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Ca^{2+}/Calmodulin System: Participation on Rat Sexual Hypothalamic Differentiation

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RODRÍGUEZ-MEDINA, M., E. CANCHOLA, M. VERGARA-ONOFRE AND A. ROSADO. *Ca^{2+}/Calmodulin system: Participation on rat sexual hypothalamic differentiation.* PHARMACOL BIOCHEM BEHAV **46**(3) 697–702, 1993.—Modifications of male rat hypothalamic sexual differentiation after neonatal administration of drugs that participate on the Ca^{2+}/calmodulin system (haloperidol, trifluoperazine, penfluridol, pimozide, and verapamil) were studied. Pups treated 72 h after birth were behaviorally tested on day 120 of extrauterine life. Five tests for homotypical behavior were conducted. Afterwards animals were castrated and tested twice for heterotypical (female) behavior under replacement hormonal therapy. Fifty percent (80% in the case of pimozide) of all treated males showed lordotic behavior compared with none of the controls. Haloperidol (39%, lordosis quotient) and pimozide (40%, lordosis quotient) were more active than the others. Results obtained with verapamil were not statistically different from the controls. Pimozide was the most active agent influencing the appetitive masculine behavior (mount latency, intromission latency, and postejaculatory interval). Verapamil was more efficient than the rest of the drugs on the consummatory behavior (mount latency, intromission frequency, interintromission interval, and ejaculatory latency). Our results support the participation of the Ca^{2+}/calmodulin system in hypothalamic sexual differentiation and in the differential modulation of the masculine and feminine behavioral patterns.

Calmodulin Hypothalamus Sexual conduct

SOME of the characteristic functions that differentiate males from females are controlled, at least in the rodents, by the brain. Many of these functions become irreversibly organized during the late prenatal (16) or early postnatal period (26) under the influence of hormones. Two of the best studied sexually differentiated brain functions are the pattern of gonadotropin secretion and sexual behavior (for a review see 30). Although there are some recent data supporting that feminization of neural structure and function is an active process (30), it is still commonly accepted that in developing fetal or neonatal mammals these brain functions have an inherently female predisposition (i.e., they will remain female, unless male differentiation is actively superimposed by the presence of aromatizable androgens or of estrogens during a critical period of sexual differentiation). Perinatal treatment of female rats with aromatizable androgens or with steroidal or nonsteroidal estrogens results in female defeminization and masculinization of sexual behavior patterns and of gonadotropin hormone release (30). On the contrary, perinatal gonadectomy or treatment of newly born male rats with androgen or estrogen antagonists results in permanent feminization of sexual behavior and gonadotropin hormone release patterns (30).

Under normal physiological conditions, testosterone levels in the serum of male rats have been shown to be elevated already before birth and to remain elevated for more than two weeks postnatal (11,27). This period of enhanced testosterone secretion is notably longer than the described "critical" developmental period when masculinization of sexual characteristics normally takes place in male rats, and can be achieved experimentally by treating female rats with testosterone, which has been more or less circumscribed to the first five days after birth (9).

Induction of modifications of the sexual parameters referred to above by manipulation of perinatal hormonal environment results in permanent changes of transmitter turnover in tuberoinfundibular dopaminergic neurons and in changes of striatal and limbic dopaminergic content (3,32).

Simerly et al. (31) and Handa et al. (10) described a sexual dimorphism in the distribution of tyrosine-hydroxylase immunoreactive cells and fibers in the preoptic region of the rat that can be reversed by a single postnatal dose of testosterone

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propionate (TP). Sex differences in dopaminergic activity have been observed in the hypothalamus of the adult rat in vivo (3) and, transiently, during early postnatal development of the rat midbrain (15).

In female rats neonatally treated with TP, phenoxybenzamine (50 μg) decreased the incidence of persistent estrus (PE) to 20% and 30% at 60 and 90 days of age, respectively. However, propanolol failed to nullify the masculinization caused by TP. These results suggest that a monoaminergic mechanism is involved in the hypothalamic masculinization of neonatal mice (15,22).

Peptide hormones, biogenic amines, or growth factors act through cell surface receptors to increase intracellular cAMP or Ca^{2+}. As far as we know, cAMP concentration can increase via activation of adenylyl cyclase or decrease by activation of cyclic nucleotide phosphodiesterase. At least one isoform of each of these enzymes requires the Ca^{2+}/calmodulin system for activity.

Calcium can enter cells through voltage-gated or receptor-mediated Ca^{++} channels or can be elevated by the intracellular action of inositol triphosphate (IP3) that is formed from phosphatidyl inositol in a pathway that is linked to occupancy of cell surface receptors. Since most cellular processes that require Ca^{++} utilize calmodulin as an intermediary, it becomes apparent that calmodulin plays a major role in controlling the level of action of all known intracellular second messengers.

Calmodulin regulates several of the effects of Ca^{2+} on synaptic biochemical processes playing an important role in the regulation of neurotransmitter turnover and release, cytoskeletal dynamics, synaptic vesicle function, and synaptic protein phosphorylation (7). Tyrosine (8,19) and tryptophan hydroxylases (14), rate-limiting enzymes in catecholamine and serotonin synthesis, have been shown to be dependent on Ca^{2+}/calmodulin system-related phosphorylation processes.

In the present work we attempt to demonstrate that the Ca^{2+}/calmodulin system plays an important role during the normal differentiation process of sexual behavior in the male rat.

MATERIALS AND METHODS

Chemicals

Analytical degree penfluridol, trifluoperazine, and pimozide were graciously donated by Janssen Pharmaceuticals (Belgium). An aqueous solution of pimozide was prepared by dissolving 20 mg of pimozide in 5 ml of tartaric acid 0.1 M in distilled water and adjusting the pH of the solution to 6.9 with sodium hydroxide. Appropriate dilutions of pimozide were prepared immediately before each experiment.

Haloperidol and verapamil were obtained from Janssen Pharmaceuticals and Knoll Pharm Co. (Mexico City) on their therapeutic parenteral presentations. Estradiol benzoate (EB) and progesterone (P4), purchased from Sigma Chemical Co. (St. Louis), were administered in sesame oil.

Since our main interest was to show the participation of the Ca/Cal complex in the process of hypothalamic sexual differentiation, we decided to use 150-μg equimolecular haloperidol doses per animal of each (16) selected drug, except verapamil. The applied dose of each agent was determined in accord with its molecular weight, relative to that of haloperidol. In the case of verapamil a preliminary group of experiments were done to determine precisely the $lethal_{(50)}$ dose for newly born pups, which was found to be 75 μg per animal. Half of this dose was then selected to be administered.

Animals

Timed pregnant Wistar rats gave birth under direct observation in our laboratory facilities. Birth time was recorded so as to give precision to the application of the indicated treatment. Pups were maintained with their mothers on a 14 : 10-h light : dark cycle.

Sexually vigorous, trained males (studs) and sexually receptive females (stimulus) of the same strain were kept on reversed lighting schedule. Stimulus females were adult, ovariectomized rats brought into behavioral estrus with s.c. injections of EB (3 μg) and progesterone (P, 2 mg), 48 and 4 h, respectively, prior to behavioral testing.

EXPERIMENT

At 72 ± 1 h after birth, male pups were randomly divided into six groups and the following treatments were applied: group 1, saline (controls); group 2, 150 μg haloperidol; group 3, 200 μg penfluridol; group 4, 190 μg trifluoperazine; group 5, 200 μg pimozide; and group 6, 37.5 μg verapamil. All pharmaceuticals were applied s.c. in 50 μl solvent in the dorsal region. Pups were weaned on days 20–22, and behavioral testing began on day 120. After being tested five times for masculine sexual behavior animals were weighed and castrated, and the obtained testes were carefully dissected free of surrounding tissues and weighed to the nearest centigram.

SEXUAL BEHAVIOR

Tests were done 120–150 days after birth during the dark phase of the cycle under dim red light. Sexual behavior of the animal subjects was observed in 1/8-in. Plexiglas cages (52 cm in diameter and 32 cm height).

The test for masculine sexual behavior took place 5–6 h after the onset of the dark phase and was evaluated by standardized techniques (23). Five tests for homotypic, masculine behavior were conducted. After a 5 min adaptation period each subject was presented with a receptive, stimulus female. The occurrence of mounts, intromissions, and ejaculatory behavioral patterns was recorded. The following measures were obtained: the occurrence of the first mount after the introduction of the females (mount latency, ML), the time between the introduction of the females and the first intromission (intromission latency, IL), the time from the first intromission to an ejaculation (ejaculatory latency, EL), the ratio between the number of mounts and the EL (mount frequency, MF), the ratio between the number of intromissions and the EL (intromission frequency, IF), and the interval between an ejaculation and the next mount or intromission (postejaculatory or refractory interval, PEI). The test was considered terminated 1) 15 min after the presentation of the female if no mount had occurred, 2) 15 min after the first mount if no intromission had occurred, 3) 15 min after the first intromission if no ejaculation had occurred, 4) 15 min after ejaculation if no new intromission had occurred, or 5) after the first intromission following an ejaculation.

The rate of copulation and its efficiency were evaluated as the interintromission interval (III) and the hit rate (HR), respectively. The interintromission interval express the ratio between the ejaculation latency and the number of intromissions, including that occurring after ejaculation. When no ejaculation occurs interintromission interval is obtained by dividing observation time (30 min) between the number of intromissions. The hit rate express the efficiency of the consummatory mechanism (2) and is obtained as the ratio be-

tween the number of intromissions and the total number of mounts plus intromissions in a given test.

Psychologists distinguish two components in male sexual behavior: the motivational, or appetitive, and the consummatory (2). It has been proposed (9,30) that sexual intercourse in the male depends on one mechanism for sexual arousal (AM) and another for the execution of the copulatory pattern (CM). Beyer (2) proposes that masculine sexual behavior results from the interaction of three mechanisms: 1) a steroid-dependent arousal mechanism; 2) a modulator mechanism exerting basically inhibitory influences through monoaminergic transmitters; and 3) a neuromotor mechanism, comprising the neuromotor elements involved in either appetitive behavior or copulation.

In the present work we considered ML, IL, and PEI as useful parameters to evaluate the effect of the tested drugs on the motivational or arousal mechanism and modification of MF, IF, and EL as parameters indicating a possible participation of the tested drugs on the consummatory mechanism.

For the study of feminine sexual behavior, all animals were castrated and tested twice for heterotypic, feminine sexual behavior under replacement therapy with EB, 3 μg, and progesterone, 2 mg, applied 48 and 4 h, respectively, before the test.

Studs were introduced into the observation cages and allowed to adapt for 5 min. Receptive, stimulus females were introduced, and the stud males were allowed to mount them until they achieved one or two intromissions. The stimulus females were then removed and the experimental, castrated males were introduced. In all cases, testing continued until the experimental males received 10 mounts. Lordosis behavior was recorded as the lordosis quotients (LQ, ratio of the presentation of the lordosis behavior was recorded as the lordosis position over the number of mounts). Solicitation behavior, although observed, was not registered.

Statistical analysis was realized in a 286 PC, IBM compatible, BPM microcomputer using the MicroStat ii statistical package. Measures of copulatory behavior from each drug group were submitted to nonparametric Kruskal–Wallis one-way analysis of variance. Mann–Whitney *U* tests were also performed to attest the statistical significance obtained by comparing the effects of each independent treatment against the others.

RESULTS

No significant differences were found between the adult body weights of control and treated animals. However, significant differences ($p < 0.05$ or less) were found in adult testes weights between the controls (2.00 ± 0.11 g) and the rats treated with trifluoperazine (1.72 ± 0.14 g), pimozide (1.64 ± 0.20 g), or haloperidol (1.50 ± 0.39 g).

Masculine Sexual Behavior

The modifications induced by the neonatal treatment with inhibitors of the Ca/Cal system in the parameters that have been related to the motivational and consummatory components of masculine sexual behavior are summarized in Tables 1 and 2. Pimozide was the most active pharmacological agent to influence the motivational or appetitive sexual behavior, followed by penfluridol and trifluoperazine. As shown in the values indicated in Table 1, pimozide was the most active drug on mount latency and intromission latency, and was second only to penfluridol over the postejaculatory interval. Treatment of newly born rats with haloperidol did nor modify any of the motivational-related parameters of masculine sexual behavior. Verapamil, an inhibitor of the slow calcium channels, was only effective on one of these parameters—namely, intromission latency, in which case it was almost as effective as pimozide.

On the contrary, verapamil was regularly more efficient than the rest of the drugs on the parameters related to consummatory behavior. As shown in the results presented in Table 2, of the parameters indicating this type of behavior, verapamil was the most active on mount frequency, which was indeed increased over the controls, and on the interintromission interval, and was second only to pimozide on ejaculation latency and intromission latency. Haloperidol was once more ineffective in inducing any modification of the consummatory parameters of masculine sexual behavior (Table 2). Of all the drugs tested, only pimozide was equally effective on both the motivational and the consummatory components of the male sexual behavior.

Feminine Sexual Behavior

Results are summarized in Table 3. Approximately 50% (80% in the case of pimozide) of all treated males showed lordotic behavior, compared with none of the 10 controls ($p < 0.02$). However, there were notable differences between the tested drugs in their ability to induce feminine sexual behavior (lordosis) on treated males under the stimulus of mounts (LQ). Haloperidol (39%) and pimozide (40%) were more active than penfluridol (18%) and trifluoperazine (26%). The results obtained with verapamil (4%) were not statistically different from the controls.

TABLE 1

EFFECT OF NEONATAL TREATMENT WITH INHIBITORS OF THE Ca^{2+}/CAL SYSTEM ON THE APPETITIVE SEXUAL BEHAVIOR OF MALE RATS

	CONT 7	HALO 11	TRIF 11	PENF 10	PIMO 10	VERA 5	*H*	*p* <
ML	0.65_a	$1.1_{a,b}$	1.8_c	1.5_b	2.7_d	1.5_b	58.1	0.0001
IL	0.60_a	$1.2_{a,b}$	$2.8_{b,c}$	2.6_c	4.1_d	$3.3_{c,d}$	87.3	0.0001
PEI	4.00_a	5.00_b	$5.7_{b,c}$	6.3_c	5.9_c	4.70_b	55.0	0.0001

Numbers indicate mediana values obtained from the number of independent experiments. Whenever necessary Tables 1 and 2 express time as decimal minutes. The *H* values obtained from the Kruskal–Wallis nonparametric analysis of variance and the statistical significance of these values are given. Different subscript letters following the mediana values indicate the existence of statistically significant differences ($p < 0.05$ or less) between independent groups as calculated by the Mann–Whitney *U* test. ML = mount latency, IL = intromission latency, PEI = postejaculatory interval.

TABLE 2

EFFECT OF NEONATAL TREATMENT WITH INHIBITORS OF THE Ca/CAL SYSTEM ON THE CONSUMMATORY SEXUAL BEHAVIOR OF MALE RATS

	CONT 7	HALO 11	TRIF 11	PENF 10	PIMO 10	VERA 5	H	$p <$
MF	0.68_a	0.45_b	0.65_a	$0.40_{b,c}$	0.31_c	0.82_d	22.4	0.001
IF	3.29_a	2.08_b	1.20_d	$1.33_{c,d}$	0.89_e	1.45_c	98.0	0.0001
III	0.30_a	0.46_b	0.82_c	0.76_c	1.25_d	0.69_c	95.2	0.0001
EL	3.78_a	4.20_b	$6.88_{c,d}$	6.15_c	7.27_d	6.20_c	60.2	0.0001
HR	0.82_a	0.77_a	0.65_b	0.68_b	0.67_b	0.58_c	27.4	0.001

Numbers indicate mediana values obtained from the number of independent experiments. The H values obtained from the Kruskal–Wallis nonparametric analysis of variance and the statistical significance of these values are given. Different subscript letters following the mediana values indicate the existence of statistically significant differences ($p < 0.05$ or less) between independent groups as calculated by the Mann–Whitney U test. MF = mount frequency, IF = intromission frequency, III = interintromission interval, EL = ejaculatory latency, HR = hit rate.

Careful observation of the treated animals indicated that all tested drugs were equally active in inducing sedation on the treated pups. Haloperidol was, however, almost completely unable to induce any significant modification on masculine sexual behavior, while the effect of verapamil on inducing heterotypic sexual behavior was also nil. This allowed us to eliminate the role played by the sedative effect of the tested drugs as a principal cause of the modifications observed in the differentiation of sexual behavior.

DISCUSSION

While the binding to calmodulin of the drugs tested is indisputable and has provided important tools for the study of Ca^{2+}/calmodulin-dependent actions, some questions may rise about the concept that all the agents tested were considered to be inhibitors of the calcium/calmodulin system, particularly in the case of verapamil, a blocker of the slow Ca^{++} channels. However, the interesting observation that the Ca^{++} channel blockers (felodipine, nifedipine, diltiazem, prenylamine, etc.) interact with calmodulin, as determined by NMR spectroscopy (4,12), leads to the possibility that calmodulin may act as an intracellular site of action of Ca^{++} channel blockers, perhaps even accounting for their major pharmacological effects (29).

One may agree with the fact that the specificity of some of the drugs is not as adequate as desirable and limits the usefulness of these compounds as direct pharmacological antagonists (16). Many of the calmodulin antagonists have great potency as receptor antagonists, including dopaminergic, α-adrenergic, serotonergic, and muscarinic cholinergic receptors (28). However, many of the nondirect-calmodulin activities of these drugs are focused upon other parameters of the calcium metabolism (i.e., inhibition of the calcium-dependent protein kinase C (35); interaction with some other Ca^{++} binding proteins (20); displacement of Ca^{++} from membrane sites; and blockade of both passive and active Ca^{++} transport processes, including non-calmodulin-regulated Ca^{++} transport by the endoplasmic reticulum (6).

We may mention that the applied dosage of the drugs tested was selected as appropriate for calmodulin antagonism (29), trying to emulate their calmodulin-binding activity, and that in most of the obtained results the order of inhibitory potency of the compounds tested paralleled their anticalmodulin potency (29,34).

The differences in activity of the tested drugs upon the homotypic and heterotypic sexual behavior support the hypothesis that the propensity of intact male rats to display masculine copulatory behavior can be modified independently of

TABLE 3

EFFECT OF NEONATAL TREATMENT WITH INHIBITORS OF THE Ca/CAL SYSTEM ON THE PRESENCE OF FEMININE SEXUAL BEHAVIOR OF MALE ADULT RATS

	Mounts per subject	Responders	LQ responders	LQ all subjects
Controls	10	0/10	0%	0%
Haloperidol	10	6/10	39/60 = 65%*	39%*
Penfluridol	10	5/10	18/50 = 36%†	18%‡
Trifluoperazine	10	5/10	26/50 = 52%*	26%†
Pimozide	10	8/10	40/80 = 50%*	40%*
Verapamil	10	4/10	4/40 = 10%‡	4%

Proportions of sexually active animals and proportion of tests in which subjects were active were analyzed by the Fisher and χ^2 tests. LQ = lordosis quotient. *$p < 0.001$; †$p < 0.01$; ‡$p < 0.05$ when compared with the saline-treated control group.

the tendency to exhibit feminine sexual behavior (9,30,33). Thus, haloperidol was as potent as pimozide in inducing the appearance of feminine sexual behavior on the treated males, but was completely unable to induce any modification on the development of masculine sexual behavior. On the contrary, verapamil and penfluridol, unable to modify feminine sexual behavior of the adult males, induced a significant modification of the homotypic sexual behavior of the treated subjects.

It has been shown that central dopaminergic systems are involved in the sexual differentiation of the brain. Therefore, the possibility exists that this differential effect could be due to the selective participation of biogenic amines on the sexual differentiating system. Several pieces of evidence suggest that manipulation of perinatal hormonal environment results in permanent changes of transmitter turnover in tubero-infundibular dopaminergic neurons (31) and in changes of striatal and limbic dopaminergic content (15) in adult rats.

Our results could support this differential participation of biogenic amine receptors. In effect, chlorpromazine is as effective as an NE antagonist at α-adrenoceptors as at DA receptors. Some neuroleptic drugs, including haloperidol and pimozide, seem to be much more specific in their DA antagonist effects, since they have little effect on NE turnover in the central nervous system. The results of the present experiments show that haloperidol and pimozide treatment during the immediate postnatal period enhance the capacity of displaying feminine sexual behavior in castrated, adult rats (Table 3). In contrast, the expression of masculine sexual behavior was unaffected by haloperidol (Tables 1 and 2). On the other hand, two of the drugs tested, penfluridol and verapamil, were unable to induce feminine sexual behavior in castrated, adult rats (Table 3), while significantly modifying the expression of normal masculine sexual behavior (Tables 1 and 2).

The activity of the tested drugs on the motivational or appetitive sexual behavior (LM, III, and PEI) appears to be related to their efficiency as inhibitors of calmodulin-dependent phosphodiesterase (34) and as ligands for the Ca^{2+}/calmodulin complex: Penfluridol was the most active, followed by pimozide and trifluoperazine, while haloperidol was only marginally effective. In addition, verapamil, an inhibitor of the slow calcium channels, induced effects that were comparable, although less important, to those elicited by the anticalmodulin drugs. On the other hand, the activity of the drugs on the consummatory behavior seems not to be related to their anticalmodulin activity. On this type of behavior verapamil was regularly more efficient than the rest of the drugs. In adults, distinct types of DA antagonists disrupt the sexual behavior of humans and animals, selectively affecting the appetitive or the consummatory components (17). It has been proposed that these differential effects of dopamine receptor antagonists on the sexual behavior of male rats (23) depends upon their site of action in the brain. Blockade of mesolimbic DA receptors interferes mainly with the appetitive or motivational component of male sexual behavior, while mesostriatal DA may be required for the normal expression of the consummatory component (23). It may be proposed that the differential effects of Ca^{++}/calmodulin system antagonists presented herein may also be related to their site of action on the brain, but confirmation of this proposal must await further investigation.

Finally, we would like to discuss briefly some of the mechanisms that may explain how the drugs tested can influence the sexual differentiation of the hypothalamus.

Some genetic activity of CaM has been demonstrated. It seems that CaM participates in the regulation of histone H4 mRNA synthesis. The mRNA for MLCK increases 5–10-fold in passing from G1 to S phases of the cell cycle. Finally, increases in CaM concentration are related to observed increases in the level of beta-actin mRNA, and a decrease in the levels of both alpha- and beta-tubulin mRNA (18).

cAMP has been shown to cause neuronal differentiation in the infant rat brain (24) and in neuroblasts in cell culture (36). Oligodendrocyte development is regulated, in part, by factors that utilize cAMP as a second messenger (25). It is also known that cAMP can move to the nucleus, where it becomes associated with the chromatin (13); this may lead to permanent repression or derepression (1).

Phosphorylation of elongation factor 2, EF2, by CaM-dependent protein kinase III suppress the ability of EF2 to support protein synthesis in a reconstituted system (21). These results suggest that intracellular calcium inhibits protein synthesis in mammalian cells via CaM-dependent protein kinase III–catalyzed phosphorylation of EF2. Finally, anticalmodulin drugs prevent cells from progressing through the S phase in a selective and reversible fashion (5).

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