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ORIGINAL ARTICLE

Oestrogen-Independent, Experience-Induced Maternal Behaviour in Female Mice

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Nulliparous female mice that have not experienced mating, pregnancy or parturition show near immediate spontaneous maternal behaviour when presented with foster pups. The fact that virgin mice display spontaneous maternal behaviour indicates that the hormonal events of pregnancy and parturition are not necessary to produce a rapid onset of maternal behaviour in mice. However, it is not known how similar maternal behaviour is between virgin and lactating mice. In the present study, we show that naturally postpartum females are faster to retrieve pups and spend more time crouching over pups than spontaneously maternal virgin females, and that these differences diminish with increased maternal experience. Moreover, 4 days of experience with pups induced pup retrieval on a novel T-maze. Furthermore, the effects of experience on subsequent maternal responsiveness are not dependent on gonadal hormones because ovariectomised females with 4 days of pup experience show pup retrieval on a novel T-maze similar to that of postpartum mice. Four days of maternal experience also induced T-maze pup retrieval in ovariectomised aromatase knockout female mice that was not significantly different from the maternal responsiveness of ovariectomised wild-type littermates. These data suggest that maternal experience can induce maternal behaviour in females that have never been exposed to oestradiol at any time in development or adulthood. Finally, ovariectomised pup-experienced females continue to retrieve pups on a novel T-maze 1 month after the initial experience, suggesting that, even in the absence of oestradiol, maternal experience produces long-lasting modifications in maternal responsiveness.

Key words: maternal behaviour, aromatase, social behaviour, oestrogens, epigenetic.

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At the time of birth, female rodents ensure the survival of their altricial young with the display of maternal behaviours (retrieval, licking, nursing) (1). Nulliparous rats and mice will also show maternal behaviour when presented with foster pups (1, 2). In the absence of hormone stimulation, continual exposure to pups initiates maternal responsiveness. Virgin female rats require 6–8 days of pup exposure to show maternal behaviour (3). By contrast, mice of many strains show 'spontaneous' maternal responses to pups within the first 15 or 30 min of pup presentation (2, 4–12), suggesting that the onset of maternal behaviour in mice is independent of hormonal mediation (8, 9, 11, 13, 14). Although some studies note that postpartum and virgin mice spend similar amounts of time with pups (14–16), detailed comparisons between postpartum and virgin females have not been reported. In the present study, we examined spontaneous maternal behaviour in the inbred C57BL/6J (B6) strain of mice. We report that naturally postpartum females and spontaneously maternal females respond differently toward pups, and that differences diminish as virgin female mice gain experience with pups.

During the postpartum period, the mother–pup interaction induces maternal motivation, or an increase in approach behaviours that help the female gain access to offspring (1, 17, 18). For example, postpartum rats and mice will traverse a novel environment containing pups and retrieve them back to their nest (19–21) or press a lever to obtain pups (22, 23). Although initial studies indicated that, when tested under these demanding conditions, virgin rats and mice were not responsive to pups (19, 20, 23–25), there is recent evidence to suggest that maternal motivation can be induced in virgin rats, although only after long periods of pup exposure (26–28).

In the present study, given that the maternal responsiveness of virgin females increased across the test day, we investigated

whether maternal experience would affect subsequent maternal motivation in B6 mice (29, 30). To directly assess whether the effects of maternal experience on maternal motivation were dependent upon circulating hormones, we examined whether ovariectomised (OVX), pup-experienced female mice, similar to postpartum mice, were willing to traverse a novel T-maze to retrieve pups. In addition, we determined that the effects of pup experience on maternal motivation persist in the complete absence of oestradiol. Finally, in rats, experience with pups has been found to produce long-lasting modifications in subsequent maternal care and maternal motivation (26–28, 31–33). Thus, we examined whether experience with pups produced long-lasting effects on maternal care and motivation in B6 mice.

Materials and methods

Subjects and housing

The background strain of all mice used for these studies was C57BL/6J. All mice were originally of Jackson Laboratory origin and were bred and maintained in the University of Virginia School of Medicine, Animal Facility. Aromatase-knockout (ArKO) mice and wild-type (WT) littermates were produced using heterozygous breeding pairs and were genotyped for disrupted or WT Cyp19 gene, as previously described (34). ArKO mice were backcrossed more than ten times into C57BL/6J. Female mice were nulliparous females, naive to pups (except for their own littermates), weaned at 21 days of age, single-sex group-housed until the beginning of each experiment (60–100 days of age), and individually housed thereafter. Mice were maintained under a 12 : 12 light/dark cycle (lights off 1200 h) and received food (#7912; Harlan Teklad, Madison, WI, USA) and water *ad lib.* Ovariectomies were conducted under isoflourane anaesthesia. After gonads were removed mice were given an s.c. injection of 0.9% sodium chloride (for rehydration), a topical analgesic (0.25% bupivacaine) and kept warm until they awakened. All females recovered from surgery for at least 1 week. A separate group of mice served as foster dams that provided stimulus pups. These females were paired with a male and allowed to give birth naturally. All procedures were in compliance with the University of Virginia Animal Care and Use Committee.

Experiment 1

Mice were randomly assigned to either the postpartum (n = 9) or the virgin (n = 10) group. Postpartum females were housed with a male of the same strain until 24–48 h before parturition. On the day of birth (postpartum day 0 = PP0), litters were culled to four pups, and maternal behaviour testing began PP1. On each test day, before the start of testing, pups were briefly removed and scattered in the cage. To compare the spontaneous maternal responsiveness of virgin female mice with the natural onset of maternal behaviour in postpartum females, postpartum females remained with their pups at the end of the 2-h maternal behaviour test. Virgin female mice were presented with four stimulus pups at the start of maternal behaviour testing, and these pups were removed at the end of the 2-h test period and returned to a lactating dam.

Experiment 2

In Experiment 1, 4 days of pup exposure (2 h per day) facilitated retrieval behaviour in virgin mice. Therefore, we tested the hypothesis that 4 days of experience (2 h per day) would also induce retrieval behaviour on a novel T-maze, and that the effect of maternal experience on subsequent maternal behaviour was not dependent on ovarian hormones. Female mice were randomly assigned to the postpartum group (n = 9), the pup-experienced group (n = 10) or the naive group (n = 11). The pup-experienced and pup-naive groups comprised nulliparous OVX virgin females. Pregnant mice were monitored for parturition and newborn pups were removed within 2–8 h of birth. Pup-experienced and postpartum females received four consecutive days of pup exposure (2 h per day), whereas pup-naive females were not exposed to pups. Pup exposure for postpartum females began the day they gave birth. Dams were exposed to their biological pups for 2–8 h on PP0 and, for the next 3 days, they received stimulus pups (obtained from a donor female) for 2 h each day. At the start of each 2-h pup exposure, four pups were scattered in the cage. At the end of the 2-h period, upon removal of the pups, all females had retrieved the four pups to the nest location. During the exposure phase, pup-naive females were not exposed to pups, rather their cage tops were opened and their bedding was disturbed at the beginning and end of the 2-h period. On the fifth day, 24 h after the last pup exposure, all females were tested for pup retrieval on a novel T-maze. To examine whether retrieval behaviour on the T-maze was related to general differences in anxiety between the groups, all females were tested on the elevated plus maze (EPM) on the day after the T-maze retrieval test.

Experiment 3

The results of Experiment 2 suggest that circulating oestradiol is not required for experience-effects on motivation; however, we could not rule out that synthesis of oestradiol in the brain produces effects on maternal behaviour (35, 36). To investigate whether the effects of maternal experience on maternal motivation would persist in the complete absence of oestradiol, we used female mice with a targeted mutation in the Cyp19 gene (aromatase knockout mice) (36). ArKO mice are deficient in aromatase, the enzyme necessary for oestradiol biosynthesis. ArKO mice (n = 6) and their WT (n = 8) littermates were OVX. All females were tested for maternal behaviour in the home-cage for 2 h per day on test days 1–4 (as described above). On test day 5, all females were tested for retrieval behaviour on the T-maze.

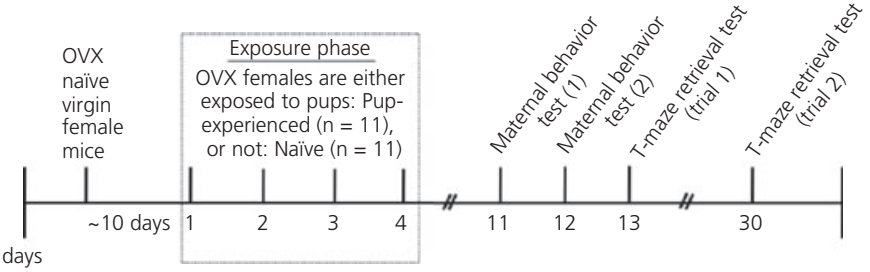

Fig. 1. Methods for Experiment 4. Timeline of pup exposure and test schedule. OVX, ovariectomised.

Note that ArKO mice do not differ from WT mice on measures of anxiety (37); therefore, we did not examine whether T-maze retrieval behaviour was related to general differences in anxiety between these two groups.

Experiment 4

OVX nulliparous mice were randomly assigned to the pup-experienced group (n = 11) or the naive group (n = 11) for the exposure phase (Fig. 1). One week after the exposure phase, mice were tested for maternal behaviour in the home cage on two consecutive test days. The results of Experiment 1 indicated that, by the third day of maternal behaviour testing, differences in pup retrieval between postpartum and virgin females had diminished. Thus, we investigated whether, by test day 3, retrieval behaviour would also be facilitated in the T-maze. To determine whether maternal experience had a lasting effect on maternal motivation, we examined pup-retrieval on the T-maze 1 month after the first experience with pups (trial 2). All females remained individually housed, and were not exposed to pups in the 1-month interim between trials 1 and 2. Before the start of the T-maze test, all females were briefly exposed to pups (1 h of pup stimulation followed by 1 h of pup deprivation) (28).

Maternal behaviour testing

Tests were conducted in the dark phase of the light/dark cycle under dim red light. Animals were habituated to the test room for at least 24 h before testing. Tests began with the placement of four stimulus pups (all the same age between 2 and 7 days old) in the areas of the cage farthest from the female's nest. A 15-min retrieval test was conducted during which the following behaviours were scored: latencies to retrieve each pup to the nest, sniff/lick all pups in the nest, and crouch over all pups in the nest. Forty-five minutes after the presentation of pups, each female was observed continuously for 15 min. Behaviour was scored once every 15 s for a total of 60 observations; during this time, hovering, sniffing and licking, or crouching over the pups were recorded. Hovering was defined as an upright posture over pups (so that pups had access to the female's ventral surface), including actively sniffing/licking the pups or engaging in self-grooming. By contrast, crouching was recorded when females were in a quiescent, immobile posture, with all four limbs supporting a slightly arched or highly arched posture over the pups (38). This detailed 15-min observation was followed by an additional 1-h observation during which behaviour was scored once every 3 min for a total of 20 observations.

T-Maze retrieval test

The walls and floors of the T-maze apparatus (67.3 × 11.4 × 8.3 cm) were clear Plexiglas, upon which a removable wire mesh top was fitted. The vertical runway measured 48.3 cm in length and opened into a horizontal runway that measured 67.3 cm in length. A goal box (11.4 × 12.7 cm) was attached to the end of the vertical runway, which could be closed off from the rest of the T-maze by a clear Plexiglas guillotine door. Three stimulus pups were scattered in the horizontal arm of the T-maze: one pup was placed in the middle of the horizontal arm and one pup was placed at each end of the horizontal arm. At the start of the retrieval test, each female was placed into the goal box of the T-maze with her nest material. After a 10-min habituation period, the Plexiglas door was removed and the 15-min retrieval test began. The following behaviours were scored: latency to emerge (all four paws) from the goal box, latency to sniff the first pup, and latency to retrieve each pup to the goal box. The test ended after 15 min, or when the female had retrieved all three pups to the goal box. The maze was thoroughly cleaned with 95% alcohol between each test.

EPM

The floors and walls of the EPM (ENV-560A; Med Associates, Inc., St Albans, VT, USA) were black polypropylene. Each runway measured 6.1 cm in width and 34.9 cm in length and was raised 71.25 cm from the floor. The walls on the closed arms were 20.3 cm in height. The outer arms were marked with a piece of white tape that indicated 'outer' open arms (39). At the start of the test, each mouse (pup-experienced, n = 10; naive, n = 11; intact postpartum, n = 9) was placed in the centre of the EPM, facing an open arm, and allowed to explore the maze for 10 min. A video camera mounted above the EPM recorded all test sessions, and all sessions were subsequently scored for the following behaviours: time spent in the open arms (s), time spent in the outer open arms (s), time spent in the closed arms (s) and number of times the mouse crossed from one arm to the other. An animal was considered in an arm if all four paws were inside the arm.

Statistical analysis

Data from Experiments 1 and 3 were analysed using a 2 × 4 ANOVA, with reproductive status as the between factor and test day as the repeated factor, followed by trend analysis across test day. Data from Experiment 4 were also analysed this way, except that a 2 × 2 ANOVA was used. Data from Experiment 2 were analysed using a one-way ANOVA. To examine differences between groups on a given test day, a post hoc Tukey–Kramer test was conducted. For the number of pups retrieved, nonparametric statistics were used.

Results

Experiment 1: Differences in the quality of maternal behaviour between postpartum and virgin female mice diminish as a result of maternal experience

All female mice retrieved pups to the nest and crouched over pups during the first 15-min test on each test day. Postpartum females were faster to retrieve (first pup: $F_{1,51} = 64.3$, $P < 0.01$, Fig. 2A; all pups: $F_{1,51} = 26.67$, $P < 0.01$, Fig. 2B) compared to virgin females. There was a main effect of test day on latency to retrieve (first pup: $F_{3,51} = 12.52$, $P < 0.01$; all pups: $F_{3,51} = 8.24$, $P < 0.01$), as well as a significant interaction between test day and reproductive status on latency to retrieve (first pup: $F_{3,51} = 11.14$, $P < 0.01$; all pups: $F_{3,51} = 4.01$, $P < 0.02$). Post-hoc analyses revealed a significant trend for all females to retrieve pups faster across test day ($P < 0.05$). Postpartum females retrieved the first pup faster than virgin females on test day 1, and all pups faster than virgins on test days 1–2 ($P < 0.05$).

There was a significant main effect of reproductive status ($F_{1,51} = 15.78$, $P < 0.01$; Fig. 2C) and test day ($F_{3,51} = 4.78$, $P < 0.01$) on latency to crouch over all pups in the nest. There was a trend for all females to crouch over pups more quickly as the test day advanced ($P < 0.05$). Postpartum females were faster to crouch over pups on test day 2 ($P < 0.05$).

The total time spent with pups differed across test day ($F_{3,51} = 3.64$, $P < 0.02$; Table 1). There was a significant trend toward more time with pups across test day ($P < 0.05$). Compared to postpartum females, virgin females spent significantly more time licking pups ($F_{1,51} = 16.57$, $P < 0.01$) on days 2 and 4 of testing

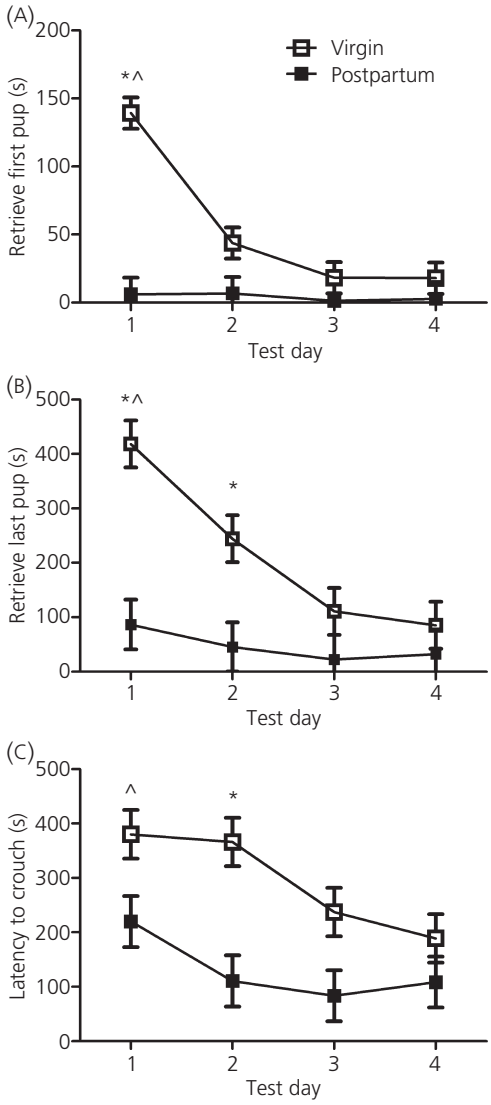

Fig. 2. Latency (s) to the onset of maternal behaviour on test days 1–4. Differences between virgin (n = 10) and postpartum (n = 9) females fade over test day as virgins respond more quickly to pups. (A) Latency (s) to retrieve the first pup on test days 1–4. (B) Latency (s) to retrieve all pups on test days 1–4. (C) Latency to crouch over all four pups in the nest. *Significantly different from postpartum group. ^Significant trend across test days.

($P < 0.05$). An inverse relationship between virgins and postpartum females was seen for crouching behaviour. There were significant main effects of reproductive status ($F_{1,51} = 15.19$, $P < 0.01$) and test day ($F_{3,51} = 6.90$, $P < 0.01$). There was a trend toward more crouching behaviour as the test day advanced. This pattern of maternal responding continued in the second hour of observation (data not shown).

Experiment 2: Maternal motivation is dependent upon experience with pups rather than ovarian hormones

A significant main effect of pup experience on latency to retrieve each of the three pups ($F_{2,27} = 9.57$, 6.81, 5.81, $P < 0.01$, respectively) from the ends of a novel T-maze was detected (Fig. 3A). Pup-naive females were significantly slower to retrieve pups compared to postpartum and pup-experienced females. There were no

Table 1. The Frequency of Observed Maternal Behaviours Across Day.

Group	Test day	Frequency pup contact (mean ± SE)	Frequency licking (mean ± SE)	Frequency crouching (mean ± SE)
Postpartum (n = 9)	1	45.1 ± 3.3	12.6 ± 3.8	28 ± 5.5
	2	58.9 ± 3.3	7.8 ± 3.8*	50.8 ± 5.5
	3	56.8 ± 3.3	17.4 ± 3.8	36.4 ± 5.5
	4	59.2 ± 3.3	3.1 ± 3.8*	56 ± 5.5
Virgin (n = 10)	1	55.6 ± 3.1	25.3 ± 3.6	19.2 ± 5.3
	2	59.5 ± 3.1	29.5 ± 3.6	27.1 ± 5.3
	3	58.7 ± 3.1	27.3 ± 3.6	27.4 ± 5.3
	4	59.9 ± 3.1	19.8 ± 3.6	38.3 ± 5.3

Number of observations (out of 60 total) in contact with pups, licking or crouching during the 15 min observation. There was a statistically significant trend across test days to spend more time with pups, less time licking and more time crouching. *Significantly different from virgin group on the same test day.

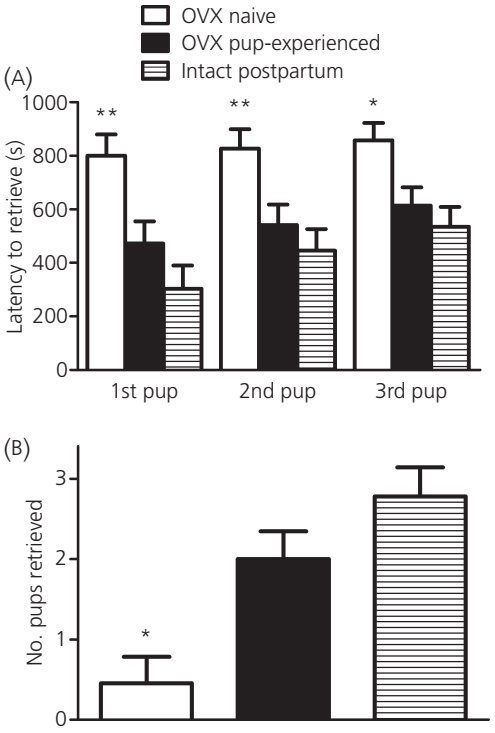

Fig. 3. Retrieval responses on the T-maze after 4 days of pup exposure (pup-experienced, n = 10; intact postpartum, n = 9) or no pup exposure (naive, n = 11). Pup-experienced and pup naive females were ovariectomised (OVX). (A) Latency (s) to retrieve each pup from the far ends of the T-maze. (B) Number of pups retrieved from the far ends of the T-maze. **Significantly different from postpartum and pup-experienced groups. *Significantly different from the postpartum group.

Table 2. Exploratory Behaviours on the EPM.

Group	n	Time in open arms (s) (mean ± SE)	Time in outer open arms (s) (mean ± SE)	Time in closed arms (s) (mean ± SE)	Arm-arm crosses (mean ± SE)
Intact postpartum	9	70 ± 12	25 ± 8	328 ± 13	37 ± 2
OVX pup experienced	10	80 ± 12	36 ± 7	364 ± 12	41 ± 2
OVX naïve	11	86 ± 11	40 ± 7	354 ± 12	42 ± 2

Activity on the elevated plus maze. Mean ± SE time (s) spent in open, outer open, and closed arms. Number of crosses between arms during the 10-min test. There were no significant differences between groups. OVX, ovariectomised.

significant differences in retrieval latencies between postpartum and pup-experienced females. Pup-naive females also retrieved significantly fewer pups than postpartum and pup-experienced females ($H = 1.6236$, d.f. = 2, $P < 0.01$; Fig. 3B).

Because differences in pup retrieval on the T-maze might be associated with differences in anxiety behaviour between females, we examined the behaviour of these subjects on the EPM. There were no significant differences in the amount of time spent in the open ($F_{2,27} = 0.47$, $P = 0.62$), the closed ($F_{2,27} = 2.05$, $P = 0.14$) or the outer open arms ($F_{2,27} = 1.19$, $P = 0.31$; Table 2). Additionally, there were no significant differences in crosses between groups ($F_{2,27} = 1.80$, $P = 0.185$).

Experiment 3: Maternal experience can potentiate maternal motivation in the absence of oestradiol

Overall, the maternal responsiveness (retrieval, licking, and nursing) of naive OVX WT mice was not significantly different from naive OVX ArKO mice. The latency to retrieve was not significantly different between WT and ArKO females across the four test days, although both groups retrieved pups faster as the test day advanced (first pup: $F_{1,36} = 22.08$, $P < 0.01$, Fig. 4A; all pups: $F_{1,36} = 11.16$, $P < 0.01$, Fig. 4B). There was also a main effect of test day on latency to crouch over all pups in the nest ($F_{1,36} = 11.32$, $P < 0.01$; Fig. 4C).

There were no significant differences in maternal interactions between WT and ArKO females and pups in the nest. During the 15-min observation, all females displayed significantly less licking across test day ($F_{1,36} = 7.08.30$, $P < 0.01$; Table 3) and significantly more crouching across test day ($F_{1,36} = 8.69$, $P < 0.01$). This pattern continued in the subsequent hour of observation (data not shown).

Because the results of Experiment 2 indicated that pup experience induced high levels of maternal responsiveness in OVX virgin mice, we also examined whether 4 days of pup experience would facilitate pup retrieval on a novel T-maze in ArKO mice. There were no significant differences between WT and ArKO mice on latency to retrieve pups retrieval on the T-maze or in the number of pups retrieved (Fig. 5).

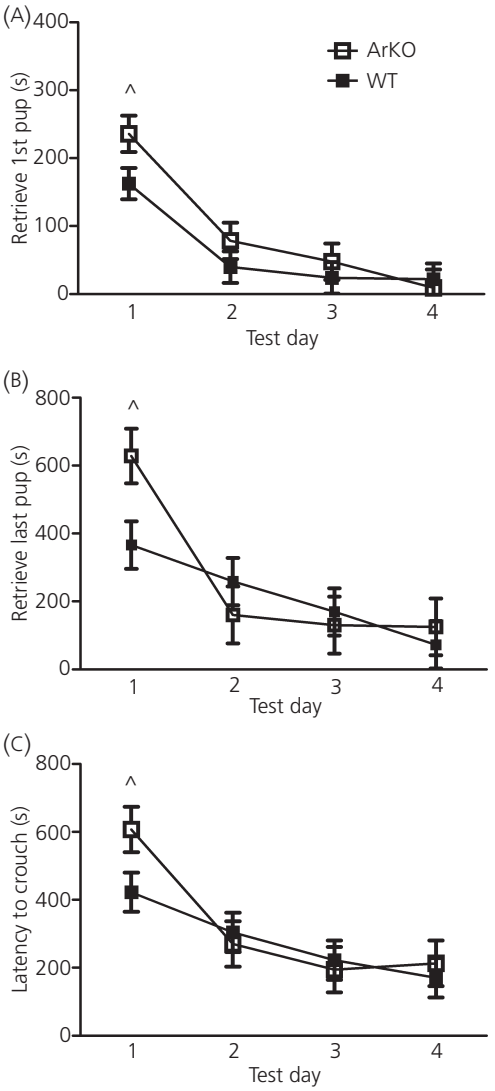

Fig. 4. Latency (s) to the onset of maternal behaviour on test days 1–4 in ovariectomised wild-type (WT; n = 8) and aromatase knockout (ArKO; n = 6) mice. (A) Latency (s) to retrieve the first pup on test days 1–4. (B) Latency (s) to retrieve all pups. (C) Latency (s) to crouch over all four pups in the nest. ^Significant trend across test days.

Experiment 4: Effects of pup experience are stable

Pup-experienced females were faster to retrieve (first pup: $F_{1,20} = 5.88$, $P < 0.05$, Fig. 6A; all pups: $F_{1,20} = 6.65$, $P < 0.02$, Fig. 6B) than pup-naive mice. There was a significant main effect of test day on latency to retrieve (first pup: $F_{1,20} = 10.76$, $P < 0.02$; all pups: $F_{1,20} = 7.11$, $P < 0.02$). There was a significant trend to retrieve first and all pups faster across test day ($P < 0.05$). Pup-experienced females retrieved the first pup faster on test day 1 ($P < 0.05$). There was an effect of status and test day ($F_{1,20} = 9.38$, 9.81, $P < 0.01$, respectively) on latency to crouch over all four pups (Fig. 6C). Pup-experienced females crouched over pups faster on days 1–2 ($P < 0.05$).

During the 15-min observation, pup-naive females displayed significantly more licking than pup-experienced females ($F_{1,20} = 11.87$,

Table 3. The Frequency of Observed Maternal Behaviours in Wild-Type and ArKO Mice Across Day.

Group	Test day	Frequency pup contact (mean ± SE)	Frequency licking (mean ± SE)	Frequency crouching (mean ± SE)
Wild-type (n = 8)	1	55 ± 2.1	21.9 ± 3.4	22 ± 5
	2	55.3 ± 2.1	20.2 ± 3.4	21.8 ± 5
	3	59.5 ± 2.1	20.5 ± 3.4	32.5 ± 5
	4	58.8 ± 2.1	12.9 ± 3.4	44.4 ± 5
Aromatase knockout (n = 6)	1	54 ± 2.5	30.7 ± 3.9	18 ± 5.7
	2	56.2 ± 2.5	27.5 ± 3.9	21.5 ± 5.7
	3	59.2 ± 2.5	19.7 ± 3.9	34.2 ± 5.7
	4	58.2 ± 2.5	8.2 ± 3.9	43.5 ± 5.7

Number of observations (out of 60 total) in contact with pups, licking, or crouching during the 15 min observation. There was a statistically significant trend across test days to spend less time licking and more time crouching.

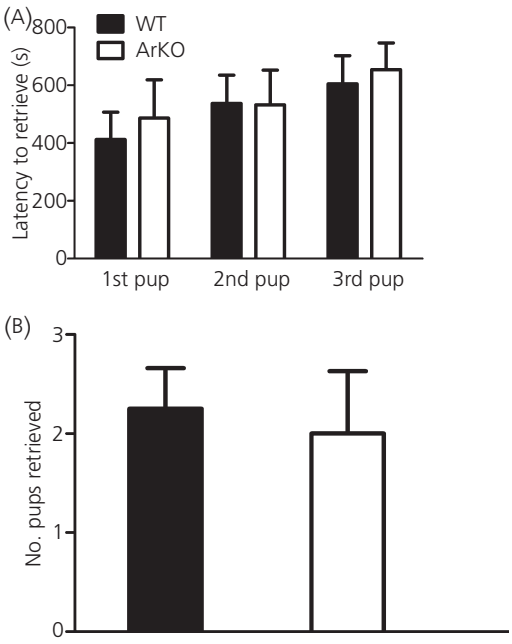

Fig. 5. Retrieval responses on a novel T-maze after 4 days of maternal behaviour testing. Wild-type (WT; n = 8) and aromatase knockout (ArKO; n = 6) females were ovariectomised. (A) Latency (s) to retrieve each pup from the far ends of the T-maze. (B) Number of pups retrieved from the far ends of the T-maze.

P < 0.01; Table 4). Inversely, pup-experienced females show significantly more crouching than pup-naive females ($F_{1,20}$ = 12.12, P < 0.01). This pattern continued in the subsequent hour of observation (data not shown).

Two days of pup exposure did not facilitate pup retrieval on the T-maze. Females that were naive to pups during the exposure phase were slower to retrieve the each pup ($F_{1,20}$ = 5.96, 7.56, 9.62 P < 0.05, respectively) from the ends of the T-maze compared to

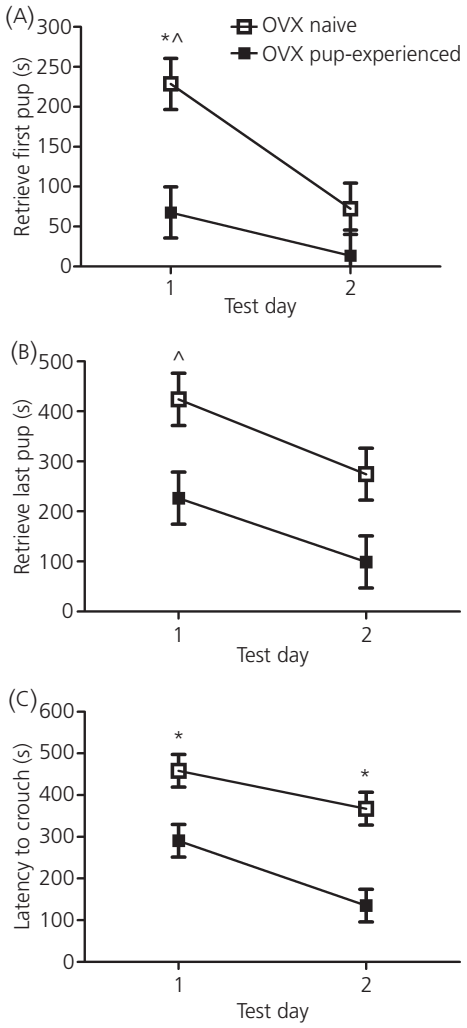

Fig. 6. Latency (s) to the onset of maternal behaviour on test days 1–2. All females were ovariectomised (OVX). Naive females (n = 11) were not exposed to pups before maternal behaviour testing. Pup-experienced (n = 11) females were exposed to pups for 2 h per day for 4 days. (A) Latency (s) to retrieve the first pup on test days 1–2. (B) Latency (s) to retrieve the last pups on test days 1–2. (C) Latency (s) to crouch over all four pups in the nest on test days 1–2. *Significantly different from pup-experienced group. ^Significant trend across test days.

Table 4. The Frequency of Observed Maternal Behaviours in OVX Maternally Experienced and Naive Mice Across Day.

Group	Test day	Frequency pup contact (mean ± SE)	Frequency licking (mean ± SE)	Frequency crouching (mean ± SE)
Pup exposure (n = 11)	1	59 ± 1.3	15.4 ± 2.4*	40.4 ± 3.1*
	2	58.8 ± 1.3	10.7 ± 2.4*	45.2 ± 3.1*
Naive (n = 11)	1	55.4 ± 1.3	28.9 ± 2.4	19.5 ± 3.1
	2	57.5 ± 1.3	28.2 ± 2.4	26 ± 3.1

Number of observations (out of 60 total) in contact with pups, licking, or crouching during the 15 min observation. *Significantly different from naive group on the same test day.

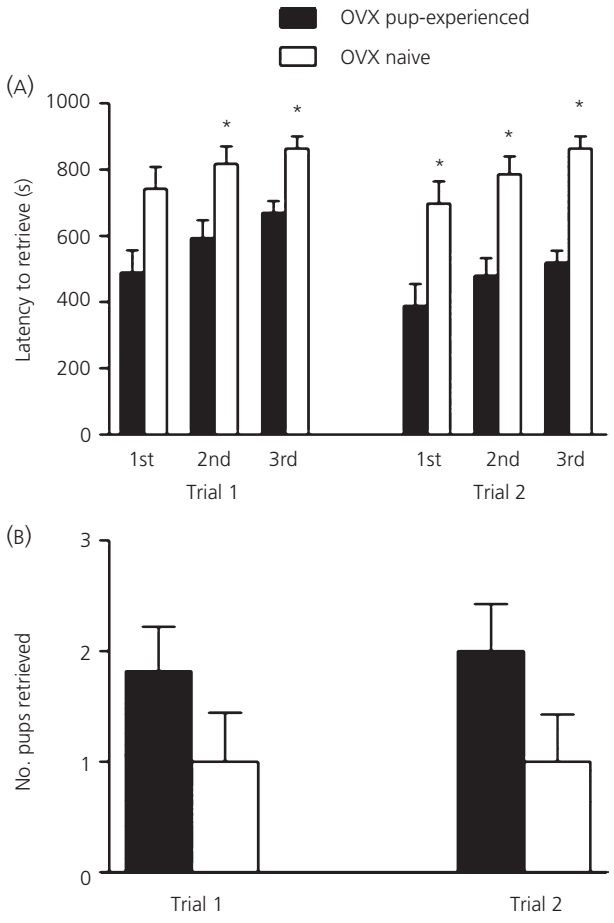

Fig. 7. Retrieval responses on the T-maze after 2 days of maternal behaviour testing. Pup-experienced (n = 11) females received 4 days of pup exposure 1 week before maternal behaviour testing. Pup-naive (n = 11) females were not exposed to pups prior to the 2 days of maternal behaviour testing. Pup-experienced and pup naive females were ovariectomised (OVX). Trials 1 and 2 were separated by 30 days. (A) Latency (s) to retrieve each pup from the far ends of the T-maze. (B) Number of pups retrieved from the far ends of the T-maze. *Significantly different from the pup-experienced group.

pup-experienced females (Fig. 7A). There was no effect of trial on latency to retrieve each pup. There were no differences in the number of pups retrieved (Fig. 7B).

Discussion

In the present study, we report that virgin females are slower to retrieve and crouch over pups, and also spend more time licking/grooming pups and less time crouching over pups compared to postpartum females. However, as the test day advanced, virgins retrieved pups faster, spent less time licking/grooming, and more time crouching over pups. Similar changes in maternal responding across the first few days postpartum have previously been reported in primiparous mice and, therefore, as virgin females gain more experience with pups, their behaviour more closely resembles that of a postpartum female (40). We speculate that crouching behaviour is not affected by the ability to lactate because OVX pup-experienced females in Experiments 3–4 spent similar amounts of time crouching as did postpartum lactating females in Experiment 1. Furthermore, it has been reported that crouching behaviour is not dependent on the ability to nurse because sensitised virgin rats show 'nursing' behaviour that is more similar to postpartum suckled than nonsuckled rats (41). Finally, although other studies have reported that postpartum and virgin females spend similar amounts of time with pups (14–16), the present study is the first to quantify pup-retrieval, licking/grooming, and crouching behaviour.

Unlike postpartum female rodents, virgin females do not show high levels of maternal motivation (16, 19–21, 23, 25, 30). The results of the present study are in agreement with these reports, and indicate that OVX naive females are not responsive to pups on a novel T-maze. It has been suggested that experience with pups, in the absence of hormone stimulation, is not sufficient to induce maternal motivation in virgin females (20, 23, 25, 30). However, we found that, even in the absence of circulating ovarian hormones (OVX), pup experience induced maternal motivation in virgin mice. One possibility is that differences exist in the sensitivity of different mouse strains to pup experience (20, 23, 30). In support of this idea, note that wild mice are unresponsive to pups even after multiple experiences with pups (42).

Although removal of the ovaries eliminates the majority of circulating steroid hormones, it is still possible that oestradiol synthesised in the brain (36, 43) during maternal experience could have facilitated pup retrieval on the T-maze. To rule out this possibility, we assessed maternal responsiveness in OVX ArKO mice given 4 days of pup experience (2 h per day) and then tested them on the T-maze along with OVX WT littermates. The fact that there were no significant differences in maternal responsiveness between groups in the home cage or on the T-maze indicates that the effects of maternal experience on maternal motivation were not dependent on oestradiol. Furthermore, the fact that ArKO female mice, which have not been exposed to oestradiol at any point during development, are responsive to pups in the home cage and on the T-maze suggests that oestradiol is not necessary for spontaneous maternal behaviour. The results obtained in Experiment 3 did not address whether the maternal responsiveness of ArKO mice is equivalent to mice that have been exposed to circulating oestradiol (intact virgins) or parturitional hormones (intact postpartum). On the basis of the results of Experiment 2, we speculate that the retrieval behaviour of ArKO females on the T-maze would not be significantly different from intact postpartum females. However, it is important to emphasise that intact postpartum females do not need 4 days of pup experience to show high levels of motivation (10, 20, 21, 25, 30); therefore, the presence of oestradiol would certainly facilitate maternal responsiveness. What is significant about the present data is that they are the first to demonstrate that, in B6 mice, maternal experience, even in the complete absence of oestradiol, is sufficient to induce high levels of maternal responsiveness.

The T-maze retrieval task requires that the female overcome her fear of a novel environment to retrieve pups; therefore, a relevant question is whether pup-experienced and postpartum females show a general reduction in anxiety that is related to their increased

motivation to retrieve pups on the T-maze. In the present study, we report that there are no significant differences in activity on the EPM between OVX naive, OVX pup-experienced and intact postpartum females. This finding is surprising considering that motherhood in rodents has frequently been associated with a general reduction in anxiety (1, 44), which has been attributed both to the hormonal events of parturition, as well as mother–pup interaction (45–47). It is important to note, however, that anxiolytic responses on the EPM are dependent upon continual pup exposure. For example, as little as 4 h of pup deprivation can eliminate differences in EPM behaviour between virgin and lactating rats (46). Therefore, because the EPM test was conducted 24 h after the last pup exposure, it is possible that intact postpartum females did not show the typical anxiolytic response on the EPM in that they were not continually exposed to pups. Therefore, differences in pup retrieval on the T-maze are not related to a general reduction in anxiety, although they might be related to a reduction in anxiety to pup stimuli, specifically (27).

The data of the present study indicate that ovarian hormones, particularly oestradiol, are not required for maternal responsiveness, although this does not preclude a role for peptide hormones such as oxytocin, vasopressin and prolactin, all of which can affect maternal behaviour through direct actions on the brain (6, 43, 48–59). However, it is important to emphasise that the facilitatory effects of oxytocin and prolactin on maternal behaviour are dependent upon intact ovaries or the co-administration of oestradiol (8, 49, 52, 56). Therefore, although these hormones play an important role in maternal behaviour, it is unlikely that the facilitatory effects of pup-experience in OVX mice are related to peptide hormones.

In Experiment 4, we addressed two questions about the effects of maternal experience on maternal motivation. First, how many days of pup exposure are necessary to produce effects on maternal motivation and, second, are the effects on maternal motivation long lasting? The results of Experiment 4 indicate that, although 2 days of pup exposure facilitates pup retrieval in the home cage, OVX naive females with 2 days of maternal experience did not respond to pups on the T-maze. Furthermore, 1 month after the initial exposure, OVX females that had a total of 6 days of pup experience (2 h per day) continued to respond to pups on the T-maze, whereas females with 2 days of pup experience (2 h per day) were still unresponsive. Therefore, subtle differences in pup exposure can influence the duration of experience-induced modifications in maternal behaviour.

The experience of interacting with pups likely produces modifications in gene transcription and the neural circuits that regulate maternal responsiveness so that, during subsequent maternal interactions, pup stimuli come to elicit maternal responsiveness more effectively. The medial preoptic area (MPOA) is the critical neural region that responds to hormonal and sensory inputs from pup stimuli to affect the display of maternal behaviour (22, 60–69). Importantly, MPOA neurones show increased responsiveness as measured by increased Fos gene (70) and Fos protein expression (12) when virgin or OVX female mice are interacting with pups. MPOA interaction with the mesolimbic dopamine (DA) system is critical for maternal responsiveness as well as the consolidation of maternal experience (71). Pup-experienced rats show significantly more DA release into nucleus accumbens in response to pups than naïve females (72) and blockade of DA D1 and D2 receptors in nucleus accumbens inhibits the consolidation of maternal responsiveness, such that DA antagonist treated females require significantly more pup exposure to induce maternal behaviour than control females (73). The mesolimbic DA system is also involved in maternal responding in mice. For example, maternal behaviour can be reinstated in olfactory bulbectomised mice following systemic treatment with apomorphine, a DA receptor agonist (74), and dopamine transporter knockout mice that are constantly exposed to supraphysiological amounts of DA show severe impairments in maternal behaviour (75). Furthermore, hyperactivity of the mesolimbic DA system is associated with naturally occurring maternal neglect in some strains of mice (76).

Although it is likely that the same neural circuits regulate maternal responsiveness in rats and mice, it is possible that the mechanisms through which maternal experience is consolidated are different. For example, in rats, the experience of interacting with pups facilitates subsequent maternal responsiveness by increasing the activity of neural circuits that regulate approach, as well as by decreasing activity in circuits that regulate pup avoidance (1). By contrast, in mice, the occurrence of spontaneous maternal behaviour suggests that neural circuits that regulate maternal responsiveness are already sensitive to pup stimuli. Therefore, experience with pups might specifically increase the sensitivity of neural circuits that regulate maternal motivation. This might explain why subtle differences in experience can impact maternal responsiveness in mice but not in rats. The molecular mechanisms through which maternal experience modifies these pathways will be resolved by our ongoing research.

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