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Predation threat exerts specific effects on rat maternal behaviour and anxiety-related behaviour of male and female offspring

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ABSTRACT

Differences in the rate of maternal behaviours received by rodent offspring are associated with differential programming of molecular and behavioural components of anxiety and stress-related functions. To determine the degree to which maternal behaviours are sensitive to environmental conditions, Long–Evans rat dams were exposed to the odour of a predator (cat) at two different time points during the first week postpartum. Exposure on the day of birth (DOB), but not the third day following birth, increased levels of maternal care in predator-exposed dams relative to dams exposed to a control condition across the first 5 days post-partum. As adults, female offspring of dams exposed on DOB exhibited a less-anxious phenotype in a novel open-field, spending more time in the center and less time displaying thigmotaxis. In contrast, under the same conditions, male offspring showed the opposite behavioural response, consistent with an increasingly anxious phenotype. Results from a subsequent stressor test (response to a predator odour) were consistent with the notion that the rearing effects were specific to anxiety-related behaviours in offspring. Accordingly, we showed that rearing conditions did not affect GR mRNA or NGFI-A expression in the hippocampus of offspring or cross-fostered offspring. The dissociation between stress and anxiety, as well as the sex-specific alterations in behaviour, may reflect the specificity inherent to neural programming in the face of naturalistic early life conditions.

1. Introduction

As newborns, rodent pups perceive limited sensory information from their environment and depend heavily on their mother for this information. During the first two weeks of life, pups depend on the mother for nutrients and tactile stimulation (collectively referred to as maternal care) for survival. Not surprisingly, variations in maternal care are thought to be an essential link between the environment and the programming of neural systems during this early critical period of development [1–3]. In rodents, maternal behaviour is characterized primarily by licking and grooming (LG) and arched-back nursing (ABN). Differences in rates of LG-ABN have been shown to regulate the development of endocrine, emotional, and cognitive responses to stress [4–6].

A number of years ago, it was demonstrated that a neonatal rat 'handled' daily, (i.e. removed from its mother and placed in a new cage for 15 min; early handling (EH)) exhibited decreased stress reactivity in adulthood; this is the basis for much of the work examining effects of alterations in early life on offspring development [7]. These responses were hypothesized to be the consequence of increased maternal behaviour from the dam towards the pups upon their return, rather than a consequence of the stimulation received from the procedure itself. Subsequent studies have shown variations in maternal behaviours occur naturally in the rodent population and that these traits follow a normal distribution [8]. Female rats that show high rates of LG and ABN (i.e. greater than one standard deviation above the mean) produce the same behavioural and endocrine profile in the offspring as EH when compared to females that show low levels of LG-ABN (i.e. greater than one standard deviation below the mean) [8,9].

As adults, the offspring of mothers who exhibit low levels of LG-ABN show significantly increased behavioural fearfulness in a variety of paradigms (e.g. activity/eating in novel environment, shock-probe burying test) [5,10]. These differences in fear reactivity are associated with a reduction in $GABA_A$ receptor density and binding in amygdaloid nuclei as well as in the locus ceruleus [5,11,12]. When presented with an acute stressor, offspring raised by high LG-ABN mothers show decreased CRF mRNA expression in the PVN as well as reduced plasma ACTH and corticosterone (CORT) when compared to offspring of low LG-ABN mothers [6]. These changes in the hormonal response to stress are partly a result of enhanced negative feedback in the form of increased glucocorticoid receptor (GR) mRNA levels within the hippocampus [6]. When taken together, these results suggest that maternal care serves to

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program neural circuits mediating fear and stress responding and this is manifested in adulthood as a change in vulnerability to stressful stimuli.

Despite the fact that individual differences in rodent maternal behaviour appear to be highly stable under standard laboratory conditions (i.e. high LG-ABN dams remain as such across multiple litters and this trait is stably transmitted from one generation to the next), there is significant evidence to suggest that maternal behaviour is sensitive to manipulation [5,8,9,13–17]. For instance, when exposed to intermittent gestational stress, dams that had previously been classified as high LG-ABN mothers reduce the levels of active maternal care towards their subsequent litters [13]. Similarly, Macaque mother–infant dyads, maintained under variable foraging demand conditions (where time/effort required to obtain food is unpredictable), display significant disruptions in mother–infant interactions compared to those maintained under low-demand conditions. The repercussions of these events were evident well into adolescence with offspring showing increased anxiety and decreased social interactions [18].

Given that maternal care regulates the development of a number of neural and behavioural parameters, and natural variations in maternal behaviours exist, variation in maternal care may serve to shape offspring development in accordance with the demands of the current environment. While rat mothers face a number of potential environmental challenges (e.g. food limits, weather, quality of potential nest sites), the factor that is most significant to them and their young is predation [19]. Because of the importance of detecting predators prior to contact, adult rats display well-characterized behavioural [20–22] and physiological [23–25] responses to the odours of various predators. We previously hypothesized that predation threat on the day of birth would alter maternal responses to offspring. In accordance with this hypothesis, a one-hour presentation of predator (cat) odour on the day of birth increased both LG and ABN across the first 5 days of lactation [17]. Natural populations of predators and prey vary in cycles, with one predator population cycle encompassing more than one prey population cycle [19,26] meaning that the levels of predation threat experienced by adult rat offspring would likely be predicted by the levels incurred during the perinatal period. Under such circumstances, predation threat-induced changes in maternal care could serve as a barometer for current environmental conditions and alter pup development so that offspring could deal more effectively with the threat of predation in adulthood.

In the present study we explored the developmental consequences of predation threat-induced maternal behaviour on the development of stress responses and anxiety in adult offspring of both sexes. We also examined the specificity of the parturient period for sensitivity to predation threat-induced changes in maternal care.

2. Methods

2.1. Animals and husbandry

Long–Evans hooded rats were purchased from Charles River Canada (St. Constant, Quebec). Throughout all experimental procedures, rats were housed in a standard colony room in Plexiglas cages (22×21×44 cm) with wire lids and free access to food and water. Cages were lined with wood shavings and provisioned with a piece of black PVC tubing for environmental enrichment. The colony room was maintained under a 12 h L:D cycle with lights OFF at 0930 h and temperature maintained at 21±2 °C. Cage maintenance was performed by the same person, once per week, at 0830 h. Before being paired for breeding, animals were housed in same-sex pairs but while pregnant, females were housed singly. All research procedures were performed in accordance with guidelines proposed by the Canadian Council on Animal Care and approved by the Dalhousie University Committee on Laboratory Animals.

2.2. Breeding and experimental exposure conditions of dams

Approximately 3 weeks after arrival, virgin females were bred with sexually experienced males. During breeding, males were removed from their home cage and placed into the females' cage, where they remained for five days. At the end of day five, the males were returned to their home cage where they spent at least two days between pairings with the two batches of females. Dams were checked daily for litters beginning on GD20 and only females found with litters before 1000 h and containing 9–13 pups were used in the present experiments. All breeding and experimental exposure procedures (described below) were identical for the three experiments.

Fig. 1 represents a schematic of the experimental timeline for the three experiments. Females were exposed to one of 2 experimental exposure conditions (predator; control) on the day of parturition (post-partum day (PPD) 0) (or on PPD3 for Experiment 1 only) and exposure on PPD0 took place within 5–6 h after giving birth. Since the predator condition involved potentially volatile biological odours, exposures could not take place within the colony room due to the risk of exposing other animals and of cross-contamination between conditions. For both predator and control conditions, at the beginning of an odour condition, the female and her pups were transported in the home cage to a separate test room. All efforts to minimize disturbing the dam and her pups were made including covering the home cage with a towel during transport, and using a test room that was in close proximity to the colony room. Dams were habituated to being transported to the test room beginning on GD16. Exposure conditions lasted for 60 min during which a 1 cm piece of cat collar (Burgham, Safety Stretch Collar; odour stimulus) was affixed to the underside of the home cage wire lid via an alligator clip. For the predator exposure, females were exposed to a 1 cm piece of cat collar cut from a collar that had been worn by a female domestic cat (reproductively active) for 2 weeks [22]. For the control exposure, females were exposed to a 1 cm piece of cat collar cut from a clean cat collar. The exposure stimulus was always placed at the end of the home cage opposite to the end where the dam had made her nest. During the 60 min exposure, rats and pups were left undisturbed. A red light illuminated the test room throughout all habituation and exposure sessions.

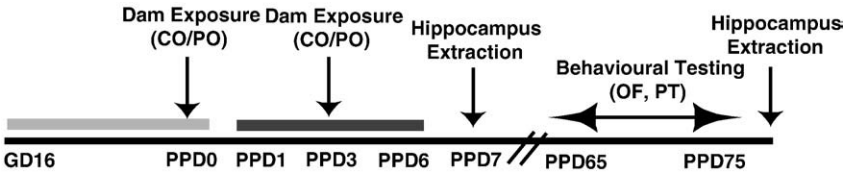

Fig. 1. Timeline of experimental procedures. F0 females were mated and beginning on gestational day (GD) 16, were habituated for 20 min each day until parturition (postpartum day (PPD) 0) to an odour exposure test room (light grey bar). On PPD0, approximately 5–6 h following giving birth, dams and litters were moved to the exposure room and exposed for 60 min to an odour condition (PO — predator odour; CO — control odour). Following odour exposure on PPD0, some pups were cross-fostered onto dams that had been exposed to the other odour condition and some were sham cross-fostered (cross-fostered back onto same condition). For Experiment 1 only, some dams and litters were exposed on PPD3. For all dams, maternal behaviour was observed daily in within the colony room from PPD1–5 (dark grey bar). On PPD7, hippocampus was extracted from pups. Others were grown to adulthood so that behaviour in a novel open-field (OF) and predator odour (PT) stress test could be assessed. At the completion of behavioural testing, adults were sacrificed and hippocampus removed.

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Care was taken to avoid contamination of exposure conditions and to preserve the integrity of the cat odour. Following collection of the collars from the cats, collars were placed in double ziplock bags and frozen at −20 °C until time of use. Collars were warmed to room temperature before use. Control collar was treated identically except that it was frozen following removal from the package and briefly handled by the experimenter.

2.3. Experiment 1 — maternal behaviour of dams exposed to acute predator odour exposure early in lactation

2.3.1. Measurement of maternal behaviour

Maternal behaviour was recorded from PPD1 through PPD6 as performed previously in our lab [17]. Approximately half of the dams were exposed on PPD0 to predator (n=5) or control (n=6) and the rest were exposed on PPD3 (n=7 to control and n=6 to predator). For those exposed on PPD3, the 60 min exposure took place in the morning prior to any maternal behaviour recording. Thus, for the PPD3 dams, maternal behaviour recorded on Days 1–2 represents pre-exposure behaviour (ex. PPD1–2), while maternal behaviour recorded on Days 4–6 represents post-exposure behaviour.

Dams and pups were observed during four ten-minute observation periods each day within the home cage in the colony room during the subjective night of the rat, under red illumination. Two observations were made between 0930 and 1300 h and two were made between 1400 and 1900 h each day for each animal. The frequency of licking and grooming (LG) of pups and arched-back nursing (ABN) events displayed was measured. LG was defined as behaviour made by the dam toward the pup including licking (i.e., licking while the dam is crouched over her pups; licking a single pup while holding it in her paws) [9]. LG was scored as bouts with a bout being defined as a continuous sequence (at least 2 s) of licking and grooming behaviours with pauses of no greater than 2 s (e.g. a greater than two second delay in such behaviours would result in 2 bouts being recorded). ABN was defined as a nursing posture characterized by the dam being immobile over the litter with her back conspicuously arched (high upright dorsal arch posture) and her lags splayed outwards [9,27]. ABN was distinguished from blanket nursing (i.e. dam displaying low dorsal arch posture) and passive nursing (i.e. dam laying beside pups) [27]. During each of the four daily observation sessions, trained experimenters (blinded to exposure condition) recorded LG and ABN continuously for the 10-minute period for each animal in turn. For each dam and for each behavioural measure, a total daily score was computed by summing across the 4 separate observation periods each day. The effect of acute predation threat on the maternal care of F0 dams was assessed using a separate mixed-design analysis of variance (ANOVA) for each dependent measure. Exposure condition (control, predator) was treated as a between-subject factor while postpartum day (PPD1–2, PPD3–4, and PPD5–6) was treated as the within-subject factor.

Other time in contact with pups was also recorded and was defined as any time that the dam was located in the nest area in physical contact or close proximity to the pups (but not licking or grooming them) or when the dam was nursing but not in the ABN posture. There was no difference between control and predator-exposed dams in other contact (defined as nursing that is not ABN, retrieving, sitting and standing on pups), as reported previously [17].

2.4. Experiment 2 — NFGI-A nerve growth factor inducible-protein A (NGFI-A) mRNA levels in the hippocampus of PND7 offspring of predator-exposed dams

2.4.1. Cross-fostering of offspring

Because we exposed the dam to the PO in the home cage with the newborn pups present, a concern was potential direct effects of the odour upon pup development. A PO-induced fear response in the neonate was not considered an issue because such responses have been undetectable in neonates until PND14 due to the lagging development of the amygdala (a critical component of the PO-induced stress/fear circuit) [28]. Nonetheless, we could not ignore the fact that olfactory processing occurs at this time point [29] and the unique odour associated with the PO stimulus could potentially have long-lasting effects on the development of the HPA axis over and above the changes in maternal behaviour coming from the dam.

Breeding and exposure of dams to CO and PO were described above. To discriminate effects of the exposure condition (CO, PO) on pups and indirect effects mediated through the dam (via changes in maternal behaviour, or other means), approximately 1 h following the exposure condition on PND0, 3 pups (a combination of 2 male and 1 female, or 2 females and 1 male) were cross-fostered to a dam that was exposed to the same (i.e. CO to CO or PO to PO) condition or to a dam that had been exposed to a different condition (i.e. CO to PO or vice-versa) to generate sample sizes of 6 for each cross-fostering condition. Handling of pups alone has been shown to affect maternal behaviour [9]. To circumvent any non-specific effects due to the cross-fostering procedure, one pup was always removed with the 3 cross-fostered pups but replaced to its original litter (sham cross-fostered). For identification purposes, cross-fostered animals were tattooed on their right front paw to distinguish between cross-fostered, sham cross-fostered (who were received a tattoo mark on their left front paw) and non-cross-fostered littermates (no tattooing). This procedure was performed by an experienced experimenter and took approximately 20 s for each pup. Pups were returned to the dam immediately following the tattooing and remained there until PND7 when they were sacrificed.

2.4.2. Extraction of PND7 hippocampus and NGFI-A mRNA expression analysis

Pups of dams exposed to CO or PO or cross-fostered on PPD0 were removed from dams as a group, and placed under a 60 W lamp. Each pup was quickly decapitated with a sharp pair of large scissors. Following decapitation, brains were quickly removed and placed onto a piece of Plexiglas kept cold on dry ice. The hippocampus was microdissected by making a sagittal incision along the midline to separate the two hemispheres. The cortical structures were peeled away to reveal the hippocampus, which was then dissected and flash-frozen in liquid N_2. Samples were maintained at −80 °C until RNA extraction and subsequent quantitative PCR (qPCR).

Frozen rat brain samples (one hemisphere only; randomly selected from each brain) were homogenized using a Polytron homogenizer and total RNA was isolated using an RNeasy Mini Kit as per the supplier's (Qiagen Inc., Mississauga, ON) instructions. Total RNA (1.5 µg) was reverse transcribed using Stratascript RT reverse transcriptase with random hexamers $pd(N)_6$ according to the supplier's (Stratagene, Cedar Creek, TX) instructions, with the exception that a final dNTP concentration of 1 mM was used. A 1.5 µL aliquot of the reverse transcription reaction was used as a template for quantitative PCR using a Stratagene MX3000p thermocycler in a total volume of 20 µL with Brilliant SYBR Green QPCR Master Mix (Stratagene). The following pairs of primers were used for quantitative PCR analysis:

rCYPHA qPCR-F1: 5′-ATGGTCAACCCCACCGTGTTCTTC-3′
rCYPHA qPCR-R1: 5′-ATCCTTTCTCCCCAGTGCTCAGAG-3′
rNGFI-A F1: 5′-GAACAACCCTACGAGCACCT-3′
rNGFI-A R1: 5′-AGTGTTGCCACTGTTGGGT-3′.

Thermal cycling conditions were identical for each primer pair and were as follows: a single cycle of 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 18 s, and elongation at 72 °C for 30 s. Melting curves were generated from 60 °C to 94 °C at the end of the PCR protocol to ensure the amplification of a

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single product. The PCR products were then separated on a 2.5% agarose gel and visualized by ethidium bromide staining to ensure the formation of a single product at the appropriate size. The product size for NGFI-A was 165 bp and CYPHA — 136 bp. Relative C_T values were obtained by the $\Delta\Delta C_T$ method [30] using a threshold of 10 standard deviations above background for C_T.

2.5. Experiment 3 — stress responding in adult offspring reared by a dam exposed to predator odour on the day of giving birth

2.5.1. Housing of offspring to adulthood

Offspring of dams that were exposed to control and predator odour on PPD0, as described above, were grown to adulthood. Offspring were weaned on PND21, housed in same-sex groups of 2 or 3, and left undisturbed, except for weekly cage changing, until early adulthood (PND 65–75). The experimental subjects were randomly selected from 6 control-exposed dams and 5 predator-exposed dams to create the following groups: males reared by control-exposed dams (CE-males; $n=9$), females reared by control-exposed dams (CE-females; $n=8$), males reared by predator-exposed dams (PE-males; $n=9$), and females reared by predator-exposed dams (PE-females; $n=7$). Each group contains pups from 3–4 different dams.

2.5.2. Behavioural testing of males and female offspring in adulthood

Rats were familiarized with being handled by the experimenter in the colony room once every day for 7 days prior to initiating the behavioural testing procedures. Handling involved picking up each rat and placing it onto the arm of the researcher for approximately 2 min, and then returning it to its home cage.

2.5.2.1. Open field. Anxiety-like behaviour was assessed using a 20-minute exposure to a novel open-field (OF) apparatus. Some data were lost to experimenter error for the open-field test and sample sizes were as follows: CE-males, $n=6$; CE-females, $n=6$; PE-males, $n=9$; PE-females, $n=7$. The OF apparatus (35.5 cm h, 79 cm w, and 79 cm l) was made of 1/4-inch black Plexiglas marked with a set of gridlines that divided the arena into nine equal areas and was covered with a clear Plexiglas lid containing ventilation holes. The following behaviours were quantified: rate and duration of rearing (standing on hind paws with body stretched upwards, unsupported by a wall), duration of thigmotaxic behaviour (body, including fur, in contact with the wall), duration of time spent, in center area (i.e., the centermost square), and spontaneous activity (as measured by line cross frequency across all areas of the open field). Due to technical difficulties, rearing behaviour could not be reliably scored for 3 PE-females.

2.5.2.2. Predator odour test (PT). Approximately one week following the OF testing, rats were exposed to a CO condition followed by a PO condition the following day. Exposures took place in a clear rectangular Plexiglas test arena (30 cm h×80 cm l×39 cm w), which was attached to a hide box (30 cm h×27 cm l×39 cm w) that was built from 3 black opaque walls (to increase darkness) and one clear wall to allow for observations [31]. The hide box contained an opening (7 cm×7 cm) through which the animal could enter and exit. To ensure that rats were familiar with the testing apparatus prior to the beginning of the experiment, rats were placed individually into the test arena containing the hide-box (without any odour stimulus) for 20 min one day prior to the CO exposure.

During experimental trials (CO and PO exposure), the hide-box was placed at the end of the arena opposite to where the odour stimulus was placed. PO exposure occurred via a strip of j-cloth (2.5 cm×15 cm) containing cat hair and dander. Each strip was coated with a thin layer of hair obtained fresh each day from 2–4 reproductively active, domestic cats residing in a colony room in the department under controlled conditions. The CO exposure involved presenting a clean strip of j-cloth in an identical manner. At the beginning of a stressor session rats were placed individually into the center of the testing arena facing the odour stimulus. The following non-defensive and defensive behaviours were quantified: line cross frequency (exploratory, non-defensive; the sum of lines crossed for all areas), rearing rate and duration (exploratory, non-defensive; front legs leave contact with the floor of the arena), head-out (defensive; the head of the rat, including the eyes, protrudes from the hide box for a minimum duration of 2 s), and odour stimulus contact (exploratory; bodily contact (excluding tail) with the odour source). The testing arena was divided into four virtual areas for quantification of time spent in each. In addition to the hidebox, there were 3 equal sized areas termed A3 (closest to hidebox), A2, and A1 (odour area; contained the odour stimulus at the opposite end of the arena from the hidebox). Data for one PE-female were lost due to experimenter error.

All behaviours elicited in the OF and PT sessions were recorded using a Sony 8 mm digital camera (CCD-TRV65 or CCD-TRV108) suspended above each test arena. Behaviours were later quantified with the aid of The Observer 5.0.31 software (Noldus, Netherlands), for the first 10 min of the 20 min OF session, and for the first 7 min of each of the 15 min PT sessions (CO and PO). All testing took place during the dark phase of the light:dark cycle (between 0930 h and 1800 h) under red illumination.

2.5.3. Extraction of hippocampus for glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA expression analysis

Separate CE- and PE-males and females ($n=6$/group) were also used for determining the long-term effects of maternal PO exposure on levels of GR and MR gene expression in hippocampus. Animals were killed by CO_2 asphyxiation followed by decapitation approximately one week following the PT. Brains were quickly removed and placed onto a piece of Plexiglas kept cold on dry ice, using a procedure similar to that described above for the removal of neonate hippocampus.

Parameters for qPCR were the same as those described earlier with the following primer pairs used:

rGR qPCR-F1: 5′-GCTTCAGGATGTCATTACGGGG-3′
rGR qPCR-R1: 5′-GCTTCAAGGTTCATTCCAGCC-3′
rMR F1: 5′-TCGGCGAAAGAACTGTCCTG-3′
rMR R1: 5′-TTGGTCGGAGCGATGTATGT-3′
rCYPHA qPCR-F1: 5′-ATGGTCAACCCCACCGTGTTCTTC-3′
rCYPHA qPCR-R1: 5′-ATCCTTTCTCCCCAGTGCTCAGAG-3′.

Product sizes were as follows: GR — 188 bp, and MR — 183 bp and again, relative C_T values were obtained by the $\Delta\Delta C_T$ method [30] using a threshold of 10 standard deviations above background for C_T.

3. Results

3.1. Experiment 1 — maternal behaviour

In order to determine whether there were differences among the four groups with respect to frequency of LG and ABN, a mixed-design ANOVA was performed separately for each dependent variable with Group (PPD0, PPD3) and Condition (CO, PO) as the between-subject factors and Day (Days 1–2, Days 3–4, Days 5–6) as the repeated measure.

The main effects of Group ($F(1,20)=10.79$, $p=0.004$) and a Group by Condition interaction ($F(1,20)=4.52$, $p=0.046$) were revealed for frequency of LG. Subsequent one-factor repeated measures ANOVAs were performed for each Group separately and these analyses revealed that females exposed to PO on PPD0 engaged in more LG than those exposed to CO ($F(1,9)=19.07$, $p=0.002$; see Fig. 2A). There were no significant effects in females exposed on PPD3 (see Fig. 2C).

A significant Group by Condition interaction ($F(1,20)=5.95$, $p=0.024$) was also revealed for frequency of ABN events. Subsequent

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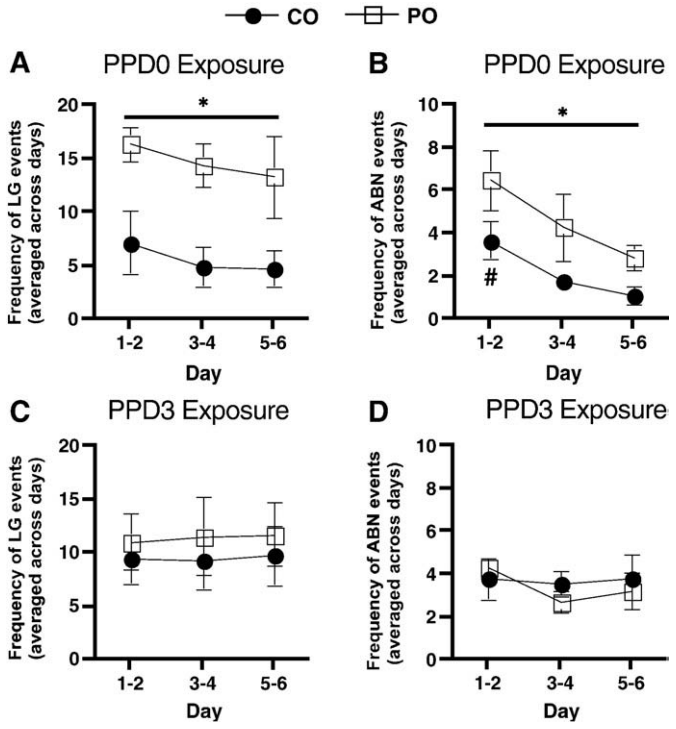

Fig. 2. Maternal behaviour is increased by predator odour (PO) exposure on post-partum day (PPD) 0 but not PPD3. Frequencies of licking/grooming (LG) bouts (A) and arched-back nursing (ABN) events (B) were significantly increased for 6 days in dams exposed to PO on PPD0 relative to those exposed to control odour (CO). A similar exposure in other dams exposed on PPD3 did not result in increased LG or ABN frequencies (C–D). *Significantly different from PO-exposed dams. #Significantly different from days 5–6.

one-factor repeated measures ANOVAs performed for each Group separately revealed that females exposed to PO on PPD0 performed more ABN events relative to females exposed to CO ($F(1,9)=7.34$, $p=0.024$; see Fig. 2B). In dams exposed on PPD0, there was also a main effect of Day ($F(2,18)=6.52$, $p=0.007$) and simple effects analyses showed that PPD0 females displayed fewer ABN events on Days 3–4 and Days 5–6 relative to Days 1–2 (p's=0.04 and 0.02, respectively). Similar to LG, there were no significant effects in females exposed on PPD3 (see Fig. 2D).

3.2. Experiment 2 — PND7 hippocampal NGFI-A mRNA expression

In order to determine whether there were effects of maternal exposure condition on NGFI-A mRNA levels in non cross-fostered neonate male and female offspring, a two-factor ANOVA was performed with Sex and Maternal Exposure (control-exposed vs. predator-exposed) as between-subject factors. Male and female pups that were cross-fostered were analyzed using a one-way ANOVA with Cross-fostering condition as a between-subjects factor. Significant interactions were followed with unpaired Student's t-tests.

There were no significant effects of Sex, $F(1,20)=3.25$, $p=0.09$, or Maternal Exposure $F(1,20)=0.10$, $p=0.76$, or an interaction, $F(1,20)=0.12$, $p=0.74$, on hippocampal NGFI-A mRNA expression levels (Fig. 3A). Analysis of cross-fostered offspring revealed no differences in NGFI-A mRNA levels in either male, $F(3,19)=0.30$ (Fig. 3B), $p=0.83$, or female, $F(3,19)=0.29$, $p=0.83$ (Fig. 3C), offspring in any of the cross-fostering conditions.

3.3. Experiment 3 — offspring behaviour in adulthood

3.3.1. Open-field behaviour

In order to determine whether there were effects of maternal exposure condition on anxiety-like behaviour of adult male and female offspring, a two-factor ANOVA was performed separately for each dependent variable from the OF test with Sex and Maternal Exposure (control-exposed vs. predator-exposed) as between-subject factors. Significant interactions were followed with unpaired Student's t-tests.

The rate of rearing was not affected by sex or maternal exposure condition (data not shown). However, rearing duration was affected by both (Sex by Maternal Exposure interaction ($F(1,21)=12.84$, $p=0.002$; see Fig. 4A). Subsequent analyses revealed that PE-females spent significantly more time rearing relative to PE-males and CE-females ($p=0.006$ and $p=0.006$, respectively). Spontaneous activity, in the form of line cross frequency, was affected by both factors. There was a significant Sex by Maternal Exposure interaction ($F(1,24)=7.29$, $p=0.013$; see Fig. 4B) and subsequent analyses revealed that both CE-females and PE-males displayed higher levels of line crosses relative to CE-males ($p=0.001$ and $p=0.005$, respectively).

Time spent engaged in thigmotaxis behaviour was significantly impacted by maternal exposure condition and sex as well (interaction, $F(1,24)=30.16$, $p<0.0001$; see Fig. 4C). CE-males displayed lower levels

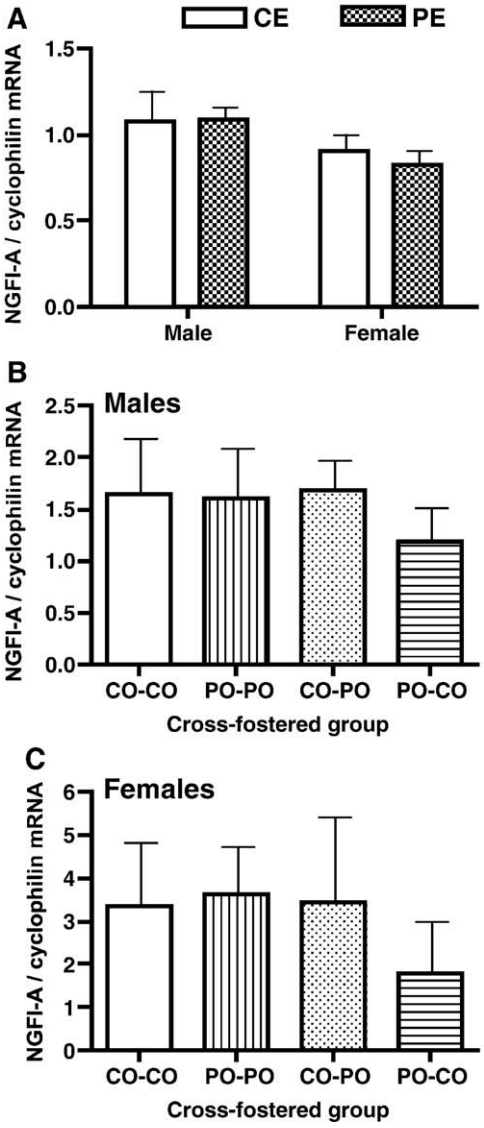

Fig. 3. Levels of NGFI-A mRNA were similar in PND7 hippocampus of males and females raised by control-exposed (CE) and predator-exposed (PE) dams (A). Additionally, offspring cross-fostered from dams exposed to control odour (CO) to those exposed to predator odour (PO), and vice versa, did not show differential levels of NGFI-A mRNA in hippocampus on PND7 (B–C).

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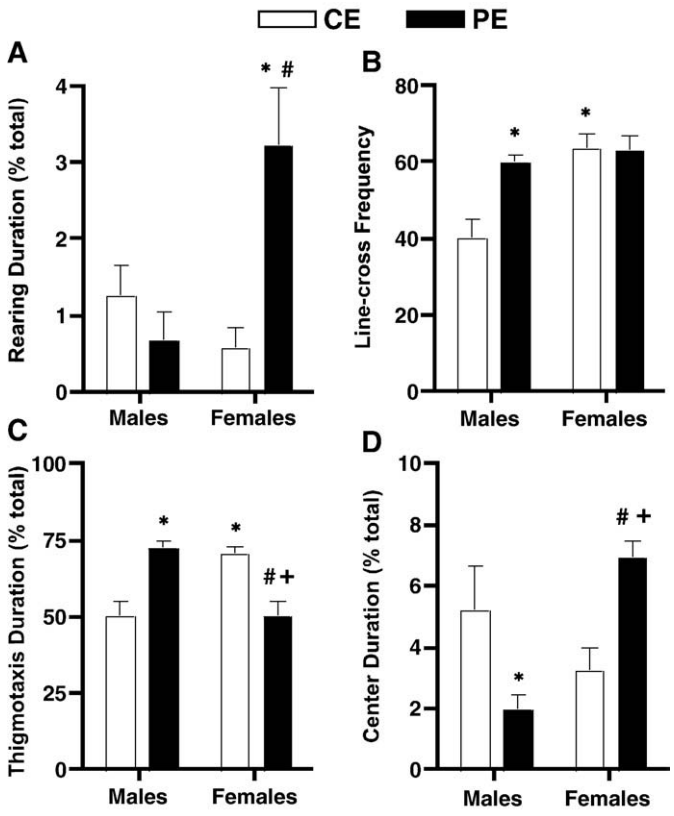

Fig. 4. Offspring reared by a predator-exposed (PE) dam displayed altered sex-specific changes in anxiety-like behaviour in a novel open-field (OF) relative to offspring raised by a control-exposed (CE) dam. Females reared by PE dams displayed more exploratory behaviour (A), less thigmotaxis (C) and increased time in the center (D) of the OF, indicative of less anxiety, relative to females raised by CE dams. In contrast, male offspring raised by PE dams displayed significantly greater hyperactivity (B) and thigmotaxis (C) and spent less time in the center (D) of the OF, suggestive of increased anxiety, relative to males raised by CE dams. *Significantly different from CE-males, #significantly different from PE-males, and +significantly different from CE-females; $p<0.05$ for all.

of thigmotaxis relative to CE-females ($p=0.004$) and PE-males ($p=0.002$), while PE-females displayed lower levels relative to PE-males ($p=0.03$) and CE-females ($p=0.003$).

The results for thigmotaxis were complementary to what was observed for duration of time spent in the center of the test arena. Again, a significant Sex by Maternal Exposure interaction was found ($F(1,24)=18.09$, $p=0.0003$; see Fig. 4D). Time spent in the center of the apparatus was lower in CE-females relative to PE-females ($p=0.002$) and PE-males spent less time in the center relative to both CE-males ($p=0.03$) and PE-females ($p<0.0001$).

3.3.2. Behaviour in the predator odour test (PT)

Dependent measures quantified for the PT were subjected to separate mixed-design ANOVAs with Sex and Maternal Exposure (control-exposed (CE), predator-exposed (PE)) as the between-subject factors and Odour (control odour (CO), predator odour (PO)) as the within-subject factor. Significant interactions were followed with paired or unpaired Student's *t*-tests, as appropriate.

3.3.2.1. Line cross frequency. Rats crossed significantly fewer lines during exposure to PO relative to CO (Odour main effect, $F(1,28)=47.58$, $p<0.0001$). A significant Maternal Exposure by Odour interaction ($F(1,28)=4.38$, $p=0.046$; see Fig. 5A) was found and subsequent analyses revealed that CE animals exposed to PO showed significantly less activity relative to those exposed to CO ($p=0.0004$). Similarly, PE animals exposed to PO showed significantly fewer line crosses than PE animals exposed to CO ($p=0.0005$). There was also a significant Sex by Odour interaction ($F(1,28)=4.87$, $p=0.036$) and subsequent analyses revealed that both males and females exposed to PO displayed fewer line crosses than males and females exposed to CO ($p=0.0006$ and $p=0.0005$, respectively).

3.3.2.2. Hidebox duration. The duration of time (percentage of total time) spent hiding was significantly increased during exposure to PO (Odour main effect, $F(1,28)=32.66$, $p<0.0001$; see Fig. 5B) relative to CO exposure. There were no significant main effects or interactions involving Maternal Exposure for the duration of time spent hiding.

3.3.2.3. Head-out duration. The duration of time (percentage of total time) spent in the head-out position was significantly greater during exposure to PO relative to CO (Odour main effect, $F(1,28)=34.72$, $p<0.0001$; see Fig. 5C).There were no significant main effects or interactions involving Maternal Exposure for duration of head-out behaviour.

3.3.2.4. Odour stimulus contact duration. There was a significant main effect of Maternal Exposure for duration of time spent in contact with the odour stimulus ($F(1,28)=6.27$, $p=0.018$). PE animals spent less time in contact with the stimulus relative to CE animals (see Fig. 5D). There was also a significant interaction between Sex and Odour ($F(1,28)=4.96$, $p=0.034$) and subsequent analyses revealed that in females only, exposure to PO reduced contact with the odour stimulus relative to CO exposure ($p=0.03$).

3.3.2.5. Odour area (A1) duration. There was a significant main effect of Maternal Exposure for duration of time spent in the odour area ($F(1,28)=4.90$, $p=0.035$; see Fig. 5E). PE animals spent less time in the odour area relative to CE animals. In addition to a significant main effect of Odour ($F(1,28)=19.01$, $p=0.0002$), there was a significant Sex by Odour interaction ($F(1,28)=7.49$, $p=0.011$). Subsequent analyses revealed that for females only, the amount of time spent in the odour area was significantly lower during exposure to PO relative to CO ($p=0.001$).

3.3.2.6. Center area (A2) duration. The duration of time spent in the center area was significantly greater in PE animals relative to CE animals (Maternal Exposure main effect, $F(1,28)=5.46$, $p=0.027$; see Fig. 5F).

3.4. Experiment 3 — GR and MR mRNA levels in adult hippocampus

Separate two-factor ANOVAs with Sex and Maternal Exposure (CE vs. PE) as between-subject factors were conducted for levels of each transcript.

There were no significant effects of either factor, alone or in combination, for either transcript (see Fig. 6). These results represent a replication of a prior experiment in which hippocampal samples ($n=6$/group) were generated in exactly the same manner. In this experiment, GR levels were also found to not differ among CE males (1.01 ± 0.07), PE males (0.78 ± 0.09), CE females (1.07 ± 0.15), and PE females (1.28 ± 0.17). As can be seen from the means, the pattern of results for GR is very similar to that observed in the present experiment.

4. Discussion

The main findings of the present study were: i) predation threat exerts long-term effects on maternal behaviour, but only if the exposure occurs within a few hours of parturition; ii) despite changes in maternal behaviour in dams exposed on PPD0, predicted effects on hippocampal NFGI-A or GR mRNA levels were not observed in offspring; iii) changes in behaviour in offspring reared by a predation threat-exposed dam were specific to anxiety-like changes.

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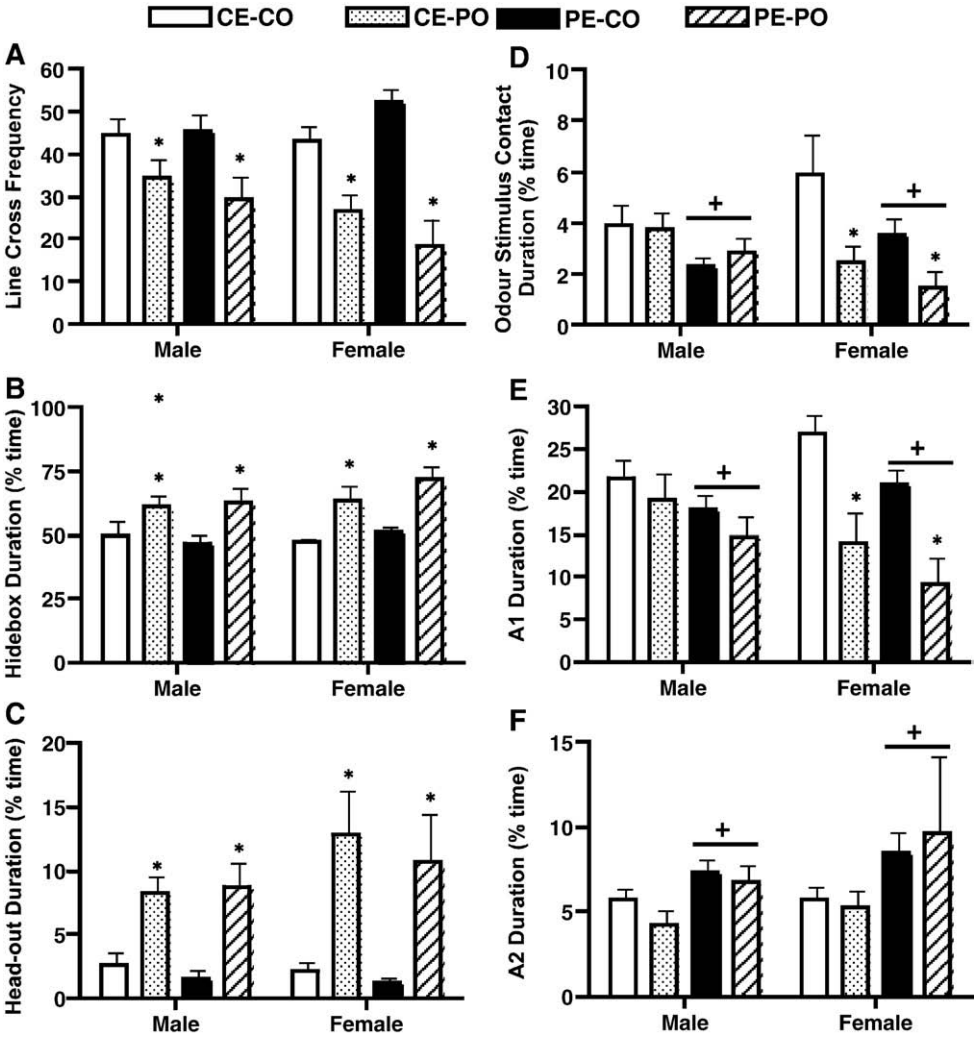

Fig. 5. Regardless of whether they were reared by dams exposed to control odour (control-exposed, CE) or predator odour (predator-exposed, PE), male and female adult offspring displayed increased defensive behaviour in response to a predator odour test. During the test, animals exposed to predator odour (PO) displayed significantly fewer line-crosses (A), spent significantly more time in the hide-box (B), and engaged in more risk assessment (head out duration) (C), relative to exposure to CO. Significant effects of rearing condition were confined to measures related to stimulus contact and space use, but were not specific to PO vs. CO. Thus, in contrast to CE animals, PE males and females spent significantly less time in contact with the odour stimulus (D), spent significantly less time in A1 (the odour area) (E), and spent significantly more time in A2 (F). *Significantly different from CO of same sex and maternal exposure, and $^{+}$significantly different from CE animals (collapsed across sex); $p<0.05$ for all.

Consistent with our previous report [17] we report that a single exposure to predation threat (cat odour) on PPD0 increases the frequency of maternal behaviours, specifically LG and ABN. This effect was specific to exposure on PPD0, as exposure to predation threat on PPD3 did not affect expression of LG and ABN. Interestingly, however, it appears that manipulation on PPD3 (control or PO) may have led to maintenance of ABN levels at a higher level across the next 3 days, as indicated by a lack of Day effect in this group (see Fig. 2). Although blunted HPA responses to stressors in lactating dams have been well documented during the postnatal week [32,33], our results suggest that the time around parturition may be particularly sensitive with respect to translating stressors into changes in maternal behaviour. This may be associated with increased oxytocin receptor (OTR) expression and activation at parturition in the female rat. Levels of OTR mRNA are significantly increased in various brain regions, including the medial preoptic area and bed nucleus of stria terminalis, both of which are important for maternal behaviour, on the day of parturition (within 90 min of birth of first pups) [34]. Although levels are lower when measured post-parturition (4–12 h following parturition) [34], in the present study, dams were exposed to predator odour within a few hours of the beginning of parturition, when levels of OTR could have been high enough to dampen acute stress

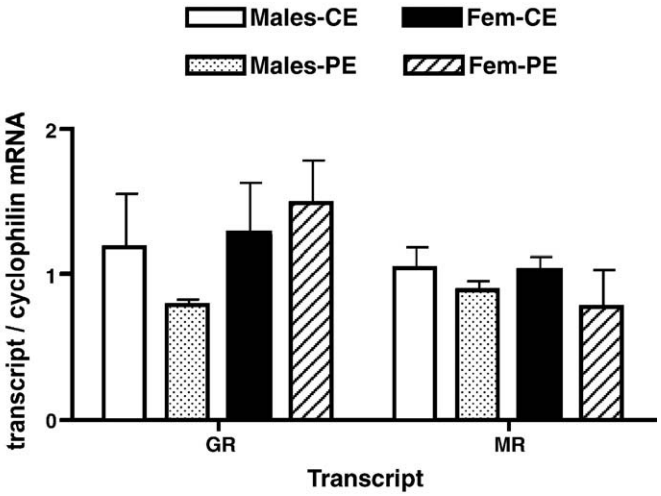

Fig. 6. Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels were similar in adult hippocampus of males and females raised by control-exposed (CE) and predator-exposed (PE) dams.

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responding [35]. Dams exposed on PPD3 would not be expected to have elevated levels of OTR.

We have shown previously that predation threat-induced increases in maternal behaviour are reliably transmitted across generations, with F1 female offspring showing high levels of maternal care towards their offspring despite not being exposed to a predation threat post-partum themselves [17]. Consistent with other work [8], we demonstrated increased ER mRNA levels in the medial preoptic area (MPOA) of F1 mothers that had received increased maternal care [17]. Our prior findings suggest that maternal behaviour is an effective conveyer of ecological information across generations, at least with respect to maternal behaviour in females. One of the objectives of the present study was to examine whether predation threat-induced alterations in maternal behaviour resulted in alterations in stress-related measures in male and female offspring.

Despite others noting alterations in GR levels in offspring raised by highly maternal dams [9,36], we did not observe increased GR mRNA in the adult hippocampus of offspring raised by PO-exposed dams, and this was consistent with the results of a previous study (Mashoodh, Sinal, and Perrot-Sinal, unpublished data). The lack of effect in GR in offspring adult hippocampus is consistent with a lack of upregulation of nerve-growth factor-inducible protein A (NGFI-A) in PND7 hippocampus. More specifically, CO-exposed neonates cross-fostered to PO-exposed dams, meaning that they experienced the increases in LG and ABN, but not the odour exposure, did not display upregulated NGFI-A mRNA levels in the developing hippocampus. This was surprising given that the tactile stimulation derived from maternal behaviour is believed to initiate a burst of excitatory neural activity triggering the activation of serotonergic neurotransmitters, which then go on to initiate cell-specific signalling cascades [37]. One consequence is the upregulation of transcription factors such as NGFI-A that can bind to the promoter region of the GR gene and ultimately program gene expression levels, through epigenetic mechanisms, that endure throughout adulthood [36,38]. Thus, NGFI-A mRNA in neonate hippocampus is a useful marker for gauging the extent to which GR levels are being 'programmed' by neonatal events. When PO-exposed neonates were cross-fostered to CO-exposed dams we failed to observe a change in NGFI-A levels, suggesting that the lack of GR 'programming' observed in adult offspring maternally exposed to PO could not be due to the opposing effects of the odour.

The discrepancy between present and past results with respect to maternal behaviour programming hippocampal GR levels in adulthood warrants discussion. Although it is possible that our results are due to low statistical power, and future studies are certainly warranted to test this possibility, there are explanations that can account for the results presented here. One issue is that LG and ABN alone are unlikely to be adequate indicators of current environmental conditions, especially considering the novelty and complexities of the peripartum period [39]. For example, although daily maternal separation (MS; 3–6 h) produces adult offspring that display higher HPA activity and heightened fearfulness [27,40–42], these effects appear to be due to differences in the imposed temporal distribution of the care, as opposed to a loss of maternal care *per se* [43]. Furthermore, the exposure to novelty necessitated by early handling (EH) paradigms has long-term consequences on offspring independent of the effects of maternal care [44]. In more ethologically relevant paradigms (described earlier), dams that spend a significant amount of time away from the litter/nest also nursed in longer, more "active" bouts [16], highlighting the importance of the duration of each bout of behaviour in addition to its frequency. Results from our previous study also highlighted the importance of duration of behaviour [17]. While the sampling procedure used here and previously [17] allowed us to detect effects of PO on maternal behaviour, it was limited by the small number of sampling sessions throughout the day, which could have resulted in missing particular aspects of the biological phenomenon exhibited by the rat dams.

The fact that rearing condition did not significantly affect adult GR levels in offspring hippocampus was consistent with a lack of effect of maternal rearing condition on typical defensive behaviour exhibited in a stress-related test (predator odour test). Given that increased maternal responsivity has been associated with decreased stress-induced HPA activation and increased GR receptor expression in the offspring hippocampus [9,45], we expected PO-induced increases in maternal behaviours to modify stress-related behaviours in a stress-based paradigm such as the predator odour test. Heightened risk assessment (i.e. head-outs), increased avoidance (i.e. hidebox time) and suppressed general activity (i.e. line crosses) following exposure to a predator odour have been classified as characteristic defensive responses to predator odour associated with activation of the HPA axis [20–23]. These behaviours were not altered in PE males or females. For a rodent whose primary concerns are survival and reproduction, the successful and swift evasion of predators encountered within the natural environment would rely on an effective behavioural response, and thus, it may be difficult to modify that. Nonetheless, there were general (regardless of whether the odour was predator or control) alterations in specific aspects of the response to predator odour in adulthood. In particular, PE animals spent less time in contact with the odour stimulus and less time in the area containing the odour stimulus (A1). Because these rearing effects were observed regardless of whether cat or control odour was present in the familiar test arena, any interpretations of these data will take into account the presence of the novel object (stimulus used to administer control or cat odour). One could argue that PE animals were more wary of the novel object and therefore, avoided it; however, PE animals also spent increased time in the center of the testing arena (A2) during both odour conditions, suggesting that they were not overly anxious. While more work is required to interpret these results, we think they are consistent with PE animals being less sensitive to the novel object.

Responding to novel environments is a critical part of adaptive strategies for dealing with environmental adversity, and therefore, we examined such behaviours in the adult offspring of dams exposed to control or PO. We demonstrated sex-specific effects of rearing by PO-exposed dams. Males reared by PO-exposed dams exhibited increased thigmotaxis and decreased time spent in the center of a novel open-field: behavioural alterations that are consistent with an increasingly anxious phenotype. In contrast, females reared by PO-exposed dams showed the opposite phenotype (less anxious). That is, they spent more time exploring the center and less time displaying thigmotaxis in the novel open field. These sex-specific changes reflect sex-specific programming of behavioural and reproductive strategies that may be critical for survival in an environment with increased threat of predation. Rodent mothers exposed to mildly challenging postnatal environments alter their maternal behaviour and modify offspring behaviour in a sex-specific manner [15,16]. Female offspring appear to be more sensitive to early environmental cues, as more subtle/modest/smaller increases in maternal behaviours (i.e. both LG and nest attendance) have the capacity to reduce anxiety-related behaviours in adult female offspring [15,16]. In male offspring, gaining independence leads to dispersal in order to reproduce whereas females remain at the nest site; therefore, sensitivity to ecological cues specified by the mother may have more adaptive value in females since they would more closely predict future environmental conditions for females than males. Adult males of many rodent species also defend a larger territory than females and an increase in predation threat could therefore have different consequences for responding to novel contexts [46].

In contrast to previous work [8,9,36], more subtle variations in the level of maternal behaviours (LG and nursing) have been shown to be predictive of responses to novelty and anxiety in the rat [47] and are thought to be important factors in producing strain differences in anxiety-related behaviours in rodents [48,49]. While there appears to be a continuum along which maternal behaviour alters anxiety-

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related (or response to novelty) behaviours in offspring, it could be that changes in maternal behaviour, specifically frequency of LG and ABN, must reach a threshold in order to affect the 'programming' of the HPA axis and thus affect stress-related functions. Supporting this hypothesis, when a mid LG (showing average levels of LG) group was introduced, only offspring reared by high LG showed decreased indices of stress during a resident–intruder test, with no observable differences between mid- and low-LG reared offspring [50]. Furthermore, selection of animals that show more subtle differences in maternal behaviour (i.e. LG and ABN that falls less than 1 standard deviation above and below the mean) failed to alter plasma CORT release following restraint stress in adult offspring [51].

In addition to pup-directed behaviours, dams are capable of passing physiological factors to their offspring (e.g. amino acids, lipids, hormones) via their milk, providing another avenue whereby the maternal environment can alter offspring development [52,53]. In the present study, this could include increased CORT levels due to exposure of the dams to the predator odour. We did not find a difference in CORT levels in breast milk between control (n=9) and predator odour-exposed (n=9) dams on PPD7 (Nelson, Mashoodh and Perrot-Sinal, unpublished data). However, we cannot rule out that levels are increased prior to PPD7 and this is something that we will investigate in the future. Indeed, previous work has demonstrated that administration of CORT via drinking water to rat dams during gestation reduces anxiety-like behaviour of female offspring [54], suggesting that increased CORT in dams exposed to predator odour may be a mechanism to consider to explain the present results. However, other work has demonstrated a similar reduction in anxiety in male offspring of dams administered corticosterone during gestation [55], suggesting that our effects may not only be mediated by CORT.

In mice, the degree of nest building activity shown by the dam is an important predictor of adult fitness and reproductive function in adult offspring [56]. Thus, the stimulation that pups receive from their mother can arise through variations in behaviours (e.g. LG, ABN, others), temporal sequences (e.g. within days and across the first week of life) and nutritional resources (e.g. composition and production of milk). The resulting combinations and permutations are likely sensitive to environmental conditions and have the capacity to alter the developmental trajectories of offspring. The increases in LG and ABN observed here may only represent a small subset of the potential changes within the maternal repertoire that occur following exposure to the PO stimulus on the day of birth that occur as a consequence of environmental complexity. Further, increases in LG and ABN may occur to compensate for maladaptive behavioural or physiological strategies (e.g. irregular feeding/foraging cycles of the dam or unusual contents of milk) that may compromise offspring development but are initiated in the presence of increased predation threat or environmental enrichment. For example, prolonged absences from, or intermittent contact with the dam, or changes in the environmental milieu (i.e. physical, thermal or olfactory challenges) may increase stress cries or ultrasonic vocalizations to which the dam must respond [57]. A more extensive analysis, including the study of many of the variables outlined above, would be useful in clarifying the dynamic interplay between environmental conditions and maternal behaviour.

5. Conclusions

The present study adds to a growing body of work indicating that maternal behaviours are plastic and can be modulated by manipulating the pre- and post-natal environments [9,13–17]. It is hypothesized that, under such circumstances, variations in maternal behaviour may serve to forecast the environmental conditions that offspring will face during the post-weaning period. Furthermore, the plasticity inherent to the neonate offspring brain suggests that it will be maximally responsive to such variations in parental behaviours, enabling offspring to be appropriately prepared for the current environment [1]. Here we show that acute predation threat increases maternal behaviour during the early post-natal period, and that long-term effects on offspring are specific to the response to novelty and anxiety-related behaviours but not stress-based responses. Together, these findings suggest that maternal behaviour has the capacity to be an effective conveyer of ecological information across generations and this transmission can be specific to particular behavioural domains. Furthermore, the parturient period appears to render female offspring particularly sensitive to such information.

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