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Injection of an Aromatase Inhibitor After the Critical Period of Sexual Differentiation

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GONZALEZ, M. I. AND M. L. LERET. *Injection of an aromatase inhibitor after the critical period of sexual differentiation.* PHARMACOL BIOCHEM BEHAV 47(1) 183–186, 1994. – We have investigated the possible role of the second week of life in the differentiation of sexually dimorphic behaviours, dependent on the androgenic aromatization, and its possible relationship with the serotonergic systems. For this purpose, 5 mg/kg of a suspension of an aromatase inhibitor, LY43578, has been intraventricularly injected to males on day 12 of life. Studies have been made in adulthood on exploratory and motor activities, anxiety, sexual motivation, and sexual performance. Indoleamine levels in the hypothalamus and corpus striatum have been measured. Sexual behaviour, exploration, and serotonergic metabolism were not affected by the treatment. Sex partner preference and anxiety in the plus-maze showed a feminized tendency in the treated group that, however, did not reach statistical significance. From these results we have confirmed the restriction of the critical period of androgenic aromatization for the organization of reproductive and exploratory behaviour.

Aromatization Sexual differentiation Serotonin Sexual behaviour and orientation Exploratory behaviour Elevated plus-maze Anxiety Critical period

CEREBRAL androgen aromatization has been described as a mechanism responsible for masculinization of the brain. Both testosterone and estradiol seem to prevent feminization of the neonatally castrated male brain (16,19), while nonaromatizable androgens, such as 5-α-dihydrotestosterone (5-α-DHT), fail to prevent feminization or induce masculine behaviour (1). However, other authors have found a masculinizing effect of nonaromatizable androgens such as 5-α-DHT (17,18)

We have shown a clear behavioural feminization and demasculinization when LY43578 (an aromatase inhibitor) was ICV injected in males (9). A connection seemed to exist between the behavioural changes and the indolaminergic alterations produced by this neonatal treatment, with extrahypothalamic areas involved (9). Serotonin (5-HT) has been proposed as a neurotransmitter involved in sexual differentiation, mainly in the hypothalamus (12). Sex differences in brain levels of 5-HT have been reported in the second week of life (5). Lordotic activity is not affected by neonatal manipulation of 5-HT (6); however, in androgenized females 5-HT seems to affect lordosis (22). It has been suggested that the reduced 5-HT concentration in the male in the second week has a functional significance in that it removes an inhibitory influence of the serotonergic system on testosterone (10).

This investigation is concerned with the possible role of the second week of life as the critical period for the differentiation of sexually dimorphic behaviours, dependent on the androgenic aromatization, and its possible relationship with the serotonergic systems. For this purpose, LY43578 has been intraventricularly injected to males on day 12 of life. Studies have been made in adulthood on various aspects of behaviour, including exploratory and motor activities, anxiety, sexual motivation, and sexual performance. A parallel analysis of indoleamines in the hypothalamus and corpus striatum has been made.

MATERIALS AND METHODS

The work was performed on Wistar rats. Five litters born on the same day were randomized, and males were injected intraventricularly on day 12 with either 2 μl saline or 5 mg/kg LY43578 (an inhibitor of aromatase; Eli Lilly and Co., Indianapolis) administered as a suspension in 2 μl saline. The females were not used in these experiments. Injections were carried out under cold anesthesia that was induced by placing pups at −20°C for 45 min. The skin was retracted and a microsyringe was introduced to the lateral ventricle 3–3.5 mm lateral from bregma and 2 mm ventral from the surface of the skull, through the skin. These coordinates were determined by dye injections. Afterwards, the animals were returned to the mother so lactation followed its normal course. They were kept throughout life in a reversed lighting regime 12 h on : 12 hours off (lights off 0800). Temperature was maintained at

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22°C, and water and food were available ad lib. There were two experimental groups: 10 males receiving LY43578 and 10 males receiving saline.

At the age of 80–85 days, rats were submitted to behavioral testing. The order of testing was as follows: The combination of holeboard and plus-maze was applied in the morning, and the holeboard test was always first. The afternoon of the same day the animals were subjected to the sex partner preference test. The day after both masculine and feminine sexual activity were tested. All the tests were carried out under red lighting.

The holeboard was a black Perspex box (60 × 60 × 35 cm) with four holes (3.8 cm diameter) equally spaced on the floor. The latter was divided into 36 squares. Frequency and duration of head-dipping and frequency of rearing were recorded. Locomotor activity was recorded as number of line-crossings. The elevated plus-maze consisted of two open arms (50 × 10 cm) and two enclosed arms of the same size with 40-cm-high walls arranged so that the arms of the same type were opposite each other. The apparatus was plastic and elevated to a height of 62 cm. The measures recorded were frequency and duration of arm visits, separately for open and closed arms. Number of boluses were counted at the end of the test. Both the holeboard and the plus-maze were thoroughly cleaned at the end of every test. Each of these tests was carried out for 5 min.

The test for sex partner preference was carried out in a circular arena of 90 cm diameter, surrounded by a 30-cm-high wall. Two small wire-mesh cages (15 cm^2) were fixed into the wall such that the front of each cage was flush with the wall and the two cages were opposite each other. They contained two stimulus animals, an intact sexually experienced male and a receptive female (ovariectomized bearing a SC 7-mm Silastic implant containing oestradiol benzoate, Sigma Chemical Co., St. Louis). During the 10-min test the following measures were taken: frequency and duration of visits to an area (30 × 15 cm) in front of each stimulus animal investigating it.

Feminine sexual activity was tested by placing animals with intact sexually experienced males and noting their lordotic responses to 10 mounts. The test was terminated after 15 min if there were no mounts, in which case a score of zero was entered for that animal. Masculine behaviour was observed by placing each animal in an arena and 5 min later introducing a receptive female (as described for the sexual orientation test). The mount latency, intromission latency, number of mounts, number of intromissions, ejaculation latency, and refractory period were noted. The test was terminated after the refractory period, after 30 min if there was no ejaculation, or after 15 min if there were no mounts.

All the animals were decapitated one week after the testing. The encephalons were extracted, and the corpus striatum and

TABLE 1

EFFECTS OF LY43578 ICV INJECTED TO MALE RATS ON DAY 12 (HOLEBOARD, ELEVATED PLUS-MAZE, SEX PARTNER PREFERENCE, AND SEXUAL BEHAVIOUR TESTS) (MEANS ± SE)

	LY43578 ICV Day 12 Males (n = 10)	Control Males (n = 10)
Holeboard Test		
Locomotion		
No. crosses	188 ± 22.3	215 ± 10.45
Head-Dipping		
Frequency	8.00 ± 1.24	7.33 ± 1.25
Rearing		
Frequency	15.85 ± 2.69	13.16 ± 1.68
Duration (min)	20.57 ± 3.40	27.30 ± 6.30
Plus-Maze Test		
Open Arms		
% Time	26.12 ± 13.90	9.06 ± 1.95
Total Entries		
No.	7.00 ± 1.51	7.83 ± 1.77
Boluses		
No.	0.28 ± 0.19	0.16 ± 0.17
Sexual Orientation		
Time Investigating		
Female (s)	159.00 ± 40.80	205.66 ± 26.13
Male (s)	280.14 ± 40.30	206.33 ± 32.60
Feminine Behaviour		
% Lordosis	0 ± 0	0 ± 0
Masculine Behavior		
Mount Latency (s)	121.0 ± 35.6	151.0 ± 90.6
No. Mounts	11.4 ± 4.8	17.6 ± 9.1
Intromission Lat (s)	425.4 ± 298.9	145.6 ± 111.7
No. Intromissions	9.7 ± 4.1	7.6 ± 4.0
% Ejaculations	42%	0%

TABLE 2

EFFECTS OF LY43578 ICV INJECTED TO MALE RATS ON DAY 12 (SEROTONERGIC CONTENTS) (MEANS ± SE)

	LY43578 ICV Day 12 Males (n = 10)	Control Males (n = 10)
Medio Basal Hypothalamus		
5-HT (ng/g)	1189.6 ± 281.2	1969.2 ± 548.0
5-HIAA (ng/g)	1227.6 ± 364.0	822.7 ± 176.9
5-HIAA/5-HT	0.76 ± 0.22	0.66 ± 0.29
Corpus Striatum		
5-HT (ng/g)	676.4 ± 83.5	750.9 ± 117.8
5-HIAA (ng/g)	601.4 ± 79.6	722.4 ± 113.9
5-HIAA/5-HT	0.92 ± 0.13	1.01 ± 0.13

medio basal hypothalamus were dissected (2), weighed, and stored at −40°C until assayed for indoleamines (maximum time three weeks) by high-performance liquid chromatography–electrochemical detection (HPLC-ED), following the method described before for monoamines (7,8).

Statistics

Behavioral results were compared by means of Mann-Whitney test after Kruskal-Wallis one-way analysis of variance (ANOVA). Aminergic ratios were used as a turnover measure to give a functional approach of the amines' activity. Serotonin and 5-HIAA contents, as well as 5-HIAA/5-HT ratio, were tested for significant differences by Student's t test for independent samples.

RESULTS

Holeboard Test

The treatment had no effect on any of the parameters: locomotion, rearing, and head-dipping (Table 1).

Plus-Maze Test

The number of total entries was similar in both groups. The percentage of time spent on the open arms was higher in the treated animals, but this tendency was not statistically significant. There was no difference in the number of boluses (Table 1).

Sex Partner Preference Test

In the control group the time spent in front of the incentives was equally distributed between males and females. Time spent by the treated males in investigating the incentive male was slightly higher compared to normal males (Table 1), but statistically not significant.

Sexual Behaviour Tests

The results of tests on masculine and feminine sexual behaviour are shown in Table 1. None of the animals showed lordosis. The aromatase inhibition did not exert any obvious effect in any masculine behaviour measure.

Aminergic Contents

No differences were found in either 5-HT, 5-HIAA, or serotonergic ratio in the corpus striatum or medio basal hypothalamus (Table 2).

DISCUSSION

The critical period for sexual differentiation of the brain has traditionally been restricted, in the rat, to the 18th to 27th days after conception (14). But within the critical period, there can be "subcritical periods" depending on the function to be differentiated. Sexual behaviour, in the male, seems to be much more sensitive to androgenic treatment in the prenatal part of the period (20), and aromatization seems to play important roles both before and after birth (15). Within this variability of effects, it could be also possible that the organizing influence of aromatization could continue to a certain extent on later stages of development.

In this experiment, sexual behaviour was not altered by the treatment. As all the animals were sexually naive, both groups showed long latencies and rather high variability in the sexual parameters measured in masculine behaviour. Feminine behaviour was absent both in the treated and control groups. In the sex partner preference test, control males spent the same time investigating incentive males than females, typical behaviour in the first sexual experience (3). Treated males showed a tendency to investigate longer the incentive males, although it did not reach significance, and their sexual performance was normal, as commented on above. This lack of effect of the aromatase inhibitor administered on day 12 greatly contrasts with the dramatical demasculinization plus feminization observed when the same dose of LY43578 was injected on the first day of life (9). Therefore, the important role of aromatization on the differentiation of sexual performance seems to be restricted to the critical period. The same restriction seems to work for locomotion and exploration, measured separately in the holeboard (4).

The control of anxiety in adulthood seems to depend on the serotonergic system (11), and a serotonergic control on the development of the systems controlling anxiety has also been reported (21). A sexual dimorphism is present in hypothalamic 5-HT levels on day 12 (5), and this difference could be related to the dimorphism in the anxious response. In the previous study in which LY43578 was ICV injected on day 1 (9), striatal serotonergic metabolism showed a long-lasting sexual dimorphism, suggesting a possible role of extrahypothalamic 5-HT in the mediation of the estrogen-induced mechanisms of behavioural sexual differentiation. In the present work, we did not find any difference in the serotonergic metabolism, neither hypothalamic nor extrahypothalamic, when the aromatase inhibitor was administered on day 12, suggesting that the reported serotonergic dimorphism on that day

might be organized earlier in the development, and can not be altered by aromatization inhibition later on.

The results from the plus-maze test showed no differences in the anxious response between the treated and control groups, although the aromatase-inhibited males spent time on the open arms not significantly longer than the control males, what would represent a lower anxiety response, more frequent in female rats (13). Total number of entries was similar in both groups, pointing out a similar general activity in both groups, also supported by the similar behaviour found in hole-board. It is interesting to point out the feminized tendency of the treated group in both sexual orientation and anxiety, although it is not statistically significant.

In conclusion, taking the above provisos into account, we have confirmed that the critical period of androgenic aromatization for the organization of sexually dimorphic behaviours is restricted to an earlier period than two weeks postnatally.

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