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Central and peripheral effects of the prenatal letrozole administration in male rats: is it a good model to study human male homosexuality?

*Sandra Olvera-Hernández and Alonso Fernández-Guasti**

Department of Pharmacobiology, Centro de Investigación y Estudios Avanzados Cinvestav, Mexico, D.F., Mexico

ABSTRACT

Masculine brain sexual differentiation in rodents is due to testosterone conversion to estradiol by the aromatase complex, producing males that when adults display their typical sexual behavior, like partner preference towards the receptive female. Disruption in endocrine milieu during development by the administration of the aromatase inhibitor, letrozole (56 μg/kg/day gestational days 10-22) results in 30% of males with same-sex preference, while the rest retain preference for the receptive female. The males with same-sex preference showed high levels of experimental anxiety in the elevated plus maze test. In addition, independently of the partner preference, this letrozole treatment produced an earlier preputial separation, and feminization of the second and fourth digit length index and of the length of some pelvic bones. These results suggest that alterations in the hormonal levels of estradiol during development not only

RESUMEN

La diferenciación sexual cerebral masculina, en roedores, se debe principalmente a la conversión de testosterona a estradiol por la enzima aromatasa. Esto da lugar a que en la edad adulta se expresen las conductas típicamente masculinas, como lo es la preferencia sexual por la hembra. Alteraciones en el ambiente hormonal, como la administración del inhibidor de aromatasa letrozol (56 μg/kg/día) durante la etapa prenatal (días gestacionales 10 a 22), dan como resultado un 30% de machos con preferencia hacia individuos de su mismo sexo y el resto permanece con preferencia hacia la hembra receptiva. Los sujetos con preferencia por su mismo sexo presentan altos niveles de ansiedad experimental en el laberinto elevado en forma de cruz. Además, independientemente de la preferencia, el tratamiento con letrozol produce un adelanto en la separación prepucial, feminización del índice de longitud del segundo y cuarto dígitos y

Correspondence to:
*Alonso Fernández-Guasti
Centro de Investigación y Estudios Avanzados
Departamento de Farmacobiología
Calzada de los Tenorios, 235
Colonia Granjas Coapa, C.P. 14330, México, D.F., México
E-mail: jfernand@cinvestav.mx

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affect the central nervous system, but also other biological markers sensitive to androgen concentrations. (REV MEX ENDOCRINOL METAB NUTR. 2015;2:68-79)

Corresponding author: Alonso Fernández-Guasti, jfernand@cinvestav.mx

Key words: Brain sexual differentiation. Aromatase inhibitor. Partner preference. Pelvic bones.

en la longitud de algunos huesos pélvicos. Estos resultados sugieren que la alteración de los niveles de estradiol durante el desarrollo no sólo afecta al sistema nervioso central, sino también a marcadores biológicos que son sensibles a las concentraciones de andrógenos.

Palabras clave: Diferenciación sexual cerebral. Inhibidor de aromatasa. Preferencia sexual. Huesos pélvicos.

SEXUAL DIFFERENTIATION

In mammals, sexual differentiation results after the expression of the sex-divergent chromosomal charge: males possess XY and females XX. Genetic sex is determined at the time of fertilization by the entry of an X or a Y chromosome from the spermatozoon into the ovule. The main role of the Y sex chromosome is to induce the formation of testes from the undifferentiated gonads by the Sry gene, with the formation of Sertoli cells. Androgens are essential for normal male sex differentiation at various levels. During fetal growth, testosterone promotes the development of Wolffian ducts into the internal male genital structures such as epididymis, vas deferens, and seminal vesicles. In the external genitalia, testosterone is rapidly transformed into 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase to induce the development of the male urethra, prostate, penis, and scrotum[1]. In addition, a large body of evidence supports the notion that the testosterone secreted by the testes needs to be locally aromatized to estradiol to promote the sexual differentiation of the neural tissue destined to mediate sexual preference and behavior[2]. In rodents, exposure to androgens during prenatal period results in the organization of masculine behavior –masculinization (mount, intromission, and ejaculatory behaviors)– in the presence of a sexually receptive female and a decrease in the probability to display feminine behavior in presence of a male –defeminization (lordosis, dorsiflexion reflex posture with the consequent pelvic elevation that allows the penis entrance into the vagina)[3,4].

HUMAN MALE HOMOSEXUALITY

Human sexuality is a multidimensional phenomenon that includes several aspects of behavior and personality. Several hypotheses have been proposed to explain the etiology of human male homosexuality, for example: genetic influence, fraternal birth order effect, maternal stress, and endocrine alterations[5-10]. As with experimental animals, it is now well established that hormones, particularly sex steroids, have a paramount role in the organization of the human developing brain[11,12]. The most accepted etiology of human male (and female) homosexuality implies endocrine changes that may have occurred in early developmental stages[13,14]. Human male homosexuality has many ways of expression and because of that it is difficult to establish its precise incidence, although in general terms its prevalence ranges between 2-10%[15,16]. Additionally, most societies have imposed a discriminatory role on this behavior. Thus for example, many homosexual men could be sexually stimulated by other men but refuse to have physical contact or may even suppress fantasies or other ideas that involve sexual contact. These men, when questioned, most likely would reject a homosexual auto-definition, confounding the final estimation numbers. Additionally, as clearly stated by Simon LeVay, homosexual activity varies greatly between gay couples, in contrast to what generally defines heterosexual man-woman sexual relationships[17].

After the discovery of the important stimulatory role of sex hormones on the induction of male and female sexual behavior in animals, various authors

suspected that human male homosexuality was the result of low levels of testosterone in this population[18,19]. However, a careful analysis of the complete hormonal profile between homosexual and heterosexual men has revealed no difference[20], suggesting that this particular sexual orientation is not mediated by low adult hormone levels. In addition, very interesting results have demonstrated that "sissy boys" frequently develop as homosexual men[21] and that many homosexual men can define their particular sexual orientation even before puberty[8]. These data, together with the large information in animal studies, propose that sexual orientation in humans and sex preference in animals is established in uterus or shortly after birth.

Besides the differences in sexual motivation between gays and heterosexuals, homosexual men, as compared with their heterosexual counterparts, present a higher prevalence of pathological anxiety and moods. Interestingly, these studies reveal that 41% of homosexual men suffer from some anxiety disorder as compared with 18% in their heterosexual counterparts[22-24]. Such different prevalence is nearly fourfold in panic disorder with agoraphobia[22]. The reasons underlying such increased prevalence in the homosexual population primarily rely in social factors that include discrimination, stigmatization, and harassment[25]. No study before the present one has proposed that the increase in anxiety levels could also have biological bases, possibly underlain by similar endocrine changes.

In addition to the clear central effects of steroids during development, peripheral tissues are also sensitive to changes in the endocrine milieu. Thus, testosterone, directly or through its conversion to estrogens or 5-alpha-reduced androgens, affects not only the brain sexual differentiation process, but also modifies other morphological characteristics like the ratio between the length of the second and fourth digit (2D:4D). In humans, as in other species, this digit length ratio is sexually dimorphic: the two fingers are approximately equally long in females, but in males the fourth digit is usually longer than the second[26,27], suggesting that such ratio is a marker of prenatal exposure to androgens. Interestingly, some controversial results have proposed that homosexual men show a lower 2D:4D ratio in comparison with heterosexuals[27-29]. These characteristics seem to be accompanied by other features: homosexual men are significantly shorter and lighter than their heterosexual counterparts[30-32]. Other data support the idea that the length and width of some pelvic bones differ according to the sexual identity; there is a report where female-to-male transsexuals have a male phenotype[33]. Even though these data do not strictly relate sexual orientation with pelvic bone differences, it is now clear that gonadal hormones exert complex variable effects on the growth of the pelvic bones because, for example, when the female rats are neonatally treated with testosterone the sex difference in bi-iliac width is abolished[34]. Finally, the age at which puberty occurs also appears to differ between homosexual and heterosexual men. Thus, Blanchard and Bogaert have found that male homosexuals report achieving puberty about 2.5 months earlier than male heterosexuals[32].

ANIMAL MODELS OF HUMAN MALE HOMOSEXUALITY

To model human male homosexuality in animals is a challenge. Animal models, by definition, should cover various criteria in order to be useful. Naturally, the best animal models of human male homosexuality are those that occur spontaneously in animals. Indeed, in almost all species studied, males may have sexual encounters with other males[35]. Such sexual relationships may be of very short duration, can occur simply because males lack access to females, or be related with dominance hierarchy[36]. Even so, in some species there are a proportion of males that consistently prefer other males, even in the presence of females, suggesting that these males may be categorized as animals with same-sex preference. Such is the case of rams, which have been thoroughly studied by Anne Perkins and Charles Roselli[37,38]. In spite that these subjects may be the best animal model for the study of human male homosexuality, their percentage of occurrence is quite small (around 5-10% of the whole population) and it is not easy to have access to the facilities required to house rams and ewes.

Based on the idea of prenatal factors affecting sexual orientation, various groups, including ourselves, have used several techniques to study partner preference in rodents. As mentioned, the endocrine hypothesis sustains that sexual preference is determined by the presence of sex steroids prenatally or during the few days after delivery[13,14]. Remarkably, the establishment of a typical male-to-female sexual preference seems to be defined by the male's fetal or neonatal exposure to relatively high levels of testosterone that should be converted to estrogens[14]. That is, the testicular male hormone, testosterone, produced by the active testes of the male fetus should reach the brain to promote differentiation. However, this hormone must be converted *in situ* to estrogen to achieve its main effect. This process is named aromatization, primarily because the A ring of the androgens' structure should be aromatized. The series of enzymatic reactions in this process is termed aromatization and the group of enzymes named aromatase[2]. Consequently, the male rats that were prenatally and/or neonatally treated with aromatase inhibitors showed female sexual behavior and male preference when adults[39-42]. Additionally, Bakker, et al. reported that the neonatal administration of 1,4,6-androstatriene-3,17 (ATD) induces bisexual behavior when males were tested in a three-compartment box where they could sexually interact with either a sexually active male or a receptive female[43,44]. Interestingly, with the receptive female, the ATD-treated males displayed mount and intromissions, whereas with the male they exhibited proceptive (female sexual inviting behaviors such as hop and darting) and lordosis behaviors[45,46]. However, ATD is a steroidal compound that irreversibly binds to aromatase and possesses affinity for androgen, estrogen, and progesterone receptors[47,48]. Letrozole is a new third-generation, non-steroidal aromatase inhibitor[49]. Using high letrozole doses during the last two gestation days, Gerardin, et al. demonstrated disruption of male rat sexual behavior after castration and testosterone propionate replacement[50].

Interestingly, besides the clear central effects of testosterone (mainly mediated by estradiol), this androgen also has profound effects in the organization of the genital tract. Thus, male and female rats differ in the anogenital distance length that is usually shorter in females. Such distance is used as biomarker of overall androgen action during the masculinization programming window and serves to sex identify the newborn[51,52].

The present review is focused on the analysis of various behavioral and physical traits of adult male rats that prenatally were exposed to the aromatase inhibitor letrozole. The general method followed implied that female rats were time mated (day of mating = day 0 of pregnancy). Prenatal treatment consisted of daily subcutaneous injections of letrozole (Sigma-Aldrich, St. Louis, USA) at a dose of 0.56 μg/kg/ml (dissolved in corn oil) or vehicle to the mothers from day 10 of pregnancy until one day before delivery. A previous study from our group demonstrated that this is the effective dose to produce males with same-sex preference. Lower doses were non-effective, while higher ones precluded parturition[39]. All behavioral observations were started when the males reached the age of three months. The tests were conducted during the early dark phase of the light-dark cycle in a room under dim red light.

PARTNER PREFERENCE

Sexual preference tests measure the subject's motivation to approach and possibly interact with same or opposite sex stimuli[53]. If the interaction is permitted, these paradigms can also indicate the stimuli's rewarding or aversive properties[54].

The test used to establish sex preference has been described in detail previously[39,46]. Briefly, the apparatus consisted of a three-compartment box (60 × 30 × 40 cm each) connected by two partitions. The left and right compartments contained stimulus animals restrained with a harness in such a way that they had a limited action radius, but were able to freely display sexual behavior. Animals that served as stimuli were ovariectomized sexually receptive females and sexually experienced males (for details see Olvera Hernández et al., 2015). During the test, the experimental subjects were placed in the middle compartment and the time spent in

each compartment recorded during 15 minutes. Experimental subjects could freely move around and interact with the stimulus animals. A partner preference score was calculated by subtracting the time spent with the sexually active male from the time spent with the sexually receptive female. A positive score indicated preference for the female, whereas a negative score revealed preference for the sexually active male.

Remarkably, around 30% (29/108) of the males prenatally treated with letrozole 0.56 μg/kg showed a same-sex preference, while only five out of 51 (10%) males whose mothers were treated with oil had a spontaneous male preference (Chi-square test, $p = 0.025$). Clearly, most control animals as well as around 70% of the males neonatally treated with letrozole had a clear female preference evidenced by their permanence in the female's compartment, accompanied by typical masculine sexual behavior towards the female. Conversely, the males with same-sex preference stayed most of the testing time in the male's compartment. Same-sex males received a larger number of mounts from the stimulus male and also attempted to mount it. Within this group there were subjects that, when mounted by the stimulus male, displayed lordosis. Interestingly, even if receiving aggressive responses from the stimulus male, the experimental males with same-sex preference stayed in its vicinity. Such male-male sex preference was not the result of changes in the time the animals spent in the neutral compartment (see Olvera-Hernández, et al., 2015). From this large group of animals, we selected 30 subjects to show in detail their performance in the preference test (Fig. 1).

To date it remains an enigma why only some individuals are affected by prenatal letrozole treatment, while others of the same litter remain with a clear female preference. Usually, only one or two males displayed male-sex preference from each litter. Such variation may depend on the male's fetus position in uterus[55], although specific experiments should be performed to analyze this possibility. It is worth clarifying that other experiments using prenatal and neonatal treatment with this aromatase inhibitor did not show an increase in the number of males with same-sex preference[39].

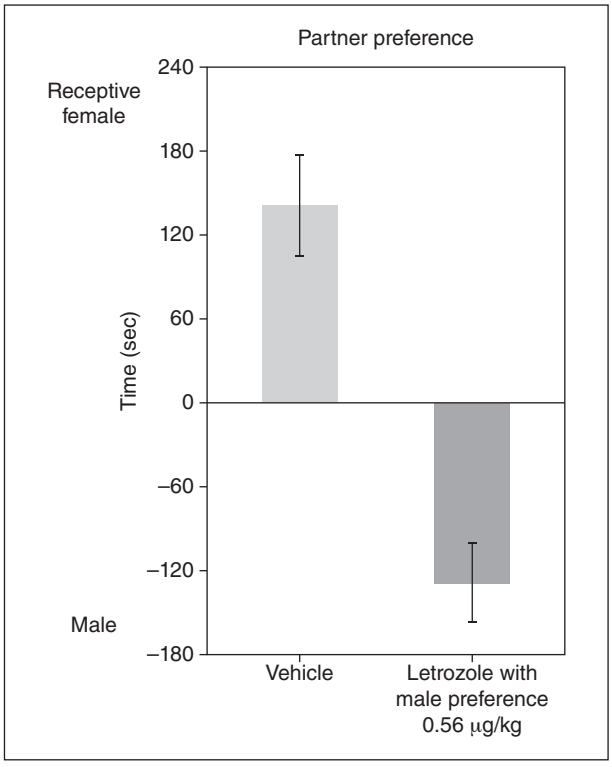

Figure 1. Partner preference of adult male rats prenatally treated with vehicle or letrozole at 0.56 μg/kg. Figure shows mean ± standard error.

A vast body of evidence and present data indicate that control male rats show a clear sexual preference for receptive females[41,56]. As mentioned, others have also reported that the aromatase inhibition during development, for example using ATD, results in males that, when adults, prefer other males[43,46]. However, it should be recalled that the results obtained with this steroidal aromatase inhibitor could be hidden by its direct actions on steroid receptors. Interestingly, Vasey proposed various criteria that should be met to conclude that the choice of a same-sex partner reflects sexual preference[36]. The present results, showing that males with same-sex preference select other males to sexually interact with, even having the possibility to copulate with receptive females, indicate that some letrozole-treated males fulfilled the criteria to be considered as having a sexual preference for other males (see Olvera-Hernández, et al., 2015, for discussion).

Besides the aforementioned aspects, other factors may influence sexual preference such as hormonal

manipulation during adulthood. Thus, several reports have analyzed the changes in partner selection after altering the adult endocrine milieu[57]. For example, previous data showed that neonatal ATD-treated males, which were castrated and given estradiol or DHT during adulthood, had same-sex preference[42]. These results are impossible to be interpreted exclusively on the basis of estrogen synthesis inhibition during development. In the present study, all males (including those with same-sex preference) were not castrated and had normal levels of serum sex steroids and gonadotropins[39]. This last observation is a notable finding that agrees with previous data reporting a similar hormonal profile in ATD-treated rats with same-sex preference[43,46] and with human data revealing that the complete endocrine profile of homosexual men did not differ from their heterosexual counterparts[20].

EXPERIMENTAL ANXIETY LEVELS

As mentioned, within the homosexual men population there is a drastic increase in all anxiety disorders that seems to result from social influences. To analyze the possible impact of biological factors determining such anxiety increase, we studied whether male rats with same-sex preference, which were prenatally treated with letrozole, or that spontaneously showed such preference (see above for methodological procedures and Garcia-Cárdenas et al., submitted), differed from males with female-sex preference.

The elevated plus maze was used to establish the experimental anxiety levels. This model consists of an elevated plus shaped maze with two opposite enclosed and two open arms (for definitions see Pellow, et al.[58]). The cumulative time spent in the open arms and the number of open-arm entries were recorded. A decrease in the time spent and number of entries into the open arms is interpreted as an anxiogenic-like response[58]. In addition to this test, a motor activity trial was made before the elevated plus maze using the same animals. The activity test consisted of placing a rat on an infrared sensor actimeter (Panlab/Harvard Apparatus). This test served to exclude putative false-positive data.

Remarkably, the results of these experiments showed that all males with same-sex preference spent less time in the open arms of the elevated plus maze than their counterparts with female preference. In addition, males with same-sex preference, regardless of their prenatal manipulation, showed much fewer entries to the open arms of this test. No change in open arms exploration could be attributed to motor impairments. All these data indicate that males with same-sex preference express higher levels of experimental anxiety assessed in this test.

In the rat, the aromatase activity is concentrated in sexually dimorphic brain areas including the amygdala (particularly the corticomedial nucleus), the bed nucleus of the stria terminalis, the paraventricular preoptic area, and the ventromedial hypothalamic nucleus[59]. Surprisingly, some of these areas, in addition to their role in male reproductive functions[60], are involved in the anxiety circuitry. Thus, Silveira, et al. in 1993, using c-Fos expression, determined that after exposing the animals to the elevated plus maze, there was an increased mark in the amygdala, the bed nucleus of the stria terminalis, the anterior hypothalamic area, the paraventricular and dorsomedial thalamic nuclei, the medial part of the entorhinal cortex, and the periaqueductal gray substance[61]. These data suggest that same-sex preference and high levels of experimental anxiety are related phenomena, putatively underlain by a poor masculinization of brain areas that participate in the regulation of both behaviors. In support, ovariectomized or females in diestrus (without ovarian steroids that reduce experimental anxiety) showed higher values of experimental anxiety than males[62].

A huge body of evidence has established that experimental anxiety levels in the elevated plus maze vary according to the endocrine condition[63-65]. As previously stated, the prenatal letrozole treatment used here does not alter the serum levels of sex steroids and gonadotropins in males showing same-sex preference, disregarding that their increased experimental anxiety is due to hormonal changes during adulthood. Furthermore, the observation that similar changes are observed in all male-oriented males (regardless of their prenatal manipulation) supports the notion that the increased experimental anxiety is associated with sex preference rather than with

Table 1. Anogenital distance and body weight at birth and at postnatal day 21 of males that prenatally received vehicle or letrozole and that as adults had female- or male-sex preference

	Anogenital distance (mm)		Body weight (g)		Subjects
	At birth	PND 21	At birth	PND 21	
Vehicle	2.44 ± 0.06	13.09 ± 0.30	5.86 ± 0.13	41.72 ± 0.80	17
Letrozole 0.56 μg/kg Female preference	2.40 ± 0.05	13.63 ± 0.31	5.99 ± 0.10	41.39 ± 1.08	21
Letrozole 0.56 μg/kg Male preference	2.42 ± 0.04	13.59 ± 0.98	6.34 ± 0.15	42.95 ± 1.76	9

Table shows mean ± standard error.
PND: postnatal day.

the causes underlying it. Needless to mention, future experiments should be made to experimentally assess these ideas.

MORPHOLOGICAL FEATURES AND PUBERTY ONSET IN MALES WITH SAME-SEX PREFERENCE

In this section we review if in males with same-sex preference, produced by prenatal letrozole treatment (for methodological details see above), there are also changes in the anogenital distance, body weight, 2D:4D finger length ratio, various bone pelvic length and width, and puberty onset. To recall, although controversial, various authors have suggested that these features differ between homosexual and heterosexual men and that they indirectly reflect the levels of androgens to which the male fetuses were exposed.

Anogenital distance and body weight

At birth and postnatal day 21, male pups were weighed and the anogenital distance (the length from the anal opening to the genitals) was determined using a vernier caliper (0.01 mm precision).

Table 1 shows the anogenital distance (AGD) and body weight at birth and at the postnatal day 21 of males prenatally treated with vehicle or letrozole and that as adults had female or male sex-preference. For these analyses it was important to include the results of males with female preference, prenatally treated with letrozole, because such treatment may affect some of these features independently of the sex preference. For these parameters, no effect of prenatal letrozole administration was found. That is, males with male preference did not differ from those that preferred females in the anogenital distance and body weight at birth or at 21 days of age. Furthermore, prenatal treatment with letrozole also lacked an effect on these parameters (Kruskal-Wallis ANOVA: $H = 0.615$, $p = NS$ and $H = 1.214$, $p = NS$).

Ratio between the length of the second and fourth digit index

To compare the putative degree of feminization induced by letrozole treatment or that accompanies male-male sex preference, a group of females with male preference was included in this and the following studied parameters. In adulthood (four months of age) we measured the length of the 2nd (2D) and 4th (4D) digits on both left and right forelimbs of control and prenatally letrozole-treated male and control untreated female rats. The digits were measured directly on restrained animals with a caliper (0.01 mm precision). Each animal's forelimb was positioned on a tainted surface. Measurements were done from the upper side of the forepaws by placing the zero point of the caliper on the basal crease proximal to the palm to the tip of the digit, excluding toenails[66]. An index 2D:4D was calculated by the length of 2D divided by 4D of both forelimbs.

Figure 2 shows the 2D:4D index obtained of both forelimbs. Clearly, as previously reported[67], females showed an index closer to 1, while males had a lower index. That is, the finger's length difference is less obvious in females than in males. Prenatal treatment

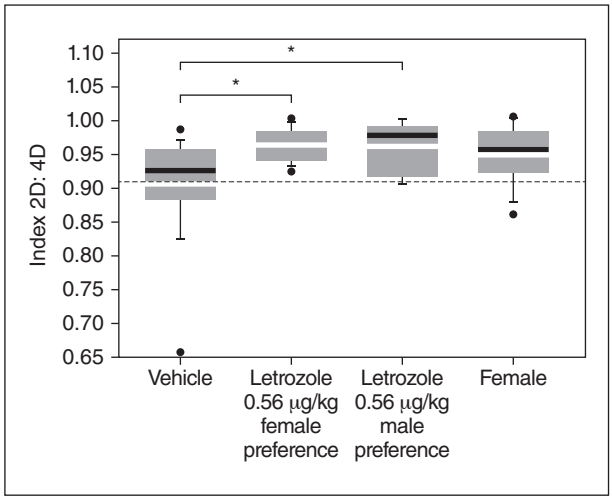

Figure 2. 2D:4D length index of both forelimbs of control males and females and of males prenatally treated with letrozole and as adults with female- or male-sex preference. Figure shows mean (white line), median (black line), and interquartile ranges. Dotted line shows mean index 2D:4D of the male control group. Black circles represent extreme values. Asterisks show comparisons between the male control group values and those of prenatal-letrozole males with female and male preference, respectively.
2D:4D: ratio between the length of the second and fourth digit.

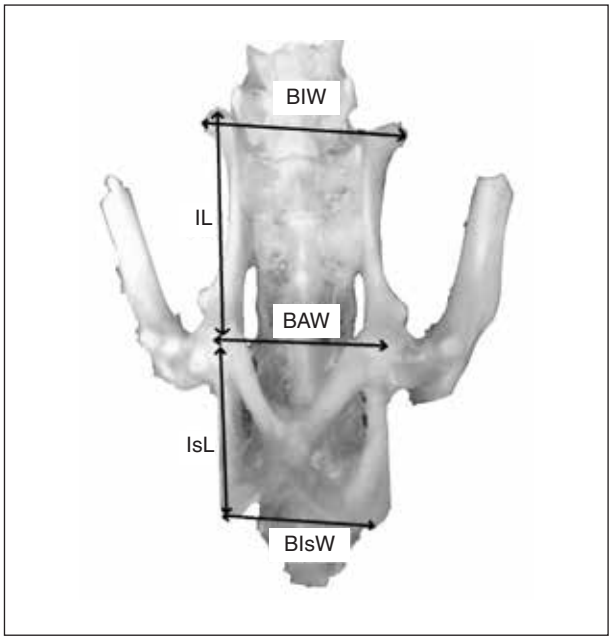

Figure 3. Rat pelvis showing the different bone length and width measured. For definitions see text.
IL: ilium length; IsL: ischium length; BIW: bi-iliac width; BAW: biacetabular width; BIsW: bi-ischial width.

with letrozole in males induces a female profile; however, no difference was found according to sex preference in the group of males. Thus, both groups treated prenatally with letrozole, regardless of their sex preference, showed a 2D:4D index higher than that of vehicle-treated males and similar to that of females. Kruskal-Wallis ANOVA found differences between vehicle and both groups of males treated with the aromatase inhibitor during development: $H = 22.169$, $p = < 0.001$; Dunn´s test $p < 0.05$.

Pelvic bones length and width

To analyze the pelvic bone length and width, rats were transcardially perfused with phosphate buffer saline and 4% paraformaldehyde. After, the pelvis was obtained and the osteo technique on hydrogen peroxide and acetic acid was performed according to Rodriguez and Zamora[68]. The following measurements were directly taken of the pelvis using a vernier caliper from the sites illustrated in figure 3 according to Huges and Tanner[69].

- Ilium length (IL): the proximal border of the iliac crest to the ilio-ischial junction.
- Ischium length (IsL): the junction of ilium and ischium to the distal border of the ischium.
- Bi-iliac width (BIW): the maximum distance between the most lateral points of the iliac crests.
- Biacetabular width (BAW): the maximum distance between the articular surfaces of the acetabula.
- Bi-ischial width (BIsW): the maximum distance between the distal points of the ischia.

Figure 4 shows the measurements of the length and width of several pelvic bones. Clearly, as previously demonstrated[34], there is a sexual discrepancy in the length of the ilium (IL), and in the bi-iliac (BIW), biacetabular (BAW) and bi-ischial (BIsW) widths, with males showing larger values than females. The ilium length and the bi-iliac width differed between vehicle and letrozole treated-males with preference for receptive females, being shorter in the latter ($H = 13.489$ and $H = 19.694$, Dunn´s test $p < 0.05$, respectively).

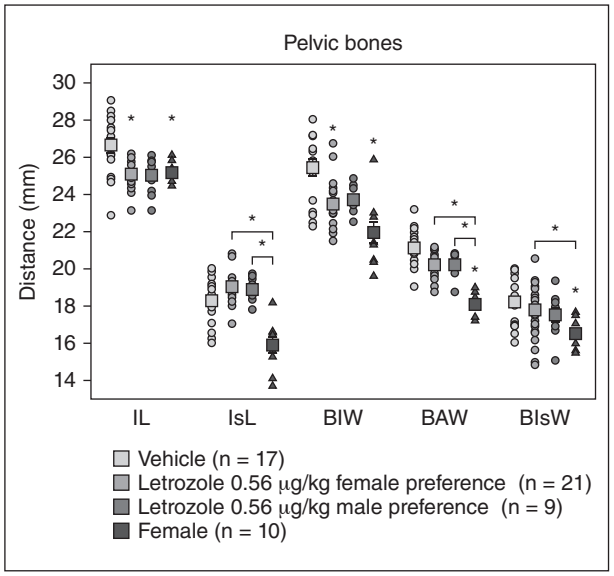

Figure 4. Pelvic bone length and width in control males and females and in males prenatally treated with letrozole with female- and male-sex preference. Figure shows raw data and means ± standard error in squares.
*Dunn´s test $p < 0.05$ versus the male control group; other comparisons are shown by brackets.
IL: ilium length; IsL: ischium length; BIW: bi-iliac width; BAW: biacetabular width; BIsW: bi-ischial width; FP: female preference; MP: male preference.

Independently of their sex preference, letrozole-treated males had a larger ischium length and biacetabular width as compared with females ($H = 22.137$ and $H = 30.280$, Dunn´s test $p < 0.05$, respectively). In no cases differences were found according to the sexual preference between letrozole-treated males.

Puberty onset

Although testicular descent and preputial separation do not strictly define puberty onset, both phenomena rely upon the increased levels of circulating androgens characteristic of this period[70], being commonly used to establish that puberty has occurred[71,72]. Since weaning, rats were examined daily and testicular descent identified when both testes were fully found in the scrotal sac and could be palpated while the males were held vertically under their forelimbs[73]. Preputial separation was established according to type W of the scale proposed by Suzuki[74].

Table 2. Days of testicular descent and preputial separation in prenatally vehicle- or letrozole-treated males with female- or male-sex preference

	Testicular descent (days)	Preputial separation (days)
Vehicle	27.35 ± 0.19	30.12 ± 0.47
Letrozole 0.56 μg/kg female preference	27.48 ± 0.33	28.71 ± 0.28*
Letrozole 0.56 μg/kg male preference	26.67 ± 0.29	28.33 ± 0.50*

Table shows mean ± standard error.
*Mann-Whitney U test, $p < 0.05$.

The day of testicular descent and preputial separation are shown in table 2. No differences in testicular descent were found between control animals and those prenatally treated with letrozole. In addition, there were no differences in this parameter between males with same-sex preference and those that preferred females (Kruskal-Wallis ANOVA $H = 3.415$; $p = 0.181$). Conversely, males prenatally treated with letrozole, regardless of their sexual preference, showed an earlier preputial separation than control subjects (Kruskal-Wallis ANOVA $H = 6.104$; $p = 0.047$; Mann-Whitney test $p < 0.05$).

IS PRENATAL LETROZOLE ADMINISTRATION TO MALE RATS A GOOD MODEL OF HUMAN MALE HOMOSEXUALITY?

The present data reveal that prenatal letrozole administration produces some features characteristic of those shown by homosexual men. We have recently reviewed and compared the effects of letrozole with those of other aromatase inhibitors on sex preference[40] and discussed these data in terms of human male homosexuality. The first great advantage of the prenatal letrozole model is, naturally, to produce male rats with an obvious male-sex preference. That is, some males treated with this aromatase inhibitor clearly prefer to stay in the vicinity of other males to sexually interact. Needless to mention,

without meeting this essential point, letrozole prenatal treatment could not be proposed as an animal model of human male homosexuality. Additionally, as mentioned, some males with same-sex preference displayed lordosis and proceptive behaviors when exposed to other males, reinforcing the idea that they are able to show typical female sexual responses[39]. Furthermore, some males with same-sex preference displayed non-contact penile erections when facing another male. Such increase in non-contact erections proves that the experimental male is sexually motivated and perceives the other male as a potential sexual partner. This conclusion is particularly interesting because lordosis and male selection in partner preference tests are typical feminine responses, while same-sex non-contact erections reveal masculine sexual arousal in the presence of another male.

Prenatal treatment with letrozole produced males with same-sex preference that, when adults, did not differ in their serum levels of testosterone, estradiol, luteinizing hormone and follicle-stimulating hormone, as compared with males, also treated with letrozole or controls, with female preference[39]. That is, the complete hormonal profile of males with same-sex preference produced by prenatal letrozole treatment is like that of males with female preference. These results are important primarily for two reasons: (i) the change in sex preference is not due to the activation effect of sex hormones in adulthood, but seems to be restricted to their organizational role before delivery[14], and (ii) this hormonal shape resembles what occurs in humans where homosexual men did not differ in their serum levels of sex steroids and gonadotropins from their heterosexual counterparts[20].

Amazingly, males with same-sex preference produced by prenatal letrozole administration or even spontaneously had higher levels of experimental anxiety evaluated in the plus maze test. This finding, as mentioned, suggests that the brain areas, organized by the influence of estradiol during development, may include those that regulate sex preference (and sexual behavior, *vide supra*) and also those that participate in the regulation of emotions[61]. Evidently, this observation should be extended to include other animal models of experimental anxiety as well as other tests aimed to evaluate depression or other mood disorders in animal models that clearly have a higher prevalence in homosexual men[22,24]. Interestingly it is an open question why some males (humans or rats), even if showing male-sex preference, do not display high anxiety levels. For men, but not for rats, most likely social, cultural, and familial reasons underlie these differences. In addition, in this study we found that some control male rats that spontaneously showed same-sex preference (i.e. without letrozole prenatal treatment) also displayed high levels of experimental anxiety (Garcia-Cárdenas et al., *submitted*). Such observation suggests that sex preference is associated with increased anxiety, regardless of the causes underlying it. In relation with the latter, the present series of experiments do not explain why some subjects display spontaneous same-sex preference.

The observations that the anogenital distance did not differ between males with same-sex preference and those with female preference nor between males treated with letrozole and controls indicate that this aromatase inhibitor lacks peripheral gross actions in the organization of the male external reproductive tract that, as mentioned above, importantly depends on androgens, particularly 5-alpha reduced[52]. To our knowledge, no evidence indicates that homosexual men possess irregularities in the genital tract development. However, some findings suggest that homosexual men tend to be lighter and shorter than heterosexuals[30-32], suggesting that sex dimorphic characteristics are under the influence of biological factors (e.g. prenatal hormones). In humans it is proposed that the lower body weight and shorter size is due to a mother immune response against components of the Y chromosome[5]. In the present study, we failed to find that male rats with same-sex preference had lower body weight than rats with female preference (regardless of their prenatal treatment). This difference with human data may rely on the age at which weight was measured (adult for humans versus at birth and weaning for rats), or alternatively may be due to the lack of an immune-based reaction in the letrozole animal model. Indeed, we agree with various authors that have proposed that human male homosexuality is a multifactorial process that is caused by very different processes[10,75,76]. Such variety of etiologies may, at least partly, explain the many ways of expression of human male homosexuality.

Prenatal treatment with letrozole reduced the male-like phenotype in 2D:4D finger ratio. That is, it produced a female-like profile that, however, did not differ between males with same-sex or female preference. This observation partly agrees with that in humans, where most groups have failed to find variations in this finger ratio between homosexual and heterosexual men, although others have found such difference[27,28,77]. The nature of such discrepancy may be due to race factors[77]. In the present study, no difference was found in any pelvic bone length and width measurement reliant on sex preference, but some effects were observed dependent on letrozole treatment. This last result indicates that the reduction in estrogen synthesis by letrozole treatment during early development marginally modifies the pelvic structure in males. A similar finding was observed for puberty onset. Thus, we here report that puberty measured as the preputial separation (but not as testicular descent) occurred earlier in males prenatally treated with letrozole, but did not differ between males with divergent sex preferences. These data suggest that the inhibition of estrogen synthesis affects one of the many factors essential for puberty onset that is controlled by a large variety of central and peripheral factors[70]. These series of results are the first that aim to experimentally relate sex preference and some peripheral characteristics.

All these data taken together suggest that prenatal letrozole treatment is a suitable model for studying human male homosexuality because it has mainly central effects with relatively poor peripheral actions. However, it should be kept in mind that no animal model can mimic the complex integration of the great diversity of factors controlling human sexuality. Furthermore, the present series of results are in line with the idea that sex preference is hormonally determined at early developmental stages.

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