Oestrogenic effects of neonatal administration of raloxifene on hypothalamic–pituitary–gonadal axis in male and female rats

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Selective oestrogen receptor modulators constitute a family of drugs that are used increasingly in the management of oestrogen-associated pathology. Raloxifene is a selective oestrogen receptor modulator that is used to treat and prevent osteoporosis in post-menopausal women. The actions of raloxifene on bone, breast, uterus and serum cholesterol concentrations have been widely analysed, but very few studies have investigated the possible actions of this drug on the central nervous system. The central nervous system of the newborn rat is very sensitive to oestrogen action. In this study a series of experiments was conducted to analyse the effects of different doses of raloxifene (50, 100, 250 or 500 µg per rat per day)

administered to neonatal rats on days 1–5 of age. Female rats treated with raloxifene showed decreased gonadotrophin secretion, hyperprolactinaemia, advanced vaginal opening, decreased body weight, persistent presence of cornified epithelial cells in vaginal smears, anovulation, inhibition of positive feedback between oestradiol and LH, and infertility. Male rats showed delayed balanopreputial separation, reduced body weight and hyperprolactinaemia. All these changes resemble those obtained after neonatal administration of oestradiol benzoate, thus indicating, for the first time, that raloxifene exerts an oestrogenic action on the hypothalamic–pituitary structures controlling reproductive function in rats.

Introduction

The major target tissues of oestrogens are the mammary gland, the reproductive tract, bone, the cardiovascular system and the central nervous system (Clark *et al.*, 1992; Turner *et al.*, 1994; Ciocca and Vargas Roig, 1995; Farhat *et al.*, 1996). In the rodent central nervous system, steroid input on hypothalamic neurones immediately after birth is critical for the organization of neural mechanisms involved in the control of neuroendocrine function, especially those serving reproductive activity (Whalen and Edward, 1969; Jacobson *et al.*, 1981; Johnson and Gay, 1983; Jean-Faucher *et al.*, 1985; Jansson and Frohman, 1987; Pozzo-Miller and Aoki, 1991; Wall and Meyerson, 1997). Administration of testosterone propionate or oestradiol benzoate to female rats induced the persistent presence of cornified cells in vaginal smears with anovulation and hyperprolactinaemia (Barraclough, 1961; Gorski, 1963; Mallampatti and Johnson, 1973; Aguilar *et al.*, 1979; Pinilla *et al.*, 1993). In addition, administration of oestradiol to male rats permanently damaged Sertoli and germ cells and the function of Leydig cells (Dhar and Setty, 1976; Gaytan *et al.*, 1986a,b; Gaytan and Aguilar, 1987). Since testosterone is converted into oestradiol in the central nervous system, it has been proposed that the oestrogenic

input on hypothalamic neurones determines the organization of the neural mechanisms involved in reproductive activity (Harlan *et al.*, 1979; Goy and McEwen, 1980; Gorski, 1990).

Raloxifene is a non-steroidal selective oestrogen receptor modulator developed as a therapeutic agent for menopausal osteoporosis (Fuchs-Young *et al.*, 1995). This drug blocks the effects of oestrogen in some tissues, such as the breast, whereas it mimics oestrogen action on bone and blood lipid concentrations (Wakeling *et al.*, 1984; Glasebrook *et al.*, 1993; Black *et al.*, 1994; Yang *et al.*, 1996; Delmas *et al.*, 1997). At present, the effects of raloxifene on the neural circuitry controlling neuroendocrine function have not been evaluated extensively. In ovariectomized rats treated with oestradiol, raloxifene inhibited the decrease in serum LH concentrations and antagonized the increase in pituitary mass and mammary gland growth (Buelke-Sam *et al.*, 1998). In addition, raloxifene antagonized the effects of oestrogen on different anterior pituitary hormones such as prolactin, LH and FSH (Draper *et al.*, 1995). In contrast, administration of a single dose of raloxifene stimulated prolactin secretion and inhibited the release of FSH (Neubauer *et al.*, 1993).

Despite the proven role of oestrogens as organizing agents of brain structures related to the neuroendocrine control of reproductive function, little attention has been given to the potential effect of raloxifene during the critical period of hypothalamic development in rodents. Raloxifene administered to pregnant rats from day 6 of gestation to day 20 post partum induced different alterations in offspring, such

as advanced vaginal opening and interruption of vaginal cycles at 10 or 14 weeks of age (Buelke-Sam *et al.*, 1998).

In the present study, experiments were designed to characterize the short- and long-term effects of neonatal administration of raloxifene in male and female rats, and special attention was given to its organizing effects of neural mechanisms involved in the control of neuroendocrine function, especially those serving reproductive activity.

Materials and Methods

Animals and treatments

Wistar rats born in our laboratory were kept under controlled conditions of light (12 h light:12 h dark, lights on at 07:00 h) and temperature (22°C), and had free access to pelleted food (Pacsa Sanders, Seville) and tap water. The day the litters were born was considered as day 1. At this stage, the litter size was adjusted to eight animals. These animals were weaned on day 21 and housed in groups of four to five animals per cage. Raloxifene chlorhydrate [2-(4-hydroxyphenyl)-6-hydroxybenzo-b-thien-3-yl-[4-[2-(1-piperidinyloxy-phenyl)-methanone hydrochloride] (Evista®, Lilly) was dissolved in saline and injected in a volume of 0.1 ml. All offspring from the same mother received the same treatment, and were pooled with pair-aged pups to be used randomly in different experiments.

Experimental designs

Females. The initial set of experiments analysed the effects of neonatal administration of raloxifene on the reproductive activity in female rats. In Expt 1, female rats (ten per group) were injected i.p. with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age. Animals were weighed each day. Trunk blood was collected after decapitation on day 20 and the ovaries were removed and weighed. In Expt 2, female rats (ten per group) were injected i.p. with raloxifene (100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age. The age and body weight at vaginal opening were recorded. After vaginal opening, vaginal smears were taken each day. The appearance of cornified cells followed by 1–2 days of dioestrus was considered as the first oestrus. Blood samples were collected after light ether anaesthesia at vaginal opening and first oestrus. In Expt 3, female rats (ten per group) were injected i.p. with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age. After vaginal opening, vaginal smears were taken each day. The appearance of epithelial cornified cells on > 12 consecutive days was considered as constant vaginal oestrus. Animals were killed at day 90 (control animals in the dioestrous phase) and trunk blood, ovaries and uteri were collected. In Expt 4, female rats (10–12 per group) were injected i.p. with raloxifene (50 or 100 µg per rat per day) or vehicle on days 1–5 of age. The animals were ovariectomized on day 90, and 14 days later were injected i.p. with 75 µg oestradiol benzoate. Blood samples were obtained by jugular vene-

puncture after light ether anaesthesia before ovariectomy and on days 14 (at 10:00 h), 15 and 16 after ovariectomy (at 10:00 and 18:00 h). In Expt 5, female rats (10–12 per group) injected i.p. with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age were paired in adulthood with control adult males of proven fertility for 21 days, and examined for a further 21 days after separation from males. The percentage of pregnancies was recorded by physical examination and final delivery of pups.

Males. In Expt 6, male rats (ten per group) were injected i.p. with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age. Trunk blood was collected after decapitation on day 20. Testes were removed and weighed. In Expt 7, male rats (ten animals per group) were injected i.p. with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age. The age and body weight at balanopreputial separation were recorded. Blood samples were obtained after light ether anaesthesia on the same day. In Expt 8, male rats (ten per group) injected i.p. with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age were maintained until adulthood (day 90) and subsequently killed. Trunk blood, testes and ventral prostate were collected. In Expt 9, male rats (15 per group) were injected i.p. with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age. In adulthood, the animals were paired with cyclic females for 21 days and the percentage of pregnancies was recorded as in Expt 5.

All experiments were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union regulations governing the care and use of experimental animals.

Histological processing

The testes and ovaries were fixed with Bouin–Hollande's fluid and processed by standard means. Section were cut at 5 µm and stained with haematoxylin and eosin.

Hormone assays

Concentrations of LH, FSH and prolactin were measured by specific radioimmunoassays using kits supplied by the NIDDK (Bethesda, MD), and hormone concentrations were expressed using LH-RP-3, FSH-RP-2 and prolactin-RP-3 as standards. All samples were measured in duplicate and all samples from each experiment were measured in the same assay. Intra-assay coefficients of variation were < 8%, and the sensitivities of the assays were 20.0, 7.5 and 10.0 pg per tube for LH, FSH and prolactin, respectively.

Statistical analysis

Data are expressed as means ± SEM. Chi-squared evaluation was used selectively for comparing data on fertility and the percentage of anovulatory cycles. All other data were analysed by one- or two-way ANOVA followed by Tukey's test.

Results

Effects of neonatal administration of raloxifene on reproductive function in females

Expt 1. Female rats injected with raloxifene (250 or 500 $\mu\text{g day}^{-1}$) showed a decrease in body weight, which was significant from day 12 of age onwards (Fig. 1a). On day 20, such a decrease was also significant in groups treated with lower doses of raloxifene (50 and 100 $\mu\text{g day}^{-1}$) (Fig. 1b). For all doses tested, raloxifene-treated females showed a significant reduction in ovarian mass (Fig. 2a) and serum LH and FSH concentrations on day 20 (Fig. 2b,c). In contrast, serum concentrations of prolactin increased (Fig. 2d).

Expt 2. Vaginal opening was advanced in female rats treated neonatally with raloxifene (100, 250 and 500 μg

Fig. 1. (a) Changes in body weight (g) in female rats treated with raloxifene (250 (●) or 500 (Δ) $\mu\text{g per rat per day}$) or vehicle (○) on days 1–5 of age. (b) Body weight (g) in 20-day-old female rats treated with raloxifene (50, 100, 250 or 500 $\mu\text{g per rat per day}$) on days 1–5 of age. Data are expressed as means $\pm$ SEM (ten animals per group). * $P \leq 0.05$ and ** $P \leq 0.01$ versus vehicle-treated rats (ANOVA followed by Tukey's test).

Fig. 2. Ovarian mass (a) and serum concentrations of LH (b), FSH (c) and prolactin (d) in 20-day-old female rats treated with raloxifene (50, 100, 250 or 500 $\mu\text{g per rat per day}$) or vehicle on days 1–5 of age. Data are expressed as means $\pm$ SEM (ten animals per group). * $P \leq 0.05$ and ** $P \leq 0.01$ versus vehicle-treated rats (ANOVA followed by Tukey's test).

day^{-1}). Serum LH and FSH concentrations decreased on the same day (Table 1).

Expt 3. Injection of raloxifene into neonatal rats permanently altered vaginal cyclicity and increased the number of cornified cells in vaginal smears (Table 2).

In adulthood, rats treated neonatally with 100, 250 or 500 $\mu\text{g raloxifene day}^{-1}$ showed a significant reduction in body weight and ovarian mass (Fig. 3a,b), whereas uterine mass decreased only in those animals injected with 500 $\mu\text{g raloxifene day}^{-1}$ (Fig. 3c). Serum LH and FSH concentrations remained unchanged in adult females treated neonatally with different doses of raloxifene (Fig. 3e), whereas an increase in prolactin was detected in females treated with doses of 250 and 500 $\mu\text{g raloxifene day}^{-1}$ (Fig. 3f).

Table 1. Age, body weight and serum concentrations of LH and FSH on the day of vaginal opening in rats treated with raloxifene (100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age

Treatment	Age (days)	Body weight (g)	LH (ng ml ⁻¹)	FSH (ng ml ⁻¹)
Vehicle	35.00 ± 0.48	110.52 ± 2.33	1.22 ± 0.21	10.50 ± 2.01
Raloxifene (µg per rat per day)				
100	25.33 ± 0.37*	60.47 ± 2.31*	0.21 ± 0.02*	3.81 ± 0.41*
250	29.00 ± 0.50*	68.81 ± 3.94*	0.17 ± 0.02*	5.02 ± 0.68*
500	24.91 ± 0.35*	49.06 ± 2.28*	0.11 ± 0.006*	4.33 ± 0.70*

Data are expressed as means ± SEM (ten animals per group).

* $P \leq 0.01$ versus vehicle (ANOVA followed by Tukey's test).

Table 2. Percentage of constant vaginal oestrus (CVO) and fertility in 90-day-old female rats treated with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age

Treatment	Number of rats tested	Percentage of rats with CVO	Percentage of fertile rats
Vehicle	11	0	100
Raloxifene (µg per rat per day)			
50	12	41.66	ND
100	10	90	ND
250	11	100	0
500	12	100	0

ND: not determined.

The histology of the ovaries in control rats showed an abundance of developing follicles and several generations of corpora lutea corresponding to those of the current and previous oestrous cycles (Fig. 4a). Animals treated with 50 µg raloxifene day⁻¹ showed similar morphological features and several generations of corpora lutea were also found, although large atretic follicles were observed occasionally (Fig. 4b). In rats treated with 100 µg raloxifene day⁻¹, the ovaries were smaller and corpora lutea were scarce. Rats treated with the highest doses of raloxifene (250 or 500 µg day⁻¹) had small ovaries in which corpora lutea were not detected, but large atretic follicles were abundant (Fig. 4c,d).

Expt 4. In the control females, serum concentrations of LH increased 14 days after ovariectomy. This increase was significantly reduced in raloxifene-treated females. Administration of 75 µg oestradiol benzoate reduced serum LH concentrations on days 15 and 16 after ovariectomy (at 10:00 h), whereas significant increases were observed at 18:00 h (positive feedback between oestradiol and LH). Positive feedback was inhibited in the group treated neonatally with 100 µg raloxifene and was significantly reduced in the group treated with 50 µg raloxifene (Fig. 5).

Expt 5. No pregnancy was detected in raloxifene-treated (250 or 500 µg per rat per day) females paired for 21 days with control males (Table 2).

Effects of neonatal administration of raloxifene on reproductive function in males

Expt 6. On day 20, male rats injected neonatally with different doses of raloxifene showed a significant reduction in body weight (Table 3). On day 20, the dose of 500 µg day⁻¹ induced a significant reduction in testis mass expressed either in absolute (16.81 ± 0.75 mg versus 24.03 ± 0.68 in controls; $P \leq 0.01$) or relative values (52.62 ± 1.90 mg per 100 g body weight versus 59.47 ± 1.83 in controls; $P \leq 0.05$), accompanied by an increase in serum FSH and prolactin concentrations (Fig. 6b,c), whereas LH concentrations were unchanged (Fig. 6a).

Expt 7. In male rats treated neonatally with 250 or 500 µg raloxifene day⁻¹, balanopreputial separation was significantly delayed, but due to growth retardation it occurred at a lower body weight than in the controls. Serum FSH and LH concentrations at balanopreputial separation were decreased in raloxifene-treated rats (Table 4).

Fig. 3. Body weight, ovarian and uterine masses (a, b, c) and serum concentrations of LH (d), FSH (e) and prolactin (f) in adult female rats treated with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle (Veh) on days 1–5 of age. Data are expressed as means $\pm$ SEM (ten animals per group). ND = not determined. * $P \leq 0.05$ and ** $P \leq 0.01$ versus vehicle-treated rats (ANOVA followed by Tukey's test).

Expt 8. Adult male rats treated neonatally with raloxifene (250 or 500 µg day⁻¹) showed a decrease in body weight, whereas testis mass and ventral prostate mass were normal (Table 5). Serum FSH and prolactin concentrations decreased in raloxifene-treated rats (Fig. 7b,c), whereas LH decreased in the raloxifene-treated rats, but the result was not significant (Fig. 7a). The testes of raloxifene-treated rats showed normal morphological features. Spermatogenesis was complete and Leydig cells in the interstitial areas were well differentiated (data not shown).

Expt 9. All control female rats paired with raloxifene-treated males showed normal pregnancies, indicating that the reproductive activity of males was maintained despite neonatal raloxifene treatment.

Discussion

Results from the present study demonstrate that neonatal administration of raloxifene permanently alters the physiology of the reproductive axis; these effects are more deleterious

Fig. 4. Micrographs from the ovaries of adult rats treated neonatally with vehicle (a), 50 µg (b), 250 µg (c) or 500 µg (d) of raloxifene. Corpora lutea are indicated by asterisks and large atretic follicles by stars. Scale bar represents 100 µm.

in females. Such a difference in the impact of raloxifene on reproductive activity in male and female rats is in agreement with the findings of studies in adult animals (Hoyt *et al.*, 1998).

The response to neonatal raloxifene treatment in female rats is characterized by: (i) a decrease in serum gonadotrophin concentration and an increase in prolactin concentrations; (ii) a decrease in body weight, and in ovarian and uterine mass; (iii) advanced vaginal opening; (iv) anovulation with persistent presence of cornified cells in vaginal smears, inhibition of positive feedback between oestradiol and LH, and absence of corpora lutea in ovaries; and (v) infertility. These findings indicate that raloxifene injected on days 1–5 of age acts as a compound with oestrogenic activity on the hypothalamic–pituitary structures controlling reproductive function. Female rats treated neonatally with oestrogens showed changes almost identical to those induced by raloxifene (Barracough, 1961; Gorski, 1963; Mallampatti and Johnson, 1973; Aguilar *et al.*, 1979; Pinilla *et al.*, 1993).

Neonatal administration of raloxifene in male rats

induced only minor changes in the reproductive axis, and adult testicular histology and fertility were normal. This finding is in agreement with data obtained in mice (Chou *et al.*, 1992). Notably, the reproductive phenotype of male rats injected neonatally with raloxifene is in contrast to the infertility and severe damage to testis structure observed after neonatal administration of oestrogens (Gaytan *et al.*, 1986a,b; Gaytan and Aguilar, 1987). Differences between both treatments may reflect distinct oestrogenic biopotency or may be the consequence of a different kinetic distribution of oestradiol and raloxifene after its administration. Raloxifene has an elimination half-life of 11–27 h (Balfour and Goa, 1998) and is dissolved and injected in saline, whereas oestradiol is administered in oil, thus ensuring prolonged release and action. This difference may indicate that neonatal oestrogenization induced a marked decrease in serum LH and FSH concentrations during the prepubertal period (Aguilar *et al.*, 1987), whereas males treated with raloxifene showed normal serum LH and increased FSH concentrations on day 20. However, prepubertal male rats treated neonatally with raloxifene showed four characteristics

Fig. 5. Serum concentrations of LH in adult female rats treated with raloxifene (50 (▲) or 100 (■) µg per rat per day) or vehicle (○) on days 1–5 of age. Blood samples (B) were obtained on day 90 (10:00 h), and at different days after ovariectomy (day 14, 10:00 h; days 15 and 16, 10:00 and 18:00 h). Animals were injected on day 14 after ovariectomy with 75 µg oestradiol benzoate (OeB) (10:00 h). * $P \leq 0.05$ and ** $P \leq 0.01$ versus values in vehicle-treated rats (ANOVA followed by Tukey's test).

similar to those obtained in neonatal oestrogenized males: (i) delayed balanopreputal separation; (ii) decreased gain in body weight; (iii) testis atrophy; and (iv) hyperprolactinaemia (Bellido *et al.*, 1985; Vaticon *et al.*, 1985; Aguilar *et al.*, 1987).

It is well known that differentiation and proliferation of Sertoli cells occurs during the 2 weeks immediately after birth (Steinberger and Steinberger, 1971; Orth *et al.*, 1988) and is critically dependent on FSH secretion (Orth, 1984). However, neonatal proliferation of Sertoli cells is essential for spermatogenesis and fertility in adulthood (Orth *et al.*, 1988). It is possible that raloxifene induced only a short-term decrease in FSH secretion in male rats followed by a rebound response with the hypersecretion observed on day 20. This may explain the preserved differentiation and proliferation of Sertoli and germ cells, as well as the normal fertility observed in raloxifene-treated male rats in adulthood.

Table 3. Body weight in 20-day-old male rats treated with raloxifene (50, 100, 250 or 500 µg per rat per day) or vehicle on days 1–5 of age

Treatment	Body weight (g)
Vehicle	39.31 ± 1.01
Raloxifene (µg per rat per day)	
50	36.41 ± 0.68*
100	32.86 ± 0.44**
250	33.44 ± 0.68**
500	32.40 ± 0.73**

Data are expressed as means ± SEM (15 animals per group).

* $P \leq 0.05$ versus vehicle; ** $P \leq 0.01$ versus vehicle (ANOVA followed by Tukey's test).

Fig. 6. Serum concentrations of LH (a), FSH (b) and prolactin (c) in 20-day-old male rats treated with raloxifene (50, 100 or 500 µg per rat per day) or vehicle on days 1–5 of age. Data are expressed as means ± SEM (ten animals per group). ** $P \leq 0.01$ versus vehicle-treated rats (ANOVA followed by Tukey's test).

Table 4. Age, body weight and serum concentrations of LH and FSH at balanopreputial separation in rats treated with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age

Treatment	Age (days)	Body weight (g)	LH (ng ml ⁻¹)	FSH (ng ml ⁻¹)
Vehicle	40.55 ± 0.33	196.67 ± 3.26	0.75 ± 0.12	24.74 ± 1.99
Raloxifene (µg per rat per day)				
250	43.66 ± 0.39*	175.12 ± 3.73*	0.17 ± 0.01*	10.82 ± 1.14*
500	45.72 ± 0.48*	165.74 ± 3.38*	0.20 ± 0.03*	7.73 ± 0.65*

Data are expressed as means ± SEM (ten animals per group).

* $P \leq 0.01$ versus vehicle (ANOVA followed by Tukey's test).

Table 5. Body weight and testis and prostate mass in adult male rats treated with raloxifene (250 or 500 µg per rat per day) or vehicle on days 1–5 of age

Treatment	Body weight (g)	Testis mass (mg)	Prostate mass (mg)
Vehicle	447.0 ± 6.0	872.30 ± 22.93	100.00 ± 6.66
Raloxifene (µg per rat per day)			
250	368.0 ± 8.0*	910.00 ± 20.00	101.62 ± 7.43
500	335.0 ± 3.0*	963.84 ± 29.53	92.72 ± 1.86

Data are expressed as means ± SEM (ten animals per group).

* $P \leq 0.01$ versus vehicle (ANOVA followed by Tukey's test).

Together, these results highlight the oestrogenic action of raloxifene on the developing central nervous system in rats. This finding opens up the possibility of new uses of raloxifene in the study of the physiology of central nervous system and new preventive actions in raloxifene treatment. Results of international trials indicate that raloxifene is an interesting drug for prevention of osteoporosis in menopausal women, showing very promising efficacy and clinical tolerance (Agnusdei *et al.*, 1999). Recent experiments have indicated that synaptic plasticity of the arcuate nucleus in adult female rats is controlled by oestrogens (Naftolin *et al.*, 1996; Horvath *et al.*, 1997a,b) and progress has been made in understanding the molecular basis of the neuroprotective properties of oestrogens (Garcia Segura *et al.*, 1998; Pike, 1999; Reibel *et al.*, 2000). In this context, promotion of clinical research on the possible disease-modifying or neuroprotective effects of oestrogens in the elderly has been proposed (Schneider and Finch, 1997). These results indicate that, in addition to anti-oestrogenic activity, raloxifene acts as an oestrogen in the central nervous system and thus support its potential use in these studies.

Nevertheless, further studies of the kinetic characteristics of raloxifene entrance in the central nervous system are necessary to clarify fully the proposed central actions of raloxifene.

Fig. 7. Serum concentrations of LH (a), FSH (b) and prolactin (c) in adult male rats treated with raloxifene (50, 100 or 500 µg per rat per day) or vehicle on days 1–5 of age. Data are expressed as means ± SEM (ten animals per group). * $P \leq 0.05$ and ** $P \leq 0.01$ versus vehicle-treated rats (ANOVA followed by Tukey's test).

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