

Neonatal Maternal Separation Alters Adult Eyeblink Conditioning and Glucocorticoid Receptor Expression in the Interpositus Nucleus of the Cerebellum

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ABSTRACT: Neonatal maternal separation alters learning and memory. Glucocorticoids also modulate adult learning and memory, and neonatal maternal separation alters forebrain glucocorticoid receptor (GR) concentrations. We used eyeblink classical conditioning to assess the effect of neonatal maternal separation on associative learning. We assessed delay eyeblink conditioning, GR expression, and total neuron number in the interpositus nucleus, a critical site of plasticity in eyeblink conditioning, in adult rats that had undergone either standard animal facilities rearing, handling for 15 min, or maternal separation for either 15 or 60 min per day on postnatal days 2–14. At 2–3 months of age, delay eyeblink classical conditioning was assessed. Brains were processed for GR immunohistochemistry, and GR expression in the interpositus nucleus was assessed using a computer-based densitometry system. Neuron counts and nuclear volumes were obtained from an alternate series of thionin-stained

sections. Maternal separation significantly impaired eyeblink conditioning in male but not female rats. Handling and maternal separation did not significantly affect interpositus neuron number and volume. However, prolonged maternal separation significantly increased GR expression in the posterior interpositus in males, and increases were correlated with eyeblink conditioning. In female rats, maternal separation and handling did not significantly alter interpositus neuron number, volume, or GR protein expression, and GR expression did not correlate with eyeblink conditioning. Thus, neonatal maternal separation produces adult deficits in eyeblink conditioning and alterations in GR expression in its neural substrate in a sex-dependent manner. © 2007 Wiley Periodicals, Inc. *Dev Neurobiol* 67: 1751–1764, 2007

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INTRODUCTION

Stress in adulthood alters learning and memory (Wood and Shors, 1998; Cordero et al., 2002; Kim and Diamond, 2002; Sapolsky, 2003; Leuner et al., 2004; Kim et al., 2006; Miracle et al., 2006; Shors, 2006a,b). Neonatal maternal separation may also

produce changes in learning and memory that persist into adulthood. For instance, prolonged daily maternal separation has been shown to either impair (Huot et al., 2002; Uysal et al., 2005) or enhance (Pryce et al., 2003) later spatial learning and memory. The effects of brief separation are less pronounced (Gibb and Kolb, 2005; Cannizzaro et al., 2006). Furthermore, whereas acquisition of fear conditioning for context and tone is not affected by either brief or prolonged maternal separation (Pryce et al., 2003; Kosten et al., 2005, 2006), adult retention of conditioned fear may be reduced following maternal separation (Meerlo et al., 1999; Kosten et al., 2005, 2006).

Neonatal maternal separation alters glucocorticoid receptor (GR) concentrations in forebrain regions such as the hippocampus (Meaney et al., 1985a,b; Ladd et al., 2004). Glucocorticoids modulate adult learning and memory, with facilitated or impaired memory performance depending on the timing and duration of glucocorticoid exposure (Rooszendaal et al., 1996; Kim et al., 2006). Thus, the effects of neonatal maternal separation on learning and memory could involve the actions of glucocorticoids in the central nervous system.

We are using a simple model system of associative learning, eyeblink classical conditioning, to assess the effects of neonatal maternal separation on adult learning and memory and its neural substrates. Eyeblink conditioning involves pairing a tone with a mild shock to the eye region, so that the tone eventually is used as a signal to predict the shock. After training, the tone (the conditioned stimulus or CS) elicits an eyeblink conditioned response (CR). The critical circuitry for eyeblink conditioning is in the cerebellum and brainstem (for reviews, see Steinmetz, 2000; Christian and Thompson, 2003). The interpositus nucleus of the cerebellum is a site of convergence for the CS and unconditioned stimulus (US), and a critical site of learning-related plasticity. For example, interpositus activity models acquisition of the CR (McCormick and Thompson, 1984a,b) and temporary inactivation of the interpositus reversibly prevents learning (Krupa et al., 1993; Nordholm et al., 1993). Indeed, rabbits trained for more than 12 months following electrolytic lesion of the interpositus fail to learn a conditioned eyeblink response (Steinmetz et al., 1993).

We examined the effect of neonatal maternal separation and handling on adult delay eyeblink classical conditioning and the interpositus nucleus. Because neonatal exposure to glucocorticoids can inhibit neuronal growth and differentiation (Balazs and Cotterrell, 1972; Ardelenu and Sterescu, 1978; De Kloet et al., 1988), we measured interpositus neuron number and volume. Additionally, because neonatal

maternal separation alters forebrain GR expression in adults (Meaney et al., 1985a,b; Ladd et al., 2004) and GRs are expressed in the cerebellar cortex and deep nuclei (Ahima and Harlan, 1990; Cintra et al., 1994), we used immunohistochemistry to assess potential changes in GR expression in the interpositus nucleus.

METHOD

Animals

Untimed pregnant Long Evans Blue Spruce rats (Harlan Indianapolis, IN, $N = 35$) arrived ~ 1 week before giving birth. Dams were housed individually in standard laboratory cages (48 cm \times 20 cm \times 26 cm), with food and water available *ad libitum* and a 12:12 h light/dark cycle (lights on at 0700 h). The day of birth was considered postnatal day (PND) 0. On PND 2 pups were culled to litters of 9–11 while maintaining a male:female ratio as close to 1:1 as possible. All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Bloomington Institutional Animal Care and Use Committee.

Maternal Separation and Handling

Each litter was randomly assigned to one of four groups: standard animal facilities rearing (control; $n = 10$ male, 8 female), gentle handling for 15 min (H-15; $n = 6$ male, 6 female), or maternal separation for either 15 min (MS-15; $n = 6$ male, 6 female) or 60 min per day (MS-60; $n = 12$ male, 9 female), on PND 2–14. Groups consisted of 3–4 litters (2–5 males and 2–3 females per litter per group) and all manipulations were initiated between 0700 and 0900 h each day. Maternal separation and handling were carried out using procedures similar to those of Huot et al. (2002). Dams were removed from the home cage and placed in an adjacent container. Pups were then removed from the home cage and placed in a Plexiglas cage (28 cm \times 17 cm \times 12 cm) lined with clean bedding. Pups in the MS-15 and MS-60 groups were taken to a nearby room and placed in an incubator (Ambient Room Temperature Incubator; Avey Incubator, Evergreen, CO) maintained at room temperature ($22.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) for the duration of the separation period. Pups in the H-15 group were taken to a nearby thermostat-controlled room maintained at $\sim 23^{\circ}\text{C}$ for the duration of the handling period. Pups were permitted to huddle with littermates during handling period for the H-15 group; only the pup that was currently being handled was prevented from huddling. On average each pup received 90 s of handling per session. On PND 28, animals were weaned and housed in same sex/same litter groups of four or fewer until eyeblink conditioning surgery.

Neonatal Corticosterone Assay

To verify the stressfulness of the manipulations, plasma corticosterone was measured in separate groups of control

($n = 15$ male, 9 female), H-15 ($n = 16$ male, 13 female), MS-15 ($n = 14$ male, 12 female), and MS-60 ($n = 17$ male, 15 female) animals on PND 2 and 12. During the stress hyporesponsive period (PND 4–14) corticosterone levels peak ~ 12 h after 60 min of maternal separation (Walker et al., 1991). Therefore, blood samples were taken 12 h after the onset of separation and handling. Pups were rapidly decapitated and trunk blood was collected in heparinized microcapillary tubes and centrifuged at 2000g for 15 min to obtain plasma. Corticosterone titers were assessed using a competitive enzyme immunoassay kit (Assay Design, Ann Arbor, MI). This assay has low cross-reactivity with other major steroid hormones, sensitivity typically <27.0 pg/mL, and coefficients of variation within and across assays of 7.7 and 9.7%, respectively.

Because there is evidence for sex-specific effects of neonatal maternal separation on adult anxiety behavior and stress response (McIntosh et al., 1999; Wigger and Neumann, 1999; Lehmann and Feldon, 2000; Kalinichev et al., 2002; DeJongh et al., 2005; Eklund and Arborelius, 2006) and adult learning and memory (Pryce et al., 2003; Kosten et al., 2005, 2006), and sex specific effects of adult stress manipulations on eyeblink conditioning (Wood and Shors, 1998; Leuner et al., 2004; Shors, 2006a,b), all analyses were completed for males and females separately. Stress-induced changes in corticosterone concentrations across time were evaluated using two-way ANOVAs (day \times group), followed by appropriate planned comparisons. Planned comparisons consisted of two-group *F*-tests done within the context of the overall ANOVA (Maxwell and Delaney, 2003), comparing the control group to each experimental group.

Eyeblink Conditioning

At 65–99 days of age, animals underwent surgery for implantation of electromyographic (EMG) and stimulating electrodes. Rats were injected with a mixture of ketamine (74 mg/kg), xylazine (3.7 mg/kg), and acepromazine (0.74 mg/kg) in physiological saline. The rats were placed in a stereotaxic apparatus, eye lubricant ointment was applied to both eyes, and the rat's skull was exposed and dried. Three holes were drilled and stainless steel screws were inserted in the skull. Rats were fitted with two Teflon-coated stainless steel EMG electrodes (0.0003") implanted in the anterior portion of the *orbicularis oculi* of the left eyelid and routed under the skin to the skull, where they were attached via gold pins to a headstage connector (Plastics One, Roanoke, VA). A ground wire was secured to two of the three skull screws and attached via a third gold pin to the headstage connector. A bipolar stimulating electrode (Plastics One) was implanted subdermally dorsocaudal to the left eye and routed to a separate plastic connector. The headstage and stimulating electrode connectors were secured to the skull and screws with dental acrylic. The surgical site was sutured around the headstage and antibiotic ointment was applied liberally to the scalp. Subcutaneous injections of 0.1 mL of Dopram and ~ 5 mL of sterile physiological

saline were administered. Rats were housed individually after surgery and handled daily for the 3 days prior to eyeblink conditioning.

After recovery from surgery (at least 5 days), eyeblink conditioning took place in operant boxes (Coulbourn Instruments, Allentown, PA) placed inside sound-attenuating chambers. A speaker for the delivery of the tone conditioned stimulus (CS) was positioned directly above the operant box and inside the sound-attenuating chamber. Each rat was placed in a conditioning chamber and EMG and bipolar connectors were plugged into a tether composed of light-weight wires and swivel connectors that allowed the rats to move freely. The rats received one 60-min adaptation session in which EMG signal was recorded during trials in which no stimuli were presented. Paired training began the next day and consisted of 10 sessions of 100 trials (1 session/day). The trials were delivered in 10 blocks of 10 trials and were 80% paired (8 paired and 2 CS-alone trial/block). An average intertrial interval of 25 s (range of 20–30 s) was used. All trials consisted of a 350-ms pre-CS period, followed by a 605-ms tone CS (2.8 kHz, 85 dB) and a 295-ms post-CS period for a total trial length of 1250-ms. During paired trials, the US (a 3.0-mA, 25-ms periocular stimulation) coterminated with the CS, producing a 580-ms interstimulus interval.

Stimulus delivery was controlled by a computer program (Spike2; CED, London, UK). Eyeblink EMG activity was amplified (1000 \times) and bandpass filtered (300–1000 Hz) by a differential AC amplifier (model 1700; A-M Systems, Carlsborgh, WA). The amplified and filtered EMG data were digitized (500 Hz), rectified, smoothed (0.01 s), and time shifted (0.01 s) by the Spike2 computer program. EMG data were saved and then analyzed using a custom data analysis program (King and Tracy, 1999) to compute the total number of trials in which a CR was detected. The threshold for detecting CRs was set at 7 standard deviations above the mean EMG activity during the pre-CS period. EMG activity during the 100-ms period immediately after CS onset was considered an alpha response to the tone and not considered a CR (Green et al., 2002). As an estimate of sensory (tone) responsivity, the average amplitude of alpha responses was calculated for each animal for each day. Trials with EMG activity during the 100-ms period immediately preceding CS onset were labeled as trials with excessive spontaneous eyelid movement and excluded from analysis. The percentage of trials in which a CR was displayed was calculated for each animal for each day. One animal was excluded from data analysis due to poor quality of EMG signal. Thus, for controls, $n = 9$ male, 8 female; for H-15, $n = 6$ male, 6 female; for MS-15, $n = 6$ male, 6 female; and MS-60, $n = 12$ male, 9 female.

Data were analyzed using two-way repeated measures ANOVAs (group \times day). To capture potential differences in CR production during early versus late training (i.e., acquisition versus asymptotic CR performance), separate repeated measures ANOVAs were performed for early acquisition (adaptation to day 5) versus late acquisition (days 6–10) for males and females, followed by planned comparisons. In addition, to rule out potential group confounds,

spontaneous blink rate and responsivity to the tone were compared across the four groups.

To assess potential differences in the stressfulness of the eyeblink conditioning procedure body weights were measured. Body weight is a sensitive indicator of either mild or short-term stress (Wellman et al., 2004; Miracle et al., 2006). Animal weights were obtained just prior to surgery and after the last training day. Average body weight was compared across groups using two-way ANOVAs (group \times day).

GR Immunohistochemistry and Quantification of GR Expression

Within 4 days of completion of behavioral testing, all rats were deeply anesthetized with urethane and transcardially perfused with cold 0.05 M phosphate buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Immediately following transcardial perfusion, the brain was removed, postfixed for 24 h, and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Frozen sections were cut coronally at 40 μ m on a sliding microtome and collected in 0.01 M PBS (pH 7.4). For each brain, two series of equally spaced sections (\sim 200 μ m apart) were collected through the entire interpositus nucleus of the cerebellum. These sections also contained sufficient coverage of cerebellar cortex region H-VI. One series was processed free-floating for immunohistochemistry, while the second series was processed for thionin staining (see later).

For immunohistochemical labeling of GR, sections were incubated for 1 h in PBS containing 3% normal goat serum and 0.1% Triton X to block nonspecific binding. Sections were then incubated for 1 h in 0.2% H₂O₂ in 50% methanol. After rinsing in PBS, sections were incubated overnight at 4°C in PBS containing 3% normal goat serum, 0.1% Triton X, and a polyclonal antibody to the rat GR (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing in PBS, sections were incubated for 30 min in PBS containing 5% NGS and biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA). After rinsing in 0.3% Triton X-100 in 0.01 M phosphate buffered saline (PBST), sections were incubated for 1 h in PBST with ABC Complex (Vector Laboratories). Staining was visualized using a nickel-intensified DAB reaction (Fig. 1). After rinsing, sections were mounted on gelatin-subbed slides, dehydrated, cleared, and coverslipped. Control sections incubated without the primary antibody were generated and demonstrated no staining. Eleven brains were eliminated due to histological processing errors (final n = control, 10 male, 7 female; H-15, 5 male, 6 female; MS-15, 4 male, 5 female; and MS-60, 7 male, 8 female).

GR expression in the interpositus nucleus was quantified using a computer-based image analysis system (MCID; Imaging Research, St. Catharines, ON, Canada) interfaced via a monochrome video camera (Sony XC-ST70; Sony, Park Ridge, NJ) with a microscope (Nikon Eclipse E600; Nikon Instruments, Melville, NY). For each animal, neurons were sampled at a final magnification of 1840 \times from a

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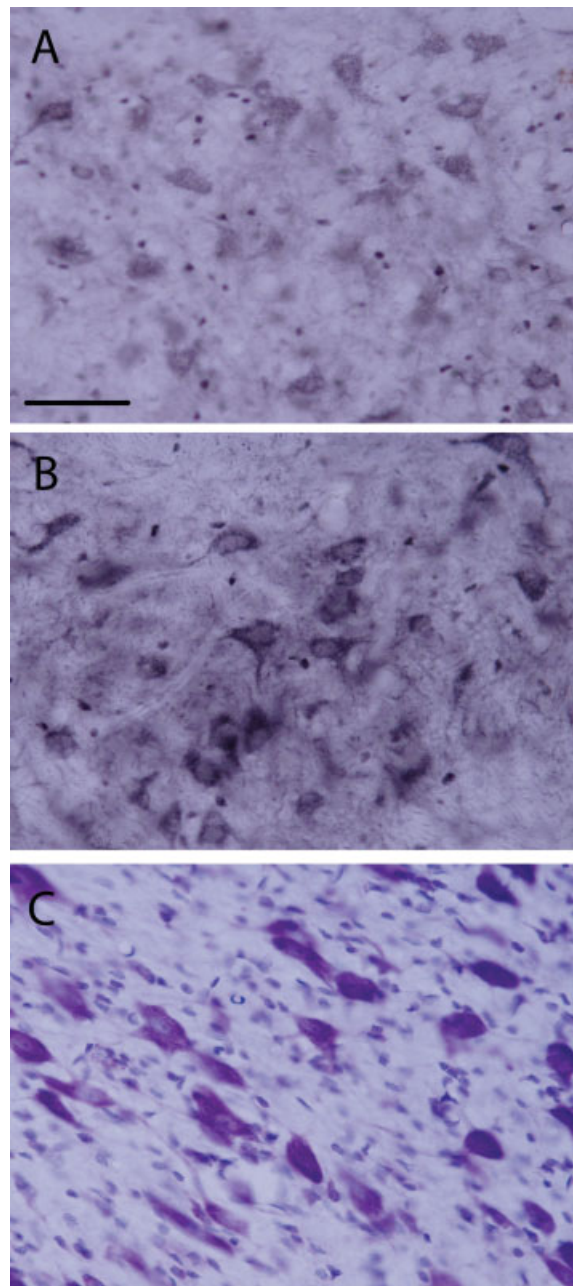


Figure 1 A, B. Digital light micrographs of GR-immunopositive neurons in the interpositus nucleus in a control (A) and an MS-60 (B) male rat. C. Thionin-stained neurons in the interpositus nucleus of a male control animal. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

125 μ m \times 125 μ m sampling frame centered mediolaterally within the interpositus. All labeled neurons contained within each sampling area were identified based on standard morphological criteria (large, multipolar soma) and the optical density of each soma was measured and expressed as average optical density per pixel, with optical density ranging from 0 (white) to 1 (black). To control for spurious dif-

ferences in staining and illumination across sections and animals, optical density measures within each section were expressed relative to white matter staining. Optical density was measured in an area of white matter averaging $1146 \mu\text{m}^2$ directly above the interpositus in each hemisphere. Relative intensity of neuronal staining was then calculated by dividing the optical density of each neuron by the optical density of the white matter in that section and hemisphere. All data were collected with the experimenter blind to condition.

Visual inspection indicated that intensity of staining of interpositus neurons varied along the anterior–posterior axis. Therefore, we divided the interpositus for each animal into three approximately equal-sized regions along the anterior–posterior axis. Relative intensity data were analyzed using two different approaches. First, mean relative intensities were computed for each animal for each region (anterior, middle, and posterior) and compared using one-way ANOVAs, followed by appropriate planned comparisons. To more carefully assess potential differences in the distribution of staining intensities, immunostaining of interpositus neurons was then categorized as being either light (having a relative intensity of at least one and a half standard deviations below the mean of controls), moderately light (between one and one and a half standard deviations below the mean), average (within one standard deviation of the mean), moderately dark (between one and one and a half standard deviations above the mean), or dark (greater than one and a half standard deviations above the mean). This method has been shown to be reliable for categorizing neurons by immunostaining intensity for subsequent frequency analyses and is sensitive to differences in protein expression assessed immunohistochemically (Osborne et al., 2007).

Estimated Neuron Number

The numerical densities of neurons in the interpositus nucleus of the cerebellum were obtained from thionin-stained sections (Fig. 1; three animals were excluded due to incomplete anterior to posterior sampling of the interpositus nucleus, thus control, $n = 8$ male, 7 female; H-15, $n = 4$ male, 6 female; MS-15, $n = 4$ male, 5 female; and MS-60, $n = 7$ male, 8 female) using an optical dissector technique similar to that described by Coggeshall (1992). Neuron counting was conducted using a computerized stereological system (StereoInvestigator; MBF Bioscience, Williston, VT) interfaced via a color video camera (Microfire; Optronics, Santa Barbara, CA) interfaced with a microscope (Nikon Eclipse 80i; Nikon Instruments). Shrinkage of sections due to histological processing was measured by focusing through each section with a $60\times$ objective and measuring the distance traveled using a stage-mounted microcator calibrated to a known standard. Shrinkage averaged $54\% \pm 0.65\%$ (average section thickness = $18.5 \mu\text{m}$); thus, the length of the dissector was set at $12 \mu\text{m}$. This length was adequate for visualizing neurons in multiple focal planes (Kim et al., 2005). This optical dissector height ($12 \mu\text{m}$) yielded an av-

erage guard zone height of $3.2 \mu\text{m}$ and a minimum guard zone of $0.5 \mu\text{m}$. For each animal, counts were made in an average of seven sections evenly spaced throughout the anterior–posterior extent of the interpositus nucleus. For each section, an average of eight samples were taken at a final magnification of $2213\times$ within a $75 \mu\text{m} \times 75 \mu\text{m}$ unbiased counting frame (i.e., only neuronal somata inside the counting frame or touching the upper or right edge of the frame, and that came into focus while focusing down through the dissector height, were counted). The medial-lateral and dorsal-ventral position of the counting frame within the interpositus nucleus of each section was systematically and randomly selected (counting grid $562 \mu\text{m} \times 274 \mu\text{m}$) by the StereoInvestigator program.

The volume of the interpositus nucleus was estimated for each animal by tracing the boundary of the interpositus nucleus for each section using the characteristic shape and thionin staining characteristics of the interpositus nucleus. These boundaries are readily identifiable in the rat. Based on the area contained within the traced contour, volumes and estimated number of neurons in the interpositus nucleus were calculated within the StereoInvestigator program. Estimates of total neuron number and interpositus volume were compared using one-way ANOVAs.

RESULTS

Neonatal Corticosterone Assay

For males, there was a significant elevation in corticosterone (Fig. 2; $F_{(3, 54)} = 4.15$, $p \leq 0.01$), and a marginal PND \times group interaction ($F_{(3, 54)} = 1.94$, $p \leq 0.08$). Planned comparisons revealed that at PND 2, corticosterone concentrations were elevated relative to control for MS-60 ($F_{(1, 33)} = 8.17$, $p < 0.01$). The increase in corticosterone approached significance for the H-15 animals ($F_{(1, 33)} = 3.75$, $p \leq 0.08$), but was not significant for MS-15 animals ($F_{(1, 33)} = 0.55$, ns). However, at PND 12, corticosterone concentrations were elevated relative to controls for all three groups ($F_{S(1, 21)} > 6.38$, $p < 0.05$).

In females, maternal separation elevated corticosterone concentrations (Fig. 2; $F_{(3, 41)} = 3.87$, $p < 0.01$) and this effect did not vary across PND ($F_{(3, 41)} = 0.36$, ns). Planned comparisons revealed that at PND 2, females corticosterone concentrations were elevated relative to control for H-15 ($F_{(1, 22)} = 5.76$, $p < 0.05$), marginally elevated for MS-15 ($F_{(1, 22)} = 3.40$, $p \leq 0.08$), but not different for MS-60 animals ($F_{(1, 22)} = 1.16$, ns). As with males, however, at PND 12, corticosterone concentrations elevated relative to control for all three groups ($F_{S(1, 19)} > 5.91$, $p < 0.05$). Therefore, separation and handling increased corticosterone concentrations for both male and female animals.

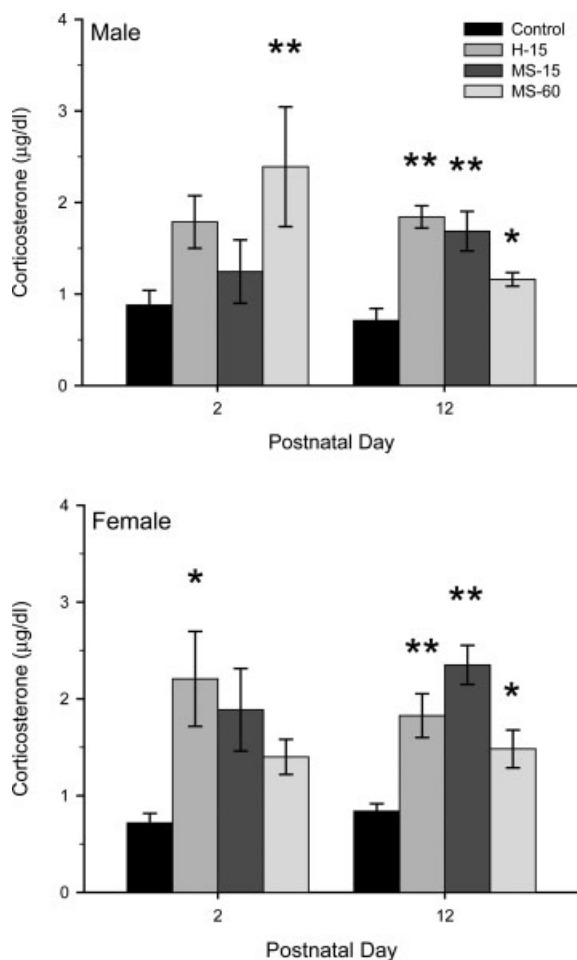


Figure 2 Mean (\pm SEM) corticosterone ($\mu\text{g/dL}$) sampled on PND 2 and 12 for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), or maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day starting on PND 2 for male animals and female animals. * $p < 0.05$ versus Control. ** $p < 0.01$ versus Control.

Eyeblink Conditioning

The baseline blink rate (Adaptation) was not different across groups (male, $F_{(3,29)} = 1.50$, ns; female, $F_{(3,29)} = 0.23$, ns). As a control measure, responsiveness to the tone before conditioning was assessed by calculating the mean amplitude of blinks that were recorded in the 100-ms period directly following the tone for each animal. Group means and standard errors were calculated and compared for male ($0.31 \text{ V} \pm 0.07$ for Control, $0.41 \text{ V} \pm 0.09$ for H-15, $0.21 \text{ V} \pm 0.09$ for MS-15, and $0.35 \text{ V} \pm 0.06$ for MS-60) and female ($0.30 \text{ V} \pm 0.06$ for Control, $0.38 \text{ V} \pm 0.07$ for H-15, $0.28 \text{ V} \pm 0.07$ for MS-15, and $0.37 \text{ V} \pm 0.06$ for MS-60) rats. There were no group differ-

ences in tone responsiveness (male, $F_{(3,29)} = 1.04$, ns; female, $F_{(3,29)} = 0.61$, ns).

For male rats, performance improved during both early and late acquisition, as indicated by increasing percent CR across day, for early (main effect of day, $F_{(5,145)} = 28.80$, $p < 0.01$; Fig. 3) and late acquisition (main effect of day, $F_{(4,116)} = 3.47$, $p \leq 0.01$). While groups did not differ in percentage of CRs during early acquisition ($F_{(3,29)} = 1.49$, ns), there was a significant difference across groups during late acquisition ($F_{(3,29)} = 3.26$, $p < 0.05$). Because there was no day by group interaction for either early or late acquisition ($F_{s(15,145)} < 1.55$, ns), planned comparisons were conducted for late acquisition comparing groups collapsed across days 6–10. MS-60 ($F_{(1,29)} = 8.71$, $p < 0.01$) and MS-15 rats ($F_{(1,29)} = 4.11$, $p \leq 0.05$) performed significantly worse than controls, with MS-60 rats performing 37% fewer CRs and MS-15 rats performing 30% fewer CRs. On the other hand, the H-15 group did not differ from controls ($F_{(1,29)} = 0.98$, ns).

For females, performance improved during both early (main effect of day, $F_{(5,125)} = 27.71$, $p < 0.01$) and late acquisition (main effect of day, $F_{(4,100)} =$

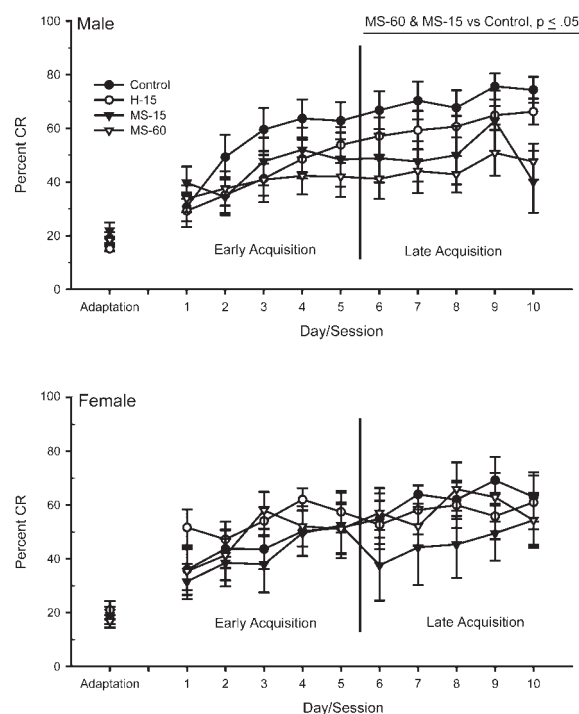


Figure 3 Mean (\pm SEM) percentage of conditioned responses (CR) for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), or maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day on PND 2–14 for male animals (above) and female animals (below).

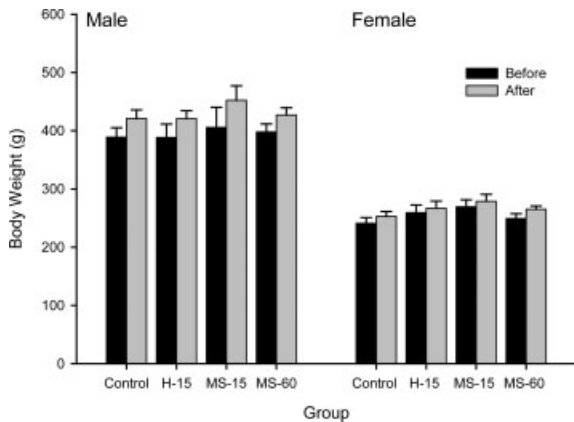


Figure 4 Mean (\pm SEM) body weight (g) before and after eyeblink conditioning for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), or maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day on PND 2–14 for male animals and female animals.

2.52, $p < 0.05$). However, there were no group differences in the percentage of CRs during either early ($F_{(3, 25)} = 0.64$, ns) or late acquisition ($F_{(3, 25)} = 0.92$, ns). There was not a day by group interaction for early ($F_{(15, 125)} = 0.62$, ns) or late acquisition ($F_{(12, 100)} = 0.72$, ns). To examine the possibility that the lack of group differences in females was due to poor performance in female controls and hence a “floor effect,” performance of control males and females was compared using two-way repeated measure ANOVAs. Males and females did not differ during early or late acquisition ($F_{S(1, 15)} < 0.89$, ns).

Both male and female rats gained weight during eyeblink conditioning (Fig. 4; main effect of day, $F_{S(3, 22)} > 14.54$, $p < 0.01$). Body weights did not differ across groups ($F_{S(3, 22)} < 1.31$, ns), nor was there a difference in weight gain across groups (group by time interaction, $F_{S(3, 22)} < 0.74$, ns), indicating that there were no group differences in the stressfulness of eyeblink conditioning for male or female rats.

Quantification of GR Immunohistochemistry

Overall, mean (\pm SEM) optical density of the white matter samples in the interpositus nucleus was 0.16 ± 0.05 , and mean white matter intensity did not vary across groups ($F_{(3, 47)} = 0.31$, ns). The mean number of neurons measured per section was 6.25 ± 0.12 . The average number of neurons measured per animal was 86.66 ± 3.33 and did not differ across groups for either male ($F_{(3, 20)} = 0.94$, ns) or female rats ($F_{(3, 22)} = 1.86$, ns).

For males, mean relative intensity of GR staining in the posterior interpositus was significantly different across groups (Fig. 5; $F_{(3, 22)} = 3.24$, $p < 0.05$). Specifically, GR staining was significantly increased by 33% in MS-60 males relative to controls ($F_{(1, 22)} = 9.05$, $p < 0.01$). H-15 males had marginally increased GR protein expression ($F_{(1, 22)} = 3.35$, $p \leq 0.08$; 22% higher than control), whereas the MS-15 males did not differ from controls ($F_{(1, 22)} = 2.13$, ns). Surprisingly, this effect was confined to the posterior region of the interpositus, as GR protein expression did not differ in the middle or anterior interpositus of male rats (middle, $F_{(3, 22)} = 0.55$, ns; anterior, $F_{(3, 22)} = 0.44$, ns).

Differences in distribution of GR protein expression paralleled those seen in the more gross measure of average relative intensity (Fig. 6). In the posterior interpositus, the distribution of staining intensities varied across groups, and this difference approached significance (for group by bin interaction, $F_{(12, 88)} = 1.75$, $p \leq 0.08$). Planned comparisons indicated that the frequency of darkly stained

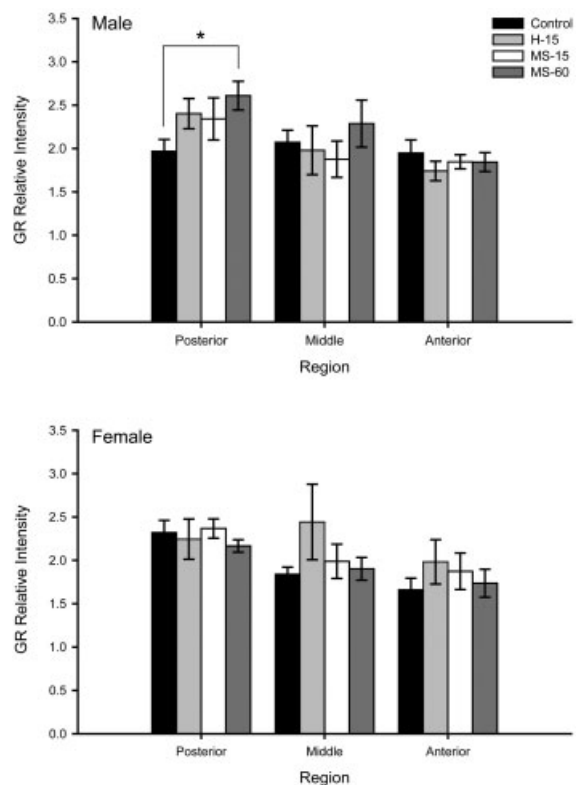


Figure 5 Mean (\pm SEM) GR relative to white matter intensity of immunostaining for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day on PND 2–14 for male animals (above) and female animals (below). * $p < 0.05$ versus Control.

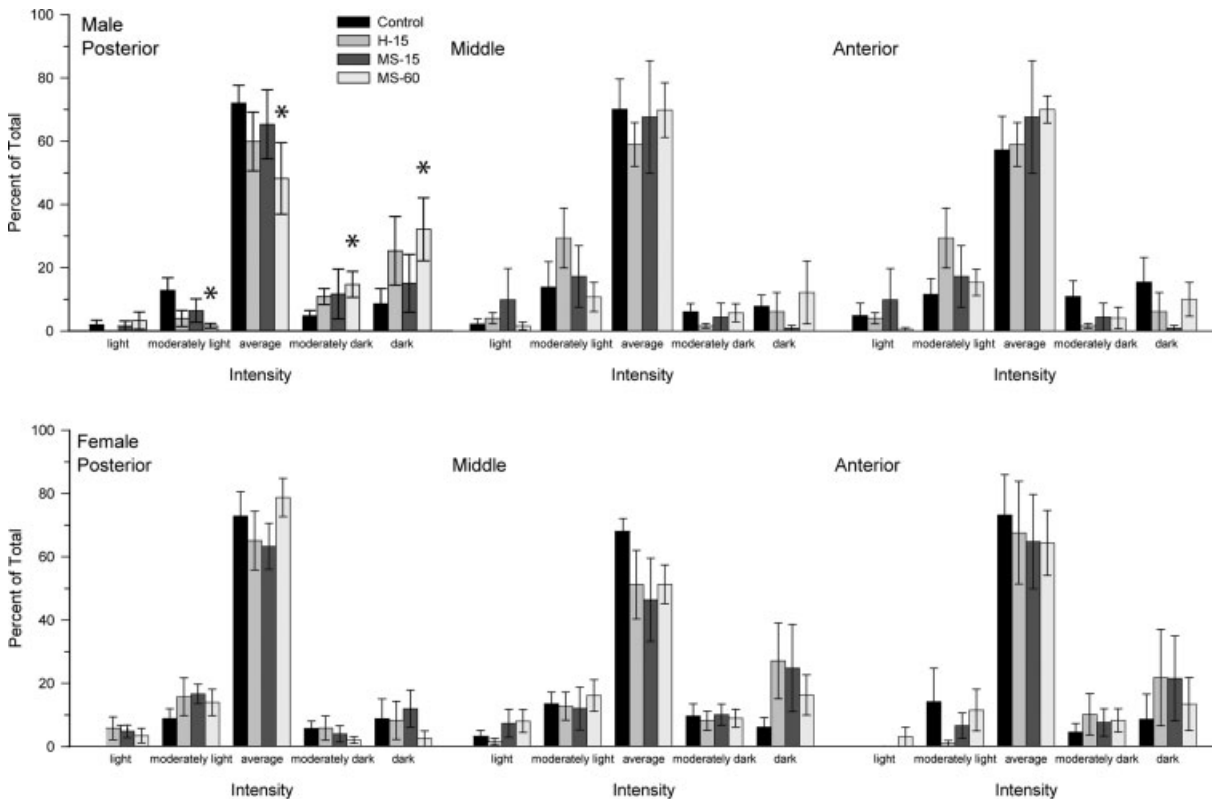


Figure 6 Histograms of the number of neurons (expressed as percent of total) categorized as light (having a relative intensity of at least one and a half standard deviations below the mean of controls), moderately light (between one and one and a half standard deviations below the mean), average (within one standard deviation of the mean), moderately dark (between one and one and a half standard deviations above the mean), or dark (greater than one and a half standard deviations above the mean) for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), or maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day on PND 2–14 in the posterior, middle, and anterior interpositus in males and females. * $p < 0.05$ versus Control.

neurons was significantly increased in MS-60 rats compared to controls, with significantly fewer neurons in the moderately light and average intensity bins ($F_{S(1, 22)} = 6.44$ and 4.45 , $p < 0.05$) and significantly more neurons in the moderately dark and dark intensity bins ($F_{S(1, 22)} = 4.90$ and 5.25 , $p < 0.05$). Additionally, in H-15 animals, there was a trend toward increased staining intensity as well, with fewer moderately light neurons relative to controls ($F_{(1, 22)} = 2.45$, $p \leq 0.08$). Planned comparisons revealed no significant differences for MS-15 or H-15 relative to control for any other intensity bins ($F_{S(1, 22)} < 2.15$, ns). As with the mean GR analyses, the effect on distribution of GR immunostaining was confined to the posterior region of the interpositus of male rats, with no differences observed in the middle and anterior portions (Fig. 6; $F_{S(12, 88)} < 0.85$, ns).

Consistent with eyeblink conditioning performance, female rats were not affected. The average

relative intensity of GR staining for female rats in the posterior, middle, or anterior interpositus did not differ across groups (Fig. 5; posterior, $F_{(3, 22)} = 0.53$, ns; middle, $F_{(3, 22)} = 1.31$, ns; anterior, $F_{(3, 22)} = 0.63$, ns). Similarly, there was not a significant difference in the staining intensities across groups in the posterior, middle, or anterior interpositus of female rats (Fig. 6; posterior, $F_{(12, 88)} = 0.73$, ns; middle, $F_{(12, 88)} = 1.03$, ns; anterior, $F_{(12, 88)} = 1.03$, ns).

Brain-Behavior Correlation

The relationship between average relative intensity of GR in the posterior interpositus of male and female animals and eyeblink conditioning averaged across late acquisition days (days 6–10) was examined. For males, there was a significant negative correlation between GR staining and percent CR (Fig. 7; $r = -0.52$, $p \leq 0.01$). On the other hand, for females, GR

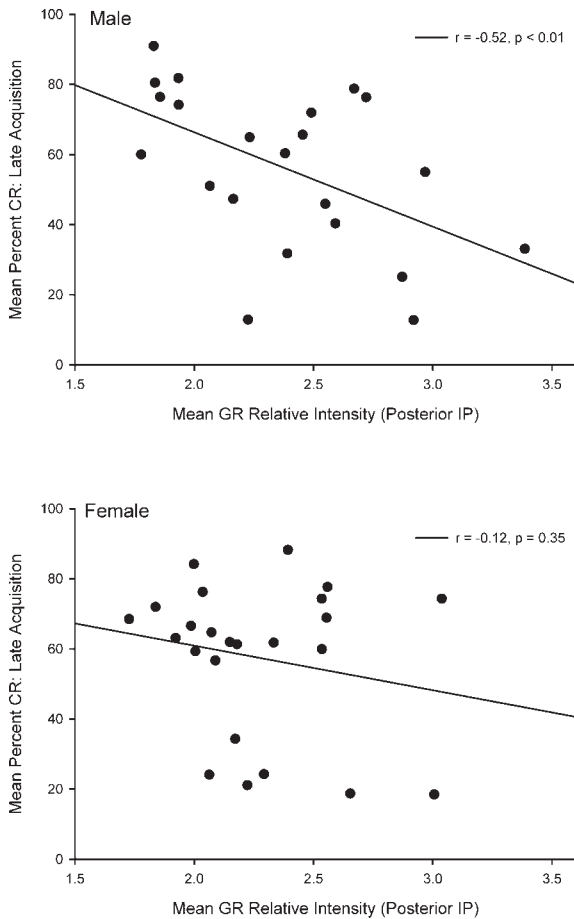


Figure 7 Linear regression analysis for mean (\pm SEM) GR intensity in the posterior interpositus and percent CR for eyeblink conditioning during late acquisition (days 6–10) for male animals and female animals.

immunostaining was not significantly correlated with average percent CR for late acquisition (Fig. 7; $r = -0.12$, ns). Thus, for male but not female animals GR protein expression in the interpositus nucleus predicts eyeblink performance.

Neuron Counts

The mean number of objects counted per frame was 6.22 ± 0.09 . The mean number of objects counted per animal was 216.36 ± 7.36 . The Gunderson coefficient of error averaged 0.07 ± 0.00 . The average number of neurons and the volume of the interpositus nucleus were compared across groups for male and female rats (Fig. 8). For male rats, neither estimated total neuron number (Fig. 8; $F_{(3, 19)} = 0.23$, ns), nor interpositus nucleus volume (Fig. 8; $F_{(3, 19)} = 0.31$, ns) varied significantly across groups. Given that GR changes were localized to posterior interpositus, we

performed an additional analysis restricted to posterior interpositus, but found no differences in neuron number for control versus MS-60 (Control 1712.80 ± 278.10 ; MS-60 1423.91 ± 299.47 ; $t = 0.71$, ns) or volume (Control $0.20 \pm 0.04 \text{ mm}^3$; MS-60 $0.17 \pm 0.02 \text{ mm}^3$; $t = 0.76$, ns). Similarly, for female rats number of interpositus nucleus neurons did not vary across the four groups (Fig. 8; $F_{(3, 22)} = 1.15$, ns). No group differences were found for interpositus volume (Fig. 8; $F_{(3, 22)} = 2.27$, ns).

DISCUSSION

The present study demonstrates a sex-dependent effect of maternal separation on eyeblink conditioning and a key component of its neural substrate, the interpositus nucleus. In males, prolonged neonatal maternal separation impaired eyeblink conditioning, and resulted in a corresponding increase in GR expression in the posterior portion of the interpositus nucleus. Brief maternal separation, with or without

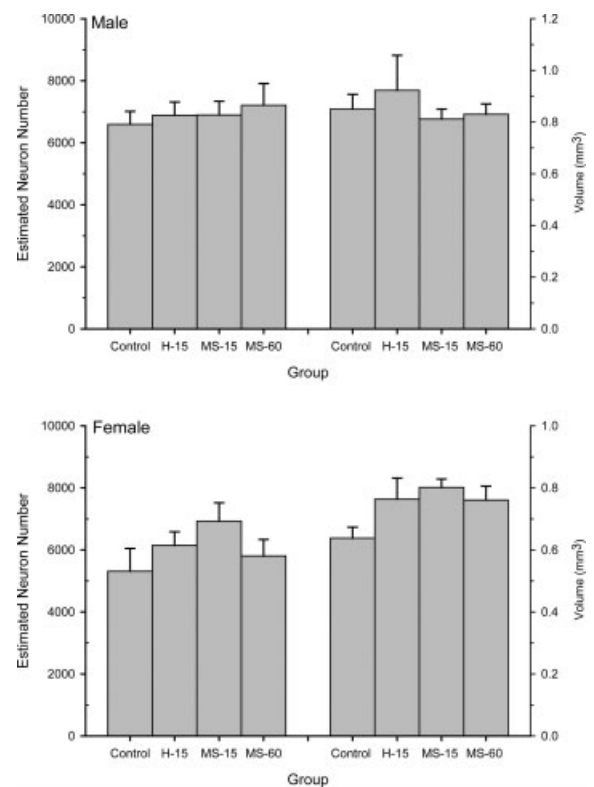


Figure 8 Mean (\pm SEM) number of neurons in and mean (\pm SEM) volume (mm^3) of the left interpositus for animals that underwent either standard animal facilities rearing (Control), handling for 15 min (H-15), or maternal separation for either 15 min (MS-15) or 60 min (MS-60) per day on PND 2–14 for male animals and female animals.

handling, had an intermediate effect on male eyeblink conditioning and GR expression. Further, eyeblink conditioning was correlated with GR expression in the posterior interpositus, with increased GR staining in posterior interpositus predicting poorer eyeblink conditioning. Interestingly, females are apparently protected from these adverse effects of maternal separation, as females did not show impaired eyeblink conditioning or altered GR expression. Maternal separation increased plasma corticosterone concentrations in both male and female rats, suggesting that the manipulation was stressful. Thus, the stress of neonatal maternal separation may have produced the alterations in GR expression and eyeblink conditioning observed in males.

Effects of Maternal Separation on Learning and Memory

The present study found that maternal separation impaired eyeblink conditioning in males. This is likely not due to altered stress responsivity modulating eyeblink conditioning. Given that body weight is sensitive to even a very mild or short-term stressor (Wellman et al., 2004; Miracle et al., 2006), the lack of group differences in body weight before and after conditioning suggests that eyeblink conditioning was not differentially stressful across groups.

Impaired eyeblink conditioning resulting from maternal separation is consistent with other studies showing separation-induced deficits in spatial learning and memory, and retention of conditioned fear (Meerlo et al., 1999; Huot et al., 2002; Pryce et al., 2003; Kosten et al., 2005, 2006; Uysal et al., 2005; Cannizzaro et al., 2006). Alternatively, other studies have found separation-induced facilitation of learning and memory, which may be due to differences in the timing or aversiveness of the various conditions (Pryce et al., 2003).

The present study is the first demonstration of an effect of neonatal maternal separation on a behavior not mediated by an HPA axis structure. The circuitry necessary for delay eyeblink conditioning is confined to the cerebellum and brainstem and is not involved in HPA axis regulation (for reviews, see Steinmetz, 2000; Medina et al., 2002; Christian and Thompson, 2003). In contrast, previous studies of spatial learning or conditioned fear engaged hippocampal and amygdala circuitry known to be important for stress responding (Eichenbaum et al., 1999; LeDoux, 2000, respectively). Therefore, the effects of neonatal maternal separation may be more widespread than previously thought. Nonetheless, it is important to

note that hippocampal and amygdalar inputs to the cerebellum play a modulatory role in some forms of eyeblink conditioning (Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999; Rorick-Kehn and Steinmetz, 2005), and therefore separation-induced changes in HPA axis structures may also play a role.

Effects of Maternal Separation on GR Expression

Our data indicate that maternal separation increases GR expression in the posterior region of the interpositus nucleus. This is consistent with previous demonstrations of altered GR expression in forebrain structures (Meaney and Aitken, 1985; Meaney et al., 1985a,b; Sapolsky et al., 1985; Dallman et al., 1991; Buijs et al., 1993; Abdulla et al., 1995; Vazquez, 1998). For instance, repeated prolonged maternal separation decreases adult hippocampal GR expression (Ladd et al., 2004). Alternatively, brief maternal separation increases hippocampal GR expression (Meaney et al., 1985a,b; Ladd et al., 2004). In the present study, brief and prolonged maternal separation both increased adult GR expression, suggesting that the mechanisms underlying separation-induced changes in GR expression in HPA axis structures such as the hippocampus may be different from those underlying changes in the interpositus.

Additionally, we found that increases in GR immunostaining in the interpositus correlated with behavioral deficits. Though this does not demonstrate causation, it is consistent with GR's modulating learning and memory (Roosendaal et al., 1996; Kim et al., 2006). Future studies can directly test the hypothesis that alterations in GRs in the interpositus nucleus of neonatally stressed rats are responsible for adult eyeblink classical conditioning deficits by blocking GRs in interpositus during conditioning in adult rats. Alternatively, alterations in GRs might not be directly responsible for the effect on behavior. For instance, the behavioral effects could be the result of an interaction between glucocorticoid and NMDA receptors, which are both plentiful in the interpositus nucleus (Araki et al., 1992) and important for learning-related plasticity (Chen and Steinmetz, 2000). Given the evidence for learning-related changes as a result of NMDA-GR interactions in the amygdala and hippocampus (Marginos and McEwen, 1995; Kim et al., 1996; Shors and Mathew, 1998; Roosendaal et al., 2003), these receptors may interact to produce the deficits in eyeblink conditioning in our study. Separation-induced changes in hippocampal and amygdalar inputs to the circuit, which also play a modulatory

role in some forms of eyeblink conditioning (Moyer et al., 1990; Kim et al., 1995; Weiss et al., 1999; Rorick-Kehn and Steinmetz, 2005), may also play a role in the deficits we have documented.

The effect of maternal separation on GR expression may exist at multiple levels of the eyeblink conditioning circuitry. GR are also expressed in the cerebellar cortex (Ahima and Harlan, 1990; Cintra et al., 1994), which provides timing-related modulatory input to the interpositus nucleus (Lavond et al., 1984; McCormick and Thompson, 1984a,b). Therefore, GR expression in cerebellar cortex may also correlate with eyeblink conditioning performance, and future studies will examine the relationship between potential separation-induced changes in GR receptors in cerebellar cortex and eyeblink conditioning.

Interestingly, the alteration in GR expression in our study was confined to the posterior interpositus, and negatively correlated with the deficits in eyeblink conditioning. Some data from the cat suggest that posterior interpositus neurons can modulate CRs (Delgado-Garcia and Gruart, 2002, 2005, 2006; Jimenez-Diaz et al., 2004), whereas studies in the rabbit suggests that lesions of posterior interpositus do not affect learning of the conditioned eyeblink response (Steinmetz et al., 1992a,b). Thus, the posterior GR effects noted here may affect CR performance rather than acquisition. Further studies in the rat should be conducted to determine the relative involvements of the anterior and posterior interpositus nucleus in eyeblink conditioning.

Sex Differences in the Effects of Maternal Separation

The effect of maternal separation on eyeblink conditioning and interpositus GRs is confined to males, providing further evidence for sex differences in stress effects in both adults and neonates. For instance, a great deal of evidence indicates sex-specific effects of maternal separation on adult anxiety behavior and stress response (McIntosh et al., 1999; Wigger and Neumann, 1999; Lehmann and Feldon, 2000; Kalinichev et al., 2002; DeJongh et al., 2005; Eklund and Arborelius, 2006). Emerging data suggest sex differences in adult learning and memory following maternal separation (Pryce et al., 2003; Kosten et al., 2005, 2006). Adult stress modulates eyeblink conditioning, with the direction of effects (facilitation, impairment, or no effect) varying with age and estrous status (Wood and Shors, 1998; Shors, 2006a,b). Interestingly, overall, these studies suggest that acute stress in adult rats facilitates eyeblink con-

ditioning in males but not female rats, a finding that is opposite the present result. This apparent contradiction suggests that organizational effects of neonatal stress may be markedly different from its activational effects in adulthood.

Given that prolonged maternal separation differentially increases stress responsivity and anxiety behavior in adult males (McIntosh et al., 1999; Wigger and Neumann, 1999; Lehmann and Feldon, 2000; Kalinichev et al., 2002; DeJongh et al., 2005; Eklund and Arborelius, 2006), the eyeblink conditioning deficits in males could be secondary to altered stress/anxiety behavior. However, this is unlikely, as body weights were equivalent across groups, suggesting no differential stress in adult males. In addition, adult stress facilitates eyeblink conditioning in males (Hodes and Shors, 2005). Therefore, if heightened stress responsiveness were responsible for our effect in males, we would expect to see facilitation of eyeblink conditioning rather than impairment. Thus, it is unlikely that differences in stress responsivity explain the deficit in eyeblink conditioning.

Possible Mechanisms

Neonatal corticosterone assays indicated that our manipulation increased corticosterone neonatally. Given that prenatal corticosterone exposure influences cerebellar development (Velazquez and Romano, 1987), it is possible that the effects of maternal separation on adult learning and memory are mediated by neonatal increases in corticosterone. However, the effect of early corticosterone exposure may depend on the timing or duration of the manipulation, as corticosterone administration on PND 18–21 did not effect delay eyeblink conditioning in males (Claffin et al., 2005).

Alternatively, the observed effects may be mediated by maternal interactions. For example, brief maternal separation results in increased maternal licking and grooming (Lee and Williams, 1975, 1977; Liu, 1997; Pryce et al., 2001), and pups that received high, naturally occurring levels of maternal care show facilitated spatial learning and memory as adults. However, prolonged maternal separation also results in increased maternal care (Pryce et al., 2001; Macri et al., 2004) and the pattern of altered maternal care is identical to that seen following brief maternal separation (Macri et al., 2004). Given that brief and prolonged maternal separation similarly increases maternal care, it seems unlikely that differences in maternal care explain the deficits in eyeblink conditioning and GR expression in interpositus, which

were less pronounced in rats experiencing brief maternal separation.

Regardless of mechanism, we have shown that prolonged neonatal maternal separation affects eyeblink conditioning performance and GR expression in the interpositus in a sex-dependent manner. This is the first demonstration that neonatal maternal separation affects a non-HPA axis structure and a behavior mediated by it. Therefore, neonatal maternal separation can have widespread (i.e., outside of the HPA axis) and yet selective (posterior interpositus) and lasting effects on brain and behavior.

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