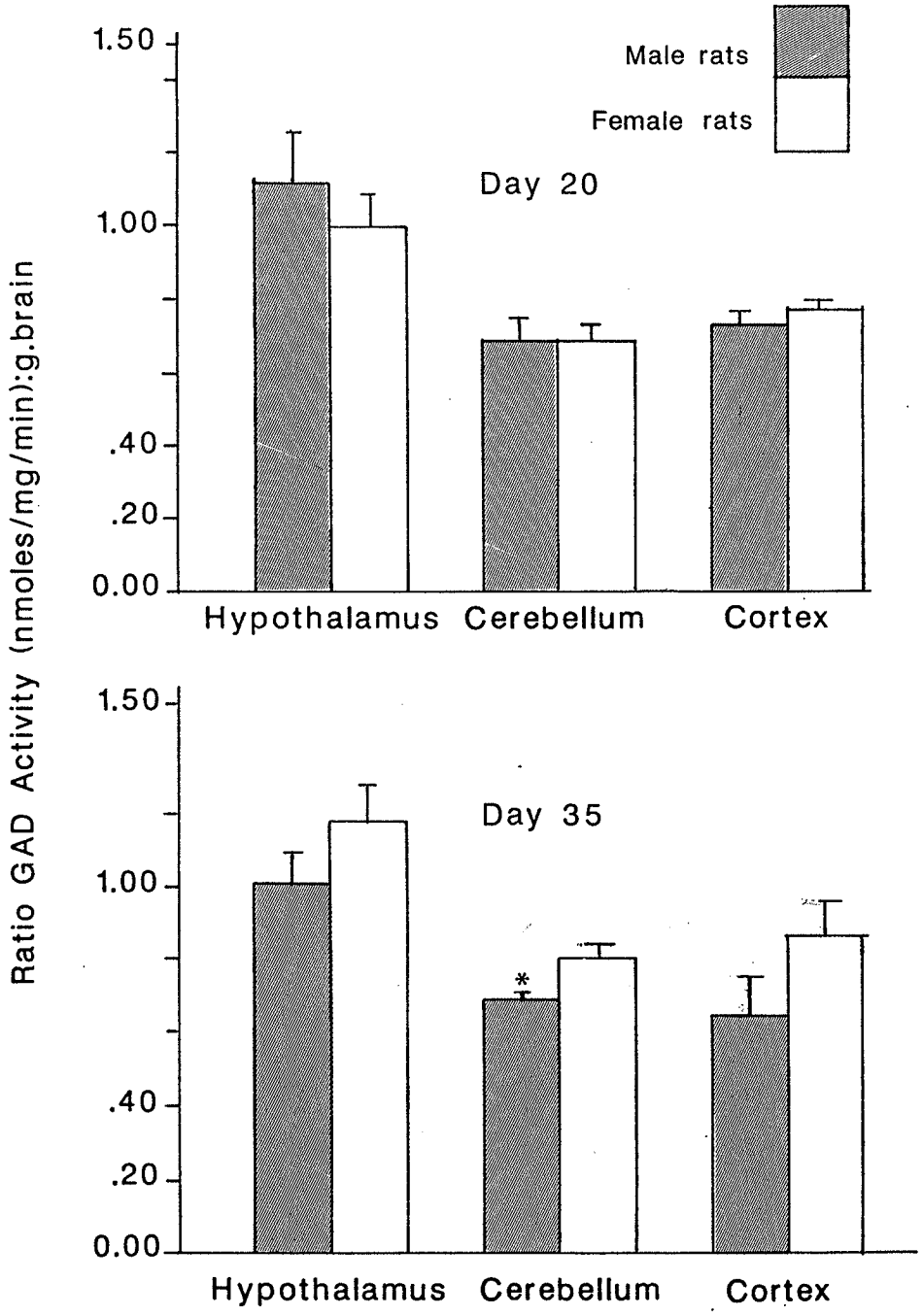

Male rats
Female rats
Day 20
Day 35
1.50
1.00
.40
.20
0.00
Ratio GAD Activity (nmoles/mg/min):g.brain
Hypothalamus
Cerebellum
Cortex
*

Figure 5

Study of the development of GABA binding in the cerebellum. Membrane preparations of cerebellum from the study on GAD activity (see Fig. 3), were tested for specific binding of [^{3}H]-muscimol as descibed in the methods. Each point represents the average of three determinations with bars representing standard deviations. Male cerebellum □ and female cerebellum ■ .

Fig.5

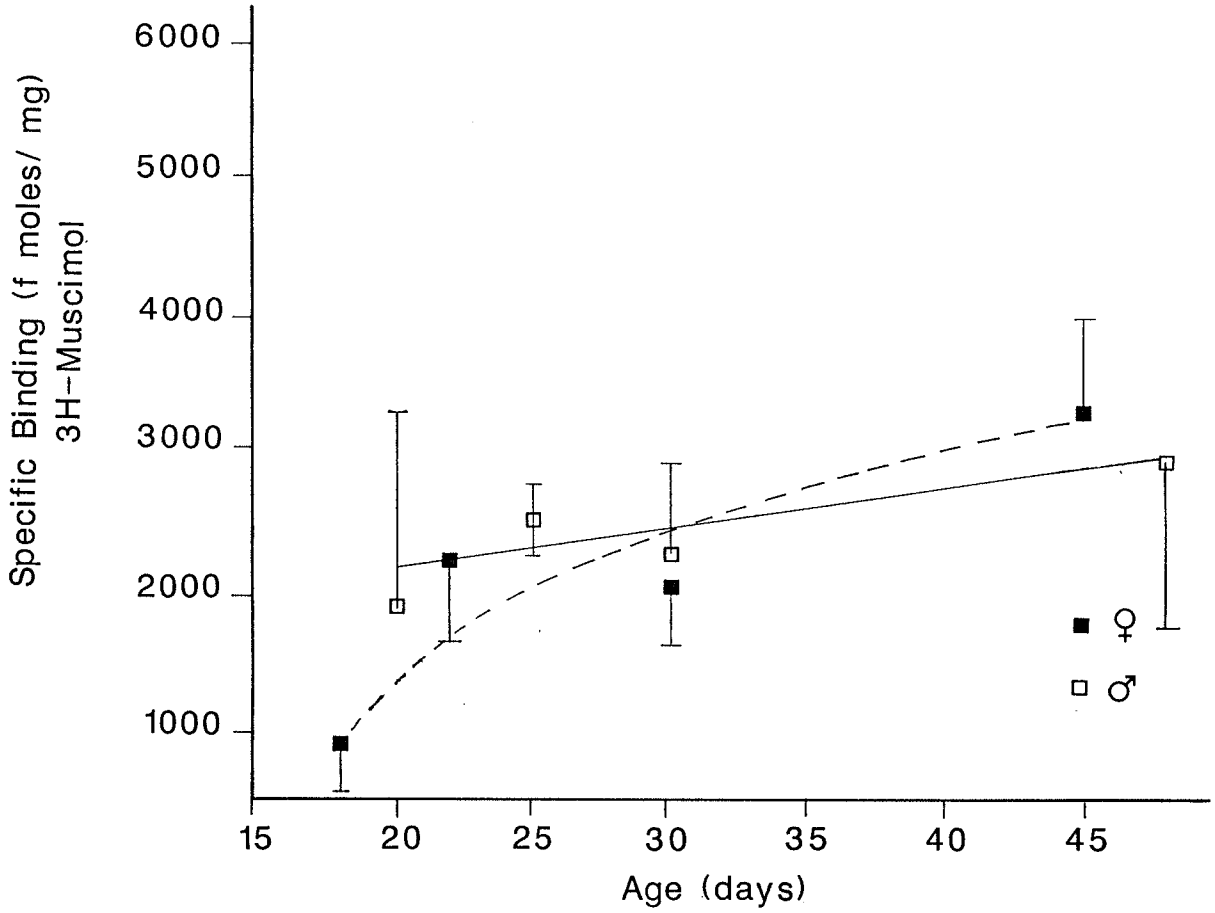
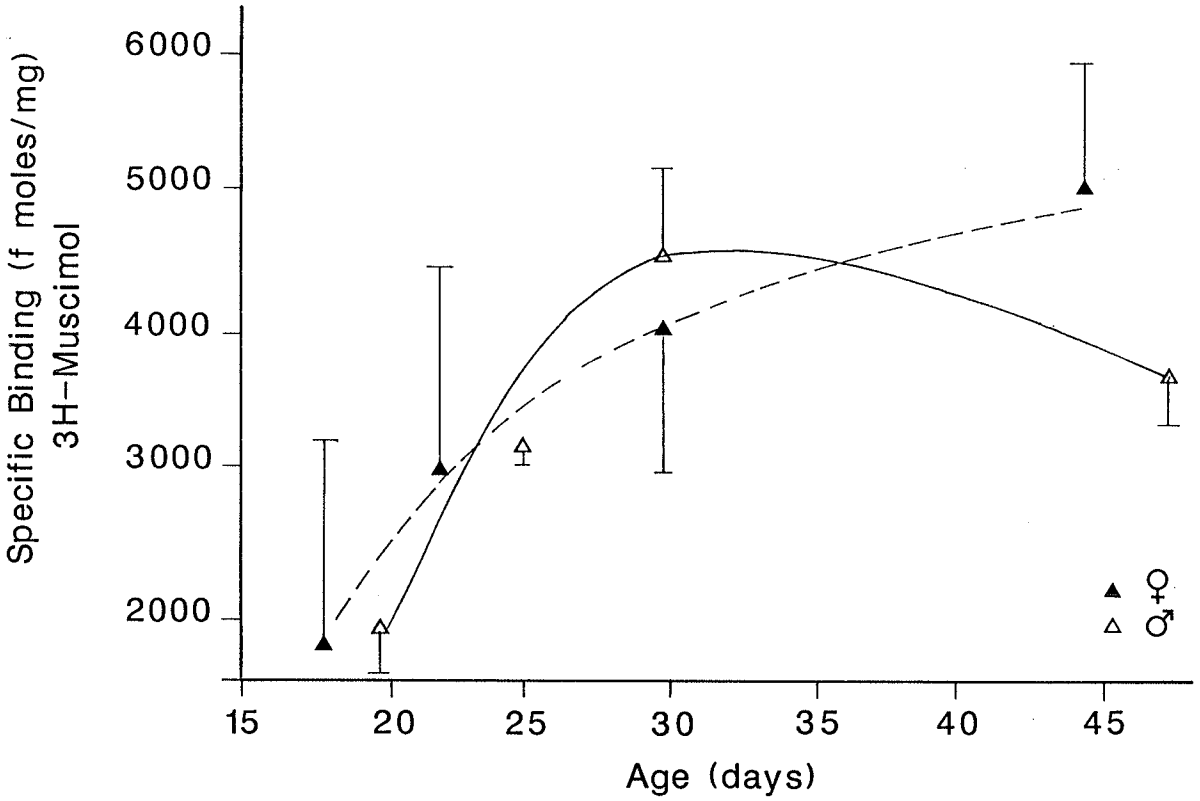

Specific Binding (f moles/ mg)
3H-Muscimol
6000
5000
4000
3000
2000
1000
15
20
25
30
35
40
45
Age (days)
♀
♂

Figure 6

Study of the development of GABA binding in rat cerebral cortex. Membrane preparations of cerebral cortex from the study on GAD activity (see Fig. 3), were tested for specific binding of [^{3}H]-muscimol as descibed in the methods. Each determination was performed in triplicate with bars representing standard deviations. Male cortex Δ and female cortex ▲.

Fig.6


Specific Binding (f moles/mg)
3H-Muscimol
6000
5000
4000
3000
2000
15
20
25
30
35
40
45
Age (days)
♀
♂

[^{3}H]-muscimol was significantly higher in female cerebellum but not in cortex (Fig. 7). At day 35, binding of this ligand appeared to be higher in the cerebellum and the hypothalamus, although the difference was not statistically significant.

5. Development of the Benzodiazepine Receptor.

The levels of BDZ binding in 2-day old rats were 660 fmoles/mg protein in the hypothalamus, 530 fmoles/mg in the cerebellum, and 700 fmoles/mg in the cerebral cortex (Table 1). Binding increased at day 12 to approximately 1200 fmoles/mg and 1350 fmoles/mg in the hypothalamus and the cortex, respectively (Table 1)

Receptor binding in the cerebellum appeared to decrease in males, but not in females, from day 18 (Fig. 8). In the cerebellum and cortex, the levels of [^{3}H]-flunitrazepam binding appeared to level off at around day 25 in both male and female rats (Fig. 9).

6. Sex Differences in BDZ Receptor.

Benzodiazepine binding was examined in male and female litter-mates at day 20 and day 35. The binding of [^{3}H]-flunitrazepam to membrane preparations from the hypothalamus and cerebellum was similar in both males and females. Male cortex exhibited slightly higher binding at both days studied (Fig. 10), but this difference was only statistically significant at day 20 ($p<0.05$) and not at day 35.

Figure 7

Sex differences in GABA binding at two different developmental stages. Specific binding of [^{3}H]-muscimol was tested in membrane preparations from the study on sexual differences in GAD activity, (Fig. 4 legend). The mean of triplicate determinations is shown with lines representing standard deviations. * $p<.02$

Fig. 7

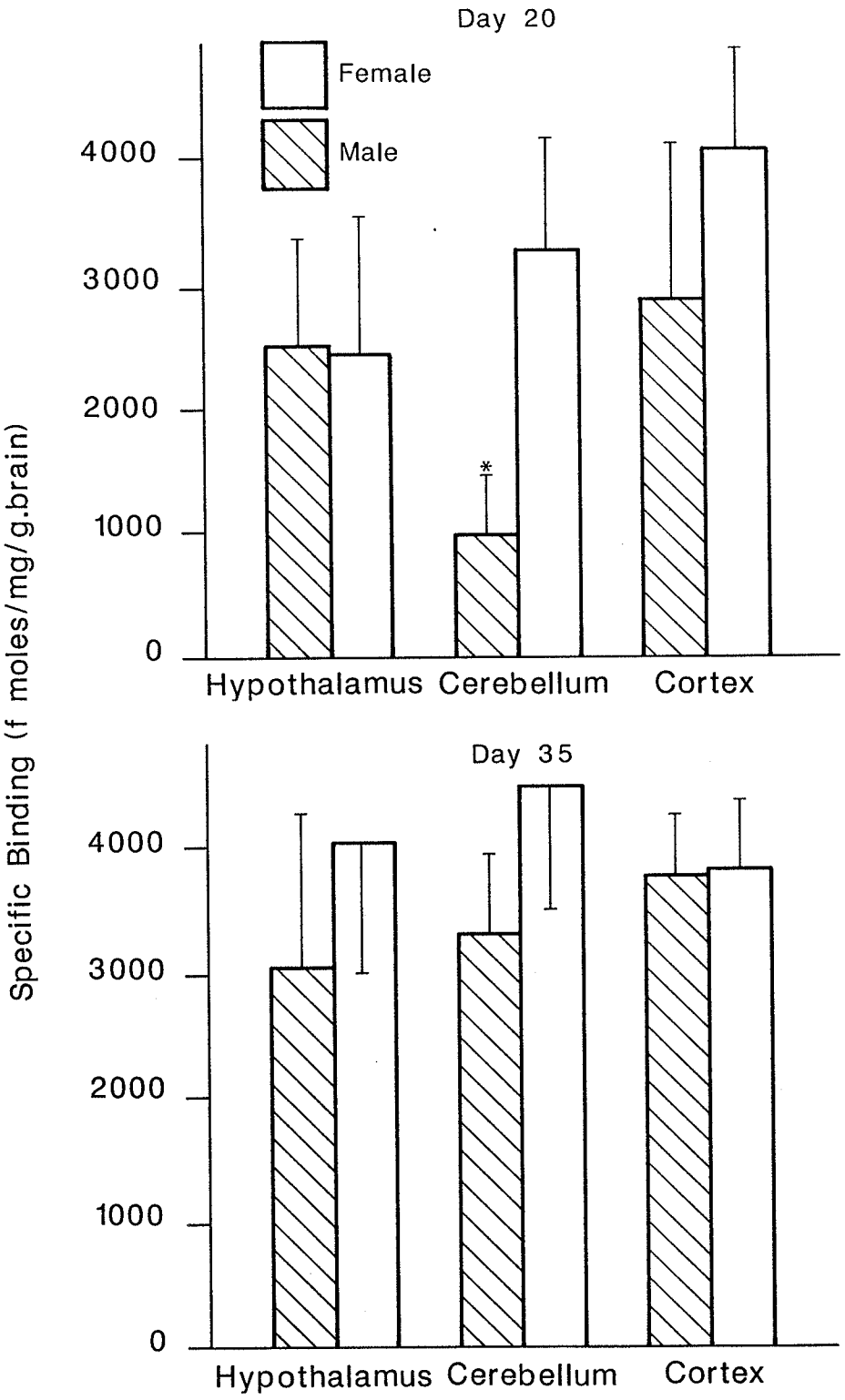

Day 20
Female
Male
4000
3000
2000
1000
0
*
Hypothalamus
Cerebellum
Cortex
Day 35
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex
Specific Binding (f moles/mg/g.brain)

Figure 8

Study of the development of BDZ binding in rat cerebellum. Membrane preparations of cerebellum from the study on GAD activity (see Fig.3), were tested for specific binding of [^{3}H]-flunitrazepam as described in the methods. Each determination was performed in triplicate with bars representing standard deviations. Male cerebellum □ and female cerebellum ■.

Fig.8

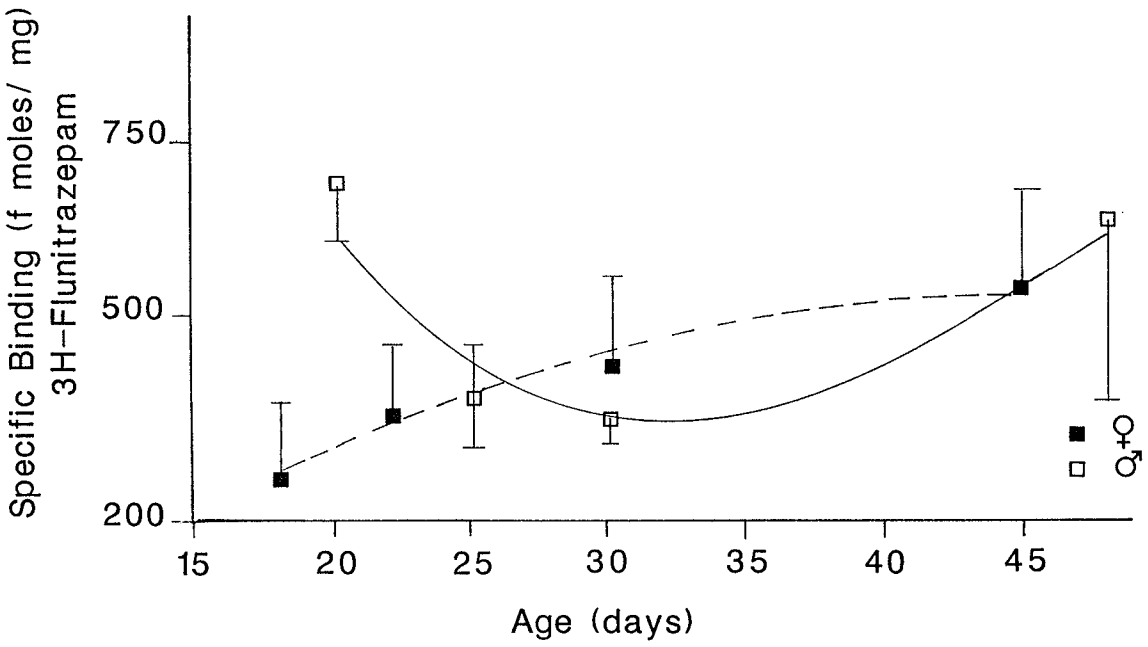

Specific Binding (f moles/ mg)
3H-Flunitrazepam
750
500
200
15
20
25
30
35
40
45
Age (days)
♀
♂

Figure 9

Study of the development of BDZ binding in rat cerebral cortex. Membrane preparations of cerebral cortex from the study on GAD activity (see Fig.3), were tested for specific binding of [^{3}H]-flunitrazepam as described in the methods. Each determination was performed in triplicate with bars representing standard deviations. Male cortex Δ and female cortex ▲.

Fig.9
BDZ Binding

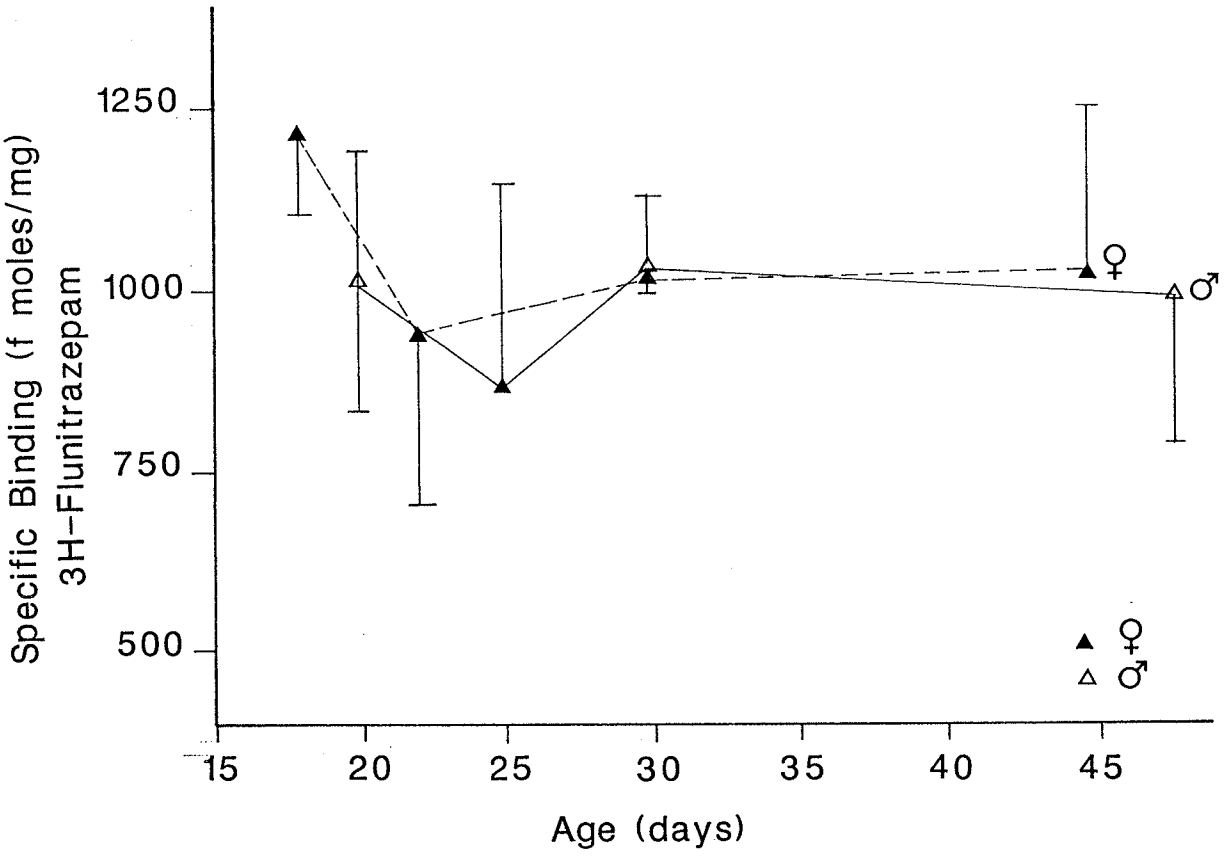
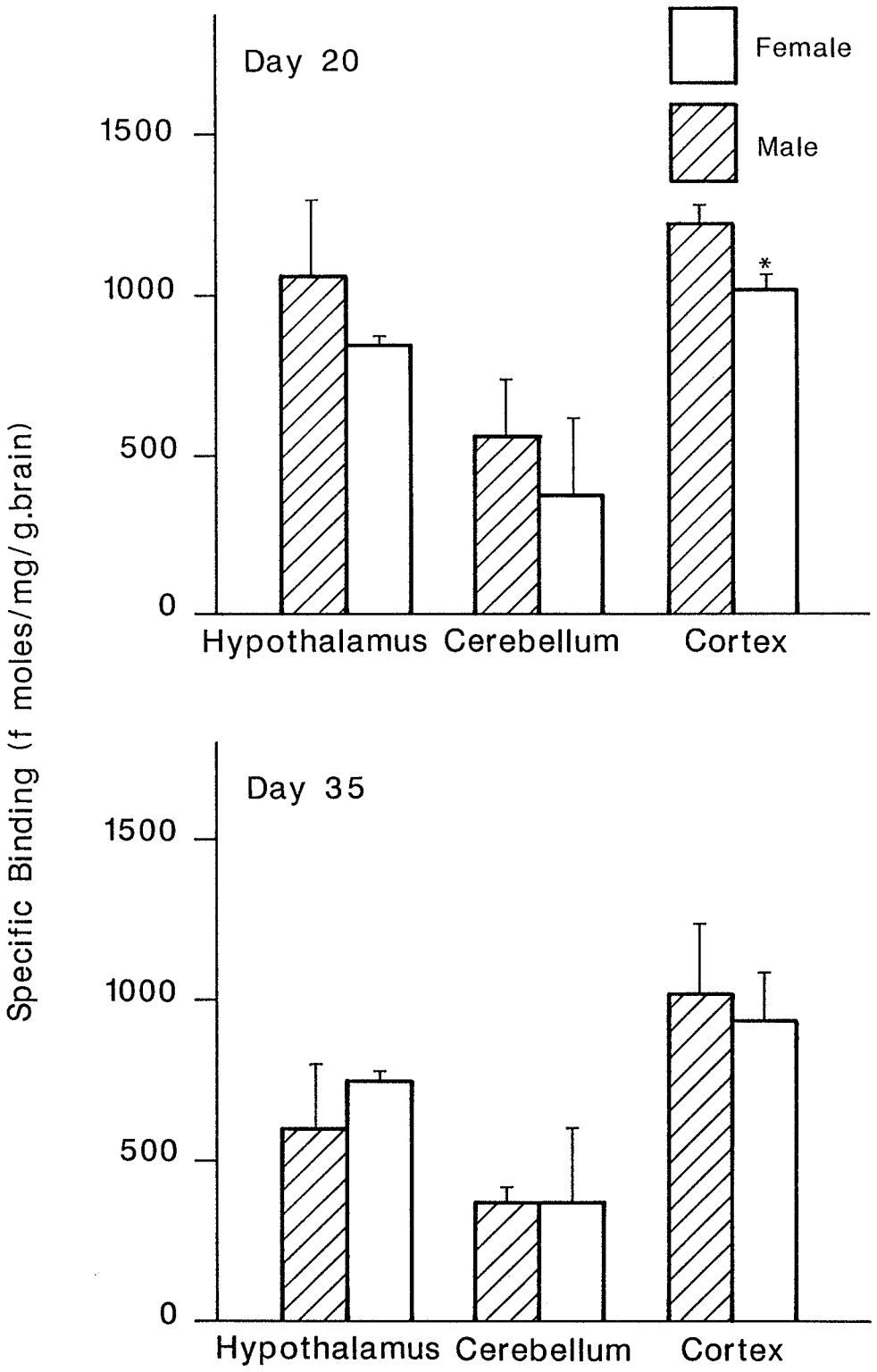


Figure 10

Sex differences in BDZ binding at two different developmental stages. Specific binding of [^{3}H]-flunitrazepam was tested in membrane preparations from the study on sex differences in GAD activity (Fig.4 legend). The mean of triplicate determinations is shown with lines representing standard deviations. * $p<.05$

Fig. 10


Day 20
Female
Male
1500
1000
500
0
*
Hypothalamus
Cerebellum
Cortex
Day 35
1500
1000
500
0
Hypothalamus
Cerebellum
Cortex
Specific Binding (f moles/mg/g.brain)

III The Effects of Hormones During Early Developmental Stages on the GABA-ergic System.

The finding that development of the GABA-ergic system appears to be different between males and females led to further studies on hormonal influences during early development, including the 'Critical Period.'

1. Glutamic Acid Decarboxylase Activity.

a) Dexamethasone Treatments. Treatment with dexamethasone did not affect significantly the GAD activity in all brain regions studied (Fig. 11).

b) Diethylstilbestrol Treatment. Figure 12 shows that the GAD activity was not significantly affected in males by diethylstilbestrol treatment. In female rats, however, GAD activity was significantly lower in the hypothalamus ($p<0.01$) and cerebellum ($p<0.005$) of treated animals.

c) Dihydrotestosterone Treatment. No significant difference was observed in GAD activity after treatment with dihydrotestosterone in all brain regins studied in both male and female rats (Fig. 13).

Figure 11

The effect of dexamethasone on GAD activity in rat brain. Pregnant female rats received 20 ug DEX in sesame oil vehicle or vehicle alone in subcutaneous injections on day 16 of gestation. After the pups were born they received 1.0 ug DEX for 3 days, then 0.4 ug DEX on alternate days until day 21. Male rats were sacrificed on day 33 and female rats on day 35. The brains were dissected the same day and assayed for GAD activity as previously described in the methods. Each bar represents the average of 5 determinations with standard deviations.

Fig.11

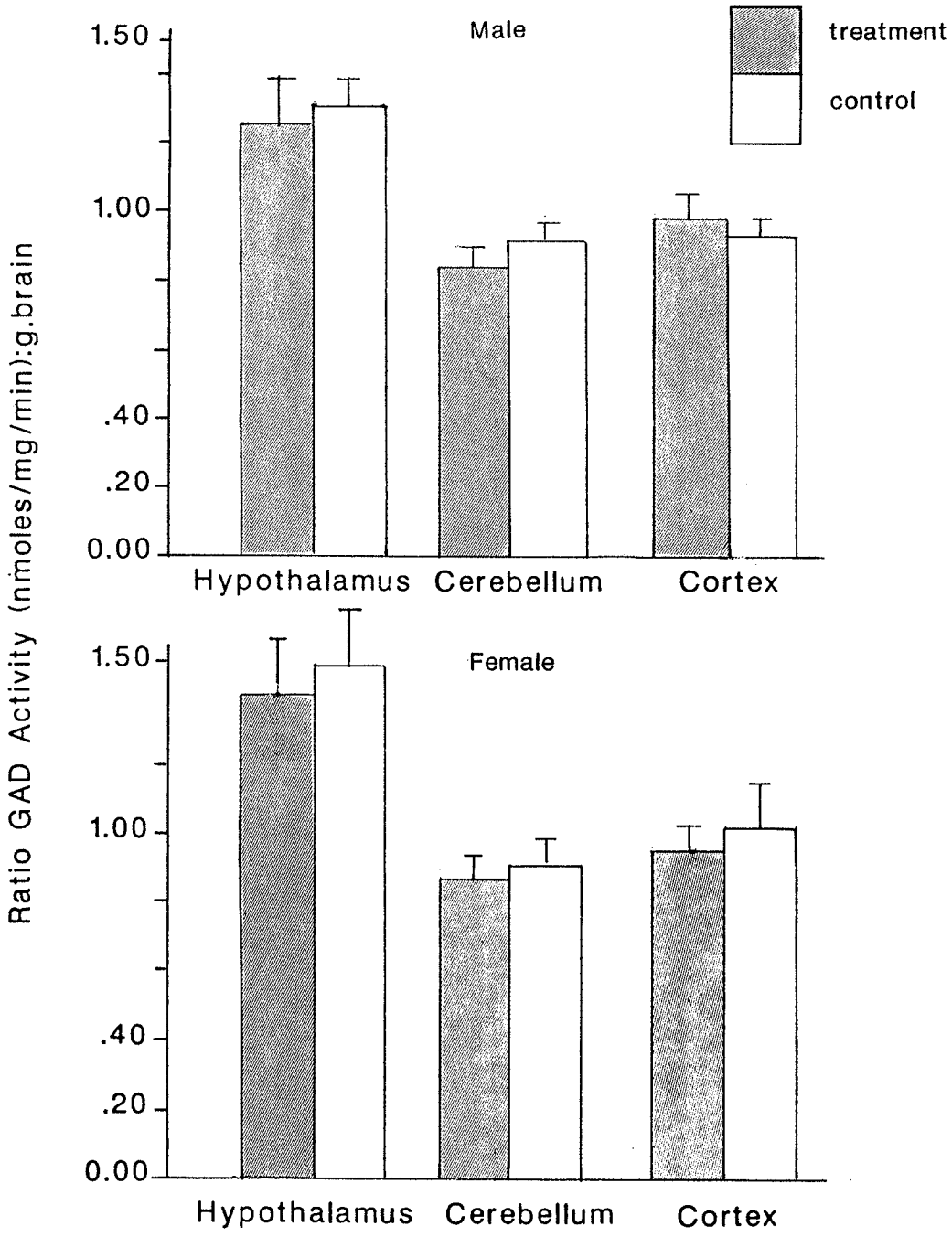

Male
treatment
control
1.50
1.00
.40
.20
0.00
Hypothalamus
Cerebellum
Cortex
Female
1.50
1.00
.40
.20
0.00
Hypothalamus
Cerebellum
Cortex
Ratio GAD Activity (nmoles/mg/min):g.brain

Figure 12

The effect of diethylstilbestrol on GAD activity in rat brain. Pregnant female rats received 1 injection of 20 ug DES in sesame oil vehicle or vehicle alone on day 16 of gestation. After the females gave birth, the pups received either 1.0 ug DES in sesame oil vehicle or vehicle alone, every day for 21 days. Male rats were then sacrificed on day 40 and female rats on day 41. For males, the bars represent the average of five determinations with standard deviations. The female treatment groups only represent the average of three determinations while the control group has five determinations in each case. * $p<.01$ ** $p<.005$

Fig.12

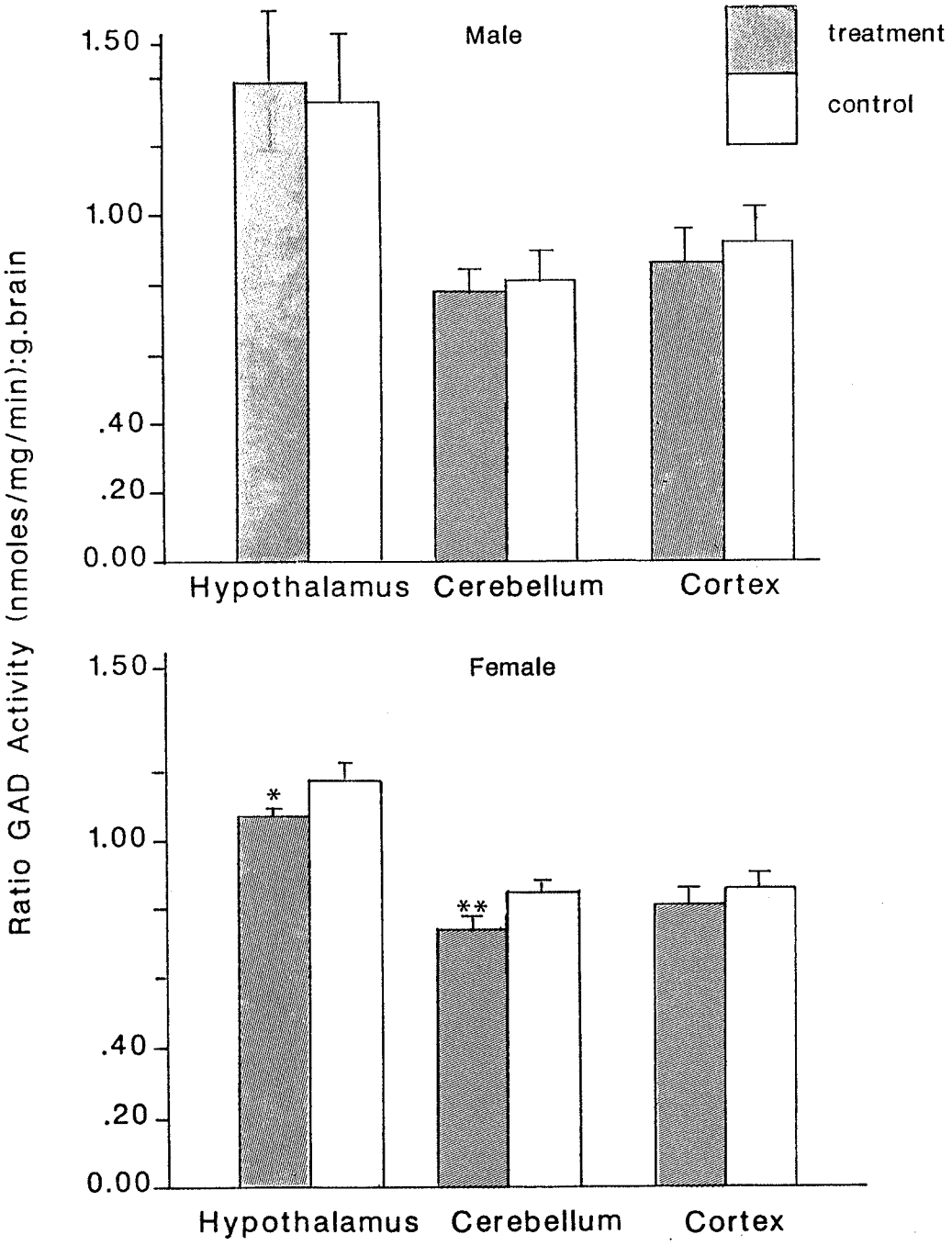
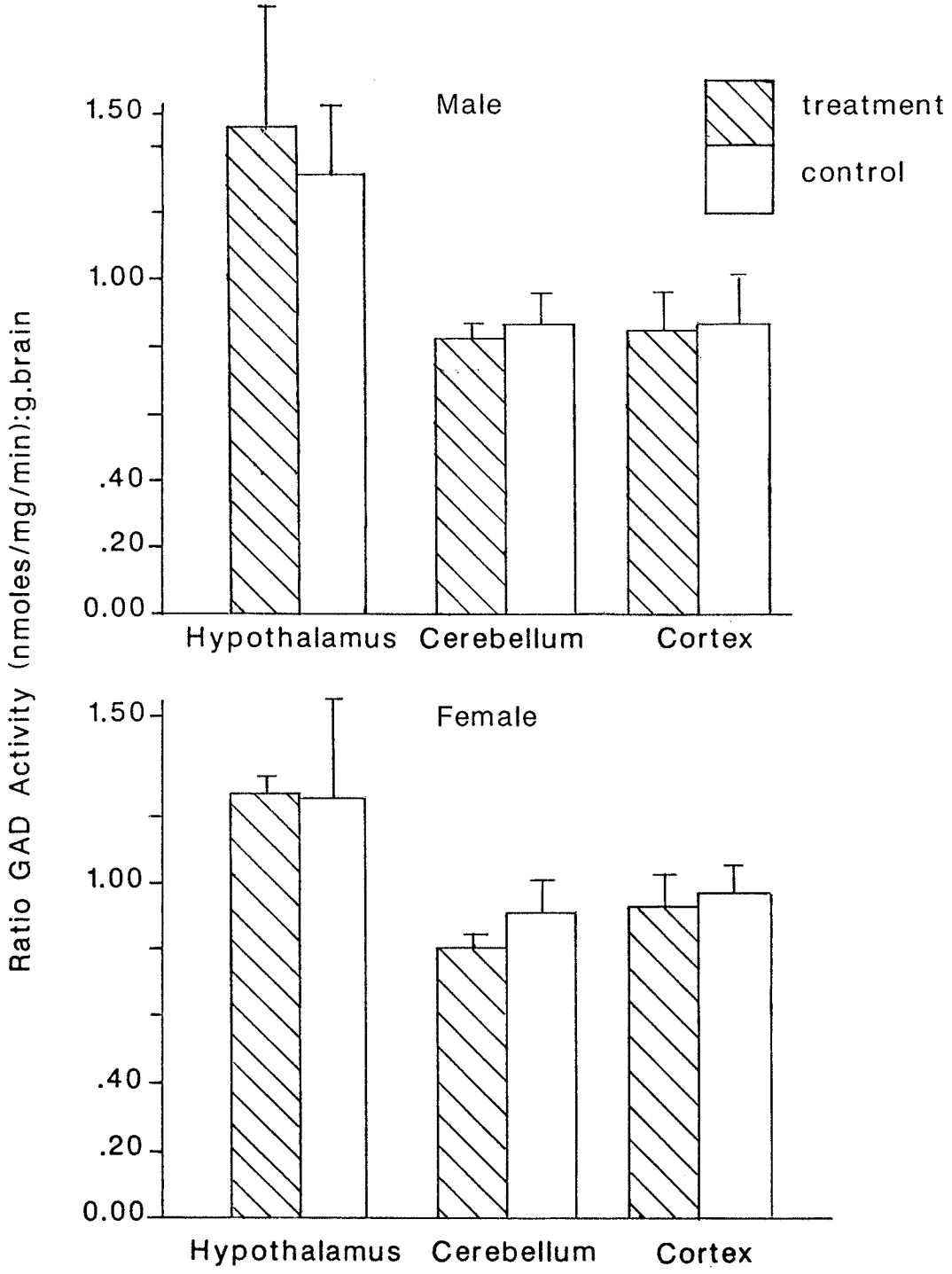

Male
treatment
control
1.50
1.00
.40
.20
0.00
Hypothalamus
Cerebellum
Cortex
Female
*
**
Ratio GAD Activity (nmoles/mg/min):g.brain

Figure 13

The effect of dihydrotestosterone on glutamic acid decarboxylase activity. Pregnant female rats were treated with 20 ug DHT in sesame oil vehicle or vehicle alone on day 16 of gestation. After birth , the pups were treated either with 1.0 ug DHT in sesame oil vehicle or vehicle alone, respectively, in the appropriate litter for 21 days. Male rats were sacrificed on day 47 and female rats on day 44 and the brain regions dissected and assayed for GAD as previously descibed. Each bar represents the average of five determinations, except in hypothalamus, where n=4 in males and n=3 in females.

Fig.13


Male
treatment
control
1.50
1.00
.40
.20
0.00
Hypothalamus
Cerebellum
Cortex
Female
1.50
1.00
.40
.20
0.00
Hypothalamus
Cerebellum
Cortex
Ratio GAD Activity (nmoles/mg/min):g.brain

2. Gamma-aminobutyric Acid Binding

a) Dexamethasone Treatments.

GABA binding,([^{3}H]-muscimol as ligand), in the hypothalamus, cerebellum and cortex was not significantly affected by treatment with dexamethasone in both males and females (Fig. 14).

b) Diethylstilbestrol Treatment.

[^{3}H]-muscimol binding was reduced slightly in all brain regions examined and significantly reduced in the cerebellum ($p<0.01$) and cortex ($p<0.05$) in male rats after treatment with diethylstilbestrol (Fig. 15). No significant difference in [H]-muscimol binding was observed in the female brain regions studied.

c) Dihydrotestosterone Treatment.

[^{3}H]-muscimol binding in the hypothalamus, cerebellum and cortex from males was not significantly affected by treatment with dihydrotestosterone (Fig. 16). In the female, however, [^{3}H]-muscimol binding increased by 42 % in the hypothalamus of treated animals relative to controls. Conversely, [^{3}H]-muscimol binding to the cortex was reduced by 28 % in treated animals.

Figure 14

The effect of dexamethasone on GABA binding in the hypothalamus, cerebellum and cortex of the rat brain. Membrane preparations from rat brain regions of dexamethasone treated and control animals (see legend Fig. 11), were stored at -20°C for up to 2 months and then assayed for specific binding of [^{3}H]-muscimol. Values are means of 5 determinations, except in the treated females and hypothalamus of control males where n=4. Vertical lines represent standard deviations.

Fig. 14

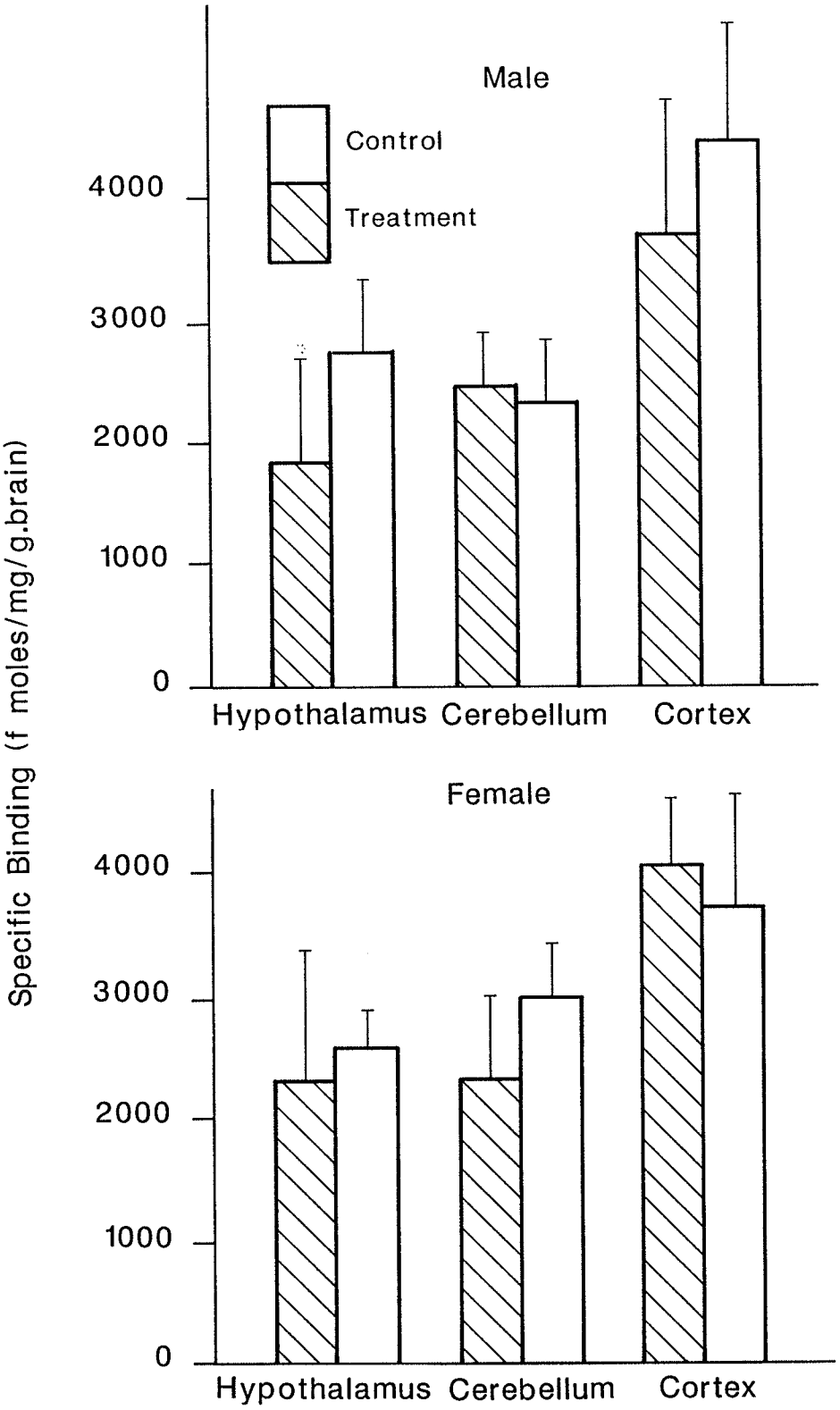

Male
Control
Treatment
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex
Female
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex
Specific Binding (f moles/mg/g.brain)

Figure 15

The effect of diethylstibestrol on GABA binding in rat brain. Membrane preparations from rat brain regions of diethylstilbesterol treated and control animals (see legend Fig. 12), are stored at -20°C for up to 2 months and then assayed for specific binding of [^{3}H]-muscimol. Values are means of 5 determinations in males and 3 determinations in females, with vertical lines representing standard deviations. ** $p<.01$, * $p<.05$

Fig. 15

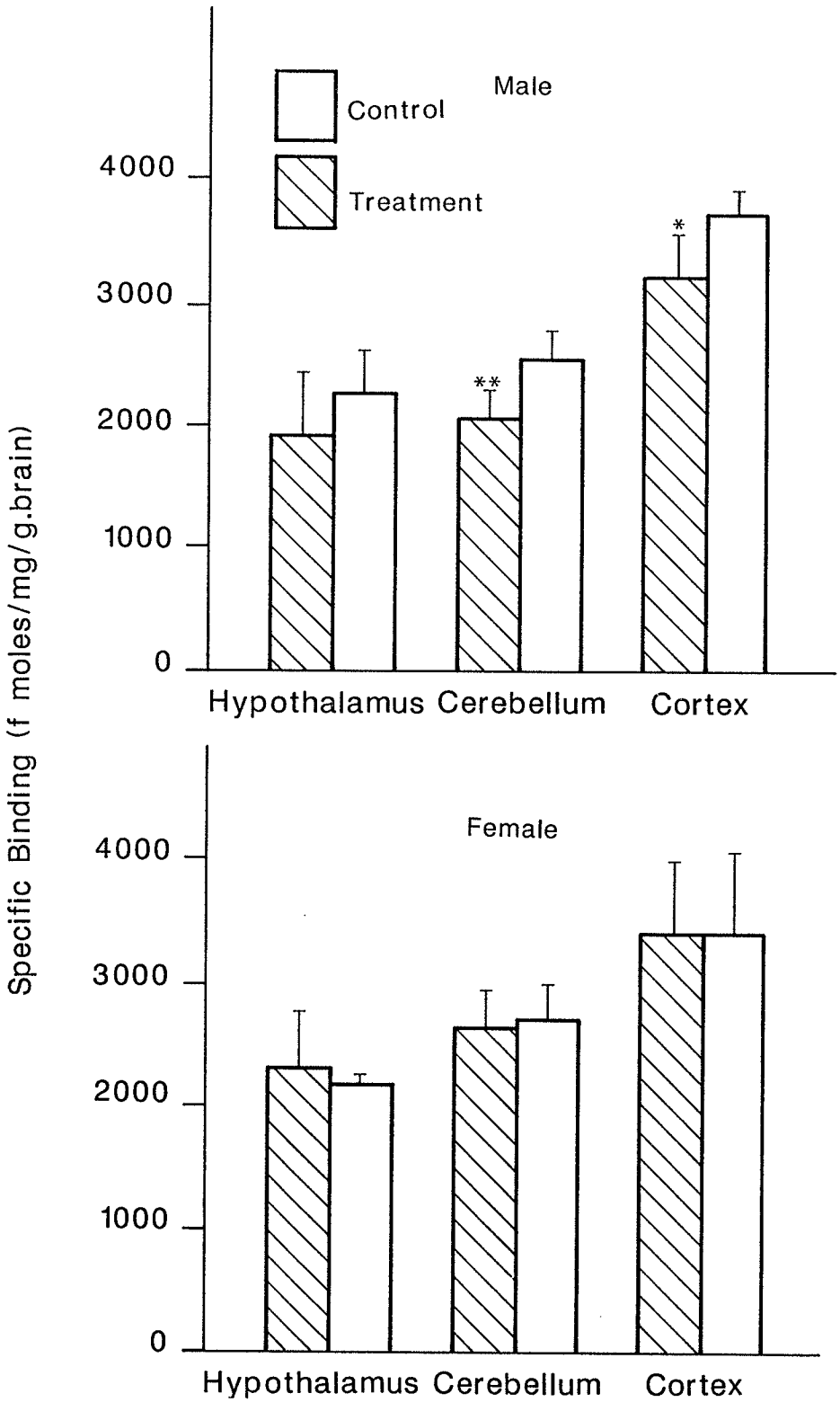
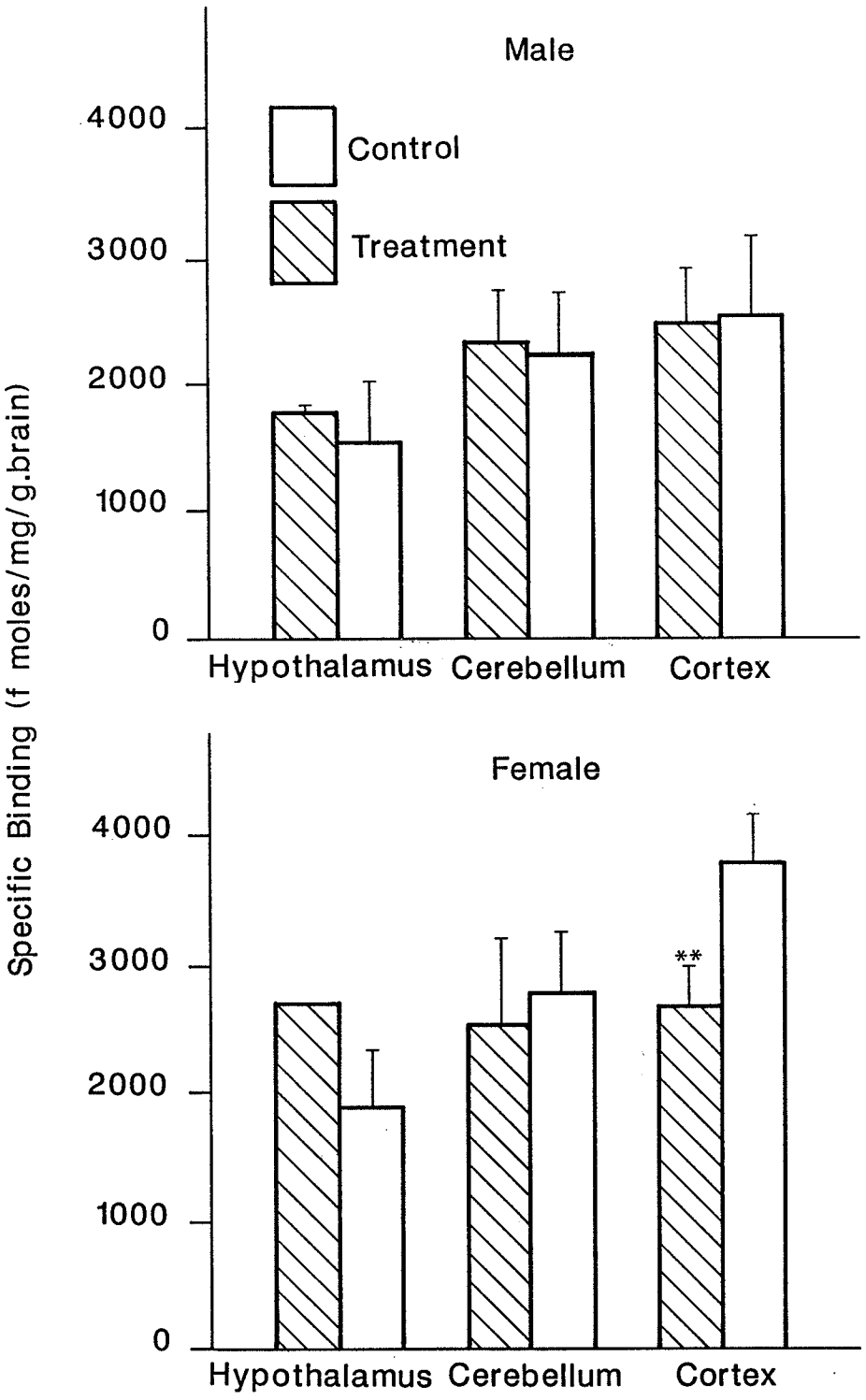

Male
Control
Treatment
4000
3000
2000
1000
0
**
*
Hypothalamus
Cerebellum
Cortex
Female
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex
Specific Binding (f moles/mg/g.brain)

Figure 16

The effect of dihydrotestosterone on GABA binding in rat brains. Membrane preparations from rat brain regions of DHT treated and control animals (see legend Fig. 13), were stored at -20° C for up to 2 months and then assayed for specific binding of [^{3}H]-muscimol. Values are means of 5 determinations, except in female hypothalamus and cortex, where n=4 for the control animals and n=3 for treated animals. ** $p<.01$

Fig. 16


Male
Control
Treatment
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex
Specific Binding (f moles/mg/g.brain)
Female
**
4000
3000
2000
1000
0
Hypothalamus
Cerebellum
Cortex

3. Benzodiazepine Receptor

a) Dexamethasone Treatments.

[^{3}H]-flunitrazepam bnding to the different brain regions was, in general, not significantly affected by the dexamethasone treatment, except in the female cerebellum, wherein binding decreased significantly ($p<0.05$) to 62 % of that seen in controls (Fig. 17).

b) Diethylstilbestrol Treatments.

No significant difference of [^{3}H]-flunitrazepam binding to the hypothalamus, cerebellum and cortex in either males or females was observed with diethylstilbestrol treatment (Fig. 18).

c) Dihydrotestosterone Treatments.

[^{3}H]-flunitrazepam binding to the different brain regions studied in either males or females was not significantly affected by treatment with dihydrotestosterone (Fig. 19).

Figure 17

The effect of dexamethasone on BDZ binding in rat brains. Specific binding of [^{3}H]-flunitrazepam was tested on the same membranes as those for GABA binding (Fig.14 legend) by the procedures outlined in the methods. The mean of quintuplicate determinations is shown with vertical lines representing standard deviations. * $p<.05$

Fig. 17

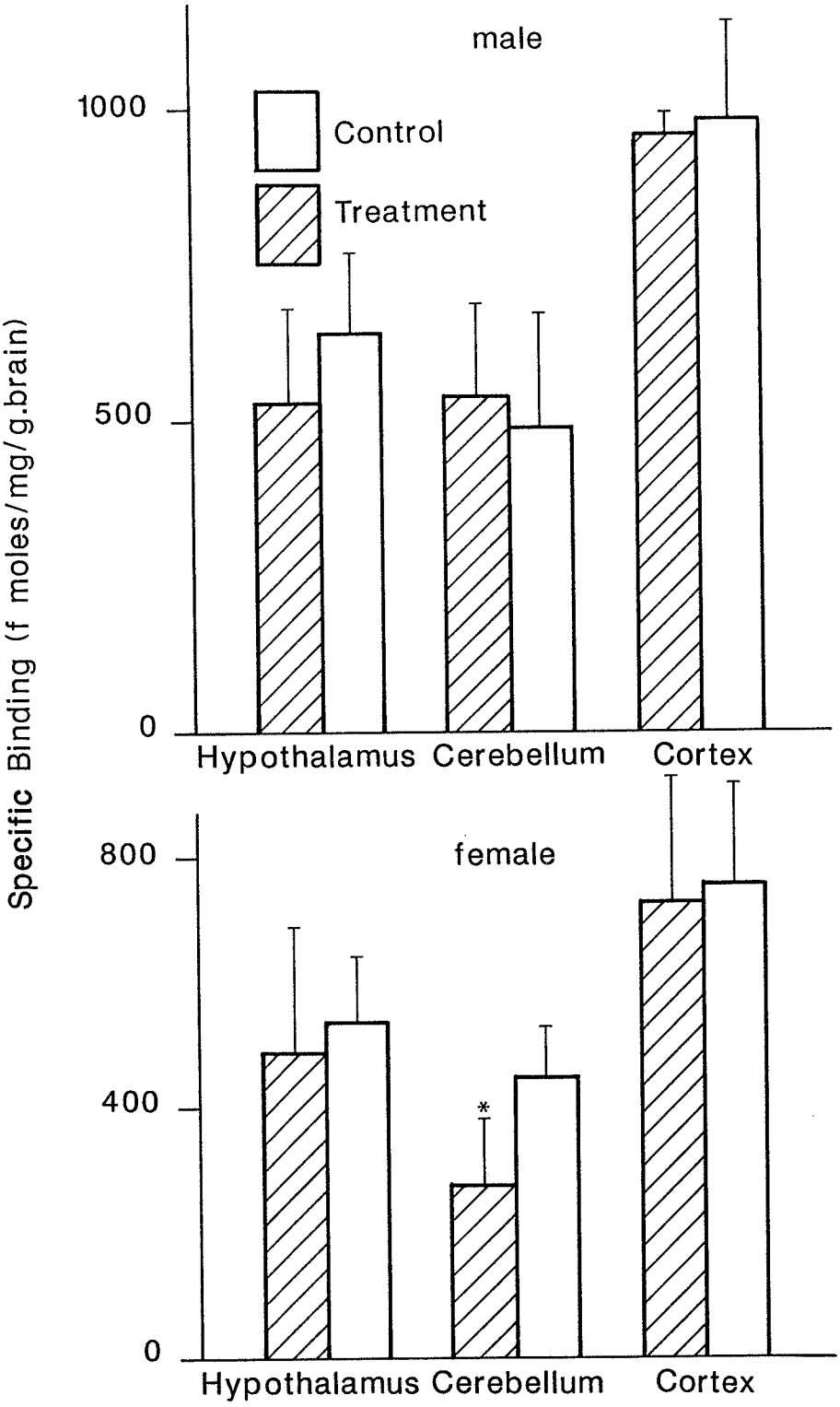

male
1000
500
0
Control
Treatment
Specific Binding (f moles/mg/g.brain)
Hypothalamus
Cerebellum
Cortex
800
400
0
female
*
Hypothalamus
Cerebellum
Cortex

Figure 18

The effect of diethylstilbestrol on BDZ binding in rat brains. Specific binding of [^{3}H]-flunitrazepam was tested on the same membranes as those for GABA binding (Fig.15 legend) by the procedures outlined in the methods. Values are means of 5 determinations for males and 3 determinations for females with vertical lines representing standard deviations.

Fig. 18

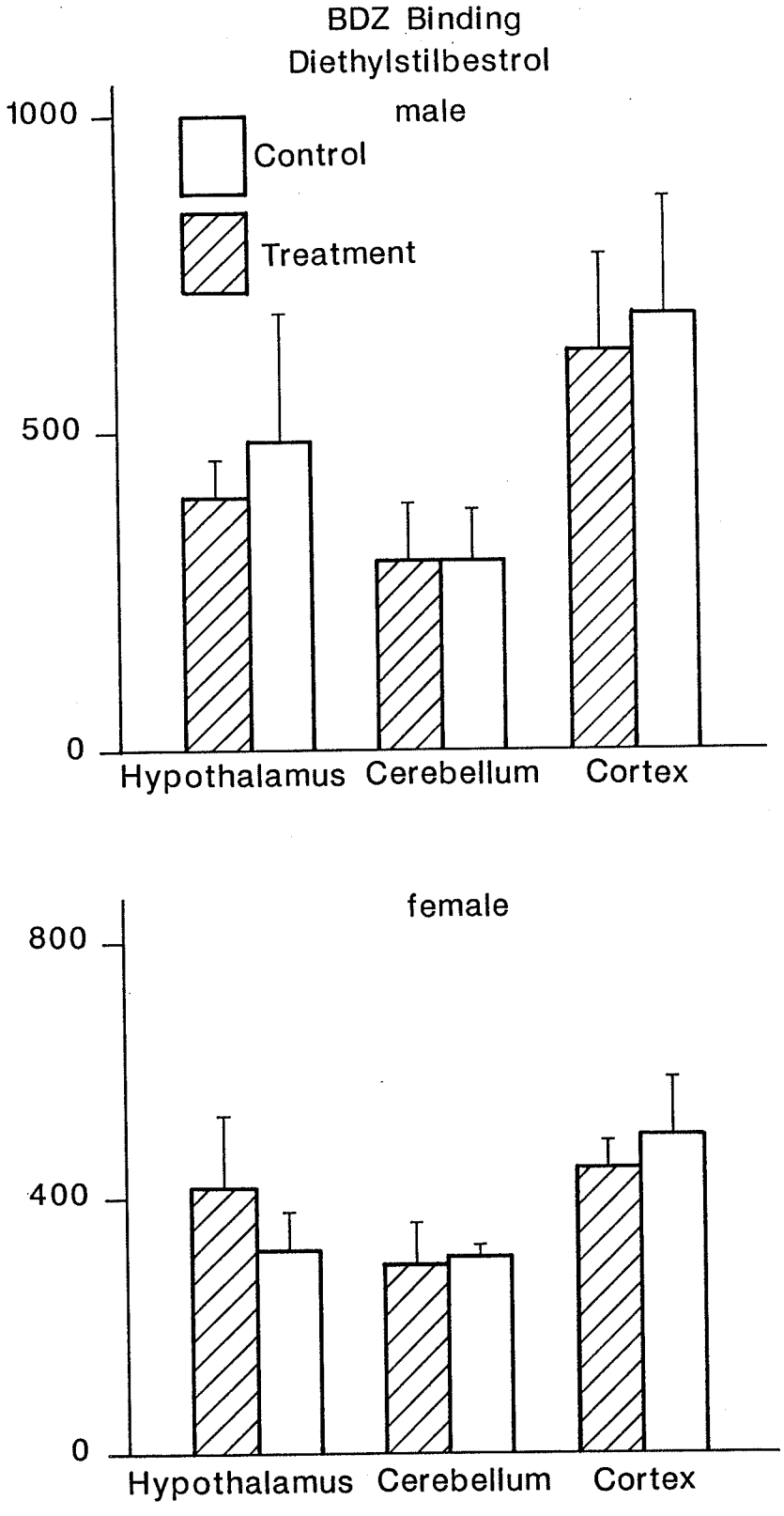
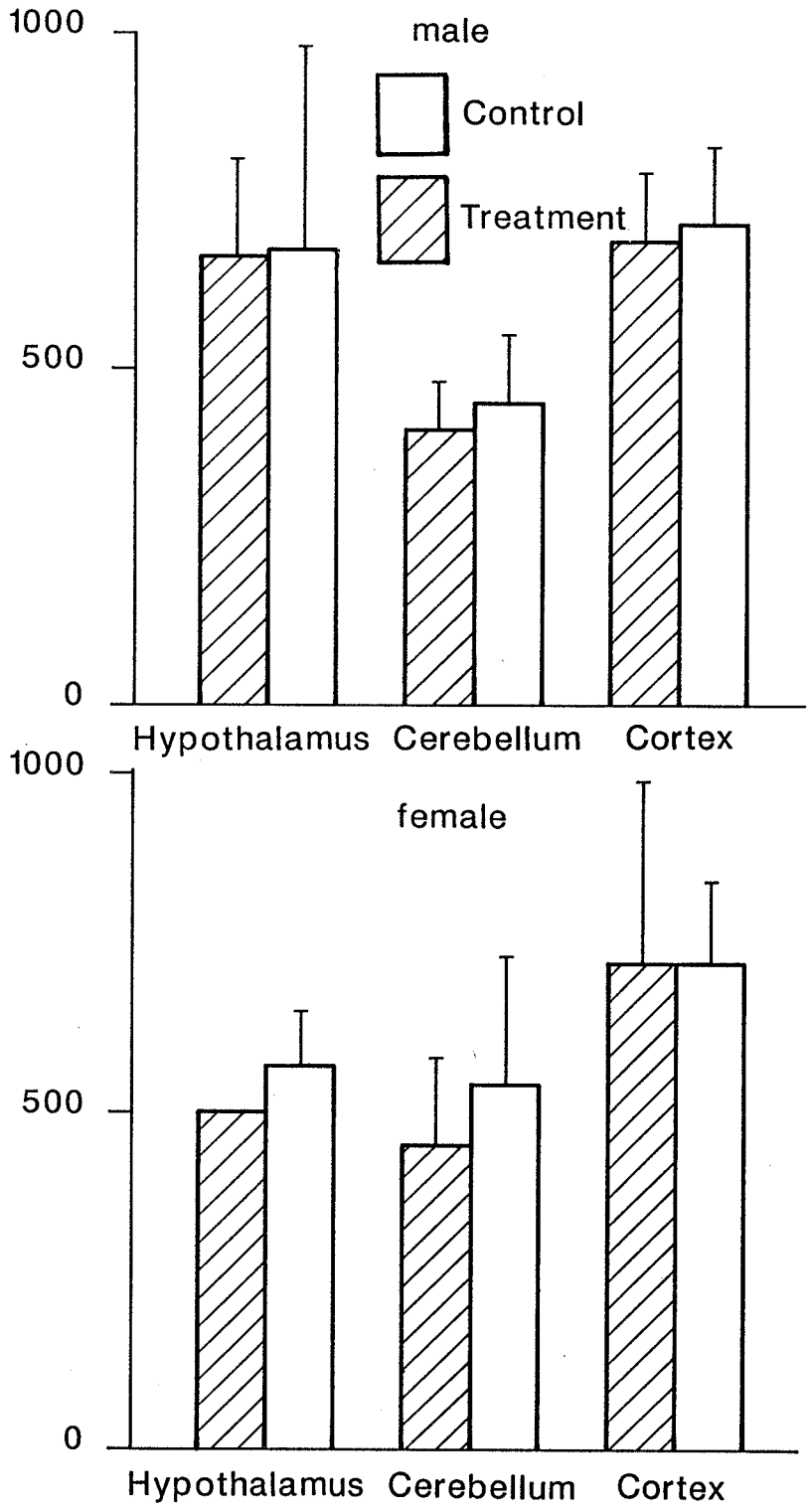

BDZ Binding
Diethylstilbestrol
male
1000
500
0
Control
Treatment
Hypothalamus
Cerebellum
Cortex
female
800
400
0
Hypothalamus
Cerebellum
Cortex

Figure 19

The effect of dihydrotestosterone on BDZ binding in rat brains. Specific binding of [^{3}H]-flunitrazepam was tested on the same membranes as those for GABA binding (Fig.16 legend) by the procedures outlined in the methods. Values are means of 5 determinations, except where otherwise indicated, and vertical lines represent standard deviations.

Fig. 19
BDZ Binding
Dihydrotestosterone



DISCUSSION

I Glutamic Acid Decarboxylase Activity in the Hypothalamus, Cerebellum, and Cortex

The hypothalamus, cerebellum, and cerebral cortex were chosen for analysis due to their relatively high levels of activity as shown in previous reports (22). GABA levels in the substantia nigra in rat as well as in other mammals are as much as two times higher than that seen other regions tested (22, 36, 43). Other areas of high activity include the globus pallidus and subthalamic nucleus. Our findings for GAD activity in the cerebellum, cortex, and hypothalamus are in agreement with previous work on these brain areas in the cat (35, 36).

The addition of sucrose to the diluting buffer for homogenizing the brain tissues was essential if a sequential testing of GAD activity and receptor binding was to be accomplished. Sucrose helps to separate the more buoyant cellular membranes from the nuclei and connective tissues which sediment faster upon low speed centrifugation (41). The addition of sucrose to the reaction mixture in our assay procedure did not affect GAD activity and was used routinely throughout the study. McGeer and McGeer (44) likewise observed that the addition of sucrose to the reaction mixture gave satisfactory results in their assay of GAD and glutaminase.

Assays of GAD activity were routinely carried out on freshly homogenized tissue samples after only a few minutes on ice. Previous studies (55) have shown that the enzyme was stable in the homogenized state at $4^{\circ}C$ for up to 24 hours.

II GABA and Benzodiazepine Binding in Hypothalamus, Cerebellum and Cortex

The most reproducible method of determining the binding of [^{3}H]-muscimol and [^{3}H]-flunitrazepam using small sample sizes was employed. With the binding assay procedures established, membrane preparations from all three regions were tested.

GABA receptor binding was tested on the frozen and thawed second pellet preparation as described by Zukin (46). The freezing and thawing and use of Triton X-100 or Nonidet P-40 as detergent has been shown to greatly reduce the amount of interfering GABA-binding modulators (22, 41, 42, 45) and thus increase the amount of ligand binding to the receptor. The phosphate buffer was chosen with the consideration of eliminating Na ion to prevent uptake by the synaptasomal membranes as reported by Enna (45).

These studies were primarily concerned with the postsynaptic GABA receptor to eliminate the number of variables changing. This was accomplished by using[^{3}H]-muscimol, a conformationally restricted agonist, as the tracer, and by making conditions less favorable for

presynaptic uptake by using sodium ion-free buffer. Several studies show this method to be effective (for review see references 20 and 46).

In the case of the benzodiazepine receptor,[H]-flunitrazepam was used as the tracer, because previous studies shown that it was more specific for the neuronal subset of diazepam binding sites (39,46). High concentrations ()10^{-6}M) of benzodiazepine blockers has been shown to displace as much as 100% of the diazepam binding found in cell culture (46). In our study R05-2807 was used at a concentration of 10^{-6} M for displacement in the determination of the specific binding. In all cases, binding was performed on membranes without washing with NP-40 since previous reports have shown that a considerable portion of BDZ receptors were removed from the membrane by detergent washings (38).

Since the procedure used herein for the binding assay of the BDZ receptor was adopted from that used for cell culture, NaCl concentration was kept at the same level to give 330 mosm as reported by Sher (49). Costa, et al. (52) also demonstrated a need for sodium and chloride in the assay of the benzodiazepine receptor.

III Studies on the Development of the GABA-ergic System.

1. Glutamic Acid Decarboxylase Activity

Our observation (Fig.1) that GAD activity increases with age is in agreement with previous reports in mouse (37). There was a marked increase of GAD activity in both males and females from day 2 to day 20 (Fig.3 and Table 1), and only a slight increase thereafter up to day 45.

The GAD activities measured at day 35 (Fig.4) indicate a 16 % increase in female cerebellum above male values and a 34 % increase in female cortex over male values. This difference indicates a fundamental sexual dimorphism in the adult animals.

2. Gamma-aminobutyric Acid and Benzodiazepine Binding

As was the case for GAD activity, testing the binding of [^{3}H]-muscimol to membranes from the hypothalamus, cerebellum and cortex gave an index of the development of these structures. There was some fluctuation in the levels of the GABA receptor in the cerebellum with age (Fig.5), however, both the cerebellum and cortex show a steady increase in specific binding with age (Figs.5 and 6).

For the BDZ receptor (Figs.8 and 9), the levels of specific binding appear to be more constant than those for GABA. The levels start high and decrease rapidly between day 2 and day 20 with only a slight decrease thereafter. This time sequence is the same as that observed for GAD activity, except that the pattern is reversed. Sher and Schrier

observed the same phenomenon in cultures of fetal mouse cerebral cortex (39).

IV The Effects of Hormone Treatments During Early Developmental Stages on the GABA-ergic System.

1. Dexamethasone Treatments.

Circulating levels of ACTH and corticosterone have been shown to selectively influence [^{3}H]-GABA receptor binding *in vivo* in the rat (32). Dexamethasone was used in our study to test the effects of glucocorticosteroids in the hypothalamus, cerebellum and cortex. DEX is more potent as a glucocorticoid than is cortisol and has been reported as a modulator of transmitter choice in superior cervical ganglia cultures from 3 day old rats (50).

The differences in GAD activity in all the brain regions studied in both males and females were not statistically significant following treatment with this hormone (Fig.11). Likewise, binding of [^{3}H]-muscimol and [^{3}H]-flunitrazepam were not significantly affected by dexamethasone treatment (Fig.14 and Fig.17) except in the female cerebellum, where binding was reduced by 38 % (Fig.17).

2. Diethylstilbestrol Treatment.

DES was used as the synthetic analog of estrogen in this particular aspect of the study since it has been shown in several studies to duplicate the effects of estradiol *in vivo* (11,51). Since DES is also a synthetic drug, it is not

metabolized in the steroid pathways. It has been shown that estrogens are the prime candidates for control of sexual differentiation in different brain regions (1,11,51).

DES treatment did not significantly affect GAD activity in male rats but the activity of this enzyme in female hypothalamus and cerebellum was significantly lower following DES treatment (Fig.12). The opposite result was seen in GABA binding studies, where GABA binding in the cerebellum and cortex in male rats was significantly reduced by DES treatment (Fig.15). DES treatment during development affects the GABA-ergic system, although the effects are different in males and females.

3. Dihydrotestosterone Treatment

Previous reports have demonstrated that DHT has a limited influence on sexual differentiation of the brain in male rats (11,48). Treatment with DHT did not significantly affect GAD activity in males (Fig.13) nor did it affect the binding of [^{3}H]-muscimol or [^{3}H]-flunitrazepam in the hypothalamus, cerebellum or cortex (Fig.16 and Fig.19).

Treatment with DHT did significantly affect GABA binding in the female cortex (Fig.16). The decrease in binding of [^{3}H]-muscimol by DHT treatment was 20%. GAD activity was not affected in the hypothalamus, cerebellum or cortex (Fig.13). Similarly, benzodiazepine binding was not significantly affected by this treatment (Fig.19).

V Summary and Conclusions

This thesis, having established a feasible method of monitoring the development of the GABA-ergic neurons *in vivo*, has demonstrated differences in the parameters of GAD activity and bindings at the GABA and benzodiazepine receptors at early stages of development of the rat. The ontogenesis of the GABA-ergic system appeared to be different between sexes and hormones were effective in modulating the eventual neurotransmitter milieu in the adult animal. Benzodiazepine binding in the cerebellum of female rats was reduced by dexamethasone treatment during early development (Fig. 17). Treatment with diethylstilbestrol for three weeks after birth lowered glutamic acid decarboxylase activity in female hypothalamus and cerebellum (Fig. 12) and reduced binding at the gamma-aminobutyric acid (GABA) receptor in the cerebellum and cortex of male rats. Dihydrotestosterone similarly reduced binding at the GABA receptor in female cortex (Fig. 16).

Many factors affect neuronal ontogenesis. With the advent of modifications in cell culture technology, systems are being developed to resemble more closely the environment *in vivo*. The influence of centrally acting hormones in concert with various growth factors and trophic factors on neuronal development and differentiation is essential in neuroscience research.

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