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Learning during motherhood: A resistance to stress

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Abstract

Hormonal and emotional responses to stress are diminished during pregnancy and the postpartum period. However, the effects of stress on learning during these stages of the female life span have not been examined. In previous studies, we have reported that exposure to an acute stressful event reduces classical eyeblink conditioning 24 h later in adult virgin female rats that are experiencing an ovarian cycle. Here we show that conditioning during late pregnancy was similarly reduced by stressful experience. However, conditioning in postpartum females was unaffected by stressor exposure. The resistance to stress during the postpartum period was evident as early as 2 days after parturition and persisted until the late postpartum period, just prior to weaning. Postpartum conditioning was unresponsive to numerous types of stressors, including brief inescapable tailshocks, swim stress, and exposure to a male intruder. The resistance to stress appears to be dependent on the presence of the offspring, because the impairment in conditioning returned when postpartum females were separated from their pups. Moreover, the resistance to stress occurred in virgin females that behaved maternally after being exposed to young pups for several days. Together, these data suggest that the presence of offspring and the nurturing and care-giving activities that they elicit protect females from the adverse effect of stress on processes involved in learning and memory.

Keywords

Postpartum; Pregnancy; Estrogen; Hippocampus; Lactation; HPA; Eyeblink conditioning; Maternal behavior; Oxytocin; Anxiety; Fear; Memory

Introduction

Females are especially responsive to stressful experience and are much more likely to suffer from stress-related mental illness (Kendler, 1998; Shors and Leuner, 2003). Even in the absence of illness, the female life span is characterized by significant fluctuations in hormonal status and the response to stress. For example, pregnancy, parturition and the postpartum period are stages of life in both rats and humans marked by reduced reactivity to stressful experience, especially those related to hypothalamic–pituitary–adrenal (HPA) responses (Thoman et al., 1970; Stern et al., 1973; Lightman and Young, 1989; Altemus et al., 1995; Neumann et al., 1998; Kammerer et al., 2002; Walker et al., 2001), fear and anxiety (Fleming and Luebke, 1981; Hard and Hansen, 1985; Toufexis et al., 1999; Heinrichs et al., 2001; Neumann, 2001; Lonstein, 2005). There are also data to suggest that learning and memory can be influenced by pregnancy and/or maternal experience (Kinsley et al., 1999; Galea et al., 2000; Brett and Baxendale, 2001; Tomizawa et al., 2003; Pawluski

et al., 2006). However, the effects of stress on learning abilities during these stages of female life have not been examined.

Classical eyeblink conditioning is an associative learning paradigm in which a conditioned stimulus (CS) of white noise predicts the occurrence of a periorbital eyelid stimulation (US), which elicits an eyeblink as an unconditioned response (UR). As the animal learns that the CS predicts the occurrence of the US, it blinks in response to the CS and thus emits a conditioned eyeblink response (CR). Learning this response has been demonstrated in most mammalian species, primarily in rabbits and humans, but more recently in mice and rats (Clark et al., 2002; Thompson, 2005). This paradigm can be used to distinguish associative from nonassociative processes and can also detect nonspecific effects on performance. Also, much of the anatomical circuitry used to learn this response has been described (Thompson, 2005). For example, the cerebellum is necessary for learning the conditioned eyeblink response and the hippocampus becomes necessary when the CS and the US are separated in time, a task known as trace conditioning (Solomon et al., 1986; Weiss et al., 1999; Beylin et al., 2001). Using this task, we have shown that exposure to an acute stressful event significantly reduces the amount of classical conditioning 24 h later in adult virgin females as they are experiencing an estrous cycle (Wood and Shors, 1998; Wood et al., 2001; Leuner et al., 2004).

Here we investigated whether the detrimental effect of stressful experience on trace eyeblink conditioning is altered during pregnancy and motherhood, times when females do not experience an estrous cycle. After establishing that exposure to an acute stressor of restraint and brief inescapable tailshocks impairs trace conditioning 24 h later in virgin and pregnant, but not postpartum females, we asked the following questions about the postpartum resistance to stress. First, does the postpartum resistance generalize to other stressful experiences? Second, are the presence of offspring and the expression of maternal behavior critical for the resistance to stress during the postpartum period? Lastly, we tested whether conditioning in virgin females induced to behave maternally would be affected by stressor exposure.

General methods

Breeding and housing conditions

Animals were individually housed on wire racks with ad libitum food and water and were maintained on a 12 h light/dark cycle. For breeding, adult virgin female Sprague–Dawley rats (60–120 days old) determined to be in proestrus using a vaginal impedance meter (Fine Science Tools, Foster City, CA) were housed overnight with an adult male. The bedding under the cage was examined for the presence of a sperm plug the next morning. If a plug was found, the female was returned to her home cage and that day designated as day 0 of pregnancy (gestation day 0; GD0). Four days before giving birth (GD18), pregnant females were transferred to individual translucent Plexiglas cages (42 × 20 × 20 cm) lined with wood chip bedding and provided with nesting material. Females were observed daily and the day of birth was designated as postpartum day 0 (PD0). Litters were culled to 10 pups (4–6 male; 4–5 female) on PD1. These experiments were conducted in accordance with the procedures outlined by the Animal Care and Facilities Committee at Rutgers University.

Vaginal cytology and determination of the estrous cycle

Stages of the estrous cycle were monitored from vaginal cytology obtained through daily vaginal swabs (Shors, 1998). Cells were removed from the vaginal tract with cotton tipped applicators, immersed in 0.9% saline and placed onto glass slides. Slides were stained with 1% Toluidine blue and cell types characterized under 10× magnification as follows: estrus—

large blue staining cornified cells, diestrus 1—small dark staining leukocytes with scattered epithelial cells, diestrus 2—similar appearance to diestrus 1 but with a lesser density of cells, proestrus—round clumped nucleated purple stained cells. Only virgin females that demonstrated at least two 4–5 day cycles with all stages of estrus were included in the experiment. Estrous cycles were not monitored in pregnant and postpartum females since these reproductive stages are characterized by persistent diestrus and the cessation of ovarian cycling (Zarrow et al., 1973; van der Schoot et al., 1978).

Surgical procedures for eyeblink conditioning

To implant electrodes for measurement of the eyeblink response, animals were anesthetized with 30 mg/kg sodium pentobarbital anesthesia (i.p.) supplemented by Isoflurane and oxygen. A headstage with four stainless steel wire electrodes was secured to the skull with screws and acrylic. The electrodes (diameter 0.127 mm) were implanted subcutaneously to emerge through and around the eyelid. Two electrodes recorded electromyographic (EMG) activity for determination of the eyeblink and two delivered the periorbital stimulation to elicit the eyeblink reflex. All rats were provided at least 3 days of recovery before training.

Acclimation to the conditioning chamber and stressor exposure

For acclimation, rats were taken directly from their home cages and placed in the conditioning chamber which consisted of an illuminated (7.5 W) inner chamber (22 × 26 × 25 cm) with metal walls and a grounded grid floor within a sound attenuating outer chamber (51 × 52 × 35 cm). Headstages were connected to a shielded, grounded cable that allowed free movement within the chamber. Acclimation occurred for approximately 45 min during which time there was no stimulus presentation. Following acclimation, groups of animals were taken into a separate room and exposed to the stressor (to be described). Additional groups of females served as unstressed controls and were kept in individual cages in the training room after acclimation. Unstressed groups were returned to their home cages at the same time as groups that had been stressed in order to ensure that all postpartum females were separated from their pups for similar amounts of time. Acclimation and stressor exposure occurred between 10:00 a.m. and 3:00 p.m.

Classical eyeblink conditioning procedures

Twenty-four hours after acclimation and stressor exposure, all rats were returned to the conditioning apparatus. The number of spontaneous blinks was evaluated in 750 ms samples (total of 22.5 s) distributed randomly over a 10-min period. Next, we evaluated potential effects of stressor exposure or reproductive status on sensitization to the cues that would be involved in the classical conditioning procedure before any conditioning occurred. To do this, rats were exposed to 10 white noise stimuli (81–82 dB, 250 ms, 5 ms rise/fall time) and blinks that occurred during the first 100 ms of the white noise were considered sensitized responses. Immediately thereafter, rats were exposed to 200 trials of trace conditioning each day for two consecutive days. These trials were presented in blocks of 10 in the following order: 1 CS alone, 4 paired trials, 1 US alone, and 4 paired trials. Paired trials consisted of a CS (the previously described white noise), followed by a 500 ms stimulus free trace interval, and then a 0.6 mA, 100 ms periorbital shock US (Fig. 1). The intertrial interval was randomized between 20 and 30 s. As animals learn this response, they blink during the trace interval and these blinks are considered adaptive CRs and are used in the present studies to evaluate learning.

Detection of eyeblinks and measurement of the conditioned response

To detect eyeblink responses, changes in EMG activity were measured via two recording electrodes that had been implanted in the obicularis oculi muscle. The electrodes were

connected to a differential AC amplifier with a 0.3–1.0 kHz band pass filter and amplified 10K. EMG signals were collected by a computer and digitized at 1000 samples/s with an A/D board.

For every trial, eyelid responses were compared to the maximum baseline EMG response occurring during a 250 ms period prior to the CS (Fig. 1). Eyelid responses were scored as eyeblinks when they exceeded the trial baseline by 4 standard deviations and were longer than 3 ms. Under baseline conditions, rats blink infrequently; however, this baseline is useful for ensuring that the background noise that is transmitted via the electrode is low, allowing for the detection of eyeblinks that occur during the trace interval. On paired trials, eyeblinks were considered CRs when they occurred 250 ms after CS onset but prior to US onset (i.e. during the trace interval) because eyelid stimulation interrupts our ability to detect the EMG response during the US itself (Fig. 1). On the CS alone trials, the recording period for measurement of the CR occurred between 250 and 1000 ms after CS onset because there was no US and thus the EMG could be recorded throughout the potential response period. This additional analysis window does not change the number of CRs that are detected because the CR begins before the US does. Eyeblink performance was calculated as the percentage of trials on which a CR was produced in response to a CS.

Measurement of the UR and pain sensitivity

Following training, a subset of rats was transferred to separate chambers for measurement of the UR (Servatius, 2000; Bangasser and Shors, 2004). Rats were exposed to 50 additional trials in which a 10 ms square-wave pulse to the eyelid (10V) was used as the US. URs were assessed during a 100 ms period beginning 50 ms after the US presentation to allow for artifact in the EMG signal from the US to dissipate. A UR was recorded when the EMG response exceeded a baseline recording by 4 standard deviations for 5 ms out of a 6 ms period. UR amplitude was recorded as the absolute value of the largest peak/trough within a UR response on US alone trials.

After measurement of the UR, the hotplate test was used to examine the pawlick withdrawal response as a measure of general analgesia (Matzel and Miller, 1989). Rats were placed on a modified hotplate consisting of an aluminum base (28.5 × 19.5 cm) maintained at 52.5°C and surrounded by opaque black Plexiglas walls 30 cm high. Latency to lick either back paw was recorded. Animals that jumped in response to the heat were excluded.

Experiment 1: effects of acute restraint and tailshock stress on trace eyeblink conditioning during pregnancy and the postpartum period

A female's reproductive state can alter the stress response. As noted, numerous studies have shown that stress-induced activation of the HPA axis is attenuated in late pregnant, parturient and postpartum rats (Stern et al., 1973; Lightman and Young, 1989; Walker et al., 1995; daCosta et al., 1996; Windle et al., 1997; Johnstone et al., 2000; Neumann, 2001; Wartella et al., 2003). In addition, postpartum females show reduced anxiety and fearfulness (Fleming and Luebke, 1981; Hard and Hansen, 1985; Toufexis et al., 1999; Neumann et al., 2001; Lonstein, 2005). However, it is unknown how, or even whether, stress affects learning during the postpartum period. In adult virgin female rats, exposure to an acute stressful event consisting of restraint and brief intermittent tailshocks dramatically impairs acquisition of the classically conditioned eyeblink response (Wood and Shors, 1998; Wood et al., 2001). Based on reproductive differences in stress responsiveness, we tested whether the impaired learning after stress would occur during pregnancy and the postpartum period.

Methods

Virgin, pregnant and postpartum females were implanted with electrodes for measurement of the eyeblink response. Electrode implantation was performed on GD13–15 for females tested during late pregnancy and on PD3–5 for postpartum females. After 3–5 days of recovery, groups of virgin ($n = 8$), pregnant ($n = 8$) and postpartum ($n = 10$) females were acclimated to the conditioning apparatus and then taken into a separate room and exposed to the stressor. For stressor exposure, rats were placed in a Plexiglas restraining tube that was located within a dark sound-attenuating chamber and 30, 1 mA, 1 s, tailshocks were delivered to the tail at a rate of 1/min. Additional groups of virgin, pregnant and postpartum females ($n = 10$ /group) were unstressed and returned to their home cage. Virgin females were acclimated and stressed in diestrus 2 and trained 24 h later during proestrus. These stages were chosen based on previous studies showing that the stress-induced impairment in conditioning is most evident in females stressed during diestrus 2 and trained 24 h later during proestrus (Shors et al., 1998; Wood et al., 2001). Acclimation and stressor exposure occurred on GD18 for pregnant females and on PD7–10 for postpartum females, times associated with attenuated HPA responses and reduced emotionality (Stern et al., 1973; daCosta et al., 1996; Windle et al., 1997; Toufexis et al., 1999). Twenty-four hours after stressor exposure, all rats were trained with the trace eyeblink conditioning paradigm.

Results

To evaluate the effects of stress on trace eyeblink conditioning during pregnancy and the postpartum period, we used a repeated measures ANOVA with reproductive status (virgin, pregnant, postpartum) and stressor exposure (stress, no stress) as the independent measures and the percentage of CRs (% CRs) over 4 blocks of 100 trials as the dependent measure. There was a significant two-way interaction between reproductive status and stressor exposure [$F(2,50) = 5.34, P < 0.01$] (Fig. 2). Post hoc analysis revealed that stressor exposure reduced the % CRs in virgin and pregnant females when compared to the unstressed controls (P values ≤ 0.05) whereas it did not affect the % CRs in postpartum females ($P > 0.05$). Reproductive status itself did not affect conditioning because the % CRs expressed by unstressed virgins was not different from that emitted by unstressed pregnant or postpartum females (P values > 0.05). To assess whether each group of females displayed an increase in responding over the training period and thus demonstrated learning, we analyzed the % CRs over the 4 blocks of training trials using a series of repeated measures ANOVAs. As illustrated in Fig. 2, virgin ($P = 0.0005$) and pregnant ($P = 0.02$) females that were not stressed increased their % CRs over blocks of training, whereas virgin ($P = 0.69$) and pregnant ($P = 0.81$) females that were stressed did not. In contrast, postpartum females, regardless of stressor exposure, increased their responding over the blocks of training ($P = 0.03$). These data suggest that the unstressed animals learned as well as postpartum animals that were stressed, whereas the stressed virgins and stressed pregnant females did not. However, we did not attempt to train animals to asymptote and thus cannot state whether they would or would not learn if they were exposed to more training trials.

Using ANOVA, we also evaluated the effects of stress and reproductive status on measures of performance that are not involved in learning the classically conditioned eyeblink response, but that might affect performance of that response. There was no effect of the stressor or reproductive status on spontaneous blinking or sensitized responding to the white noise stimulus before any training occurred (P values > 0.05) (Table 1). We also found no evidence that these manipulations affected the amplitude of the UR or analgesia as measured using the hot plate test (P values > 0.05) (Table 1).

Experiment 2: effects of acute restraint and tailshock stress on trace eyeblink conditioning during the early and late postpartum periods

In the previous experiment, we demonstrated that exposure to an acute stressful event impairs conditioning in virgin and pregnant, but not postpartum females. However, maternal females were tested at a single time point corresponding to the middle postpartum period (days 7–10). The postpartum period is dynamic and characterized by dramatic hormonal changes as well as alterations associated with maternal responsiveness, anxiety, and maternal aggression (Mayer et al., 1987; Numan and Insel, 2003; Lonstein, 2005). In addition, stress reactivity varies throughout the postpartum period with early postpartum females showing greater stress-induced HPA activation relative to later postpartum stages (Smotherman et al., 1977; Walker et al., 1995; daCosta et al., 2001; Deschamps et al., 2003). Thus, in this experiment, we tested whether the resistance to stress was evident throughout the postpartum period.

Methods

Eyeblink surgery was performed on GD18 for animals tested during the early postpartum period (PD2) and on PD10–13 for animals tested late postpartum (PD16–18). After 3–5 days of recovery, groups of early and late postpartum females were acclimated to the conditioning apparatus and then taken into a separate room and exposed to the stressor of restraint and 30, 1 mA, 1 s tailshocks (early postpartum: $n = 8$; late postpartum: $n = 13$) or were unstressed and returned to their home cage (early postpartum: $n = 9$; late postpartum: $n = 14$). Twenty-four hours after stressor exposure, all rats were trained with the trace eyeblink conditioning paradigm.

Results

For each postpartum time point, we used a repeated measures ANOVA with stressor exposure (stress, no stress) as the independent measure and the % CRs over 4 blocks of 100 trials as the dependent measure. Exposure to the restraint and tailshock stressor either during early (PD2) [$F(1,15) = 0.63$, $P > 0.05$] or late (PD16–18) postpartum [$F(1,25) = 0.02$, $P > 0.05$] did not affect the % CRs emitted during trace conditioning (Fig. 3). All groups of postpartum females showed evidence of learning as demonstrated by an increase in the % CRs over blocks of training, whether they were trained early ($P = 0.01$) or late ($P = 0.01$) during the postpartum period. As in the previous experiment, there was no effect of stressor exposure on spontaneous blinking, sensitized responding, analgesia or UR amplitude during the early or late postpartum periods (data not shown; P values > 0.05).

Experiment 3: effects of inescapable swim stress on trace eyeblink conditioning during the postpartum period

Next, we tested whether conditioning during the postpartum period was resistant to another type of stressor that did not involve shock, inescapable swim stress. This stressor is associated with reduced HPA responsiveness in postpartum females (Walker et al., 1995). As with restraint and tailshock, exposure to inescapable swim stress impairs acquisition of the conditioned eyeblink response in adult virgin females (Shors et al., 1998). We also assessed whether stress or the surgical procedure for electrode implantation affected lactation.

Methods

After acclimation to the conditioning chamber, groups of weight-matched postpartum (PD7–9; $n = 13$) and diestrus 2 virgin ($n = 11$) females were transferred to a different room and

placed in individual gray plastic cylinders (29 × 38 cm) filled with room temperature water (20–21°C) at a depth of 30 cm for 20 min. Additional groups of postpartum ($n = 13$) and virgin ($n = 11$) females were unstressed and returned to their home cage. As before, all animals were trained 24 h following stressor exposure on the trace eyeblink conditioning task. To evaluate whether eyeblink surgery or exposure to inescapable swimming had any effect on lactation, litter weights were obtained daily from subsets of animals that underwent eyeblink surgery and that were stressed ($n = 5$) or unstressed ($n = 4$) as well as from naive females without surgery (stress: $n = 4$; no stress: $n = 4$).

Results

The data were analyzed using a repeated measures ANOVA with reproductive status (virgin, postpartum) and stressor exposure (swim stress, no stress) as independent measures and the % CRs emitted over the 4 blocks of training as the dependent repeated measure. There was a 3-way interaction [$F(3,123) = 2.84$, $P < 0.05$] and post hoc analysis revealed that exposure to the swim stress decreased the % CRs that were emitted by virgin females relative to the % CRs expressed by unstressed virgin females ($P < 0.001$). In contrast, the % CRs emitted by postpartum females was unchanged by stressor exposure ($P > 0.05$) (Fig. 4). A repeated measures ANOVA indicated a significant increase in the % CRs across training blocks in unstressed virgin females ($P = 0.0005$) but no change for the virgin females that were exposed to the swim stress ($P = 0.85$). For the postpartum females, both groups emitted a greater % CRs across the 4 blocks of training trials ($P < 0.00001$). These results suggest that swim stress reduces conditioning in virgin but not in lactating female rats. Again, we did not attempt to train animals to asymptote and thus cannot state whether the stressed virgins would or would not learn if they were exposed to more training trials.

As before, spontaneous blinking, sensitized responding, UR magnitude and analgesia were not affected by stress or reproductive status (data not shown; P values > 0.05). Also, we found no evidence that the eyeblink surgery or stressor exposure affected daily litter weight gain (surgery stress: 15.8 ± 0.35 g/day; surgery no stress: 16.7 ± 1.23 g/day; naive stress: 17.1 ± 0.61 g/day; naive no stress 16.7 ± 0.67 g/day; P values > 0.05) suggesting that lactation was not compromised by the experimental manipulations. Together, these data suggest that swim stress impairs trace conditioning in virgin females but does not affect conditioning in females that are lactating.

Experiment 4: effects of male intruder stress on trace eyeblink conditioning during the postpartum period

In this experiment, we tested the effects of another type of stress, one that might be considered more ethologically salient to a postpartum female—intrusion of a novel adult male. This stressor, referred to as male intruder stress, is different from exposure to inescapable tailshocks or swimming because it does induce increases in glucocorticoid responses in postpartum, as well as in virgin, females (Neumann et al., 2001; Deschamps et al., 2003).

Methods

After acclimation to the conditioning chamber, females were returned to their transparent Plexiglas home cages for at least 30 min since separation from pups has been shown to reduce maternal aggression (Gandelman and Simon, 1980; Ferreira and Hansen, 1986). For intruder exposure, home cages of postpartum females with their litters (PD7–10; $n = 8$) or virgin females in diestrus 2 ($n = 10$) were transferred to a dimly lit observation room and an unfamiliar adult male was introduced into the cage for 30 min. The latency to the first attack and each occurrence of attacks was recorded. An attack was defined as a lunge at the male

followed by either a pin or wrestling bout. This measure was used because it has been shown to differentiate between lactating and nonlactating females more readily than other behaviors (Mayer et al., 1987). Unstressed postpartum ($n = 10$) and virgin ($n = 9$) females remained in their home cages. As before, all animals were trained 24 h following stressor exposure on the trace eyeblink conditioning task.

Results

An independent samples t test revealed that postpartum females exhibited more aggressive behavior towards the male intruder than virgin females in diestrus 2; they were faster to attack [$t(16) = 5.31$, $P < 0.0005$] and exhibited a greater number of attacks [$t(16) = -4.73$, $P < 0.0005$] (Fig. 5A). The next day, trace conditioning was assessed in all animals. Exposure to the male intruder stressor had differing effects on trace conditioning in virgin than it did in the postpartum females. Using a repeated measures ANOVA with reproductive status (virgin, postpartum) and stressor exposure (male intruder stress, no stress) as the independent measures and the % CRs emitted over the 4 blocks of training as the dependent repeated measure, there was a significant two-way interaction between reproductive status and stressor exposure [$F(1,33) = 9.11$, $P < 0.005$]. Post hoc analysis revealed that virgin females exposed to the male intruder stress emitted fewer CRs than did the unstressed virgin females ($P < 0.005$) (Fig. 5B). With respect to the postpartum females, those that were exposed to the male intruder did not respond differently than those that were not exposed to the intruder ($P > 0.05$). However, the % CRs over the 4 blocks of training trials increased for the virgin females that were not stressed ($P = 0.004$) as well as those that were stressed ($P = 0.001$). The % CRs also increased over blocks of training for the postpartum females ($P < 0.00001$). Thus, all groups in this experiment showed evidence of learning, including the virgin females that were exposed to the male intruder. These data are different from those in the previous experiments, in which virgin females that were stressed with inescapable tailshocks or swim stress did not show any evidence of learning. However, as we did not train to asymptote, it is unclear whether the virgins would eventually reach the same level of conditioned responding and thus we cannot determine whether they would eventually learn. Also, we did not expose additional groups of virgin females to the other stressors in this experiment. Thus, we cannot evaluate whether the effect of the male intruder on performance in the virgins is substantively different from that in response to the other stressors. However, it would appear that the postpartum females are resistant to the effects of the intruder stress on the overall % of CRs as well as the % of CRs that are emitted over the training period. As in the previous experiments, there was no effect of the stressor or of reproductive status on spontaneous blinking, sensitized responding, UR magnitude or analgesia (data not shown; P values > 0.05).

Experiment 5: effects of acute restraint and tailshock stress on trace eyeblink conditioning in postpartum females following pup removal

The data thus far suggest that trace conditioning is not affected by stress in females that are trained during the postpartum period. However, we do not know what aspect of the postpartum period mediates this effect. The most obvious one is the offspring themselves. Contact with pups is essential for maintaining many behavioral, neural and hormonal adaptations of postpartum females. For example, maternal responsiveness declines after pup removal (Gandelman and Simon, 1980; Ferreira and Hansen, 1986; Numan and Insel, 2003). Postpartum reductions in anxiety also dissipate when the offspring are absent (Lonstein, 2005). Similarly, the HPA axis response to stress returns about 48–72 h after separation from the litter (Stern et al., 1973; Lightman and Young, 1989; Windle et al., 1997). Here we tested whether the presence of offspring is critical for maintaining the postpartum resistance to stress. As before, we used trace eyeblink conditioning to assess their learning ability.

Methods

Pups were removed from postpartum females 7 days after giving birth. Three days later (PD10), groups of postpartum females were acclimated to the conditioning chamber and then taken into a separate room, restrained and exposed to the 30, 1 mA, 1 s tailshocks (1/min) ($n = 9$). Another group of postpartum females ($n = 9$) were not stressed and were instead returned to their home cage. One day later, all rats were trained with the trace eyeblink conditioning task.

In a separate group of postpartum females, we assessed the effect of litter removal on lactation and the resumption of vaginal cyclicity. Litters were removed on PD7. Three days later, one group of postpartum females ($n = 6$) were provided with three 4 to 5-day old foster pups. Pups had been recently fed (as determined by the presence of milk bands) and were weighed immediately prior to placement in the female's home cage. Twenty-four hours later, pups were again weighed and examined for milk bands. Estrous cycles were monitored in another group of females ($n = 4$) beginning on the day of litter removal and the number of days until a vaginal smear of cornified epithelial cells indicative of estrus was determined.

Results

In this experiment, pups were removed from postpartum females (PD7) and 3 days later, the postpartum females were either exposed to the stressor or not. The next day, they were trained on the trace eyeblink conditioning task. The data were analyzed using a repeated measures ANOVA with stressor exposure (stress, no stress) as the independent measure and the % CRs over 4 blocks of 100 trials as the dependent measure. There was a significant effect of stressor exposure on the % CRs emitted across the 4 blocks of training [$F(1,16) = 12.31$, $P < 0.005$]; postpartum females without pups exposed to the stressor emitted fewer CRs than those that were unstressed (Fig. 6). The % CRs did not increase over the blocks of training in the stressed females ($P = 0.79$). In addition, the % CRs did not increase over the blocks of training in the unstressed females, and instead decreased ($P = 0.04$). These data suggest that neither group learned. However, the unstressed females did achieve a very high level of responding (~80% at one point) and thus did appear to learn. To address this issue, we also analyzed the % CRs during the first 100 trials in blocks of 20 trials. With this analysis, there was an increase in the % CRs [$F(4,64) = 6.30$, $P < 0.0005$]. These data indicate that the animals were learning the CR during the initial block of training trials. Also, there was an overall effect of the stressor in the postpartum females during the first 100 trials of training [$F(1,16) = 7.16$; $P < 0.05$]. Thus, exposure to the stressor decreased the overall level of responding in postpartum females that were separated from their pups. Because we did not train animals to asymptote or to a specific criterion, we cannot state whether the postpartum females that were separated from their pups before the stressor would learn if they were exposed to more training trials.

As in the previous experiments, there was no detectable effect of the stressor on spontaneous blinking, sensitized responding, analgesia or UR amplitude (data not shown; P values > 0.05). Importantly, removal of the litter was also found to inhibit lactation. The weight of foster pups provided to separate groups of females deprived of pups for 3 days declined from an average of 37.3 ± 1.9 g to 34.2 ± 1.7 g in 24 h [$t(5) = 3.5$, $P < 0.05$]. None of the pups were observed to have milk bands. Estrus cycles resumed about 9 days after the litters were removed. However, at the time of stressor exposure and conditioning, the females without their litters did not cycle.

Experiment 6: effects of acute restraint and tailshock stress on trace eyeblink conditioning in maternally behaving virgin females

Upon giving birth, primiparous rats immediately begin to express maternal behavior (Rosenblatt, 1967). In contrast, cycling virgins will avoid young pups and may even express infanticide (Wiesner and Sheard, 1933; Rosenblatt, 1967; Fleming and Rosenblatt, 1974). However, with time, virgin females can be induced to behave maternally. This process is known as sensitization or concaveation (Wiesner and Sheard, 1933). After approximately 5–7 days of exposure, virgin females will begin to care for the young pups and will display most components of maternal behavior.

In this experiment, we tested whether the resistance to stress that we observed in postpartum females would occur in virgin females that were induced to be maternal. As before, we used trace eyeblink conditioning to assess learning ability. It is noted that the behavioral and hormonal responses to stress are different in females that are induced to be maternal than those in postpartum females and are more similar to those in virgins that are not exposed to pups. Unlike the postpartum female, the sensitized virgin female is not less anxious (Hansen, 1990; Ferreira et al., 2002) and the HPA response to stress is not suppressed (Schlein et al., 1974). Thus, the mechanisms whereby a sensitized female might become resistant to stress would likely not depend on a decrease in anxiety or a decrease in the HPA response.

Methods

After eyeblink surgery, adult virgin females were individually housed in Plexiglas cages (42 × 20 × 20 cm) lined with wood chip bedding and provided with nesting material. The next day, half of these animals were given three 1 to 10-day old freshly fed pups scattered in the home cage opposite to where the virgin female was positioned. The incidence of pup licking (full body or anogenital), retrieving (picking up a pup and carrying it to another location in the cage) and grouping (retrieving all three pups to the same location) was recorded during a 15-min observation period. Each morning, the pups were removed from the virgin female's home cage and replaced by 3 milk-replete foster pups that had remained with a lactating surrogate for at least 24 h and the daily 15-min maternal behavior observation was performed. Virgin rats were considered maternal when they licked, retrieved and grouped all three pups within the observation period on two consecutive days and assigned a latency corresponding to the first day of the two consecutive parental displays. Thereafter, maternal virgins continued to receive three freshly fed pups every 24 h throughout the duration of the experiment and 5 min observations were performed to ensure the continual display of maternal behavior. Females that cannibalized pups failed to become maternal within 14 days or that stopped behaving maternal were not tested.

Maternal virgins were acclimated to the conditioning apparatus on the second day the criteria for maternal behavior were met. After acclimation, maternal virgins were taken into a separate room where they were restrained and exposed to the 30, 1 mA, 1 s tailshocks ($n = 10$) or left unstressed ($n = 8$) and returned to their home cages. Additional groups of virgin rats that had not been exposed to pups were stressed ($n = 9$) or left unstressed ($n = 11$). One day later, all rats were trained with the trace eyeblink conditioning paradigm, as before. Vaginal smears were obtained daily from all animals to monitor estrous cycles.

Results

In this experiment, virgin females were induced to display maternal behavior by continual exposure to pups. They required a median of 6.2 days of continuous pup exposure before displaying maternal behavior during two consecutive daily observations. To determine the

effects of stress on trace eyeblink conditioning, we used a repeated measures ANOVA with reproductive status (virgin, maternal virgin) and stressor exposure (stress, no stress) as the independent measures and the % CRs over 4 blocks of 100 trials as the dependent measure. There was a significant two-way interaction between reproductive status and stressor exposure [$F(1,34) = 4.2, P < 0.05$] (Fig. 7). Post hoc analysis showed that virgin females without pups exposed to the restraint and tailshock stressor emitted fewer CRs than unstressed virgin females without pup exposure ($P < 0.005$). Conditioning in virgin females displaying maternal behavior and exposed to the stressor did not differ from maternally behaving virgin females that were unstressed ($P > 0.05$). Exposure to the pups did not alter conditioning ($P > 0.05$). Approximately half of the females became acyclic during the sensitization procedure and were found to be in persistent diestrus; however, the % CRS in those that continued to cycle versus those that did not cycle was not different nor was the response to stress ($P > 0.05$). With a repeated measures ANOVA, it was determined that unstressed virgin females without pup exposure increased their % CRs over the 4 blocks of trials ($P = 0.05$), whereas those that were exposed to the stressor did not ($P = 0.75$). In contrast, virgin females that were induced to be maternal increased their % CRs, irrespective of stressor exposure ($P < 0.0002$). As before, we found no evidence that stressor exposure or reproductive status altered spontaneous blinking, sensitized responding, UR magnitude or analgesia (data not shown; P values > 0.05).

General discussion

Here we show that effect of stressful experience on associative learning in females is profoundly influenced by their reproductive status. One day after exposure to an acute stressful event, trace eyeblink conditioning was significantly reduced in adult females with no previous reproductive or maternal experience (Wood and Shors, 1998; Wood et al., 2001; Bangasser and Shors, 2004; Leuner et al., 2004). Stressor exposure similarly impaired conditioning in pregnant females. However, stressor exposure did not affect trace conditioning in postpartum females. This resistance to stress was not limited to a particular stage of the postpartum period; it began as early as 2 days following parturition and persisted throughout the late postpartum period. Moreover, the postpartum resistance to stress generalized to various types of stressors including brief inescapable tailshocks, swim stress and exposure to a male intruder. The presence of offspring and the expression of maternal behavior were sufficient to establish the resistance to stress. When postpartum females were separated from their pups, the impairment in conditioning after stress reemerged. Also, there was no effect of stress on trace conditioning in virgin females that were induced to behave maternally by exposure to pups when compared to the significant reduction in conditioning in virgin females that were not exposed to pups. Together, these data indicate that postpartum females respond differently to stress than do virgin females; their level of classical conditioning was not affected by an acute stressful experience whereas that in virgins was reduced. In these experiments, we did not train the animals to asymptote or to a criterion but rather trained them for a set number of trials. Thus, we cannot state whether or not the stressed virgins would eventually learn but can state that the postpartum females learn readily, with or without previous stressor exposure.

Several recent studies report that spatial navigation learning is enhanced as a result of motherhood (Kinsley et al., 1999; Tomizawa et al., 2003; Pawluski et al., 2006). By contrast, here we found similar levels of conditioning on the trace eyeblink conditioning task in maternal and virgin females under unstressed conditions. The difference between these studies is likely attributable to the task (i.e. spatial versus classical) used to assess learning. Moreover, spatial tasks have motivational and locomotor demands which could influence performance and not learning, per se. In the present studies, we found no evidence that reproductive status or exposure to the stressful event altered nonspecific responding as

measured by increased spontaneous blinking or sensitized responding to the conditioning stimuli. In addition, we found no evidence that reproductive status or stressor exposure altered analgesia as measured with the hotplate test. The magnitude of the unconditioned response, which is used to measure the perceived intensity of the shock US (Servatius, 2000; Bangasser and Shors, 2004), was also unaffected by stress or reproductive status. Moreover, in most of the experiments, maternal females, regardless of stressor exposure, showed evidence of learning as measured by an increase in the % CRs across training blocks. In contrast, only virgin and pregnant females that were unstressed showed evidence of learning. Overall, these data indicate that motherhood does not influence performance of the conditioned response but rather modulates the effects of stressful experience on associative learning. However, there were two exceptions to these results. First, responding increased over trial blocks in virgin females exposed to the male intruder but not in those exposed to swim stress or inescapable tailshock. Thus, females exposed to a male intruder do show evidence of learning albeit at a much reduced levels relative to unstressed virgins. Second, unstressed postpartum females that had pups removed for 3 days emitted fewer responses during the last 200 trials of training. The reason for the decrease in responding is unknown but likely reflects either fatigue or some loss of electrode sensitivity. Even with these exceptions, it is clear that the effects of stressful experience on trace conditioning depend on a female's reproductive status.

Although stressor exposure impaired conditioning during late pregnancy and not during early postpartum, we cannot state the precise time when maternal rats begin to show the resistance to stress. Since the pregnant females were tested only on GD18, it is unknown whether the resistance to stress would be evident even closer to parturition. Perhaps the resistance to stress emerges coincident with the prepartum onset of maternal behavior, which occurs on the last day of pregnancy (Mayer et al., 1987; Kinsley and Bridges, 1990). Indeed, since stressor exposure impaired learning in postpartum females deprived of their pups, but not postpartum females and virgins induced to display maternal care, it is likely that the expression of maternal behaviors is important in protecting females from the detrimental effects of stress on conditioning. Alternatively, but not mutually exclusive, is the possibility that contact with pups, and the sensory stimulation they provide, mediates the resistance to stress in maternal females. For example, oral stimulation from pup licking and ventral somatosensory stimulation from pup nuzzling, both of which are necessary for maternal responsiveness and various other aspects of maternal care, could be involved (Numan and Insel, 2003; Morgan et al., 1992; Stern, 1997). Also, distal stimulation from visual, auditory or olfactory inputs may mediate the resistance to stress. However, there are a number of postpartum behavioral changes that require full pup interaction. The postpartum reduction in anxiety is not sustained by distal pup-related cues (Lonstein, 2005). Physical contact is also necessary for pups to acquire reinforcing value and for the long-term retention of maternal responsiveness (Morgan et al., 1992; Orpen and Fleming, 1987; Lee et al., 2000). Another interpretation of the present findings is that the combination of sensory stimulation provided by pups resembles that associated with a complex, enriched environment (Diamond et al., 1971). It has been shown that exposure to enriched environments can prevent or reverse many adverse consequences associated with stress (Francis et al., 2002). Thus, the enriching experiences associated with motherhood and the presence of offspring may provide the environmental features that are sufficient for inducing a resistance to stress.

The response to stress in the virgins and the absence in the various groups of maternal females suggest that a common mechanism mediates the resistance to stress. However, this cannot be verified. It cannot even be concluded that virgin, pregnant and postpartum females are experiencing the stressful events similarly. In support of this, stress-induced neuronal activity in the amygdala and the hippocampus, brain areas involved in stress-regulation and emotionality is reduced in females that are pregnant or lactating. These data suggest that the

perception of the stressful event may change across stages of reproductive life (daCosta et al., 1996; Wartella et al., 2003). In addition, there are numerous indications that emotional responses to stress depend on a female's reproductive status. As noted, the expression of fear and anxiety is reduced during the postpartum period (Fleming and Luebke, 1981; Hard and Hansen, 1985; Toufexis et al., 1999; Neumann, 2001; Lonstein, 2005). It could be argued that the effects of stress on conditioning are mediated by changes in anxiety after exposure to the stressor. However, we have not been able to demonstrate that such a relationship exists. For example, chronic treatment with the antidepressant Prozac (fluoxetine) prevents the effects of stress on classical eyeblink conditioning and does so without altering anxiety-related behaviors, as measured in the elevated plus maze (Leuner et al., 2004). In the present experiments, we found that stress did not affect classical conditioning in late postpartum females or virgins that were induced to behave like mothers and yet these females reportedly are as anxious as virgin females without pup exposure (Hansen, 1990; Ferreira et al., 2002; Lonstein, 2005). Thus, the effects of stress on learning do not seem directly related to alterations in anxiety.

The most obvious candidate for mediating the resistance to stress would be alterations in the HPA responses, especially since they are known to be suppressed during the postpartum period (Thoman et al., 1970; Stern et al., 1973; Lightman and Young, 1989; Neumann et al., 1998; Johnstone et al., 2000; Walker et al., 2001). However, there are a number of situations in which postpartum females are behaviorally reactive to stress despite diminished HPA activation. For example, both postpartum and virgin females exhibit enhanced startle responses to auditory and contextual stimuli associated with footshock after fear conditioning (Toufexis et al., 1999) and similar levels of immobility in the Porsolt forced swim test (Walker et al., 1995). In previous studies, we have shown that the detrimental effect of stress on learning in virgin females occurs even in the absence of glucocorticoids (Wood et al., 2001). The data presented here also indicate that glucocorticoids are probably not necessary for the absence of the stress effect in maternal females. First, stress did impair conditioning during late pregnancy despite the fact that glucocorticoid responses would be reduced at this time (daCosta et al., 1996; Neumann et al., 1998; Johnstone et al., 2000). Conversely, stress did not affect conditioning in early postpartum females even though they are known to show an enhanced HPA response to stress, when compared to their responses during other stages of the postpartum period (Smotherman et al., 1977; Walker et al., 1995; daCosta et al., 2001; Deschamps et al., 2003). Also, postpartum females reportedly show a normal HPA response to the stress of a male intruder (Deschamps et al., 2003), yet conditioning in these females was also resistant to the stressful experience. Finally, we found that learning in virgin females that were induced to display maternal care was resistant to the stressor even though their HPA response to stress is reportedly normal (Schlein et al., 1974). These data suggest that changes in the release of glucocorticoids after the stressful experience do not account for the absence of the stress effect on learning in the maternal female.

Instead, ovarian hormones and in particular estrogen are likely to be involved in these effects of stress on learning in females during different phases of reproductive life. It has been shown that their presence is necessary for the stress-induced impairment of classical eyeblink conditioning in virgin females (Shors and Leuner, 2003). Specifically, conditioning in females that are ovariectomized or treated with an estrogen receptor antagonist was unaffected by exposure to an acute stressful event, whereas that in untreated females was severely disrupted (Wood and Shors, 1998; Wood et al., 2001). Moreover, there is a positive relationship between absolute levels of estradiol and the impact of stress on eyeblink conditioning. High estrogen levels that occur during the stage of proestrus and late pregnancy are associated with impaired performance following acute stress whereas low estrogen levels observed during the postpartum period are not (Shors et al., 1998; Wood et

al., 2001). It is noted that although necessary, the presence of estrogen is probably not sufficient for the effects of stress on classical eyeblink conditioning. In the present studies, levels of conditioning were reduced by stress in postpartum females that had litters removed for three days. The vaginal cytology of these females was similar to females in diestrus, suggesting that their estrogen levels were likely low. Moreover, levels of conditioning in virgin females that are stressed during estrus, when estrogen levels are reduced, are also reduced provided that they are trained in estrus (Wood et al., 2001). A final point to consider is that cyclical fluctuations in estrogen associated with an estrous cycle are not necessary for the reduced conditioning after stress. Levels of conditioning were reduced in pregnant females and in postpartum females that had litters removed, neither of which were experiencing ovarian cycles. In addition to estrogen, other possible mediators of these differing effects of stress on learning include oxytocin and prolactin, since they fluctuate across reproductive life and interact with estrogen (Neumann, 2002; Torner and Neumann, 2002; Numan and Insel, 2003). The receptors for both neuropeptides are expressed in the hippocampus (Tribollet et al., 1988; Bakowska and Morrell, 1997), a brain structure affected by stress (McEwen, 1999) and involved in the acquisition of trace memories (Solomon et al., 1986; Weiss et al., 1999; Beylin et al., 2001).

Collectively, these results indicate that females are differentially affected by stressful experience depending on their stage of reproductive life. They further suggest that this type of learning is preserved during motherhood, at least to the extent that it resists disruption after exposure to a stressful event. Interestingly, the resistance to stress is evident in maternally behaving virgin females, even though they do not have reproductive experience, thus highlighting the importance of the offspring in manipulating the stress response. Certainly, motherhood is a time of new and oftentimes stressful experience. It may be that this resistance to stress reflects another of the many adaptations that occur to increase the likelihood that mothers and their offspring survive.

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Fig. 1.

Trace of EMG response. On paired trials, CRs are those eyeblinks that occur after the CS is terminated and prior to the onset of the US. This period is known as the trace interval and is depicted as a hatched line from 500 ms to 1000 ms from the beginning of the recording period. Trace conditioning was assessed by measuring significant changes in the EMG response that occurred in response to the CS and during the trace interval. Performance was calculated as the percentage of trials that a CR was produced in response to a CS.

Fig. 2.

Postpartum conditioning is unresponsive to acute restraint and tailshock stress. Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Virgin (diestrus 2), pregnant (GD18) and postpartum female rats (PD7–10) were exposed to an acute stressor consisting of restraint and brief intermittent tailshocks or left unstressed. Animals were trained 24 h later on the associative learning task of trace eyeblink conditioning. Trace conditioning was impaired in virgin and pregnant females exposed to the stressor. Postpartum conditioning was unaffected by stressor exposure.

Fig. 3.

Trace conditioning is resistant to acute restraint and tailshock stress throughout the postpartum period. Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Exposure to the restraint and tailshock stressor either during early postpartum (PD2) or late postpartum (PD16–18) did not affect trace conditioning 24 h later.

Fig. 4.

Postpartum conditioning is also resistant to inescapable swim stress. Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Virgin (diestrus 2) and postpartum (PD7–10) female rats were exposed to 20 min of inescapable swim stress or left unstressed and trained 24 h later on the trace eyeblink conditioning task. Although swim stress reduced conditioning in virgin females, it did not affect conditioning in postpartum females.

Fig. 5.

Male intruder stress reduces trace conditioning in virgin but not postpartum females. (A) An adult male was placed into a female's home cage for 30 min. The latency ($\pm$ SEM) and number of attacks ($\pm$ SEM) emitted by the female towards the intruder were recorded. Unstressed females remained in their home cages. Postpartum females exhibited more aggressive behaviors towards the intruder male than did the virgin females; they were faster to attack and exhibited a greater number of attacks. (B) Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Exposure to the male intruder stressor reduced overall levels of trace conditioning in virgin females but not in postpartum females.

Fig. 6.

The presence of pups is necessary for maintaining the postpartum resistance to stress. Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Pups were removed from postpartum females 7 days after giving birth (PD7). Three days later (PD10), groups of postpartum females were exposed to the acute stressor of restraint and tailshock or left unstressed. Exposure to the stressor reduced trace conditioning in the postpartum females that had been separated from their pups.

Fig. 7.

Trace conditioning in virgin females that display maternal behavior is resistant to acute restraint and tailshock stress. Data are represented as a mean percentage of CRs $\pm$ SEM with the first 100 trials divided into block of 20 trials and the remaining 300 trials divided into blocks of 100. Virgin females were induced to display maternal behavior through continual exposure to pups. They were either exposed to the restraint and tailshock stressor or not and trained 24 h later along with virgin females that were not exposed to pups. Exposure to the stressor reduced trace conditioning in virgin females that were not exposed to pups but did not affect trace conditioning in virgin females that were exposed to pups and behaved maternally.

Table 1

Percentage (%) of spontaneous eyeblinks, % of sensitized eyeblinks, UR magnitude and pawlick withdrawal latency in no stress and stressed virgin (diestrus 2), pregnant (GD18) and postpartum (PD7–10) females

	% spontaneous eyeblinks	% sensitized eyeblinks	UR amplitude (mV)	Pawlick latency
Virgin no stress	6.5 ± 0.73	34 ± 7.78	2.6 ± 0.44	8.1 ± 0.70
Virgin stress	5.4 ± 0.89	35 ± 11.18	1.8 ± 0.39	9.8 ± 1.22
Pregnant no stress	7.0 ± 1.5	38 ± 7.58	2.2 ± 0.27	10.3 ± 1.09
Pregnant stress	6.7 ± 1.7	27 ± 10.0	2.0 ± 0.3	8.7 ± 0.6
Postpartum no stress	5.3 ± 1.33	17 ± 6.68	1.6 ± 0.38	12.3 ± 1.76
Postpartum stress	5.3 ± 1.81	36 ± 12.4	2.0 ± 0.46	11.3 ± 2.23

Data are represented as means ± SEM.