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Changes in Hypothalamic Calmodulin Concentration Induced by Perinatal Hormone Manipulation in the Rat

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RODRIGUEZ-MEDINA, M. A., M. VERGARA, M. E. CHAVARRIA, A. ROSADO AND A. REYES. *Changes in hypothalamic calmodulin concentration induced by perinatal hormone manipulation in the rat.* PHARMACOL BIOCHEM BEHAV **61**(4) 445–450, 1998.—Calmodulin (CaM) presence and concentration was determined (RIA) in the rat hypothalamus (2, 6, 12, 24 h and 90 days after birth) in vehicle-treated animal (controls), in testosterone propionate (TP)-treated females (30 μg/rat subcutaneously 1 h after birth) and in tamoxifen-treated males (200 μg/rat subcutaneously 1 h after birth). CaM concentration, either as total content/hypothalamus or as concentration per mg ww, was significantly higher in both male and female adult rats than in newborn subjects. CaM concentration/mg protein increased with age, being two times higher in adult males and greater than three times higher in adult females than in their respective newborns. Two, 12, and 24 h after birth CaM concentration was significantly lower in control females than in control males. This relation was reversed in adults in which CaM concentration was higher in females. The application of TP to the females and tamoxifen to the males, induced a significant decrease in CaM/mg protein, both in the newborn (2 and 6 h) and in the adult animals. In adults, treated females had CaM concentrations similar to those found in control males. Our data suggest: first, a lasting effect of newborn hormonal treatment upon the CaM concentration in rat hypothalamus; second, that CaM is preferentially synthesized in the adult female hypothalamus, indicating an important role of this protein in female reproductive function. © 1998 Elsevier Science Inc.

Calmodulin concentration Hypothalamus Testosterone treatment Tamoxifen treatment Sexual differentiation

TWO of the brain-controlled sexual functions that differentiate males from females—the pattern of gonadotropin secretion and sexual behavior (13)—become irreversibly organized under the influence of hormones during the late prenatal (5) or early postnatal period (14), at least in rodents. Perinatal gonadectomy of male rats, or the application of androgen or estrogen antagonists like tamoxifen to these animals, results in permanent feminization of the sexual behavior patterns and of the gonadotropic hormones release pattern (27,29). On the contrary, female defeminization and masculinization of sexual behavior patterns and of gonadotropic hormones release can be induced by perinatal treatment of female rats with aromatizable androgens, or with steroidal or nonsteroidal estrogens (13). Some of these behavioral and endocrinological sex-related differences have been ascribed to the development of some, mainly anatomic, dimorphic characteristics that clearly separate males from females (19,25).

We have recently shown that neonatal administration of some drugs that interfere with the normal functioning of the Ca^{2+}/calmodulin system (haloperidol, trifluoperazine, penfluoridol, and pimozide) is capable of inducing significant modifications in the hypothalamic sexual differentiation of the male rat. These induced behavioral changes are particularly important on the motivational and appetitive sexual behavior of the

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adult animal (23). Similar changes have been observed in the testosterone masculinization of female rats (M.A. Rodríguez-Medina, M. Vergara, E. Canchola, and A. Rosado, unpublished observations). We have also found that application of Ca^{2+}/calmodulin system inhibitors to adult, ovariectomized, estrogen-primed rats, inhibits the progesterone induced facilitation of lordosis behavior (3). These results suggest that calmodulin participates effectively, not only in the regulation of the lordotic behavior in the adult female rat, but also plays an important role in the biochemical processes that mediate hypothalamic sexual differentiation in these animals.

To contribute to the understanding of the physiological mechanisms that regulate the perinatal sexual differentiation of rat hypothalamus, we studied the changes in calmodulin hypothalamic levels during normal growth and differentiation of neonatal (2, 6, 12, and 24 h after birth) and adult (90 days) male and female rats, and the modifications that may occur by the neonatal treatment of female rats with testosterone and of male rats with tamoxifen.

METHOD

Timed-mated Sprague–Dawley rats were individually housed in a temperature and light-regulated environment (14L:10D). The gestation period under our experimental conditions was previously recorded as 23 days ± 6 h (23). The time of birth for each pup was carefully registered so as to give precision to the application of the indicated treatment. One hour after birth the animals were weighed and separated according to their sex. Male and female rats were randomly divided into two equal groups. The control group received a subcutaneous (SC) injection of 50-μl sesame oil; the experimental group received a SC injection of 30-μg testosterone propionate in 50-μl sesame oil (females) (1) or tamoxifen, 200 μg per animal in 50-μl sesame oil (males) (7).

After treatment, pups were maintained with their mothers on a 14L:10D cycle and were randomly selected to be studied at five different times after birth (2, 6, 12, or 24 h, and 90 days of age). Newborn rats were carefully weighed, sacrificed by decapitation, and utilized only for the determination of the hypothalamic biochemical parameters, including calmodulin concentration measurements. Adult rats were weighed and randomly divided into two groups. One group was submitted to carefully controlled male and female sex behavioral tests, following the procedures previously described (3,23), to check the efficiency of the applied treatment. The other group was sacrificed by decapitation and utilized for the determination of the hypothalamic biochemical parameters. In the case of adult female rats, the stage of the estral cycle was determined by daily vaginal cytology (26), and the animals were always sacrificed when clear evidences of estrus were detected.

The hypothalami were obtained as previously described (23) following the procedure proposed by Vangala et al. (28). The isolated hypothalami were placed in preweighted Eppendorf tubes containing 1 ml of isotonic buffer (NaCl 0.14 M, Tris-HCl—tris-hydroximethyl amino methane hydrochloride—0.01 M, magnesium acetate 0.0015 M, pH 7.4). In the case of newborn rats, three to five hypothalami obtained from animals under the same conditions were pooled and used for each determination. Wet weight (ww) of the samples was determined as previously described (23). To use each hypothalamus as its own duplicate, hypothalami were carefully divided into two equal parts and each half was processed individually. Five to six individual samples, or pools of samples as indicated, were processed for each variable studied.

Quantification of calmodulin (CaM) was done using a commercial double-antibody radioimmunoassay kit purchased from DuPont NEN Research Products (Boston, MA). The kit instructions were carefully followed, including the precaution of working with similar protein concentrations for all the assayed samples. The only differences were in the initial homogenization procedure and in the fact that the homogenized tissues were preserved in a deep freezer (−40°C) before the assay. These modifications in the protocol were similar to those applied by Ruiz de Elvira et al. (24) and did not affect the reliability of the procedure. Protein determinations in the homogenized tissue and in the extraction fractions were done by the procedure of Lowry et al (17).

TABLE 1

GENERAL PARAMETERS OF MALE AND FEMALE VEHICLE TREATED RATS (CONTROLS), AND OF EXPERIMENTALLY TREATED FEMALES (TP, 30 μg/RAT) AND MALES (TAMOXIFEN, 200 μg/RAT)

	Female Controls	Female Testosterone	Male Controls	Male Tamoxifen
Newborn animals**	(n = 28)	(n = 27)	(n = 28)	(n = 27)
Body weight (g)	7.2 ± 0.9*	7.2 ± 0.8*	7.3 ± 0.9*	7.5 ± 0.4*
Hypothalamic weight (mg)	16.8 ± 3.2*	15.1 ± 2.1*	17.6 ± 3.4*	16.8 ± 3.1*
Protein (μg/Hypothalamus)	51.0 ± 8.2*	54.0 ± 10.8*	48.0 ± 10.5*	50.8 ± 7.1*
Hypothalamus (% body weight)	0.23 ± 0.04*	0.21 ± 0.04*	0.24 ± 0.03*	0.22 ± 0.03*
Adult animals	(n = 7)	(n = 7)	(n = 7)	(n = 7)
Body weight (g)	241 ± 10*	236 ± 11*	331 ± 19†	323 ± 21†
Hypothalamic weight (mg)	58.6 ± 7.2*	46.3 ± 8.2*	51.2 ± 7.2*	60.3 ± 14.2*
Protein (μg/Hypothalamus)	98.6 ± 7.2*	174.6 ± 27.2†	162.1 ± 29.2†	118.0 ± 27.1*
Ovarian weight (mg)	57.3 ± 8.7*	26.4 ± 9.3†	—	—
Testis weight (g)	—	—	1.53 ± 0.09*	1.28 ± 0.2†
Hypothalamus (% body weight)	0.24 ± 0.002*	0.21 ± 0.0016†	0.014 ± 0.0012‡	0.019 ± 0.001†

**Includes data from the animals sacrificed 2, 6, 12, and 24 h after treatment, because there were not significant differences among them.

Numbers show the mean ± the standard deviation of the number of cases shown as n (In the case of newborn animals pools of three to five hypothalami were used for each determination.)

*,†,‡Different superscripts indicate statistical significant differences among data in the same row (one-way ANOVA), except in the case of gonadal weight in which case the Student t-test was used.

Statistical analysis were performed using a compatible PC microcomputer and the Microstat II statistical Package (Ecosoft Inc., Indianapolis, IN). Significance of differences in mean values of the general parameters (Table 1) was studied by one-way analysis of variance (ANOVA) for each parameter tested. In the case of gonadal weights, group comparisons were carried out by the Student's *t*-test. Significance of differences in the mean values of CaM concentration (Figs. 1–3) were assessed using a 4×5 two-way ANOVA. In all ANOVA studies we used 95% confidence limits for post hoc comparison between groups using the Tukey test. To assess the significance of differences in the proportion of rats showing sexual behavior (Table 2) we used the nonparametric Mann-Whitney *U*-test and χ^2 tables.

RESULTS

The general characteristics of the samples used in this study are shown in Table 1.

Although the effect of the applied treatment on the sexual behavior of the adult rat was not one of the main objectives of this study, it was considered necessary to check that the dose of the tested drugs, and the time of their application would efficiently induce the previously described behavioral modifications (7,10). Tables 2 and 3 indicate that the treatment of newborn females with testosterone and of newborn males with tamoxifen, indeed induced the expected modifications in the sexual behavior of the adult rats (7,14,27).

The concentrations of CaM per mg protein (CaM/mg protein) in the hypothalami of the studied rats are depicted in Fig. 1. In general it can be seen that, in control rats, the amount of CaM/mg protein increased with age, being almost two times higher in adult than in newborn males, and more than three times higher in adult than in newborn females.

In untreated, control animals, CaM/mg protein was significantly lower in females than in males at 2, 12, and 24 h after birth. This relation was reversed in the adults, in which case CaM concentration was significantly higher in estrous females than in males (Fig. 1). It can also be seen that treatment, either testosterone in the females or tamoxifen in the males, decreased the concentration of CaM/mg protein; although such decrease only reached the level of statistical significance in the newborn and in the adult animals. This effect of the endocrine treatment was particularly important in the adult females, in which CaM concentration was almost three times lower in the testosterone treated than in the control animals (Fig. 1). We must mention, however, that some of these differences were due to changes in the protein concentration of the hypothalamic tissue.

If CaM is expressed as total content per hypothalamus (Fig. 2), or as CaM/mg wet weight of the hypothalami (Fig. 3),

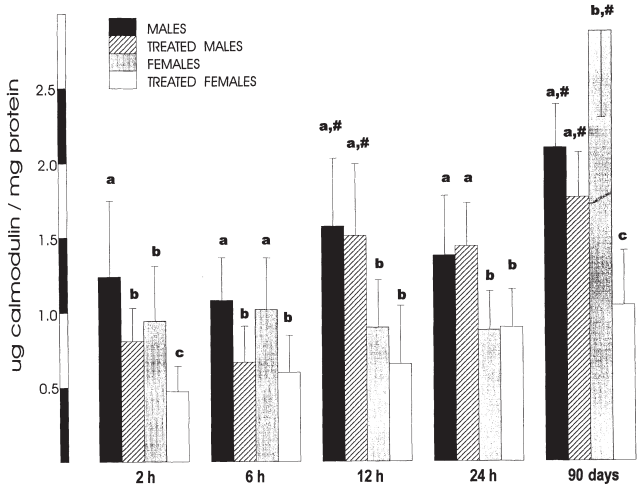

FIG. 1. Calmodulin concentration per mg protein in rat hypothalamus. Male and female rats (1 h after birth), were randomly divided into two equal groups. The control groups received a subcutaneous (SC) injection of 50-μl sesame oil. The treated female group received an SC injection of testosterone propionate (30 μg in 50-μl sesame oil) while the treated male group received an SC injection of tamoxifen (200 μg per animal in 50-μl sesame oil). The animals were sacrificed at the time indicated under the bars, the hypothalami were extracted (28), washed, and homogenized in Tris-buffer, pH 7.4 and calmodulin concentration was determined using a commercial double antibody radioimmunoassay kit. The bars indicate the mean ± the standard deviation of the indicated group of animals. The number of determinations is indicated in the Method section. The results of the two-way ANOVA indicate that the difference in the mean values among the different groups and between the different levels of time is greater than would be expected by chance, after allowing for the effects in differences in the other parameter ($p < 0.01$ and $p < 0.001$, respectively). There is a statistically significant interaction between time and groups ($p < 0.002$), indicating that the differences observed in each group depends on the sampling times. [a,b]Different letters above the columns indicate the existence of statistical significant differences ($p < 0.05$) among the data in the same group (two-way ANOVA). #Indicate the existence of statistical significant differences ($p < 0.05$) when compared with similar data in the previous time (two-way ANOVA).

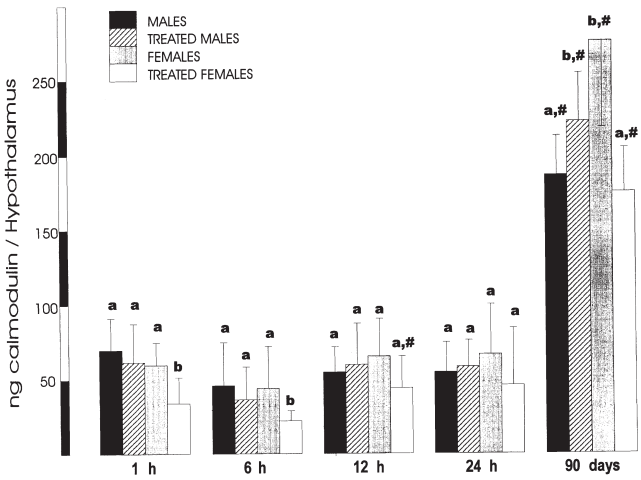

FIG. 2. Total calmodulin concentration in rat hypothalamus. The wet weight of each hypothalamus was measured (23) and the calmodulin concentration per hypothalami was calculated. All other observations are as indicated in Fig. 1. The results of the two-way ANOVA indicate that the difference in the mean values among the different groups and between the different levels of time is greater than would be expected by chance, after allowing for the effects in differences in the other parameter ($p < 0.005$ and $p < 0.001$, respectively). There is a statistically significant interaction between time and groups ($p = 0.006$), indicating that the differences observed in each group depends on the sampling times. [a,b]Different letters above the columns indicate the existence of statistical significant differences ($p < 0.05$) among the data in the same group (two-way ANOVA). #Indicate the existence of statistical significant differences ($p < 0.05$) when compared with similar data in the previous time (two-way ANOVA).

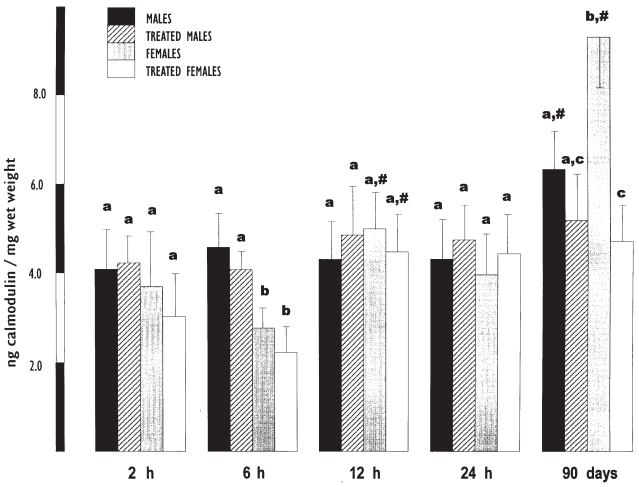

FIG. 3. Calmodulin concentration per mg of wet weight of the rat hypothalamus. The wet weight of each hypothalamus was measured (23) and the calmodulin concentration was calculated per mg of wet weight. All other observations are as indicated in Fig. 1. The results of the two-way ANOVA indicate that the difference in the mean values among the different groups ($p = 0.087$) is not great enough to exclude the possibility that the difference may be due to random sampling variability. However, the differences among the different times is highly significant ($p < 0.001$), and there is a statistically significant interaction between groups and time ($p = 0.008$). [a,b]Different letters above the columns indicate the existence of statistical significant differences ($p < 0.05$) among the data in the same group (two-way ANOVA). #Indicate the existence of statistical significant differences ($p < 0.05$) when compared with similar data in the previous time (two-way ANOVA).

the differences remain but somewhat modified: (a) differences in CaM concentration in control animals could be observed only in the adults, being significantly higher in the females. In fact, CaM is significantly higher in control adult females than in any other of the studied groups; (b) both newborn and adult females showed a decrease in CaM concentration induced by the testosterone treatment; (c) there is virtually no difference in calmodulin concentration in both control and tamoxifen-treated males, from the date of birth until adulthood, However, when CaM is calculated as total content per hypothalamus, a significant increase could be observed in the tamoxifen-treated adults; finally (d) calmodulin concentration in the adult animals was modified in accordance with the expected effect of the hormonal treatment: tamoxifen-treated males tend to have a CaM content (ng/hypothalamus) similar to that observed in the control females, while in PT-treated females CaM content (ng/hypothalamus) or concentration (expressed per mg protein, or per mg ww), remains low and does not differ significantly from that observed in adult control males.

DISCUSSION

Data about calmodulin concentration in nervous tissue are numerous but show wide variations, going from 0.5 μg/mg protein in the spinal medulla (9), to 48.9 μg/mg protein in the diencefalus (15). Determinations performed in the hypothalamus are much more scarce, but show the same wide variation, from 0.99 to 18.2 μg/mg protein (9,16). Some of this variation may be due to differences in the methods used for the quantification; i.e., Egrie et al. (9) used an enzymatic assay, while Hoskins et al. (15) and Loullis et al. (16) used RIA and HPLC. Our results in control rats, from 0.79 μg/mg protein in the newborn females to 2.83 μg/mg protein in the adult females, are in accord with those published by Egrie et al. in the pig (9) and by Gnegy et al. in the rat (11).

In situ hybridization studies (20,22) have shown that two CaM mRNAs are strongly expressed in the cerebellum, hippocampus, and the cerebral cortex of the adult rat brain, but failed to show any significant level of expression in the hypothalamic region. This may be related to the lower concentration of this protein found in the hypothalamus, when compared to that reported in the cerebellum or in the hippocampus.

TABLE 2

MASCULINE SEXUAL BEHAVIOR IN VEHICLE TREATED RATS (CONTROLS), AND IN EXPERIMENTALLY TREATED MALES AND FEMALES

Behavioral Pattern	Female Controls ($n = 7$)	Female Testosterone (30 μg/rat) ($n = 6$)	Male Controls ($n = 7$)	Male Tamoxifen (200 μg/rat) ($n = 8$)
Mount (%Ss)*	60[a]	100[b]	100[b]	100[b]
Mount and intromission (%Ss)*	32[a]	100[b]	100[b]	100[b]
Ejaculatory behavior (%Ss)*	0[a]	100[b]	100[b]	50[c]
Ejaculation (%Ss)*	0[a]	0[a]	100[b]	50[c]
Mount latency (min)†	8.9 ± 3.9[a]	0.7 ± 0.36[b]	0.08 ± 0.05[c]	0.74 ± 0.2[b]
Intromission latency (min)†	—	0.7 ± 0.36[a]	0.08 ± 0.05[b]	0.74 ± 0.2[a]
Ejaculation latency†	—	—	4.12 ± 1.24[a]	14.8 ± 1.1[b]
Postejaculatory interval (min)†	—	—	4.17 ± 0.6[a]	7.8 ± 0.2[b]
Mounts per min†	0.1 ± 0.04[a]	0.84 ± 0.3[b]	0.72 ± 0.3[b]	0.40 ± 0.28[b]
Intromissions per min†	—	0.93 ± 0.2[a]	0.35 ± 0.3[b]	0.67 ± 0.18[c]
Interintromission interval (min)†	—	1.08 ± 0.3[a]	0.3 ± 0.09[b]	1.48 ± 0.2[c]
Copulatory efficiency†	0[a]	0.57 ± 0.22[b]	0.79 ± 0.14[c]	0.62 ± 0.12[b]

Ss = subjects.

* χ^2 test.

† Numbers show the mean ± the standard deviation of the number of cases shown as n (Mann–Whitney U-test).

[a,b,c] Different superscripts indicate statistical significant differences ($p < 0.05$) among data in the same row.

TABLE 3

FEMININE SEXUAL BEHAVIOR IN VEHICLE TREATED RATS (CONTROLS), AND IN EXPERIMENTALLY TREATED MALES AND FEMALES

Behavioral Pattern	Female Controls ($n = 7$)	Female Testosterone (30 μg/rat) ($n = 6$)	Male Controls ($n = 7$)	Male Tamoxifen (200 μg/rat) ($n = 8$)
Lordosis (%Ss)*	100[a]	100[a]	14.28[b]	100[a]
Receptivity Coefficient† (#lordosis/10 mounts × 100)	99.2 ± 1.1[a]	42.6 ± 6.5[b]	2.0 ± 2.4[c]	68.9 ± 5.4[d]

Ss = subjects.

* χ^2 test.

† Numbers show the mean ± the standard deviation of the number of cases shown as n (Mann–Whitney U-test)

[a,b,c]Different superscripts indicate statistical significant differences ($p < 0.05$) between the data in the same row.

It has also been reported that the levels of mRNAs in cerebellar tissue increase during early postnatal development, being three- to fivefold higher in the adult than in the newborn rat (4,20). Our data point to a similar age-related increase in the CaM concentration of the rat hypothalamus.

The importance of CaM for hypothalamic function in the adult rat, may be reflected in the observation that its concentration was significantly higher in both male and female adult rats than in newborn subjects, when expressed either as total content/hypothalamus (Fig. 2), or as CaM/mg ww (Fig. 3). This must be contrasted with the fact that the hypothalamic weight was 7–10-fold higher in the newborn animals than in the adults, when estimated as percent of the body weight. However, when CaM is expressed per mg ww, the level of significance of this increase in the male barely reaches the limits of significance ($p < 0.05$), suggesting that changes in the male rat could also be explained by a proportional increase between CaM concentration and hypothalamic size (Table 1).

Proteins play a predominant role in most biological events. Adequate function of differentiated tissues depends on the presence and concentration of specific, metabolically active proteins. Therefore, proteins that are indispensable for the normal function of a given tissue must be not only synthesized, but also preferentially protected against changes in their required tissue concentration. From this point of view, our results about CaM/mg protein (Fig. 1) are important. Calmodulin seems to be more important for the differentiating male hypothalamus; i.e., female CaM concentration per mg protein is lower in neonates in all the times studied, except in the 6-h period (Fig. 1). These requirements are reversed in the adult, in which calmodulin concentration is significantly higher in the females. This high concentration of hypothalamic CaM, along with the low protein content of the adult female hypothalamus (Table 1), indicate that CaM is preferentially synthesized in the adult female hypothalamus, pointing out to an important role of this protein in the female reproductive function.

Calmodulin synthesis and redistribution among intracellular compartments of neuronal cells can be easily induced in response to different kinds of stimulation, such as neuronal activity (12), NGF (2), hormonal stimulation (8), etc. It is possible that some of the changes in CaM concentration found by us may be partially explained on basis of particular responses to specific stimuli. This may be particularly important in the female, in which hypothalamic CaM concentration depends on the phase of the estrous cycle (M.A. Rodríguez-Medina, M.E. Chavarría, A. Reyes, M. Vergara and A. Rosado, unpublished observation). Therefore, to obtain results that could be compared with the permanent estrus observed in the testosterone-treated female rats, all our control female subjects were sacrificed during the estrous phase of the cycle.

Hormonal treatment of neonate rats decreased CaM synthesis in both males and females (significant decrease in calmodulin concentration per mg protein, with no modifications in the total protein content of the hypothalamus at 2 and 6 h) (Fig. 1). These results will support the described effects of neonatal treatment with calmodulin inhibitors on the development of sexual behavior in the male rat (23). However, from 12 h after birth until adulthood, no significant differences on CaM/mg protein were observed between controls and tamoxifen-treated males. On the contrary, TP treatment of the neonate females, in addition to the described decrease in CaM/mg protein at 2 and 6 h, induced a highly significant decrease in the calmodulin hypothalamic concentration in the adult animals.

Under normal physiological conditions, testosterone levels in the serum of male rats are already elevated before birth (21,30) and remain elevated for more than 2 weeks postnatally (6). However, some data have suggested that androgens can exert their inductive action on the developing brain during a very short period of 12 h or less (1). These results are also consistent with the hypothesis that androgen action can be divided into two phases: a brief phase (about 3 h in length) of androgen uptake by neurons, followed by a more prolonged phase (about 9 h) of intraneuronal biochemical differentiation.

In reference to the sexual differentiation of the brain, we conclude that masculinization and defeminization of sexual brain functions in the rat are irreversible processes once they have been accomplished. Therefore, modification of the hormonal environment during the permissive period must produce irreversible changes in the transcription of genetic information of the involved hypothalamic cells. The lasting effect produced in the reproductive physiology of the rat by short-term pharmacological treatment in the newborn animal may be partially explained by a modification in the neurotubule assembly of the developing hypothalamic region (18). This may be induced by the changes in the calmodulin concentration observed in the newborn animal, and by a permanent modification of the CaM expression, which is clearly observable in the adult; it is perceivable that the tamoxifen-treated males had a total CaM content similar to that found in the control females, while in the testosterone-treated adult females CaM concentration was similar to that found in control males.

Although it is not possible to completely explain our previous results on the development (23) and expression (3) of sexual behavior on basis of the observed variations in the hypothalamic CaM concentration, it is certain that CaM concentration changes significantly and differentially during hypothalamic sexual differentiation, and that hypothalamic functions related to reproductive physiology of the adult rat may be strictly dependent on CaM concentration. In this research effort we studied the hypothalamus as a whole; however, given the detail currently available regarding the organization of sexually dimorphic circuits in the hypothalamus, we look forward to combine immunohistochemical quantitative analysis and recombinant DNA techniques to determine the distribution of CaM among the hypothalamic nuclei, as well as to study the participation of CaM-regulated enzymes in the acquisition and establishment of the sex-specific reproductive physiology in the rat.

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