Ano-genital distance as a factor in determining puberty acceleration in mice

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Summary. The daily exposure of newly born female mice to the urine of lactating mice with a small ano-genital distance accelerated the onset of first oestrus while the urine from donors with a large ano-genital distance was without effect in advancing puberty. The rate of growth of the mice exposed to the urine of lactating mothers was greater than that of a control sample but it was only those mice exposed to the urine of lactating females with a small ano-genital distance which continued to grow, after the cessation of treatment at 21 days of age, at a faster rate. There was no difference in the mass of the uterus when the mice were killed when adult but there were significant age-dependent differences in the mass of the ovaries and adrenal glands. The mice exposed to the urine from lactating mothers (with both large and small ano-genital indices) had smaller ovaries than the control mice while the adrenal glands of mice exposed to the urine of lactating mothers with a large ano-genital index were of greater mass than those of the control mice and mice exposed to mothers with a small ano-genital distance. Exposure to the urine of lactating mothers had no effect on the subsequent activity of the mice when tested in an automated activity recorder. The results confirm that urine from lactating mothers accelerates the onset of puberty and suggests that the effects are restricted to the urine from mothers with a small ano-genital index.

Introduction

In the mouse the presence of adult conspecifics can modify the rate of onset of puberty and the factors responsible are contained in the urine (Vandenbergh, 1967, 1969; Fullerton & Cowley, 1971). Adult male urine accelerates while that of adult females slows the rate of sexual maturation. A change in the reproductive state of the female may, however, accelerate or retard the age of onset of puberty. The urine of pregnant and lactating mice is associated with the early onset of first oestrus while urine from virgin females delays puberty (Cowley & Wise, 1972; Drickamer & Hoover, 1979). The effects that have been observed in changing the rate of sexual development may have their counterparts in modifying the behaviour of mice. Infant mice exposed to urine from late lactational mothers were less active than mice exposed to the urine from mothers at post-partum oestrus (Cowley & Pewtress, 1986). More surprising was the finding that urine from inactive mothers, collected while lactating, affected target mice by reducing activity levels while urine from active mice enhanced the activity of the recipients. The recipients were exposed to the urine early in life and the changes in activity were observed when adult (Cowley & Smale, 1986).

In this experiment attention is directed to the effects of exposing mice, during the first 21 days of life, to the urine of adult female conspecifics that differ in respect of their ano-genital distance. The measure has been used as an index of masculinity (Short, 1972) and in respect of mice and rats is known to be modified, *in utero*, by the hormonal secretions of fetuses of the same or opposite sex (Clemens, 1974; vom Saal & Bronson, 1978; Meisel & Ward, 1981). The sexing of infant rats and

mice in laboratory practice is often based on the observation that the ano-genital distance in males is greater than that in females although the reliability of the method when dealing with values that lie between the extremes is open to question. The paper describes a method developed to improve the reliability of the measurement of the ano-genital distance.

Materials and Methods

Adult TO strain (Theiler Original) female mice that had been housed in groups of 8 in 6 large cages (45 × 28 × 13 cm) under identical laboratory conditions were used. Three of the cages provided donors (see below) and the mice in the other 3 cages were mated to provide the female infants that constituted the recipients of the study. All mice were mated with colony males of the same strain and the allocation of the mice, as donors or recipients, was random.

Donors. From each cage allocated as donors the 2 mice with the largest and the 2 mice with the smallest ano-genital distance were selected to provide, during lactation, the urine to which the recipients were exposed. The ano-genital distance was measured by projecting an enlarged image of the rump of the mouse onto a television monitor. A bi-convex lens of focal length 18.3 cm was positioned between the mouse and a television camera mounted 90 cm directly above the animal. The camera was fitted with a mirror mounted immediately in front of the lens and inclined at an angle of 45° and this facilitated positioning mouse, lens and camera in an appropriate line of sight. A perforated plastic tube (30 mm i.d., 100 mm long) was attached to the rotating arm of a retort stand about 95 cm below the camera. By placing the tube at an incline of 30° the mouse could readily be encouraged to enter head first and thereafter the tube was rotated through a further angle of 60°. In this inverted position the mouse was firmly held by the tube and provided a clear enlarged picture of the rump on the monitor. A V section cut into the tube was lined with plastic edging to prevent abrasion of the tail region and the tail itself was held extended at an angle of 90° to the vertical line of the tube. The mouse was prevented from reversing back up the tube by placing two fingers over the end while avoiding obscuring the projected view of the ano-genital region on the screen. Vertical and horizontal scales of equivalent units were inscribed on the screen and the distance from the anus to the ano-genital papilla (phallus) was measured with a pair of dividers. The magnification facilitated recording and small wriggling movements did not interfere with the taking of measurements which were made by a second person who was unaware of the history of the animal. The mice were introduced to the procedure twice before the final measurement of the ano-genital distance was taken.

The urine was collected at 4–5-day intervals from lactating donors. The post-parturition oestrous period and the 2 days immediately before weaning, at 21 days of age, were not used for collecting urine. The urine was obtained by holding the mouse over a Petri dish and gently squeezing the flanks of the animal. The urine was diluted with distilled water in the ratio of 10 parts water to 1 part of urine. The urine and distilled water was kept at –17°C except when thawed and painted on to the dorsal surface of the nose of the recipients. A fine artists paint brush, and using 4 or 5 gentle strokes with the brush in a drip-free condition, was used for applying each of the treatments. The urine collected from donors with a large ano-genital distance was mixed together and kept in separate containers from that of the urine of mice with a small ano-genital distance.

Recipients. The recipients were the offspring of mothers that had been housed in the same conditions as the donors. The mice were mated so as to give birth 1 week after the donors. Mice born on the same day were weighed and thereafter mixed together and 5–6 female young were randomly returned to each mother. Male young were discarded and litters were subsequently examined at intervals to ensure that the sex of the young had been reliably established. The mothers and infants were housed in plastic cages (33 × 15 × 13 cm high) in a room away from the males. Food and water were available at all times and three tungsten lights (3 × 60 W) provided a 12 h light/12 h dark cycle with lights on at 06:00 h. Temperatures were maintained at 21°C.

The recipients were exposed to the urine from donors (or distilled water in the control condition) from Day 1 of age and the treatments were continued until the mice were weaned at 21 days of age. The mice were allocated on a random basis to the three treatment groups and infants in any one cage received the same treatment. Once weaned the mice were kept in their same cage units and examined daily for vaginal introitus. Vaginal smears were taken for 10 consecutive days from vaginal opening. The number of mice exposed to each of the three treatments during the suckling period are shown in Table 1 together with the mean body weight $\pm$ s.e.m. at the time that treatment was discontinued (21 days of age).

At 80 days of age 12 mice from each treatment were tested for 2 min on 5 consecutive days using an automated activity recorder (PANLAB Acti System, ORMED Ltd, Welwyn Garden City, Herts, U.K.) and thereafter they were killed and the uterus, left ovary and left adrenal were removed and weighed on a microbalance. A second independent sample of mice ($N = 30$) was subjected to the same activity testing procedure when older and the animals were killed and tissues removed at 130 days of age.

Statistical analysis. One way analysis of variance and Duncan's New Multiple Range Test were carried out with the use of SPSS (2nd edition) and on a DEC 1091 Computer.

Results

Growth of mice

Table 1 shows the number and mean weight $\pm$ s.e.m. of the mice at 21 days of age when treatment ceased. The table also shows the mean weight $\pm$ s.e.m. and number of mice in the treatment groups that were killed when 90 or 130 days of age and from which the uterus, adrenals and ovaries were removed. At birth the weight differences between samples were negligible but by the end of the treatment at 21 days of age there were marked differences between the groups ($P < 0.01$). The mice exposed to the urine from mothers with large and small ano-genital indices were heavier at this age than the mice used as a control ($P < 0.05$) in both instances. The growth rates after the discontinuation of treatment also showed differences between samples, and at 90 days of age the mean differences in weights were marked ($P < 0.004$). The mice exposed to the urine of mothers with a small ano-genital index were heavier than the control mice and those exposed to the mothers with a large ano-genital index ($P < 0.05$ for both). The mean difference between the mice exposed to the urine of mothers with a large index did not differ significantly from that of the controls. The weight of the mice kept for testing at about 130 days of age showed that the samples had retained the same relative positions in body mass as at the earlier age though the mean weight differences between treatments were not significant.

Table 1. Effects of exposing mice to urine from lactating females during the suckling period on their body weight, age at vaginal introitus and onset of first oestrus

Donor groups	Body weight (g)			Vaginal introitus (days)	First oestrus (days)
	21 days	90 days	130 days		
Large ano-genital distance	14.3 $\pm$ 0.4 (28)	32.3 $\pm$ 0.6 (12)	35.2 $\pm$ 0.7 (11)	27.4 $\pm$ 0.5 (28)	29.8 $\pm$ 0.5 (28)
Small ano-genital distance	14.2 $\pm$ 0.2 (27)	35.6 $\pm$ 0.8 (12)	37.6 $\pm$ 1.1 (9)	26.4 $\pm$ 0.4 (27)	28.3 $\pm$ 0.5 (27)
Water control	13.1 $\pm$ 0.3 (32)	33.6 $\pm$ 0.6 (12)	36.3 $\pm$ 1.1 (10)	27.8 $\pm$ 0.6 (32)	30.3 $\pm$ 0.6 (32)

Values are mean $\pm$ s.e.m. with the number of mice in parentheses. Some animals were killed at 90 and others at 130 days of age.

Fig. 1. The mean mass ($\pm$ s.e.m.) of (a) adrenal and (b) ovary expressed as a percentage of body weight for the mice killed at 90 and 130 days of age. The mice were exposed from 1 to 21 days of age to the urine of mothers with a small (▨) or large (■) ano-genital index or to a control (water) condition (□). The numbers of mice in each of the treatment groups are shown in Table 1.

Vaginal opening and first oestrus

There were some indications that the treatments had an effect on the age of vaginal introitus (Table 1) but the mean difference in age of opening did not reach an acceptable level of significance ($0.1 > P > 0.05$). The mean age of first oestrus differed between treatments ($P < 0.03$). The mice exposed to the urine of mothers with a small ano-genital index had an earlier onset of oestrus when compared with the control sample ($P < 0.05$). There were no other significant differences between treatments.

Uterus, ovary and adrenal weights

The uterus, left ovary and left adrenal glands were removed and the weights of the tissues expressed as a percentage of the body weight of the mice. An analysis of variance showed that at 90 days of age there was only an overall treatment effect in the weight of the adrenal glands ($P < 0.02$), with the adrenal weight of the mice exposed to the urine of mothers with large ano-genital index being greater than that of the control mice ($P < 0.05$) and of the mice exposed to the urine of mothers with a small ano-genital distance ($P < 0.05$). The mean difference in adrenal weight between young exposed to the urine of mice with a small index and the control sample did not differ significantly and nor did the adrenal glands differ in mass, between treatments, in mice killed at 130 days of age (Fig. 1a). There were no statistically significant differences between the treatments in the mean weight of the uterus and ovary at the younger age. Mice killed at 130 days of age also did not differ in the weight of the uterus but there was a significant difference in the mean weight of the ovary ($P < 0.02$). The ovary weight of recipients treated with urine from mothers with large and small ano-genital distances, while not differing between themselves, were lighter than those of the control mice ($P < 0.05$ for both groups). Figure 1(b) shows that, at the younger

and older age, the ovary mass was similar between treatments although it was only in the mice killed at the older age that the differences were statistically significant.

Activity

There were no significant differences between the mean activity scores of the mice in the different treatments at 80 and 120 days of age.

Discussion

The experiment was undertaken to ascertain whether urine from donors with large and small ano-genital indices would, when applied to recipients early in life, subsequently modify the rate of sexual maturation and the activity of the mice when adult. The results show that it is the infants exposed to the urine of donors with a small ano-genital index that show an accelerated onset of puberty—an effect which is absent in infants exposed to the urine of mice with a large index. There was no real evidence that the urine from the donors accelerated the mean age at vaginal opening. The reliability of vaginal opening as an index of sexual maturation in the mouse has been questioned (Kennedy & Mitra, 1963; Vandenberg, 1967; Fullerton & Cowley, 1971) and in female mice isolated from the presence of males or their odours, vaginal opening often occurs in the absence of ovulation (Vandenberg, 1973; Bronson & Desjardins, 1974). The number of mice and the composition of the social group may modify the concentration of plasma gonadotrophins and so disrupt pubertal cycles (Stiff *et al.*, 1974). An accelerated gain in weight during treatment was apparent in both urine-treated samples but it was only the mice exposed to the urine of the small index donors that maintained the advantage when adult. The growth-promoting and puberty-accelerating properties of the urine differ between treatments although both features show enhancement in relation to the infants treated with the urine from small index donors. The short-term accelerated growth in the infants treated with urine from donors with the large indices is not associated with enhanced sexual maturation and, indeed, the growth rate after the end of treatment was less than that of the control mice.

The exposure of young mice to urine may have a differential effect on the suckling stimulus but in other studies (Cowley & Wise, 1972; Fullerton, 1977) there has been a failure to observe changes in the responsiveness of mothers to infants that have been exposed to urine of male and female donor mice. It may be necessary, however, to embrace a wider range of behavioural categories; the change in rate of growth suggests that there may be differences in the let down of the mothers milk and/or in the responsiveness of the infants, in the different treatments, to the olfactory and other stimuli that elicit nipple search attachment, and in suckling behaviour (Pedersen & Blass, 1981). What is of interest is to enquire as to how the urine from the donors may differ in composition. McClintock (1983) has suggested that prolactin-dependent compounds can lengthen the oestrous cycle of rats through the action of odours from lactating donors. The action of prolactin on the ovary has been reviewed by McNeilly *et al.* (1982), providing evidence that during hyperprolactinaemia the increase in prolactin may inhibit granulosa cell oestrogen secretion by interfering with the aromatization of androgens. In the present study, the mass of the ovaries of the mice treated with urine was smaller at both ages than that of the control sample and the differences were appreciable when comparisons were made at the older age. However, the exposure of infant mice to the urine of donors at the post-partum oestrus and donors at a late stage of lactation did not provide evidence of ovarian atrophy (Cowley & Pewtress, 1986), although as in the present study the onset of puberty was accelerated. In the rat, prolactin plasma concentrations are as high, if not a little higher, shortly after birth (4–7 h) than they are at 10 days *post partum* (Nagasawa & Yanai, 1972) and if the pattern is similar in the mouse then it would indicate that it is not simply an

elevated prolactin concentration that is operative in producing the condition necessary for the increase in weight of the ovaries, and early sexual maturation. The question as to whether the urine acts to modify steroidogenesis in the infant recipients, including concentrations of androstenedione, needs to be considered and the conditions may be homologous to those in which puberty is accelerated after exposure of infant female mice to adult male conspecifics or their urine (Vandenbergh, 1967, 1969; Cowley & Wise, 1972).

The effect of treatment on the mass of the adrenal glands was contrary to that observed for the ovaries. The mass is little different between groups at the older age but at the younger age the mice receiving the urine from the mothers with large ano-genital distances were very much heavier. The pattern of change may reflect on some reciprocal action between the ovary and adrenal tissues and this has been well documented for the mouse (Parkes & Deanesly, 1960). In man, in which the fetal adrenal has much the same intrinsic properties and is subject to the same pituitary regulatory mechanisms as in postnatal life (Winter, 1985), prolactin may act as a growth factor in increasing adrenal mass although the results have been questioned and the complexity of interactions between ACTH, oestrogens and prolactin and the long-term changes in the gland are manifold (review by Hornsby, 1985). Exteroceptive factors, including the handling or gentling of infant rats, have also been subsequently associated with an increase in adrenal mass, elevated concentrations of ACTH and corticosteroids in the blood of adult rats (reviewed by Archer, 1973; Daly, 1973). The findings have often been interpreted in terms of the gentling being stressful but we have questioned the appropriateness of this (Cowley & Widdowson, 1965). The handling effects may be due to a transfer of urine from the mothers, by the handler, to the infants. The absence of an increase in adult activity changes, which has been well documented in studies of handling in rats, may relate to the differences in species used but it may also relate to the particular criteria selected. When a behavioural measure (levels of activity) was used in selecting donors instead of a morphological criterion (ano-genital distance), a striking change in the activity of the recipient mice was observed. The urine from inactive donors suppressed the activity of adult recipients that had been exposed to the urine in infancy and there was no evidence of habituation to the test situation. Similarly, the urine of active donors enhanced the long-term effect on the activity of the mice (Cowley & Smale, 1986) and this showed, over 4 days, rapid habituation to the test arena. The composition of the urine reflects the neuroendocrine state of the donor and provides, through the action of pheromones, a basis for modifying and hence controlling the physiological state and development of the recipient.

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