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BINDING AND METABOLISM OF TESTOSTERONE IN THE RAT BRAIN DURING SEXUAL MATURATION—I. MACROMOLECULAR BINDING OF ANDROGENS

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SUMMARY

Sections of the cerebral cortex, hypothalamus and hypophysis of three, six and nine week old male rats were incubated with tritiated testosterone, and its binding to macromolecules in the cytosol and nuclear supernatant was determined by gel filtration (Sephadex G25).

The hypophysis of the adult male rat has a much stronger affinity for testosterone than that of the immature animal. Binding of the hormone in the adult rat hypothalamus differed slightly from that of the immature rat, but this difference was not significant. Binding of the hormone at the cortical level was not influenced by the animals' age.

The elution patterns of bound radioactivity after gel filtration (Sephadex G 200) of cytosols from tissues of immature and adult animals were compared. When distinct differences between the tissues were found, age did not appear to be an important factor of qualitative macromolecular modification.

In the experimental conditions used, sexual maturation of the male rat appeared as a phenomenon which parallels or requires a prior increase in the quantity of androgen binding macromolecules in the diencephalon. Binding by macromolecules in the hypophysis was particularly pronounced.

INTRODUCTION

A negative feedback by hormones at the diencephalic level occurs in both adult animals and humans. These mechanisms have been found to be functional in children [1, 2], in animals prior to puberty [3–6] and in the foetus [6–8].

Moreover, the preferential binding of estrogens at the diencephalic level has been established in adult animals [9–11] as well as in prepubertal animals [12–14] and in the foetus [14]. The majority of the work on diencephalic binding of androgens has been carried out in adult animals [15–19] and the foetus [14] rather than in young growing animals [20].

These two observations suggest that sexual maturation is a step in the course of the hypothalamic-hypophyseal-gonadal functional process but does not act as its initiator. This functional stage has been studied mainly at the hypophyseal level in their anatomical [21], histological [22] and endocrinological aspects [23]. We have used a biochemical approach and have studied the macromolecular binding of testosterone and/or its metabolites in the hypophysis and hypothalamus of the male rat during puberty, the cerebral cortex being used as a control reference tissue.

MATERIAL AND METHODS

Biological material

Sprague–Dawley strain rats of different ages were used. The appearance of spermatozoids in the seminiferous tubules was taken as evidence of completion of puberty. A histological study of testicular smears was made with a phase-contrast microscope (Table 1).

In our experiments three stages of development were studied. Three week old immature rats (20 rats were used for each experiment), pubertal, 6-week-old rats (15 rats were used for each experiment,) 9-week-old mature rats (10 rats were used for each experiment). Animals were castrated 24 h or 48 h prior to the experiments.

The animals were killed by decapitation, and the following organs or parts of organs were removed: fragment of frontal cortex; the entire hypothalamus, between 1 and 2mm in thickness, limited anteriorly by

Table 1. Morphological data of Sprague Dawley strain rats studied

Age in weeks	Mean weight of hypophysis in mg ± S.D.	Mean weight of animal ± S.D. in g	Testicular histology
3	3·42 ± 0·15	42 ± 5	Spermatogonia
4	—	80 ± 10	Spermatogonia plus spermatocytes, primary and secondary
5	—	120 ± 8	Spermatogonia plus spermatids at Golgi phase
6	7·38 ± 0·62	165 ± 10	Spermatogonia plus spermatids at acrosomial phase
9	10·36 ± 0·70	280 ± 12	Spermatogonia plus complete spermatozoids
12	—	330 ± 18	Spermatogonia plus complete spermatozoids in large quantities

S.D. = Standard deviation.

the optic chiasm, laterally by the hypothalamic fissures and posteriorly by the mammillary bodies; the entire pituitary, removed from the sella turcica after section of the stalk.

Tritiated testosterone

[1,2-^{3}H]-testosterone: specific activity = 42·6 Ci/mmol [1,2,6,7-^{3}H]-testosterone: specific activity = 91 Ci/nmol. The purity was checked before use by paper chromatography.

The following media were used: Incubation medium: Krebs III medium [24].

Homogenization media: Medium I: Tris–HCl pH 7·4 50 mM, EDTA 1·5 mM, Mercaptoethanol 2 mM, $MgCl_2$ 2 mM, KCl 10 mM, Saccharose 250 mM. Medium II: Tris–HCl pH 7·4 50 mM, EDTA 1·5 mM, Mercaptoethanol 2 mM, $MgCl_2$ 2 mM, KCl 10 mM, NaCl 1 M.

Elution media

Medium IV: Medium I without saccharose but with 2 μg of cold testosterone per l. Medium V: Medium II with 2 μg of cold testosterone per l.

These media contained a concentration of cold testosterone one hundred times that of the incubation medium.

Method of incubation

After decapitation and dissection, the tissues were immediately placed in the Krebs III solution at 4°C and sliced into smaller fragments. After 30 min pre-incubation at 4°C, the tissues were incubated for 2 h at 37°C in a Krebs solution containing tritiated testosterone with constant agitation in a 99·5% oxygen atmosphere. The testosterone concentration was 20ng/ml which is about four to five times the physiological concentration in rat plasma [25, 26].

Sub-cellular fractionation

After incubation, each tissue was thoroughly rinsed with the first medium (100ml) on a fine cloth filter, then homogenized for one min in a glass–Teflon Potter–Elvehjem homogenizer.

Fractionation of the homogenates in a Beckman centrifuge (type 3-40) at 800 g_{av} during 10 min yielded a pellet of crude nuclei and a cytoplasmic supernatant. The latter when centrifuged for 90 min at 100,000 g_{av} gave the "cytosol" fraction plus a pellet containing the insoluble parts of the cell (microsomes, mitochondria, etc...). The nuclear pellet was washed twice, suspended in medium II and agitated for 30 min. This homogenate was then fractionated. After a 15 min centrifugation at 20.000 g_{av}, a pellet (chromatin and nuclear membranes) and a soluble fraction (proteins and nucleic acids of low mol. wt.) were obtained. All these procedures were carried out at 0°–4°C.

Measurement of the radioactivity

A portion (0·1 ml) of the homogenates and of the total cellular fractions was dissolved in 4 vol. (0·4 ml) of a Packard solubilizer (soluene) at 40°C for 24 h. The soluene fluorescence was neutralized by 2 vol. (0·2 ml) of glacial acetic acid and three vol. (0·3 ml) of ethanol after which fourteen ml of scintillator (PPO = 5 g, dimethyl POPOP = 0·3 g toluene 1 l.) were added.

With the eluted fractions obtained by gel filtration two different types of measurements were made depending on the presence or absence of proteins in the fraction. In the absence of proteins the measurement was made on evaporated aliquots, redissolved in 0·5 ml of 100% ethanol with addition of 14·5 ml of scintillator. When the fraction contained proteins an extraction according to the following procedure was used: the fraction was first extracted four times by three volumes of chloroform which were pooled and evaporated. The residue was redissolved in 0·5 ml of 100% ethanol and counted in 14·5 ml of scintillator.

All samples were counted in a Tri-Carb scintillation spectrometer (Model 3320) and corrected for quenching by internal or external standards. All samples were counted for the time required to ensure an error of less than 5%.

Table 2. ^{3}H-Steroid uptake with respect to age of the rats was expressed by the radioactivity content of homogenates and 100,000 g_{av} pellets in d.p.m. 10^3 per mg wet wt. ± S.D. Tissues were incubated for 2 h in Krebs medium containing 20 ng of [1,2,6,7-^{3}H]-testosterone per ml. Rats were castrated 48 h before use. Values are averages of at least two experiments

Age of animals in weeks	(A) Homogenates			(B) 100000g_{av} Pellets		
	Cerebrum	Hypophysis	Hypothalamus	Cerebrum	Hypophysis	Hypothalamus
3	46·0 ± 9·0	48·9 ± 1·6	48·2 ± 16·4	2·5 ± 1·2	6·3 ± 1·3	3·6 ± 0·2
6	44·2 ± 2·2	60·2 ± 8·5	43·4 ± 9·9	1·5 ± 0·1	11·4 ± 2·2	3·0 ± 0·9
9	50·7 ± 13·0	58·8 ± 15·3	48·2 ± 16·4	1·6 ± 0·4	9·1 ± 3·3	3·3 ± 1·0

After 2 h of incubation the radioactivity found in homogenates was about 10% of the total radioactivity of the incubation medium.

During cellular fractionation the radioactivity of the homogenates (100%) was distributed as follows (average values): cytosol: 60%; 100,000 g_{av} pellet: 10%; nuclear pellet: 25%; 5% losses were recovered after ethanol washing of the polyallomer centrifuge tubes.

Gel filtration

The amount of bound hormone was measured by chromatography of an aliquot on Sephadex G25 "coarse" gel saturated with a concentration of unlabeled testosterone one hundred times higher than that of the incubation medium. The total radioactivity of the filtered subcellular fraction was distributed into two radioactive peaks: a peak of bound steroids eluted just after the void volume and a peak of free steroids eluted later. The ratio bound radioactivity/free radioactivity depended on the organ and the subcellular fraction studied but not on the age of the animals. After 2 h of incubation at the hormonal concentration used, the following average ratios were found:

Cellular fraction	Cerebrum	Hypophysis	Hypothalamus
Cytosol	0·002	0·011	0·003
Nucleus	0·026	0·126	0·042

Qualitatively, the presence of ^{3}H-steroid macromolecular complexes was determined by chromatography of an aliquot on Sephadex G200 gel similarly saturated. The saturation of the gel with unlabelled testosterone was used to saturate the Sephadex binding sites for testosterone, to reduce the quantity of non-specific low-affinity hormone–protein complexes and eventually to limit the degradation of the specific hormone–protein complexes.

Two types of glass columns were used: Small columns with a diameter of 0·6 cm and a height of 80 cm for the aliquots. Large columns with a diameter of 2·5 cm and a height of 100 cm for the total extracts. Using Sephadex G200, these columns were first filled with silicone coated glass beads in order to decrease the pressure at the bottom of the column [27], therefore increasing the speed of elution and consequently limiting the degradation of the steroid–receptor complexes in relation to time.

Protein and DNA analysis

Protein concentrations of the cytosolic fractions were measured by the method of Lowry *et al.* [28]. A serum albumin solution was used for the standard curve.

DNA concentrations of the nuclear fractions were analysed by the method of Schneider [29], using calf-thymus DNA as the standard.

RESULTS

Hormone tissue uptake

The radioactivity found in the homogenates of cerebral cortex and hypothalamus showed no difference in hormonal uptake between the three age groups studied after 2 h of incubation (Table 2A). Only the hypophysis of the immature rat presented a much lower hormone uptake than those of the pubertal or adult rat but the differences were not significant ($0·5 < P < 0·10$).

Since the microsomes are rich in 5α-reductase [30], the tritiated steroid content of the 100,000 g_{av} pellet obtained after fractionation of the preceding homogenate was measured (Table 2B). In the hypothalamus and cerebral cortex there was no significant difference in the level or radioactivity with respect to age. However, the hypophysis pellet was found to contain more radioactivity than the two other tissues regardless of age. In addition, 6-week-old rats showed a significantly greater uptake than that of 3-week-old rats ($0·02 < P < 0·05$).

Influence of age on the amount of bound hormone. (a) Study of tissues from rats castrated 24 h previously and incubated with 20 ng of [1,2-^{3}H]-testosterone per ml (Table 3).

The average values obtained in the cytosol fractions

Table 3. ^{3}H-Steroid binding to macromolecules with respect to age of the rats was measured by the radioactivity bound to the first-eluted peak from Sephadex G25 chromatographic column. This radioactivity is expressed in d.p.m. per mg of protein ± S.D. in the cytosol fraction and in d.p.m. per mg of DNA in the nuclear fraction. Tissues were incubated in Krebs medium containing 20 ng of [1,2-^{3}H]-testosterone per ml. Rats were castrated 24 h before use. Values are averages of at least two experiments. No S.D. is given for nuclear fractions because samples of the same age were combined before extraction

Age of animals in weeks	(A) Cytosol			(B) Nuclear supernatant		
	Cerebrum	Hypophysis	Hypothalamus	Cerebrum	Hypophysis	Hypothalamus
3	1283 ± 91	3632 ± 365	1411 ± 60	3075	3498	2310
6	1348 ± 91	3535 ± 400	1989 ± 322	2706	3960	2025
9	1127 ± 349	6233 ± 688	1975 ± 342	4551	4686	1729

(Table 3-A) showed hormone binding related to age and the type of the tissue studied. The hypophysis bound significantly more radioactivity than the hypothalamus or the frontal cortex which showed approximately the same results. The adult rat hypophysis (9-week-old) showed a binding almost twice as high as that of immature (3-week-old) and pubertal (6-week-old) rats ($0{\cdot}001 < P < 0{\cdot}01$). Due to the small quantities of radioactivity present in the nuclear extracts (Table 3-B), corresponding fractions of the same age groups were pooled for analysis. Consequently, no statistical comparison could be done for this fraction. However, it seems that the average values obtained are not significantly different in the different age groups. Here again the hypophysis presented a steroid binding definitely higher than that of the other two tissues.

(b) Study of tissues from rats castrated 48 h previously and incubated with 20 ng of [1,2,6,7-^{3}H]-testosterone per ml (Table 4).

[1,2,6,7-^{3}H]-testosterone was used to facilitate recognition for the same steroid concentration of the differences previously noted. 48 h after castration, a more pronounced binding in the nuclear fraction could be expected.

The results obtained for the cytosol fraction (Table 4-A) of the hypophysis showed a significant difference ($0{\cdot}001 < P < 0{\cdot}01$) between the 3 and 6-week-old rats with the latter showing a binding twice that of the former. The values obtained for the 6 and 9-week-old rats showed no appreciable difference. As found previously, the hypothalamic cytosol in the 6 and 9-week-old animals bound a greater quantity of hormone than that of three week old rats but the differences were still not significant. At the three age levels studied the cerebral cortex cytosols showed no differences.

The nuclear fraction of the hypophysis and hypothalamus showed a higher binding than that which was observed in the animals tested 24 h after castration when results were corrected for the relative specific radioactivity of the labeled testosterone used.

In the nuclear fraction of the hypophysis, the binding in relationship to age parallels that observed for the cytosol. In the hypothalamus, 6-week-old rats bound more radioactivity than 3 and 9-week-old rats. The hypothalamic binding was higher than that of the cerebral cortex which was minimal at any age.

Influence of age on the pattern of macromolecular binding

The elution patterns from Sephadex G-200 chromatography of cytosols of the different tissues from 3- and

Table 4. ^{3}H-Steroid binding to macromolecules with respect to age of the rats was measured by the radioactivity bound to the first-eluted peak from Sephadex G25 chromatographic column. This radioactivity is expressed in d.p.m. per mg of protein ± S.D. in the cytosol fraction and in d.p.m. per mg of DNA in the nuclear fraction. Tissues were incubated in Krebs medium containing 20 ng of [1,2,6,7-^{3}H]-testosterone per ml. Rats were castrated 48 h before use. Values are averages of at least two experiments. No S.D. is given for nuclear fractions because samples of the same age were combined before extraction

Age of animals in weeks	(A) Cytosol			(B) Nuclear supernatant		
	Cerebrum	Hypophysis	Hypothalamus	Cerebrum	Hypophysis	Hypothalamus
3	1424 ± 572	4310 ± 80	2063 ± 435	9840	18018	24775
6	728 ± 188	7748 ± 76	3259 ± 938	8856	33924	40390
9	982 ± 143	8018 ± 142	3602 ± 428	10455	32472	27615

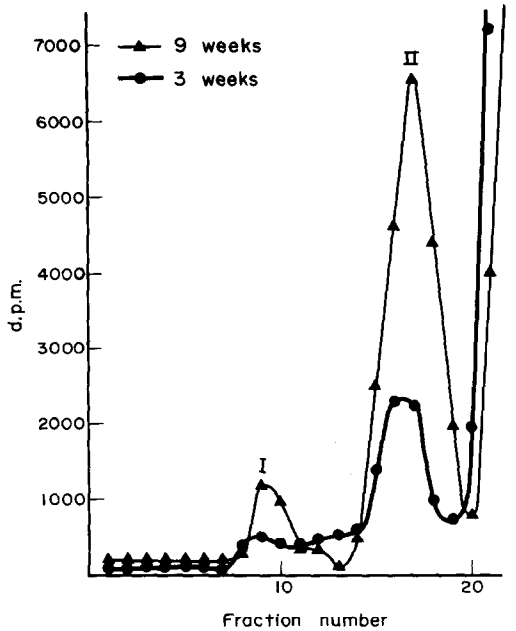

Fig. 1. Gel filtration on Sephadex G200 of pituitary cytosolutions from immature (3-week-old) and mature (9-week-old) rats. The cytosols were obtained by subcellular fractionation of tissues incubated for 2 h in Krebs medium containing 20 ng of [1,2,6,7-^{3}H]-testosterone per ml. The same quantity of radioactivity (0·20 μCi) was applied to the column in each case.

nine-week-old rats were compared. The diagrams presented here are typical results.

(a) *Hypophysis.* In the adult rat two peaks of radioactivity were clearly distinguished: a peak eluted just after the void volume (peak I), smaller than the second (peak II) which just precedes elution of the free radioactivity. For the same quantity of radioactivity, the immature rat showed lower peaks than the adult, peak I not being well defined. However, the surface ratio peak I/peak II was not modified by age.

(b) *Hypothalamus.* In the adult rat, as in the hypophysis, similar peaks were found but the presence of a third intermediary peak (III) suggests some differences.

In the immature animal, the first and second peaks were well characterized while the third peak appeared as a rather ill defined hump.

(c) *The cerebral cortex.* In both the immature and adult animal only the low, ill defined intermediary peak (III) of the hypothalamus was found, the surface area of which was not modified with age.

DISCUSSION

The aim of this work was to study the uptake and binding of testosterone by the diencephalon, the hypophysis and the cerebral cortex of the male rat during sexual maturation. These tissues removed from immature, pubertal and adult animals were studied under identical experimental conditions. Age had no influence on the uptake of the hormone by the tissues (Table 2-A). Only the hypophysis of the immature rat showed a somewhat lower uptake but this difference was not significant ($P > 0{\cdot}05$). In contrast, the high radioactivity of the 100,000 g_{av} pellet (Table 2-B) appeared as a much more specific phenomenon. The hypophysis fixed more radioactivity than the hypothalamus and cortex and there was a significant difference ($0{\cdot}02 < P < 0{\cdot}05$) in uptake between the 3- and six-week-old animals. Interpretation of the preferential binding, considering such factors as the type of tissue and the age of the animal requires, as the first step, isolation and analysis of the pellet components, specifically of mitochondria and microsomes.

As expected, utilisation of the [1,2,6,7-^{3}H]-testosterone (specific activity 91 Ci/mmol) made the results even more evident than when [1,2-^{3}H]-testosterone (specific activity 42·6 Ci/mmol) was used. Extension of the castration period from 24–48 h altered bound hormone levels with respect to the tissues and cellular fractions considered: with regard to the nuclear fraction (Table

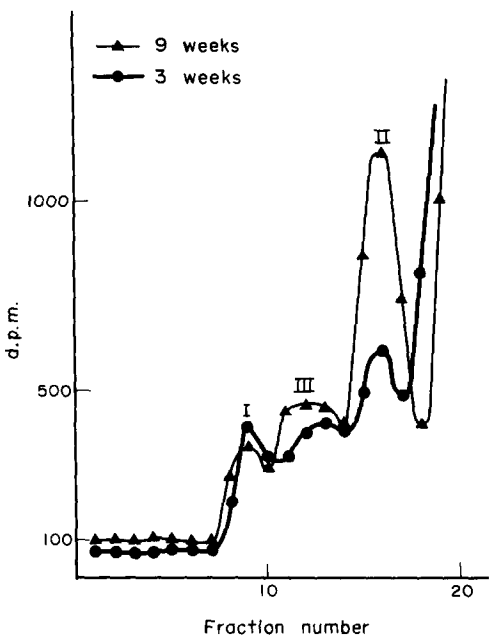

Fig. 2. Gel filtration on Sephadex G200 of hypothalamic cytosols from immature (3-week-old) and mature (9-week-old) rats. The cytosols were obtained by subcellular fractionation of tissues incubated for 2 h in Krebs medium containing 20 ng of [1,2,6,7-^{3}H]-testosterone per ml. The same quantity of radioactivity (1 μCi) was applied to the column in each case.

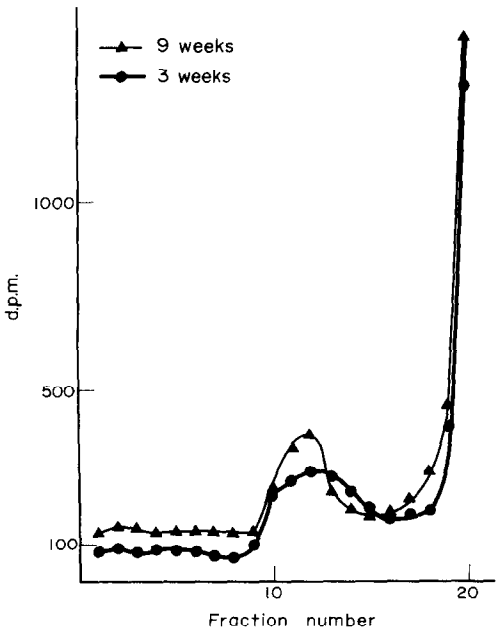

Fig. 3. Gel filtration on Sephadex G200 of cerebral cortex cytosols from immature (3-week-old) and mature (9-week-old) rats. The cytosols were obtained by subcellular fractionation of tissues incubated for 2 h in Krebs medium containing 20 ng of [1,2,6,7-^{3}H]-testosterone per ml. The same quantity of radioactivity (0·70 μCi) was applied to the column in each case.

4-B) important increases in the quantity of bound hormone in the hypothalamus and hypophysis compared to cortex were accompanied by definite differences depending on the age of the animals. In the case of the hypophysis the steroid binding by the nuclei in the 6- and 9-week-old rats was twice that found in the 3-week-old animals. In the hypothalamus the steroid binding by the nuclei in the 6-week-old animals was higher than that of the 3- and 9-week-old rats which gave almost identical values. This last result, which is greatly at variance with those found 24 h after castration (Table 3-B), requires further confirmation.

As for the cytosol fraction (Table 4-A), when the results are corrected for the difference of specific activity of the two radioactive steroids used, the quantity of bound steroid remained the same in the cortex and decreased little in the hypothalamus when compared with the hypophysis. In the latter tissue the quantity of bound radioactivity is similar in 6- and 9-week-old animals whereas, in the first case (Table 3-A), i.e. 24 h after castration, animals of 3 and 6 weeks of age showed similar results. All the rats were taken from the same strain and were thus identical except for the castration time. This leads us to suppose that the variation of the steroid binding in the hypophysis depended greatly on a precise endocrine state which changed rapidly in relation to time. A systematic study of this age period would be necessary to determine if the variation was due to a rapid synthesis or an activation of macromolecules that bind androgens already present in the tissue and under what conditions this reaction would take place.

Comparative study of the chromatographic diagrams of the hypophysis (Fig. 1), hypothalamus (Fig. 2) and the cerebral cortex (Fig. 3) cytosols from immature and adult animals revealed no qualitative differences with respect to age. This would suggest that neither the hypothalamus nor the hypophysis contain any molecules specific to the adult. The quantitative increase of bound radioactivity is approximately proportional in the observed peaks between the adult and immature rats, suggesting a parallel specificity of the corresponding binding molecules. On the contrary, the differences in the chromatographic diagrams from the three tissues are probably due to functional differences and depend on the androgens involved.

Lobl and Shapiro [20] showed the preferential concentration of testosterone in the hypothalamus of 3-day-old female rats when compared with 45-day-old pubertal rats by measuring the radioactivity in a chloroform extract of the total homogenate. The experimental conditions used in the present study showed no difference in the steroid uptake between the hypothalamus of immature or adult animals, suggesting a sex-difference. Moreover puberty of the male rat seemed to be accompanied by an increased hormonal binding, specifically in the nuclear fraction (Table 4). The statistically non-significant difference observed in the steroid binding with respect to age was probably due to a dilution of a small quantity of active cells in the total hypothalamus. Debeljuck *et al.* [23] have shown that testosterone propionate injections significantly reduced the release of gonadotropins in male rats after LH-RH treatment. Pelletier and Ortavant [31], using the preceding experimental procedure found time-dependent biphasic lowering of the plasma LH level in the adult castrated goat. They attributed the first decrease to a direct steroid influence on the hypophyseal cells. Kamberi and McCann [32] obtained with intra-hypophyseal implants results which were highly similar to those obtained with testosterone propionate injections. Finally, Schally *et al.* [33] incubated hypophyseal rat slices in LH RH with and without ethynyl oestradiol and found significant inhibition of gonadotropin release (FSH and LH) when the tissue was incubated in the presence of ethynyl oestradiol. Their results are in agreement with the

suggestion of the preceding authors that steroids have a direct influence on hypophyseal cells.

In light of the present study, the role of the hypophysis which shows a significant increase in hypophyseal macromolecules that bound androgen at puberty could have an important role in the sensibility alteration of the gonadostat as described by Kulin *et al.* [2], whereby the hypophyseal level of androgen binding macromolecules in the male rat plays a role in the functional antagonism between the hypothalamic and sex hormones in the hypophyseal cells. Further studies are necessary to clarify this point.

From the experimental data collected, it appears that the hypothalamus as well as the hypophysis of the young animal have the same potential of androgen binding macromolecules as the adult animal but the functional differences between the immature and adult animals could be due to quantitative modifications of these binding macromolecules, particularly in the hypophysis.

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