eminence still contains clusters of big catalase granula in its superficial layers (fig. 2).

Discussion. In previous studies on the histochemical characteristics of puberty in the mediobasal hypothalamus it was found that prolactin administration[8], castration[10] and suprachiasmatic nuclei destruction[9] in one way or another resulted in a retardation of the described cell displacements[2-6] but in all cases the arrival of catalase granula at day 45 in the arcuate nucleus was completed. No catalase granula remained present in the intermediate area or median eminence in these cases except in the prolactin administration study. On the other hand, administration of monosodium glutamate postnatally from day 4 until day 11 accelerated the displacement[7].

Administration of melatonin did block the displacement of catalase granula, but only partially. Such a differential answer to melatonin administration in prepubertal rats is unexpected. A retardation of the neuronal migratory events could be expected on the basis of descriptions of a delayed sexual maturation by others[13]. These results suggest that melatonin indeed causes a delay of maturation of the hypothalamus-pituitary axis.

Melatonin action not only contributes to the termination of phasic LH secretion, but also participates in the entrainment of the LH surge to the photoperiod in the female rat[18]. Melatonin performs an acute inhibitory effect on pituitary LH and FSH responses to LHRH[19,20]. Since binding of melatonin to a cytoplasmic receptor was found in several gonadal and endocrine tissues[21], as well as in brain tissue[22], it cannot be concluded whether a direct or an indirect action of melatonin is involved. The differential effect of melatonin administration on the displacement of catalase positive cells is under investigation now.

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Crowding during pregnancy delays puberty and alters estrous cycles of female offspring in mice

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Summary. Chronic crowding of mice during late pregnancy resulted in offspring of lowered birthweights and, in the females, delayed puberty and altered estrous cycles. Plasma corticosterone in the crowded dams was elevated acutely, lending some support to the hypothesis of adrenocortical mediation of prenatal stress effects.

Key words. Crowding stress; pregnancy; puberty; estrous cycle; corticosterone; birthweight.

Exposure of rodents during pregnancy to stressful environmental conditions has been shown to produce a variety of effects on the endocrine systems of male offspring, including accelerated fetal testosterone surge[1], underdevelopment of adrenal and testis[2], lower testosterone secretion[3] and lower stress-induced corticosterone and prolactin secretion[4]. All these endocrine effects have been reported in the rat but impairment of sexually-differentiated behavior in prenatally stressed males has been reported in both rats[5] and mice[6,7]. Of the few studies examining the effects of stress during pregnancy upon the female offspring the following consequences have been reported: impairment of sexual receptivity in rats[8] and mice[9], lengthening of the combined estrus-metestrus stages of the vaginal cycle in rats[10], and delay of vaginal opening in mice[11]. However, to date, there has been no thorough study of the effects of stress during pregnancy upon reproductive development of the female offspring. It has been suggested that the effects of stress during pregnancy upon male offspring are mediated by exposure of the fetus to maternal pituitary-adrenal products[2]. We have recently examined this suggestion giving experimental evidence for this hypothesis in both male[6] and female[12] offspring. The hypothalamo-pituitary-gonadal axis develops prenatally in both rats and mice; LH, FSH and PRL are all secreted by the fetal pituitary[13-15] and, estrogen receptors are present in fetal rat hypothalamus[16]. Although development of hypothalamo-pituitary function is not completed until early postnatal life[17], it is clear that any abnormality in the maternal endocrine profile during late pregnancy is likely to have some impact on the development of these systems in the fetus. The purpose of this study was to examine the effects of chronic crowding during pregnancy upon the onset of puberty and the estrous cycle of female offspring, to provide a more complete documentation of the effects of prenatal stress on

Table 1. The effects of chronic crowding stress during the final third of pregnancy upon the onset of puberty in female offspring housed in groups of 3–6. Data given are as means ± SEM

	Number of mice	Vaginal opening Age (days)	Body weight (g)	First estrus Age (days)	Body weight (g)
Females from mice undisturbed during pregnancy	31	30.64 ± 0.39	20.67 ± 0.30	31.16 ± 0.47	21.01 ± 0.33
Females from mice stressed during pregnancy	38	31.87* ± 0.34	21.82** ± 0.19	32.24* ± 0.37	22.23** ± 0.18

*Significant difference $p < 0.025$ (t-test). **Significant difference $p < 0.001$ (t-test).

Table 2. The effects of chronic crowding stress during the final third of pregnancy upon the estrus cycle of adult female offspring housed individually. Data given are as means ± SEM

	Number of mice	Days of pro-estrus	Days of estrus	Days of metestrus	Days of diestrus	Cycle length (days)
Females from mice undisturbed during pregnancy	15	3.60 ± 0.32	6.40 ± 0.34	6.73 ± 0.33	4.27 ± 0.27	5.11 ± 0.18
Females from mice stressed during pregnancy	15	2.60* ± 0.27	6.80 ± 0.34	7.07 ± 0.37	4.47 ± 0.39	5.20 ± 0.21

*Significant difference $p < 0.025$ (t-test).

reproductive development in the female rodent. To discover whether crowding was stressful, plasma corticosterone was measured in both crowded and control pregnant females.

Materials and methods. Females used were virgin 'TO' mice (A. Tuck and Sons, Battlesbridge, Essex) obtained two weeks prior to mating, or were virgin 'TO' females bred in our own laboratory. They were housed in groups of 10 in large plastic cages (42 × 25 × 11 cm) allowed ad libitum supply of food (Labsure animal diet, Christopher Hill, Ltd, Dorset) and water, and maintained on a reverse lighting regime (red lights on 12.00–22.00 h) at 18–23 °C. At 10–12 weeks of age these females were placed individually into small plastic cages (30 × 13 × 11 cm) with a male and observed daily for the appearance of a vaginal plug, which was deemed to indicate day 0 of pregnancy. Males were then removed and females were left undisturbed until day 12 of pregnancy when treatments began. Pregnant females were assigned at random either to the crowding or control group. On day 12 females in the crowding group were placed in a large cage containing 25–28 males. The total housing density was 30 animals per cage (35 cm^2/animal). Further details of this procedure are given elsewhere[6,7]. On day 17 females were rehoused individually to ensure undisturbed births. Control females remained individually housed during pregnancy (390 cm^2/animal).

In the study of puberty onset, offspring were derived from 9 crowded mice and 9 controls, and offspring used in the study of the estrous cycle were derived separately from 8 crowded and 8 control dams.

At birth (day 0) all pups were weighed and a litter mean calculated. Litters were randomly culled to 8 pups and fostered within 13 h to an untreated female that had given birth within the previous 24 h. Foster mothers and litters were left undisturbed until weaning on postnatal day 21 when offspring were weighed and rehoused in groups according to sex. To assess puberty, females were housed in large cages in groups of 3–6 according to age and treatment. From day 25, animals were examined daily between 10.00 and 12.00 h for vaginal opening. The second successive day of vaginal opening was recorded, as occasionally vaginal membranes reclose after the first day. Vaginal smears were then taken daily until the first estrus was recorded. Adult females used in the study of the estrous cycle were housed in non-sibling groups of 8–10 in large cages from weaning until 8 weeks old. They were then rehoused singly, and after a further week daily vaginal smears taken from them for 21 days. In both experiments smears were taken between 10.00 and 12.00 h by the lavage method, and stained and classified as previously described[12]. Contact with male mice or their bedding was avoided, as this is known to influence female mouse reproduction[18].

In the study of plasma corticosterone, blood samples were taken from pregnant females immediately before crowding, then at intervals of 1, 4 and 8 h and 1, 2 and 3 days, with samples taken from control females at equivalent times. Blood was collected from the retro-orbital sinus under ether anesthesia, within 3 min of disturbing the animals, heparinized, centrifuged and stored at −20 °C. No animal was sampled more than once in 24 h, and their offspring were not used in experiments. Corticosterone in the plasma samples was determined by RIA as described elsewhere[19,20].

Body weight data up to day 21 were analyzed using the litter as the unit of analysis[21]: all other analyses were based on the individual. Comparison of means were made using Student's t-test, and proportional differences by Fisher's Exact Probability. The Mann-Whitney U-test was used when the criteria for the t-test were not met.

Results. Table 1 shows the effects of crowding during late pregnancy on the onset of puberty. Vaginal opening and first estrus were both significantly delayed in the experimental females, and their body weight was significantly higher on the days in question than that of their control counterparts.

The effects on the estrous cycle of a similarly treated group of females are shown in table 2. Experimental females showed fewer pro-estrus smears over the 21-day observation period than controls. Proportional analysis showed that amongst these experimental females more mice (86.6%) showed cycles apparently lacking a nucleated-cell pro-estrus stage than their control counterparts (46.6%: $p = 0.025$).

Data on body weight of offspring at birth and weaning and for

Table 3. Plasma corticosterone concentrations ($ng \cdot ml^{-1}$) ± SE in control and crowded pregnant mice. Figures in brackets are numbers of individuals. Time zero was 10.00 h on day 12 of pregnancy

Time (h)	Control	Crowded
0	643 ± 91 (5)	–
1	786 ± 24 (3)	860 ± 132 (2)
4	448 ± 83 (3)	2039* ± 861 (3)
8	565 ± 36 (2)	1564 ± 910 (3)
24	916 ± 93 (4)	1085 ± 110 (3))
48	1799 ± 224 (3)	1577 ± 640 (3)
72	1652 ± 113 (5)	1561 ± 201 (3)

*$p = 0.05$, Mann-Whitney U.

mortality were analyzed by combining the two experiments. Birthweight of experimental pups, at 1.54 ± 0.04 g was significantly lower than controls (1.70 ± 0.04 g: $p < 0.005$). The difference at weaning was not significant: experimental 10.09 ± 0.38 g; control 11.02 ± 0.45 g.

5.6% of control litters had one or more pup deaths at or after birth, compared with 27.7% of experimental litters.

Corticosterone levels in maternal plasma are shown in table 3. Circulating corticosterone was elevated for 8 h after introduction to the crowding cage, but the difference was only significant at 4 h.

Discussion. The results of this study clearly show that the stress of crowding pregnant female mice with aggressive males leads to a delay in the onset of puberty, as indicated both by vaginal opening and first estrus, in the female offspring. Although lighter in weight at birth, prenatally stressed pups gained weight faster than controls, under the care of untreated foster-mothers, so that by weaning their weight deficit had been eliminated. The fact that by puberty they were heavier than controls is a reflection of their delayed sexual maturation, not of continued faster growth. The real significance of this result is that weight gain, an important determinant of puberty[22], can not have been the cause of their retarded sexual development. As these offspring of crowded females also showed alterations to the estrous cycle, an endocrine change is a more likely cause of both results. Politch and Herrenkohl[11], using restraint as a stressor, have also reported delayed puberty in mice and alterations to the estrous cycle of both mice[11] and rats[10]. The nature of the presumed endocrine change has not been directly investigated, but some clue may be gained from the alteration to the vaginal cycles; in both our study and another[10] the estrogen-dependent phase of the cycle is shortened, suggesting an attenuated or abbreviated estrogen peak; a feature which would also be consistent with delayed puberty.

The mechanism by which stress during pregnancy alters offspring development is widely believed[2, 6, 12] to involve the elevation of maternal adrenal steroids such as corticosterone, which crosses the placenta[23] and can act directly on the fetus. For this reason, and because the stressor we employed could be expected to be milder than many used[1, 10, 11] we thought it necessary to verify that it did, in fact, stimulate maternal corticosterone secretion. Table 3 shows that the corticosterone elevation was only transient, and although our infrequent blood-sampling may have allowed occasional surges to go undetected the face-value interpretation is that a single, brief exposure to raised corticosterone levels on day 12 is sufficient to alter offspring development. Corticosterone levels in maternal circulation in the mouse rise substantially during pregnancy, and during the later stages of pregnancy much of the corticosterone in maternal circulation is of fetal origin[24]. The 4-fold increase in corticosterone detected in this study is likely to be almost exclusively from the maternal adrenals.

Raised adrenocortical activity during pregnancy could act on the offspring peripherally as this has been shown[25] to cause adrenal underdevelopment in offspring. Several authors[26–29], have pointed out the importance of the adrenal in the timing of puberty, and that corticosterone secretion is closely associated with gonadotrophin regulation[30–32] and the estrous cycle[19], showing a larger diurnal peak at the pro-estrus stage (the most affected in this study) than any other. It is thus not difficult to see that pituitary-adrenal underdevelopment could produce the results reported here though perhaps surprising that such brief elevation of plasma corticosterone could be responsible for poor fetal adrenal growth. There is some evidence[33] that newborn rats whose mothers were stressed during pregnancy do have abnormally small adrenals, but this may be compensated for[2] in young adulthood.

A central mode of action of corticosterone, through prolactin (PRL) is also possible: even short exposures to this steroid which has pronounced effects on development, might radically alter adult function of neuroendocrine systems such as that controlling PRL secretion. PRL can be suppressed by corticosterone and other adrenal products[34] and offspring of restraint-stressed rats are PRL-deficient[4, 35]. Adequate PRL is required for normal body growth[36] and ovarian development[37] and in the rat administration of exogenous PRL hastens puberty[38]. In the mouse, however, PRL may delay puberty[39]. It is thus not possible at present to be more precise as to the details of the endocrine factors which, caused by prenatal stress, result in alterations to female as well as male sexual development in mice.

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