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Sexual differentiation of oestradiol–LH positive feedback in a marsupial

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The surge of LH that induces ovulation in mammals showing spontaneous ovulation is precipitated by the positive feedback of increasing oestrogens from the developing follicles in the ovary. In eutherians, exogenous oestrogens can mimic this effect by eliciting an LH surge in females, but not usually in males. The absence of a positive LH response in eutherian males is either due to an acute suppression by the secretory products of the testes during adulthood or the permanent disabling of the system by testosterone during early development. This phenomenon is examined in tammar wallabies, *Macropus eugenii*. The results show that the oestradiol–LH positive feedback response is sexually dimorphic in this marsupial. A surge in plasma LH occurred between 15 and 28 h after injection of 2.5 μg oestradiol benzoate kg^{-1} in 13 of 16 intact females and 4 of 4 ovariectomized females, but in none of 11 intact males. Five females each implanted with a 100 mg testosterone pellet 3 months earlier failed to produce an LH surge. Four males castrated in adulthood and three adult males castrated before puberty also failed to show an LH surge. However, three males castrated 24–26 days after birth showed an unambiguous LH surge when challenged with oestradiol benzoate during adulthood. Thus, in tammar wallabies, the ability to generate an LH surge to oestradiol is a sexually dimorphic response that is suppressed in the male by the organizational effects of the testes in early life and presumably supplemented by an inhibitory effect of circulating testosterone in adulthood.

Introduction

In adult female mammals, a surge of LH induces changes in the preovulatory follicle resulting in ovulation and formation of the corpus luteum. In spontaneously ovulating species, the preovulatory LH surge is precipitated when rising concentrations of oestrogen begin to have a positive feedback effect on LH secretion; progesterone may also facilitate this positive feedback in some species (Fink, 1988). A preovulatory-like discharge of LH can be evoked artificially by the administration of exogenous oestrogens in females, but the same hormonal regimen is almost always ineffective in producing a positive LH response in males (Booth, 1979).

The inability of male eutherian mammals to produce a positive LH surge in response to oestrogen is attributed to permanent or temporary effects of testicular hormones acting on the central nervous system (reviewed by Booth, 1979; MacLusky and Naftolin, 1981; Gorski, 1985, 1991). In most non-primate eutherian males, the oestradiol–LH positive feedback system is rendered permanently inoperative by changes in the organization of the brain pathways induced by testicular hormones during a critical period in early neurological development (for example, rat, Neill, 1972; sheep, Short, 1974; Karsch and Foster, 1975). In contrast, the oestradiol–LH positive feedback system in the adult male primate, while potentially operational, remains suppressed by products secreted by the testes in adulthood (rhesus monkeys, Karsch *et al.*, 1973; pig-tailed macaques, Steiner *et al.*, 1978; humans, Barbarino *et al.*, 1983, Gooren, 1986). The marmoset monkey is an exception in that testicular hormones have no apparent inhibitory effect on positive feedback at any stage of development (Hodges and Hearn, 1978; Hodges, 1980). Despite intense interest in the influence of the testis on the oestradiol–LH positive feedback system across a wide range of eutherian mammals, there is no information available for marsupial mammals.

The reproductive physiology and endocrinology of marsupials, including the relationship between oestradiol and LH, is best understood in tammar wallabies, *Macropus eugenii*. Females have a postpartum oestrus within 1–3 h after parturition (Rudd, 1994a) that coincides with an increase in plasma oestradiol concentration (Harder *et al.*, 1984, 1985; Shaw and Renfree, 1984). An LH surge occurs

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8–16 h after oestrus and this precedes ovulation by about 24 h (Sutherland *et al.*, 1980; Tyndale-Biscoe *et al.*, 1983; Harder *et al.*, 1985; Renfree and Lewis, 1996). The preovulatory discharge of LH is presumably triggered by an increasing oestradiol concentration, as a single injection of oestradiol benzoate produces an LH surge in ovariectomized adult and intact peripubertal female tammar wallabies (Horn *et al.*, 1985; Williams, 1990). In contrast to females, there are no data on the effects of oestradiol on LH release in males.

The aim of this study was to determine whether there is a sex difference in the LH surge response to oestradiol in tammar wallabies and, if so, to assess whether the difference is mediated by testicular activity during development or adulthood.

Materials and Methods

Animals

Tammar wallabies descended from animals caught on Kangaroo Island, South Australia, were maintained in a breeding colony located at Monash University, Melbourne, Australia. The animals were housed in grassed outdoor enclosures with trees and artificial shelters and were provided with compressed lucerne cubes and water *ad libitum* and fresh vegetables each weekday. All surgical and experimental procedures conformed to Australian National Health and Medical Research Council (1990) guidelines and were approved by Institutional Animal Experimentation Ethics Committees.

Surgery

Young tammar wallabies remain in the mother's pouch throughout most of the 9 month lactation period, and thermoregulation does not develop until around 150 days post partum. Male pouch young aged 24–26 days were therefore anaesthetized by hypothermia, whereas prepubertal (14.5 months) and adult (over 2.5 years old) animals were anaesthetized by injection of sodium pentobarbitone i.v. (60 mg ml^{-1} in saline; Abbott, Kurnell, NSW) as described by Renfree and Tyndale-Biscoe (1978). All of the castrated males and the females implanted with testosterone were used in a parallel behavioural study, for which full details regarding anaesthesia, surgical procedures, mortality and postoperative care have been presented (Rudd *et al.*, 1996). Ovariectomies were performed according to Renfree and Tyndale-Biscoe (1978).

Oestradiol administration and collection of blood samples

Oestradiol benzoate (Intervet, Lane Cove, NSW) in arachis oil was injected i.m. at 2.5 μg kg^{-1}. Blood samples were taken via an indwelling 18G catheter (Jelco, Johnson and Johnson, Arlington, TX) inserted into the lateral tail vein and collected in 2.5 or 5.0 ml syringes containing 50–100 μl heparinized saline (125 iu heparin sodium ml^{-1}). The catheter was kept patent by the administration of 100 μl heparin into the catheter after each sample. All blood samples were stored at 4°C until centrifugation at 1700 *g* for 15 min. Separated plasma was stored in glass vials at –20°C until assayed.

General experimental protocol

The LH surge response to oestradiol benzoate was initially tested in four intact adult females and four intact adult males. The LH response to oestradiol was subsequently tested in five females implanted with a 100 mg testosterone pellet 3 months earlier, in four adult ovariectomized females, and in ten males castrated either 24–26 days after birth ($n = 3$), before puberty at 14.5 months of age ($n = 3$), or in adulthood ($n = 4$). Adult males and females were gonadectomized 5–6 months before the oestradiol challenge experiments, but the day 24–26 males were castrated 3 years earlier and 14.5-month-old males at least 18 months earlier. Hence, some of the original group of 13 animals castrated after birth and some of the six animals castrated before puberty died of natural causes before testing (see Rudd *et al.*, 1996). Each oestradiol challenge included four intact females as positive controls, with three or four intact (control) males included in experiments using the males castrated at 24–26 days and 14.5 months of age. Experiments were performed while the females were in seasonal quiescence when plasma progesterone concentrations are low (Tyndale-Biscoe and Renfree, 1987); there are no apparent seasonal effects on oestradiol–LH positive feedback in female tammar wallabies (Horn *et al.*, 1985). All animals were over 3 years of age when tested.

Animals were injected i.m. with 2.5 μg oestradiol benzoate kg^{-1} between 21:00 and 22:00 h. The dose was based on that used by Horn *et al.* (1985) and Williams (1990), which achieved physiological concentrations of LH as described in a number of reports (Sutherland *et al.*, 1980; Tyndale-Biscoe *et al.*, 1983; Fletcher *et al.*, 1988; Williams, 1990; Hinds *et al.*, 1996). A 5 ml blood sample was taken immediately before the injection (time 0); additional samples (2.5 ml) were collected at 2–3 h intervals from 9 to 28 h after injection, during which time the animals were kept in small (6–8 m^2) outdoor pens to facilitate catching. All samples were measured for plasma LH. Plasma testosterone concentrations were also analysed in four intact females and in all control males, castrated males and testosterone-treated females from the time 0 samples.

Radioimmunoassays

Plasma LH was measured using the tammar radioimmunoassay of McFarlane *et al.* (1997). This assay system uses a ^{125}I-labelled hCG tracer in conjunction with the monoclonal antibody 518B7 (Dr J. Roser, Davis, CA) raised against bovine LH and with purified tammar wallaby LH (ME-14B) as the reference standard. The sensitivity of the assay was 1.75 ng ml^{-1} and the intra- and interassay coefficients of variation were 6.7% ($n = 8$) and 11.8% ($n = 4$), respectively.

Plasma testosterone concentrations were measured using a radioimmunoassay validated for the tammar wallaby

(Williamson *et al.*, 1990). The antiserum (6050) (Dr R. I. Cox, Prospect, NSW) was raised in sheep and had a crossreactivity of 100% with testosterone, 31% with 5α-dihydrotestosterone, 30% with 4-androsten-3β,17β-diol, 3.5% with 4-androsten-17β,19-diol-3-one and 1.3% with androstenedione. The efficiency of extracting [1,2,6,7-^{3}H]testosterone from wallaby plasma was 91.6%. The sensitivity of the assay was 0.07 ng ml^{-1} and the intra-assay coefficient of variation was 6.9% (n = 8).

Statistical analyses

A rise in plasma LH concentration was defined as a surge when the putative surge encompassed at least three consecutive sampling periods that were significantly greater (Student's t test; $P < 0.05$) than the LH concentration before the oestradiol injection. Additionally the data were ranked by aligning the LH peaks using analysis of variance (ANOVA) to compare peak LH concentrations with basal concentrations and between groups. All data were tested for equal variance and normal distribution and log transformed if necessary. Statistical analyses were performed using the SAS statistical package (SAS Institute Inc., Cary, NC).

Results

Effect of oestradiol on plasma LH in females

Intact females. Administration of 2.5 μg oestradiol benzoate kg^{-1} triggered a surge of plasma LH in 13 of 16 intact females. Plasma LH concentrations were significantly increased relative to basal concentrations (2.0 ± 0.3 ng ml^{-1}; mean ± SEM) for a period of at least 6 h ($P < 0.0001$), with peak concentrations of 22.2 ± 2.2 ng ml^{-1} at 15–28 h after injection (n = 13). The LH profiles were similar in all females that responded after injection of oestradiol. In two groups (Fig. 1a,b), five of eight females showed marked peaks (20.3–34.5 ng ml^{-1}), one female showed a small peak (5.8 ng ml^{-1}) and no response was seen in the remaining two females. In the remaining two groups, seven of the eight females had a marked LH peak (range 10.5–34.9 ng ml^{-1}) and one showed no response (data not shown).

Ovariectomized and testosterone-implanted females. Oestradiol benzoate elicited an LH surge in all four ovariectomized females. Plasma LH concentrations were significantly increased relative to basal concentrations (3.4 ± 0.7 ng ml^{-1}) for 4 h ($P < 0.001$), peaking at 14.5 ± 4.3 ng ml^{-1}. Two of the ovariectomized females showed marked LH peaks of 12.3 and 33.4 ng ml^{-1} at 16 and 20 h after injection, respectively (Fig. 1c), although the remaining two females showed smaller LH peaks of 6.6 and 5.5 ng ml^{-1} at 24 and 26 h after oestradiol treatment, respectively. The apparent suppression of plasma LH after oestradiol treatment until the onset of the LH surge was not significant ($P > 0.05$).

In contrast to the intact and ovariectomized females, there was no significant effect ($P > 0.05$) of oestradiol benzoate on plasma LH in the testosterone-implanted females, and concentrations remained low throughout the sampling period (Fig. 1d).

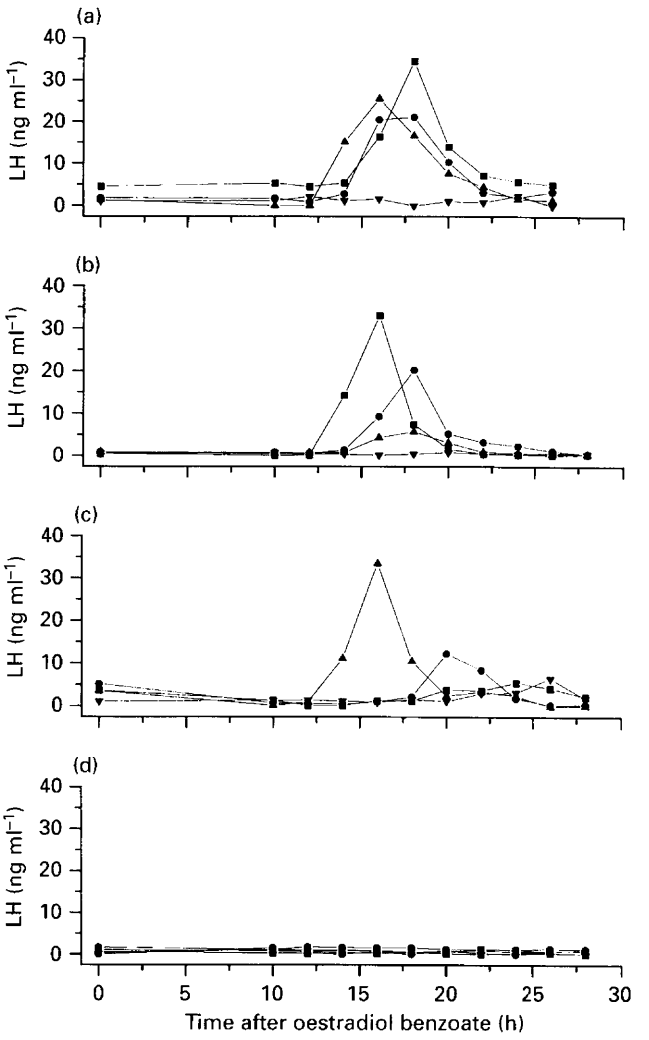

Fig. 1. Individual plasma LH profiles of tammar wallabies after injection of 2.5 μg oestradiol benzoate kg^{-1} i.m. at time 0. (a,b) Two groups of four intact (control) adult females, (c) four adult ovariectomized females, and (d) five females implanted with 100 mg testosterone pellet 3 months before injection of oestradiol.

Effect of oestradiol on plasma LH in males

Intact males. Oestradiol benzoate administration had no significant effect on plasma LH concentrations in intact males (n = 11). The LH profiles were similar in all three groups tested. In one group of four males, plasma LH concentrations of < 1.75–3.4 ng ml^{-1} were measured over the entire sampling period (Fig. 2a). In the remaining seven males, comprising two groups, plasma LH concentrations never exceeded 3.0 ng ml^{-1} (data not shown). Basal LH concentrations were 2.1 ± 0.5 ng ml^{-1} (n = 11).

Castrated males. All four adult castrated males failed to produce a positive LH response to oestradiol, with plasma LH concentrations decreasing significantly ($P < 0.0001$)

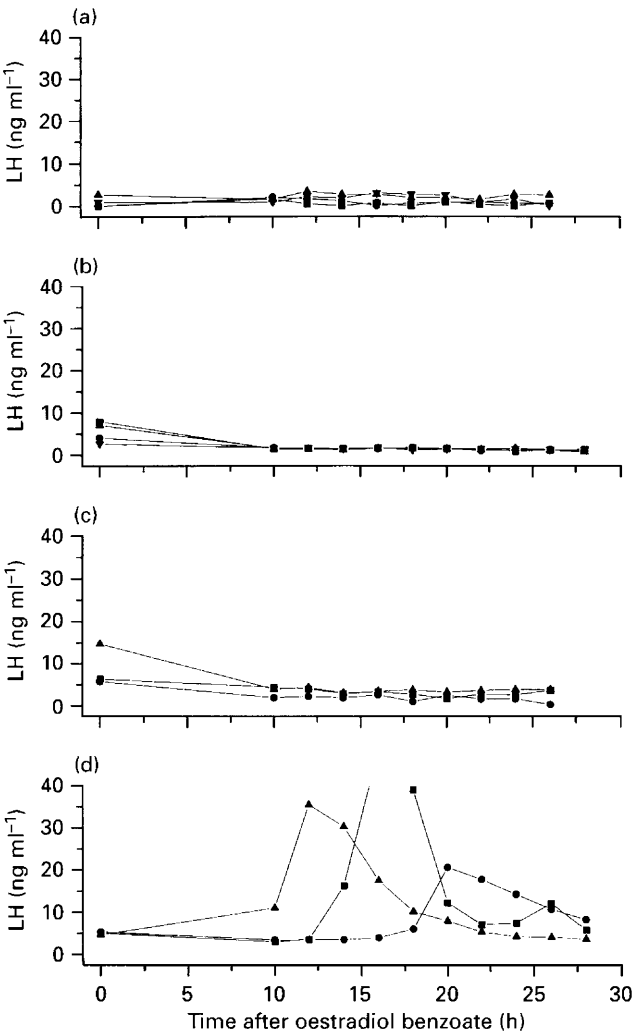

Fig. 2. Individual plasma LH profiles of tammar wallabies after injection of 2.5 μg oestradiol benzoate kg^{-1} i.m. at time 0. (a) Four intact (control) adult males, (b) four adult castrated males, (c) three males castrated before puberty at 14.5 months of age, and (d) three males castrated 24–26 days after birth.

from 5.4 ± 1.25 ng ml^{-1} (mean ± SEM) before injection to < 2.0 ng ml^{-1} for the entire sampling period after injection (Fig. 2b). Similarly, all three males castrated at 14.5 months of age failed to show a positive LH response to oestradiol, with plasma LH concentrations decreasing ($P < 0.001$) from 8.9 ± 3.1 ng ml^{-1} before injection to < 4.0 ng ml^{-1} throughout the sampling period (Fig. 2c). In marked contrast, all three males castrated at 24–26 days of age produced a substantial surge of LH in response to oestradiol benzoate. Plasma LH concentrations were significantly ($P < 0.0001$) higher for 8 h relative to basal concentrations (5.0 ± 0.2 ng ml^{-1}), with peak concentrations of 34.8 ± 5.1 ng ml^{-1} (range 20.7–48.2 ng ml^{-1}) measured at 12–20 h after injection (Fig. 2d).

Resting plasma testosterone in males and females

Plasma testosterone concentrations were below the sensitivity of the assay, that is < 0.07 ng ml^{-1}, in the four intact females tested, and 1.1 ± 0.54 ng ml^{-1} (mean ± SD) in the five testosterone-implanted females. Plasma testosterone concentrations were 0.61 ± 0.29 ng ml^{-1} in the intact males ($n = 11$), 0.08 ng ml^{-1} in one of the males castrated at 14.5 months of age, but were below the assay sensitivity, that is < 0.07 ng ml^{-1}, in the remaining nine castrated males.

Discussion

There is a clear sexual dimorphism in the response of adult tammar wallabies to an oestradiol challenge, with an LH surge triggered only in females. The absence of an LH surge response to oestradiol in the male can be explained by the permanent disruption of the positive feedback pathways due to an organizational effect of testicular hormones during development, which appears to be supplemented by an inhibitory effect of circulating testosterone in adulthood.

The LH responses showed considerable variation with up to a sixfold difference in peak LH concentrations measured among certain individuals. Nevertheless, this is consistent with the response of ovariectomized females, as seen in the present study and in the study of Horn *et al.* (1985) in which differences in peak LH concentrations of around sevenfold were recorded. After challenge with oestradiol, 13 of 16 females showed a surge in plasma LH compared with none of 11 intact males. In those females that responded positively, plasma LH concentrations were increased typically for 6–10 h, similar to the duration of the preovulatory LH surge in normal females (Sutherland *et al.*, 1980; Tyndale-Biscoe *et al.*, 1983). The interval of 15–28 h from oestradiol administration to the LH peak in intact females is also comparable to adult ovariectomized tammar wallabies as seen in this study and in the study of Horn *et al.* (1985), as well as some eutherian species, including sheep (Short, 1974; Karsch and Foster, 1975) and marmoset monkeys (Hodges and Hearn, 1978). However, in most eutherians, the interval from oestradiol administration to the LH peak is greater, ranging from 36 to 48 h in rhesus monkeys (Karsch *et al.*, 1973) and pig-tailed macaques (Steiner *et al.*, 1978), 55 h in rats (Neill, 1972) and 60–72 h in pigs (Lantz and Zimmerman, 1974).

In rhesus monkeys and pig-tailed macaques, the inability of the intact male to generate an LH surge is entirely due to inhibition by the adult testis (Karsch *et al.*, 1973; Steiner *et al.*, 1976; Norman and Spies, 1986; Steiner *et al.*, 1976, 1978). Thus, male monkeys castrated in adulthood are capable of generating oestradiol-induced LH surges that are indistinguishable from those in females. This is in marked contrast to tammar wallabies, in which a single injection of 2.5 μg oestradiol benzoate kg^{-1} triggers an LH surge in adult ovariectomized females (Horn *et al.*, 1985), but the same dose does not produce a positive LH response in males castrated before puberty at 14.5 months of age or in adulthood. Hence the positive but not the negative feedback mechanisms in adult male tammar wallabies remain unresponsive to oestradiol, even after the inhibitory influence of the adult testis is removed. This implies that the testicular hormones have an irreversible inhibitory effect on the oestradiol–LH positive feedback system during early life.

Permanent disruption of the LH surge system in male tammar wallabies can be prevented by castration relatively soon after birth. Males castrated early in pouch life at 24–26 days of age showed a pronounced surge of LH in response to oestradiol in adulthood, with peak LH concentrations similar to, or even exceeding, those recorded in the most responsive female wallabies. However, the absence of positive feedback in males castrated at 14.5 months of age indicates that the oestradiol–LH positive feedback system in the male is rendered permanently inoperative by the testes some time between 26 days and 14.5 months of age. This pattern is similar to that seen in most non-primate eutherian males in which the positive feedback system is permanently disabled by the organizational effect of testicular steroids during early development.

Although sexual differentiation of the oestradiol–LH positive feedback system occurs between 26 days and 14.5 months of age, it is likely that the critical period is considerably narrower. In neonatal male tammar wallabies, the testosterone concentration in the testis increases sharply after birth, and remains high until 40 days of age (Renfree *et al.*, 1992). This relatively brief period is associated with significant androgen-dependent somatic sexual differentiation (Shaw *et al.*, 1988; Renfree *et al.*, 1995; Lucas *et al.*, 1997; Ryhorchuk *et al.*, 1997) and coincides with the onset of rapid neurological development (Renfree *et al.*, 1982). If the permanent loss of positive feedback in male tammars is due to testosterone as it is in eutherians, it is likely that this process occurs between the first 26 and 40 days of the 9 month pouch life.

Despite the positive feedback mechanism being permanently disabled in males during early postnatal life, it is likely that circulating testosterone alone would be able to prevent an oestradiol-induced LH surge. This is evidenced by the lack of response in the testosterone-treated females, in which plasma testosterone concentrations were similar to those in adult male tammars outside the breeding season (Catling and Sutherland, 1980; Inns, 1982; Rudd *et al.*, 1996). The mechanism by which circulating testosterone suppresses the ability to generate an LH surge to oestradiol is unknown. However, Knobil (1974) reported that female rhesus monkeys with oestradiol implants gradually lost the ability to generate LH surges to oestradiol injections. This was attributed to a progressive increase in sensitivity to the unchanging concentrations of oestradiol in plasma and concomitant reduction in LH secretion. It is possible that testosterone acts in a similar way in tammar wallabies, although further investigation is clearly warranted.

Inhibition of the positive feedback response of oestradiol on LH by circulating testosterone has also been recorded in female rats (Klawon *et al.*, 1971). In rhesus monkeys (Karsch *et al.*, 1973) and pig-tailed macaques (Steiner *et al.*, 1978), positive feedback is suppressed by circulating testicular hormones, although testosterone is probably not the suppressive agent in the macaque (Steiner *et al.*, 1978). No inhibition of positive feedback by the testes is evidenced in marmoset monkeys (Hodges and Hearn, 1978; Hodges, 1980). The significance of these interspecies differences is difficult to explain.

There are fundamental differences in the way in which sexual differences in positive feedback and reproductive behaviour are controlled in tammar wallabies. Male-type sexual behaviour is determined by the presence of circulating testosterone in adulthood and there is no apparent organizational effect of the testicular hormones on the brain systems controlling masculine behaviour during early development (Rudd *et al.*, 1996). Similarly, the characteristic birth posture and parturient behaviour of tammar wallabies, normally associated only with parous females (Renfree *et al.*, 1989), can be induced easily in intact males as well as in nulliparous females treated with $PGF_{2\alpha}$ (Hinds *et al.*, 1990; Shaw, 1990). Hence, sex differences in the expression of male-type sexual behaviour and female-type parturient behaviour are determined by the adult hormone milieu and, unlike the oestradiol–LH positive feedback system, there is no evidence that the neural mechanisms controlling reproductive behaviour are sexually differentiated. There are also no gross morphological sex differences in the brain regions that are important in the control of male-type behaviour, including the anterior hypothalamic–preoptic area and the accessory olfactory bulb (Rudd, 1994b).

In tammar wallabies, the loss of oestradiol–LH positive feedback is permanently imprinted in the developing male early in life and probably within 6 weeks post partum. Circulating testosterone may supplement this effect in adult males, or override the absence of imprinting in females, but castration cannot resurrect the oestradiol–LH positive feedback response in a brain masculinized by testicular hormones in early life.

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