Neuroscience 177 (2011) 56–65

NEONATAL CORTICOSTERONE ADMINISTRATION IMPAIRS ADULT EYEBLINK CONDITIONING AND DECREASES GLUCOCORTICOID RECEPTOR EXPRESSION IN THE CEREBELLAR INTERPOSITUS NUCLEUS

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Abstract—Neonatal maternal separation alters adult learning and memory. Previously, we showed that neonatal separation impaired eyeblink conditioning in adult rats and increased glucocorticoid receptor (GR) expression in the cerebellar interpositus nucleus, a critical site of learning-related plasticity. Daily neonatal separation (1 h/day on postnatal days 2–14) increases neonatal plasma corticosterone levels. Therefore, effects of separation on GR expression in the interpositus and consequently adult eyeblink conditioning may be mediated by neonatal increases in corticosterone. As a first step in exploring a potential role for corticosterone in the neonatal separation effects we observed, we assessed whether systemic daily (postnatal days 2–14) corticosterone injections mimic neonatal separation effects on adult eyeblink conditioning and GR expression in the interpositus. Control uninjected animals were compared to animals receiving either daily corticosterone injections or daily injections of an equal volume of vehicle. Plasma corticosterone values were measured in a separate group of control, neonatally separated, vehicle injected, or corticosterone injected pups. In adulthood, rats underwent surgery for implantation of recording and stimulating electrodes. After recovery from surgery, rats underwent 10 daily sessions of eyeblink conditioning. Then, brains were processed for GR immunohistochemistry and GR expression in the interpositus nucleus was assessed. Vehicle and corticosterone injections both produced much larger increases in neonatal plasma corticosterone than did daily maternal separation, with the largest increases occurring in the corticosterone-injected group. Neonatal corticosterone injections impaired adult eyeblink conditioning and decreased GR expression in the interpositus nucleus, while the effects of vehicle injections were intermediate. Thus, while neonatal injections and maternal separation both produce adult impairments in learning and memory, these manipulations produce opposite changes in GR expression. This suggests an inverted U-shaped relationship may exist between both neonatal corticosterone levels and adult GR expression in the interpositus nucleus, and adult GR expression in the interpositus and eyeblink conditioning.
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Abbreviations: CR, conditioned response; CS, conditioned stimulus; EMG, electromyographic; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; NGS, normal goat serum; PBS, phosphate buffered saline; PBST, 0.3% Triton X-100 in PBS; PND, postnatal day; US, an unconditioned stimulus.

Key words: eyeblink conditioning, maternal separation, neonatal stress, neonatal corticosterone, glucocorticoid receptors, learning and memory.

Adverse early experience is associated with the development of many psychiatric disorders (Salk et al., 1985; Lizardi et al., 1995; Glod and Teicher, 1996; Cortes et al., 2005), including schizophrenia (van Os and Selten, 1998; Edwards, 2007). In animal models, adverse effects of neonatal stressors such as maternal separation include increased adult emotionality and hypothalamic-pituitary-adrenal (HPA) axis responsivity to stress (McIntosh et al., 1999; Kalinichev et al., 2002; De Jongh et al., 2005). Maternal separation also produces changes in adult learning and memory. For instance, neonatal maternal separation results in adult impairments in spatial learning and memory (Huot et al., 2002; Uysal et al., 2005; Aisa et al., 2007, 2009) and extinction of conditioned fear (Stevenson et al., 2009; Wilber et al., 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 et al., 2007). Finally, cerebellar activity is altered in humans with schizophrenia (Andreasen and Pierson, 2008), and animal models show that adverse early experience, including maternal deprivation, produces changes in the cerebellum that may contribute to schizophrenia-like behaviors (López-Gallardo et al., 2008; Laviola et al., 2009).

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). While forebrain regions such as the hippocampus and amygdala also contribute to eyeblink conditioning, the critical circuitry for delay eyeblink conditioning is within the cerebellum and brainstem (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 et al., 1993). Therefore, although alterations in many brain regions likely contribute to psychopathology,

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doi:10.1016/j.neuroscience.2011.01.010

eyeblink conditioning and the critical cerebellar circuitry provides an ideal model system for exploring the mechanisms of neonatal stress effects on adult learning and memory. Understanding how neonatal stress alters the function of this well-characterized model system may ultimately contribute to our understanding of the role of adverse early experience in the development of psychopathology.

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 et al., 2007; Wilber and Wellman, 2009a). Further, infusion of the GR antagonist mifepristone into the posterior interpositus in adults reverses separation-induced deficits in eyeblink conditioning (Wilber et al., 2010), suggesting that the increased GR expression in the posterior interpositus is responsible for the impaired adult eyeblink conditioning. Similarly, neonatal separation-induced increases in plasma corticosterone are apparent following single and repeated daily maternal separation (Wilber et al., 2007), GR expression is altered by postnatal day (PND) 15 (Wilber and Wellman, 2009b), and perinatal corticosterone exposure influences the development of several brain structures (Balazs and Cotterrell, 1972; Ardelenu and Sterescu, 1978; De Kloet et al., 1988b; Sousa et al., 1998; Roskoden et al., 2005), including the cerebellum (Velazquez and Romano, 1987). Thus, neonatal increases in corticosterone could contribute to the effects of maternal separation on GR expression, and consequently adult learning and memory. Therefore, we assessed the effects of daily corticosterone injections on PND 2–14 on adult eyeblink conditioning and GR expression in the interpositus.

EXPERIMENTAL PROCEDURES

Animals and treatment

As in previous experiments (Wilber et al., 2007, 2009, 2010; Wilber and Wellman, 2009a,b), untimed pregnant Long Evans rats (Harlan Indianapolis, IN, USA, $n=11$) 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 seven to 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 produces a slow rise in plasma corticosterone, with the largest peak in corticosterone concentration occurring nearly 12 h after a 1 h separation (Walker et al., 1991; Wilber et al., 2007). It is not possible to exactly duplicate this complicated profile with exogenous corticosterone; therefore we chose to focus on mimicking the peak corticosterone concentrations that occur in the afternoon and early evening 12 h after maternal separation. Thus, as in previous experiments (Wilber et al., 2007, 2009, 2010; Wilber and Wellman, 2009a,b), litters (four to five per group) were randomly assigned to either standard animal facilities rearing (Control; $n=8$ pups), corticosterone (Sigma, St. Louis, MO, USA; $n=14$ pups), or vehicle treatment groups ($n=10$ pups).

Previous studies have shown that neonatal administration of corticosterone in doses ranging from 1 to 5 mg/kg can produce long-lasting elevations in plasma corticosterone and changes in adult behavior (Van Oers et al., 1998; Roskoden et al., 2005). However, because pilot studies in our laboratory indicated that the 5 mg/kg dose produce extremely large and highly variable plasma corticosterone concentrations, in this study we used a smaller dose, 1 mg/kg. Corticosterone was dissolved in sesame oil and administered at approximately 1 mg/kg based on unpublished data from our laboratory on average weight for age. Injection volume did not exceed 0.05 ml and vehicle injected pups received an equivalent volume of sesame oil. Weight data from a separate group of age-matched pups were used in place of daily weighing to minimize handling and time away from the dam. Injections were subcutaneous and occurred at approximately 1800 h each day. Prior to injections dams were removed from the home cage, after which pups were placed in a Plexiglas cage (28×17×12 cm) lined with bedding. As is standard practice in our laboratory (Wilber et al., 2007, 2009, 2010; Wilber and Wellman, 2009a,b), on PND 28, animals were weaned and housed in same sex/same litter groups of two to three until surgery.

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

Eyeblink conditioning

At 12–16 weeks of age, electromyographic (EMG) and stimulating electrodes were implanted. Rats were anesthetized with i.p. ketamine (Pfizer Animal Health, Madison, NJ, USA; 74 mg/kg), xylazine (Lloyd Laboratories, Shenandoah, IA, USA; 3.7 mg/kg), and acepromazine (Butler, Dublin, OH, USA; 0.74 mg/kg) and given s.c. Rimadyl (Pfizer Animal Health, Madison, NJ, USA; 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 superior 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, USA). 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. Finally, the connectors 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), rats underwent eyeblink conditioning, which took place in operant boxes inside sound-attenuating chambers (Med-Associates; St. Albans, VT, USA). 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 (eight paired and two 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, 85 dB) 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×) and bandpass filtered (100–9000 Hz; Lynx-8 amplifier, Neuralynx; Bozeman, MT, USA), 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

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