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Glucocorticoid receptor blockade in the posterior interpositus nucleus reverses maternal separation-induced deficits in adult eyeblink conditioning

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Abstract

Previously, we showed that neonatal maternal separation impaired eyeblink conditioning in adult rats. This impairment is correlated with increased glucocorticoid receptor (GR) expression in the cerebellar posterior interpositus nucleus, a critical site of learning-related plasticity. To assess whether increased GR expression is responsible for the separation-induced learning impairment, we infused a GR antagonist (mifepristone) or vehicle into the posterior interpositus during eyeblink conditioning in adult male Long-Evans rats that had undergone control rearing or neonatal maternal separation (1 h/day, postnatal days 2–14). Rats received standard rearing (control) or neonatal maternal separation (separated; 1h/day on postnatal days 2–14). In adulthood, rats underwent surgery for implantation of recording electrodes in the obicularis oculi of the left eyelid, a bipolar stimulating electrode dorsocaudal to the left eye, and an infusion guide cannula positioned over the posterior interpositus. Then, rats underwent 10 daily sessions of eyeblink conditioning. Rats in each group received either 0.2 μ l of mifepristone (2ng in 2% EtOH) or vehicle infusion prior to each eyeblink conditioning session. Mifepristone infusions improved conditioning in separated rats, but impaired control rats' performance. Thus, separation-induced increases in GRs may mediate the learning deficit seen in adult neonatally separated rats.

Keywords

eyeblink conditioning; maternal separation; mifepristone; RU38486; glucocorticoid receptors

Introduction

Neonatal stressors such as maternal separation have adverse effects on adult behavior and cognition, including adult emotionality and hypothalamic-pituitary-adrenal (HPA) axis responsivity to stress (De Jongh, Geyer, Olivier, and Groenink, 2005; Kalinichev, Easterling, Plotsky, and Holtzman, 2002; McIntosh, Anisman, and Merali, 1999). Maternal separation also produces changes in adult learning and memory. For instance, neonatal maternal separation results in adult impairments in spatial learning and memory (Aisa, Gil-Bea, Marcos, Tordera, Lasheras, Del Río, and Ramírez, 2009; Aisa, Tordera, Lasheras, Del

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Rio, and Ramirez, 2007; Huot, Plotsky, Lenox, and McNamara, 2002; Uysal, Ozdemir, Dayi, Yalaz, Baltaci, and Bediz, 2005), and extinction of conditioned fear (Stevenson, Spicer, Mason, and Marsden, 2009; Wilber, Southwood, and Wellman, 2009). However, some forms of learning and memory may be facilitated by maternal separation; for example, adult active avoidance learning is enhanced following neonatal maternal separation (Schäble, Poeggel, Braun, and Gruss, 2007).

Eyeblink conditioning provides a simplified and well-characterized model system for exploring the mechanisms of neonatal stress effects on adult learning and memory. Eyeblink conditioning involves pairing a tone with a mild shock to the eye region (an unconditioned stimulus; US). With training, the tone (conditioned stimulus; CS) comes to predict the shock, and elicits an eyeblink conditioned response (CR). The critical circuitry for eyeblink conditioning is well understood (Thompson and Steinmetz, 2009). The interpositus nucleus of the cerebellum is a site of CS-US convergence, and a critical site of learning-related plasticity: Interpositus activity models acquisition of the CR (McCormick and Thompson, 1984a; b) and temporary inactivation reversibly prevents learning (Krupa, Thompson, and Thompson, 1993).

Neonatal maternal separation produces deficits in eyeblink conditioning and increases glucocorticoid receptor (GR) expression in the posterior region of the interpositus nucleus in adult male rats (Wilber, Southwood, Sokoloff, Steinmetz, and Wellman, 2007). Further, GR expression in posterior interpositus interneurons but not projection neurons predicts deficits in eyeblink conditioning (Wilber and Wellman, 2009a), which is consistent with the notion that impaired eyeblink conditioning may be due to altered modulation by posterior interpositus. Others have shown that glucocorticoids modulate adult learning and memory, with facilitated or impaired performance depending on the timing and duration of exposure (Kim, Song, and Kosten, 2006; Roozendaal and McGaugh, 1997; Roozendaal, Quirarte, and McGaugh, 1997), and that systemic administration of the GR antagonist mifepristone in adults blocks separation-induced deficits in object recognition memory (Aisa et al., 2007). Thus, the increased GR expression in posterior interpositus following maternal separation might be responsible for the impaired adult eyeblink conditioning. To test this hypothesis, we assessed whether mifepristone infusion into the posterior interpositus nucleus of adult rats during eyeblink conditioning can ameliorate the effect of daily maternal separation on adult eyeblink conditioning.

Materials and Methods

Untimed pregnant Long Evans rats (Harlan Indianapolis, IN, N = 16) arrived approximately 1 week before giving birth. Dams were housed individually in standard laboratory cages (48 × 20 × 26 cm), with food and water ad libitum and a 12:12 h light/dark cycle (lights on at 0700 h). On PND 2 (day of birth = PND 0), pups were culled to litters of 7–10 while maintaining a male:female ratio of approximately 1:1. 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 IACUC.

Maternal separation was carried out as described previously, to allow us to extend our previous findings (Wilber et al., 2007; Wilber and Wellman, 2009a; b). Litters (7–9 per group) were randomly assigned to either standard animal facilities rearing (Control) or maternal separation groups (1 h per day, PND 2–14; Separated). At approximately 0800 hours each day, dams were removed from the home cage, after which pups were placed in a Plexiglas cage (28 × 17 × 12 cm) lined with bedding, taken to a nearby room, and placed in an incubator (Ambient Room Temperature Incubator; Avey Incubator; Evergreen, CO). The incubator was maintained at room temperature (22.5 ± 0.5 °C) for the duration of the

separation period, during which pups were permitted to huddle with littermates. On PND 28, animals were weaned and housed in same sex/same litter groups of 2–3 until surgery.

Although whole litters were manipulated, eyeblink conditioning was examined in males only, because separation-induced changes in eyeblink conditioning and GR expression in the interpositus occur only in males (Wilber et al., 2007; Wilber and Wellman, 2009b).

At 77–147 days of age, electromyographic (EMG) and stimulating electrodes and an infusion cannula aimed at the posterior interpositus were implanted. Rats were anesthetized with *ip* ketamine (74 mg/kg), xylazine (3.7 mg/kg), and acepromazine (0.74 mg/kg) and given *sc* Rimadyl (5 mg/kg). Rats were placed in a stereotaxic apparatus, and two Teflon-coated stainless steel EMG electrodes (0.075 mm) were implanted in the anterior portion of the *orbicularis oculi* of the left eyelid and routed subdermally to the skull, where they were attached via gold pins to a headstage connector (Plastics One; Roanoke, VA). A ground wire was secured to skull screws and attached via a third gold pin to the headstage. A bipolar stimulating electrode (Plastics One) was implanted subdermally dorsocaudal to the left eye and routed to a separate connector. A 26 gauge guide cannula (Plastics One; Roanoke, VA) was implanted in the left posterior interpositus (3.8 mm posterior to lambda, 2.3 mm from midline, 4.8 mm ventral). Finally, the connectors and guide cannula were secured to skull screws with dental acrylic. Skin was sutured around the headstage. Rats were housed individually after surgery and handled three times prior to eyeblink conditioning.

After recovery from surgery (≥ 5 days), animals were randomly assigned to vehicle ($n=10$ control; $n=10$ separated) or mifepristone ($n=7$ control; $n=7$ separated) infusion groups. Prior to each session of eyeblink conditioning a dummy cannula was removed and an infusion cannula was inserted into the guide cannula, through which 0.2 μ l of mifepristone (2ng in 2% EtOH) or vehicle was pressure-injected into the posterior interpositus nucleus using a Hamilton syringe (Reno, NV) connected to the infusion cannula (Bioanalytical Systems, West Lafayette, IN). The infusion cannula was left in place for 5 min to ensure sufficient time for diffusion. Eyeblink conditioning began immediately following removal of the infusion cannula and took place in operant boxes inside sound-attenuating chambers (Med-Associates; St. Albans, VT). Rats received one 60-min adaptation session in which EMG signal was recorded during trials in which no stimuli were presented. The next 10 days consisted of daily sessions comprised of 10 blocks of 10 trials, 80% paired (8 paired and 2 CS-alone trials/block), with an average 25 s intertrial interval. All trials consisted of a 350-ms pre-CS period, followed by a 375-ms tone CS (2.8 kHz, 85dB) and a 295-ms post-CS period (trial length 1020 ms). During paired trials, the US (3.0-mA, 25-ms periocular stimulation) co-terminated with the CS, producing a 350-ms interstimulus interval.

Stimulus delivery was controlled by Spike2 software (CED, London, UK). Eyeblink EMG activity was amplified (5000 $\times$) and bandpass filtered (100–9000 Hz; Lynx-8 amplifier, Neuralynx; Bozeman, MT), then digitized (1000 Hz), rectified, smoothed (0.01 s), time shifted (0.01 s), acquired, and stored with a Power 1401 625 kHz data acquisition system (CED, London, UK). EMG data were analyzed using a custom program to compute the 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, and trials with EMG activity 14 standard deviations above the mean during the pre-CS period were discarded (Wilber et al., 2007; Wilber and Wellman, 2009a). On average 13% of trials were discarded, similar to other studies (e.g., Green, Johnson, Goodlett, and Steinmetz, 2002). EMG activity during the 100-ms period immediately after CS onset was considered an alpha response to the tone rather than a CR (Green et al., 2002; Wilber et al., 2007; Wilber and Wellman, 2009a). As an estimate of sensory responsivity, the average amplitude of alpha responses was calculated for each animal for each day. Trials with excessive spontaneous

eye movement (EMG activity during the 100 ms immediately preceding CS onset) were excluded from analysis. The percentage of trials in which a CR was displayed and the average amplified amplitude of each CR (V) were calculated for each animal for each day. Data were analyzed using three-way repeated measures ANOVAs (rearing condition $\times$ drug $\times$ day) followed by appropriate planned comparisons.

After completion of eyeblink conditioning, rats were deeply anesthetized with urethane and transcardially perfused with saline followed by 10% formalin. Brains were removed and cryoprotected. Frozen sections were cut coronally at 40 μ m, mounted on chrome alum-subbed slides, and stained with cresyl echt violet. Infusion cannula placement was verified based on standard cytoarchitectural criteria (Figure 1; Paxinos and Watson, 1998) and confined to the posterior third of the interpositus nucleus.

Results

To rule out potential confounds, rate and amplitude of spontaneous blinking during adaptation and responsivity to the tone (alpha response) were compared. Spontaneous blink rate did not vary across rearing condition or drug treatment (Figure 3; main effect of rearing condition, $F_{(1, 30)} = 0.05$, ns; main effect of drug, $F_{(1, 30)} = 0.00$, ns; rearing condition by drug interaction, $F_{(1, 30)} = 3.77$, ns). Similarly, spontaneous blink amplitude did not vary across rearing condition or drug treatment (Figure 3; main effect of rearing condition, $F_{(1, 30)} = 0.00$, ns; main effect of drug, $F_{(1, 30)} = 0.20$, ns; rearing condition by drug interaction, $F_{(1, 30)} = 3.42$, ns). Consistent with previous results (Wilber et al., 2007), there was no effect of rearing condition on responsiveness to the tone (Control Vehicle, mean = $0.11 \text{ V} \pm 0.03$ SEM; Separated Vehicle $0.10 \text{ V} \pm 0.01$; $F_{(1, 30)} = 3.35$, ns). However, there was a significant effect of mifepristone treatment on tone responsiveness (Control Mifepristone, $0.28 \text{ V} \pm 0.07$; Separated Mifepristone, $0.16 \text{ V} \pm 0.03$; $F_{(1, 30)} = 9.04$, $p < 0.01$), and this did not vary across rearing conditions ($F_{(1, 30)} = 2.39$, ns). Thus, mifepristone treatment increased the amplitude of rats' alpha responses, regardless of rearing condition.

Group differences were readily apparent when the raw data for animals in each group were examined (Figure 2), and statistical analyses agreed with visual examination of the raw data (Figure 3). Performance improved during training, as indicated by increasing percent CR across day (Figure 3; main effect of day, $F_{(9, 270)} = 21.07$, $p < 0.001$). There was not a significant main effect of drug ($F_{(1, 30)} = 0.53$, ns) or rearing condition ($F_{(1, 30)} = 0.54$, ns), and no significant interaction for day by rearing condition ($F_{(9, 270)} = 0.61$, ns) or day by drug ($F_{(9, 270)} = 0.51$, ns). However, there was a significant interaction of drug and rearing condition ($F_{(1, 30)} = 2.43$, $p < 0.05$), and this effect varied across training days (day by drug by rearing condition $F_{(9, 270)} = 2.38$, $p < 0.05$). Planned comparisons were conducted for vehicle-treated separated versus control rats for each day of acquisition. By the end of training, maternally separated rats showed significantly fewer CRs (day 9, $F_{(1, 18)} = 4.31$, $p \leq 0.05$; days 1–8 and 10, $F_{(1, 18)} < 3.78$, ns). Further, mifepristone significantly improved performance of separated rats compared to vehicle treated separated rats on acquisition days 2, 4, and 7–9 ($F_{(1, 15)} > 4.57$, $p \leq 0.05$; days 1, 3, 5, 6, and 10, $F_{(1, 15)} < 4.15$, ns). Finally, mifepristone infusions had the opposite effect on control rats. Mifepristone infusion significantly impaired performance in control rats at the end of acquisition compared to vehicle treated control rats (days 9 and 10, $F_{(1, 15)} > 4.85$, $p < 0.05$; days 1–8, $F_{(1, 15)} < 1.45$, ns).

CR amplitude increased during training (Figure 3; main effect of day, $F_{(9, 270)} = 7.99$, $p < 0.001$). There was not a significant main effect of drug (Figure 3; $F_{(1, 30)} = 0.12$, ns) or rearing condition ($F_{(1, 30)} = 0.50$, ns), and no significant interaction for day by rearing condition ($F_{(9, 270)} = 1.86$, ns) or day by drug ($F_{(9, 270)} = 1.26$, ns). However, there was a

significant interaction of drug and rearing condition ($F_{(1, 30)} = 4.73$, $p < 0.05$), and this varied across training days (day by drug by rearing condition $F_{(9, 270)} = 2.44$, $p < 0.05$). Planned comparisons were conducted for vehicle-treated separated versus control rats for each day of acquisition. Maternal separation did not significantly reduce CR amplitude ($F_{(1, 18)} < 2.18$, ns). Nonetheless, planned comparisons for separated rats that received vehicle versus mifepristone infusions showed that mifepristone significantly increased CR amplitude on acquisition days 8–10 ($F_{(1, 15)} > 4.76$, $p \leq 0.05$; days 1–7, $F_{(1, 15)} < 3.63$, ns). Finally, planned comparisons demonstrated that mifepristone did not alter CR amplitude for control rats (all $F_{(1, 15)} < 3.31$, ns).

Discussion

Consistent with our previous findings (Wilber et al., 2007; Wilber and Wellman, 2009a), this study demonstrated that maternal separation impairs eyeblink conditioning. The separation-induced impairment in eyeblink conditioning was reversed by infusion of a GR antagonist into the posterior interpositus of neonatally separated rats. Further, in control rats, blocking these receptors impaired eyeblink conditioning.

Evidence from nonrodent species suggests a topographical (i.e., to posterior) organization of the interpositus nucleus, with the anterior interpositus playing an important role in learning the CR (Steinmetz, Lavond, Ivkovich, Logan, and Thompson, 1992; Steinmetz, Logue, and Steinmetz, 1992) and the posterior interpositus modulating (Delgado-Garcia and Gruart, 2006; Sanchez-Campusano, Gruart, and Delgado-Garcia, 2007) the CR. There is evidence for a similar anterior localization of the critical circuitry for learning in the rat (Plakke, Freeman, and Poremba, 2007). We previously demonstrated that posterior interpositus interneurons show more robust changes in GR expression than projection neurons and only GR expression in interneurons is associated with eyeblink conditioning performance (Wilber and Wellman, 2009a). Together with the present data, these studies suggest that interneurons in the posterior interpositus may modulate the activity of projection neurons in the anterior interpositus and neonatal maternal separation alters that modulation via changes in GR expression.

The circuitry necessary for delay eyeblink conditioning is confined to the cerebellum and brainstem and is not involved in HPA axis regulation (Christian, 2003; Medina, Repa, Mauk, and LeDoux, 2002; for reviews see, Steinmetz, 2000). Nonetheless, neonatally, cerebellar GR expression is higher than in any other brain region (Pavlik and Buresova, 1984) and it has been suggested that this might make the cerebellum particularly susceptible to neonatal exposure to exogenous glucocorticoids (Aden, Goverud, Liestol, Loberg, Paulsen, Maehlen, and Lomo, 2008; Noguchi, Smith, Swiney, and Farber, 2008; Noguchi, Walls, Wozniak, Olney, Roth, and Farber, 2008; Pavlik and Buresova, 1984). Alternatively, neonatal separation produces changes the hippocampus (Ladd, Huot, Thrivikraman, Nemeroff, Meaney, and Plotsky, 2004; Vazquez, Van Oers, Levine, and Akil, 1996) and hippocampal inputs to the cerebellum play a modulatory role in some forms of eyeblink conditioning (Kim, Clark, and Thompson, 1995; Moyer, Deyo, and Disterhoft, 1990; Weiss, Bouwmeester, Power, and Disterhoft, 1999); therefore, separation-induced changes in the hippocampus may also play a role. Finally, note that prenatal stress associated with shipping pregnant dams, as was done in this and previous studies (Wilber et al., 2007; Wilber and Wellman, 2009a; b), could interact with the neonatal maternal separation to contribute to the effects we observed.

Little is known about the function of GRs in the cerebellum. In fact, given that increased GR expression in the hippocampus is associated with facilitated learning (Liu, Diorio, Day, Francis, and Meaney, 2000; Liu, Diorio, Tannenbaum, Caldji, Francis, Freedman, Sharma,

Pearson, Plotsky, and Meaney, 1997), opposite the impaired learning following increased GR expression we described in our earlier studies (Wilber et al., 2007; Wilber and Wellman, 2009a), the role of GRs may differ for the cerebellum and hippocampus.

The finding that neonatal maternal separation resulted in impaired adult learning is consistent with other studies showing 15 min to 3 h repeated daily neonatal separation during at least the first two or three postnatal weeks induced impairments in adult learning and memory relative to animals that underwent standard animal facilities rearing (Aisa et al., 2009; Aisa et al., 2007; Huot et al., 2002; Stevenson et al., 2009; Wilber et al., 2007; Wilber et al., 2009; Wilber and Wellman, 2009a). However, in the present study, blocking GR in separated animals results in eyeblink conditioning performance that is enhanced relative to controls (though not to the point of statistical significance), suggesting that it might be possible to enhance adult learning with a neonatal manipulation that produced a similar level of adult GR activation. In fact, some neonatal maternal separation manipulations do enhance adult learning and memory. For example, 6h maternal separation every other day from postnatal days 12–18 resulted in enhanced adult latent inhibition learning (Lehmann, Stöhr, and Feldon, 2000). Future studies could test the hypothesis that a different intensity or duration of neonatal stressor may facilitate adult eyeblink conditioning by exposing pups to a range of corticosterone doses.

Given that the neonatal separation employed in this study results in increased GR receptor expression in the posterior interpositus in adults (Wilber et al., 2007; Wilber and Wellman, 2009a; b), it is likely that mifepristone infusion into the posterior interpositus of neonatally separated rats improved eyeblink conditioning by blocking these excess GRs. It is possible that mifepristone could be diffusing into the anterior interpositus or cerebellar cortex lobule HVI and action of mifepristone on GRs in these structures could contribute to these effects. However, effects mediated by nearby structures are unlikely given that others have shown that a similar dose and volume of mifepristone infused into the central amygdala does not diffuse to the adjacent basolateral amygdala (Rooszendaal and McGaugh, 1997). Regardless, it is unlikely that mifepristone diffused outside the cerebellum, so mifepristone's effect could not be due to actions on GRs in other brain regions affected by maternal separation, such as the prefrontal cortex (Meaney, Aitken, Bodnoff, Iny, Tatarewicz, and Sapolsky, 1985; Wilber et al., 2009) or hippocampus (Aisa et al., 2007; Ladd et al., 2004; Meaney et al., 1985).

Alternatively, we observed increased amplitude of alpha responses in mifepristone-infused animals. Thus, mifepristone might increase the percentage of conditioned responses by simply modulating blink amplitude. However, this is unlikely, because whereas mifepristone increased alpha responses for both control and separated rats, it had opposite effects on learning across rearing conditions.

Finally, mifepristone also binds to progesterone receptors, albeit with markedly less affinity than GRs (Van Look and Bygdeman, 1989), and progesterone receptors are present in the cerebellum, including the deep nuclei (Curran-Rauhut and Petersen, 2002; Guerra-Araiza, Reyna-Neyra, Salazar, Cerbón, Morimoto, and Camacho-Arroyo, 2001); therefore, we cannot rule out the possibility that mifepristone's action on progesterone receptors contributes to the effects we observed. Nonetheless, given that we know maternal separation increases GR in the posterior interpositus and mifepristone has a higher affinity for GRs (Van Look and Bygdeman, 1989), it is likely that mifepristone's ability to reverse the separation effect is due to its blockade of GRs.

Interestingly, mifepristone infusion impaired performance in control rats. An inverted U-shaped relationship between GR activation and performance has been described for other

learning tasks (Roozendaal and McGaugh, 1997; Roozendaal, McReynolds, Van der Zee, Lee, McGaugh, and McIntyre, 2009). Our data suggest a similar relationship for cerebellar-dependent forms of learning and memory. Specifically, neonatal maternal separation increases GR expression, causing increased GR activation that impairs eyeblink conditioning performance. Blocking GRs in separated rats may reduce GR activation to an optimal level, thus improving performance. Blocking GRs in control rats may reduce GR activation below normal, optimal levels, thus impairing eyeblink conditioning.

In summary, neonatal maternal separation impairs eyeblink conditioning and infusion of a GR antagonist into the posterior interpositus reverses separation-induced impairments in eyeblink conditioning. Thus, separation-induced increases in GR in posterior interpositus may mediate the deficit in eyeblink conditioning seen in neonatally separated rats.

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Figure 1.

Coronal sections throughout the posterior (top) to anterior (bottom) extent of the interpositus nucleus (ip; top of each section is dorsal; from Paxinos and Watson, 1998). Cannula placement for adult rats that underwent standard animal facilities rearing (Control; circle) or maternal separation for 1 h per day on PND 2–14 (Separated; triangle) and received daily vehicle (White) or mifepristone (Black) infusions.

Figure 2.

Raw EMG traces for the last 10 paired trials on the final acquisition day for representative rats that underwent either standard animal facilities rearing (Control) or maternal separation for 1 h per day on PND 2–14 (Separated) and received daily vehicle or mifepristone infusions just prior to the training session. Rats were chosen because their raw data were near their respective group means.

Figure 3.

Above. Mean ($\pm$ SEM) percentage of conditioned responses (CR) for rats that underwent either standard animal facilities rearing (Control) or maternal separation for 1 h per day on PND 2–14 (Separated) and received daily vehicle ($n=10$ control; $n=10$ separated) or mifepristone (Mif; $n=7$ control; $n=7$ separated) infusions just prior to each adaptation and paired training session. Below. Mean ($\pm$ SEM) amplitude of conditioned responses (CR) for Control and Separated rats that received daily vehicle or mifepristone infusions. Asterisks (*) indicate significant differences relative to Control Vehicle (Veh.); daggers (†) indicate significant differences relative to Separated Veh.