GLUCOCORTICOID MECHANISMS OF NEONATAL SEPARATION EFFECTS ON ADULT LEARNING AND MEMORY

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Aaron A. Wilber

GLUCOCORTICOID MECHANISMS OF NEONATAL SEPARATION EFFECTS ON ADULT LEARNING AND MEMORY

Many studies have documented the relationship between adverse early experience and the development of psychiatric disorders. Understanding the mental health consequences of perinatal stressors is crucial to preventative treatment. Neonatal maternal separation in the rat is a good model system for assessing the effects of adverse early experience, and eyeblink conditioning is a good model for studying the relationship between neonatal stress and adult learning and memory. Previously, I showed that daily neonatal maternal separation (1h/day on postnatal days 2-14) increases plasma corticosterone levels during the first and second postnatal week. Further, I showed that neonatal maternal separation impairs adult eyeblink conditioning and produces a correlated increase in glucocorticoid receptor expression in the posterior interpositus nucleus of the cerebellum. My dissertation research is focused on characterizing the role of glucocorticoids in this effect. I measured cerebellar glucocorticoid receptor expression on postnatal day 15 and 21, and found that maternal separation (1h/day on postnatal days 2-14) prevented a normal decrease in glucocorticorticoid receptor expression in the interpositus from postnatal day 15 to 21. Further, I showed that infusion of a glucocorticoid receptor blocker into the interpositus of adult rats before training normalized eyeblink conditioning in separated rats. This suggests that the increased glucocorticoid receptor expression in the interpositus mediates the adult deficit in

eyeblink conditioning. Next, I showed that either neonatal corticosterone or vehicle injections on postnatal days 2-14,which both produced larger increases in plasma corticosterone than did neonatal separation and mimicked the separation-induced impairment in adult eyeblink conditioning, but did so by *decreasing* glucocorticoid receptor expression. This suggests an inverted-U shaped relationship between both the magnitude of neonatal stress and adult glucocorticoid receptor expression; and adult glucocorticoid receptor activation and learning. Finally, I found that blocking glucocorticoid receptors during maternal separation (1h/day on postnatal days 2-14) attenuated the separation-induced impairment in adult eyeblink conditioning and increased GR expression. Together, these experiments suggest that neonatal separation alters glucocorticoid receptor modulation of adult associative learning, and separation-induced increases in neonatal plasma corticosterone may play a role in this effect.

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Chapter 1:

Introduction and Background

Introduction

Neonatal maternal separation as a model of adverse early experience.

Adverse early experiences such as child abuse or neglect are associated with a variety of problems later in life, including the development of childhood and adult onset psychiatric disorders such as depression (Lizardi et al., 1995; Frodl et al., 2010), increased risk for adolescent suicide (Salk et al., 1985; Roy et al., 2010), post-traumatic stress disorder (Glod and Teicher, 1996; Cortes et al., 2005), and schizophrenia (van Os and Selten, 1998; Edwards, 2007). Morphological and anatomical changes in certain brain regions have also been documented in adults who experienced adverse events as infants and children. These changes include reduced hippocampal volume (Bremner et al., 1997; Stein et al., 1997; Driessen et al., 2000; Vythilingam et al., 2002), reduced amygdala volume, (Driessen et al., 2000), and reduced hippocampal white matter (Frodl et al., 2010). Brain changes following neonatal adversity have been observed as early as adolescence (Carrion et al., 2001), though most studies suggest that neonatal stress-induced brain changes are not apparent until adulthood (Bremner et al., 1997; Stein et al., 1997; De Bellis et al., 1999; Driessen et al., 2000; Carrion et al., 2001; De Bellis et al., 2001; Vythilingam et al., 2002).

Non-human animal studies have also found adverse effects of neonatal stressors on adult behavior and cognition. Neonatal maternal separation in

which rodent pups are removed from the dam daily during the first 2-3 postnatal weeks is commonly used as a neonatal stressor (Kalinichev et al., 2002; Kinkead et al., 2005; Kosten et al., 2005; Avitsur et al., 2006; Garcia-Rodenas et al., 2006; Kosten et al., 2006; Michaels and Holtzman, 2006; Aisa et al., 2008; Aisa et al., 2009a; Aisa et al., 2009c; Avitsur and Sheridan, 2009; Boasen et al., 2009; Farkas et al., 2009). Neonatal maternal separation alters adult emotionality and hypothalamic-pituitary-adrenal (HPA) axis responsivity to stress (McIntosh et al., 1999; Wigger and Neumann, 1999; Kalinichev et al., 2002; De Jongh et al., 2005; Daniels et al., 2009). Early life stress in animals using maternal separation has also been used to model the pathology seen in humans reared in adverse environments. For example, neonatal maternal separation increases adult drug intake, including increased adult ethanol (Huot et al., 2001; Miczek et al., 2008) and cocaine intake (Moffett et al., 2006; Miczek et al., 2008), possibly by modifying the reinforcing properties of drugs of abuse (Michaels and Holtzman, 2007; Moffett et al., 2007; Miczek et al., 2008), and suggesting a neonatal stress-induced vulnerability to drug abuse.

Maternal separation effects on adult learning and memory.

In addition to increased adult emotionality and increased drug intake, neonatal maternal separation also produces changes in several forms of adult learning and memory. A majority of the studies suggest that neonatal maternal separation results in adult impairments in spatial learning and memory when neonatally separated adults are compared to animals that were neonatally reared in a standard animal facilities environment (Huot et al., 2002; Uysal et al., 2005;

Aisa et al., 2007; Aisa et al., 2009a; Aisa et al., 2009c). In contrast one study found facilitated spatial learning in adults that underwent daily individual pup maternal separation (isolation) rearing compared to completely non-handled (including cage changing) rearing (Pryce et al., 2003). Neonatal maternal separation has also been associated with impaired acquisition of conditioned fear (Stevenson et al., 2009), extinction of conditioned fear (Stevenson et al., 2009; Wilber et al., 2009), object recognition (Aisa et al., 2007; Aisa et al., 2008), and inhibitory avoidance learning and memory (Kosten et al., 2007). Similarly, adult recall of conditioned fear may be reduced following maternal separation (Meerlo et al., 1999), but again, when pups are separated individually (isolated) and compared to non-handled controls, no impairment is observed (Kosten et al., 2005). Finally, when pups are separated individually (isolated) and compared to non-handled controls adult object recognition memory is enhanced (Kosten et al., 2007).

In general, both brief separation (15 minutes/day), a manipulation commonly referred to as handling (Meaney et al., 1985a; O'Donnell et al., 1994), and long separation (1-6 h/day) lead to impaired learning and memory; however, the effects of brief separation tend to be less severe. Long (either 1 or 6 h/day) neonatal separation impairs acquisition of conditioned fear (Wilber et al., 2007b; Stevenson et al., 2009), while brief (15 minutes/day) neonatal separation has no effect on acquisition, but impairs initial extinction (Stevenson et al., 2009) and recall of extinction assessed 24 h later (Wilber et al., 2009). Similarly, long neonatal separation (6 h/day) impairs both acquisition and retention of spatial learning and memory (Uysal et al., 2005), while neonatal separation for shorter

durations (2 – 3 h/day) impairs retention but not acquisition of spatial learning (Aisa et al., 2007; Aisa et al., 2009c).

The changes in adult behavior and cognition are paralleled by changes in morphology and neurochemistry of the circuitry critical for learning and emotion regulation. For example, neonatal separation alters glucocorticoid receptor concentrations and mossy fiber density in the hippocampus (Meaney et al., 1985b; Huot et al., 2002; Ladd et al., 2004; Aisa et al., 2009c). Similarly, neonatal separation results in adult alterations in the prefrontal cortex, including a developmental reorganization of the expression of the number of calbindin-D28k-immunopositive interneurons (Helmeke et al., 2008), decreased N-methyl-D-aspartic acid (NMDA) receptor expression (Ziabreva et al., 2000; Wilber et al., 2009), and increased glucocorticoid receptor expression (Wilber et al., 2009). Further, systemic injections of the glucocorticoid receptor antagonist mifepristone in adult rats reverse neonatal separation-induced deficits in adult spatial and object recognition memory (Aisa et al., 2007; Aisa et al., 2008). Together these studies suggest that changes in receptor expression and morphology in these forebrain structures suggest putative mechanisms for both the altered anxiety-related behavior, and changes in fear conditioning, extinction, and spatial learning and memory. Further, neonatal separation decreases dopamine transporter expression in the nucleus accumbens and caudate putamen (Meaney et al., 2002), suggesting a potential mechanism for the neonatal separation-induced vulnerability to drugs of abuse.

Eyeblink conditioning and neural substrates as model system.

Eyeblink conditioning provides a simplified and well-characterized model system for exploring the mechanism of neonatal stress effects on adult learning and memory. 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, or unconditioned stimulus (US). After training, the tone (the conditioned stimulus or CS) elicits an eyeblink conditioned response (CR). The critical circuitry for eyeblink conditioning is well understood, and includes structures in the cerebellum and brainstem (Figure 1.1; for reviews see, Steinmetz, 2000; Christian and Thompson, 2003; Thompson and Steinmetz, 2009). There are two critical sites of plasticity in the cerebellum: A deep cerebellar nucleus, the interpositus nucleus, and the cerebellar cortex, including lobule H-VI and the anterior lobe.

The interpositus nucleus of the cerebellum is a site of convergence of the CS signal from the mossy fibers of the contralateral pontine nucleus (Brodal, 1981; Steinmetz and Sengelaub, 1992) and US from the contralateral dorsolateral accessory olive (Brodal, 1981; Sugihara et al., 2001). There are two general types of cells in the interpositus nucleus, principal neurons and interneurons, and both cell types are present early in postnatal development (Brodal, 1981; Maricich and Herrup, 1999; Czubayko et al., 2001; Morcuende et al., 2002; Uusisaari et al., 2007). The principal cells in the interpositus nucleus project to the contralateral red nucleus (Brodal, 1981; Morcuende et al., 2002), while interneurons synapse primarily on principal neurons within the interpositus

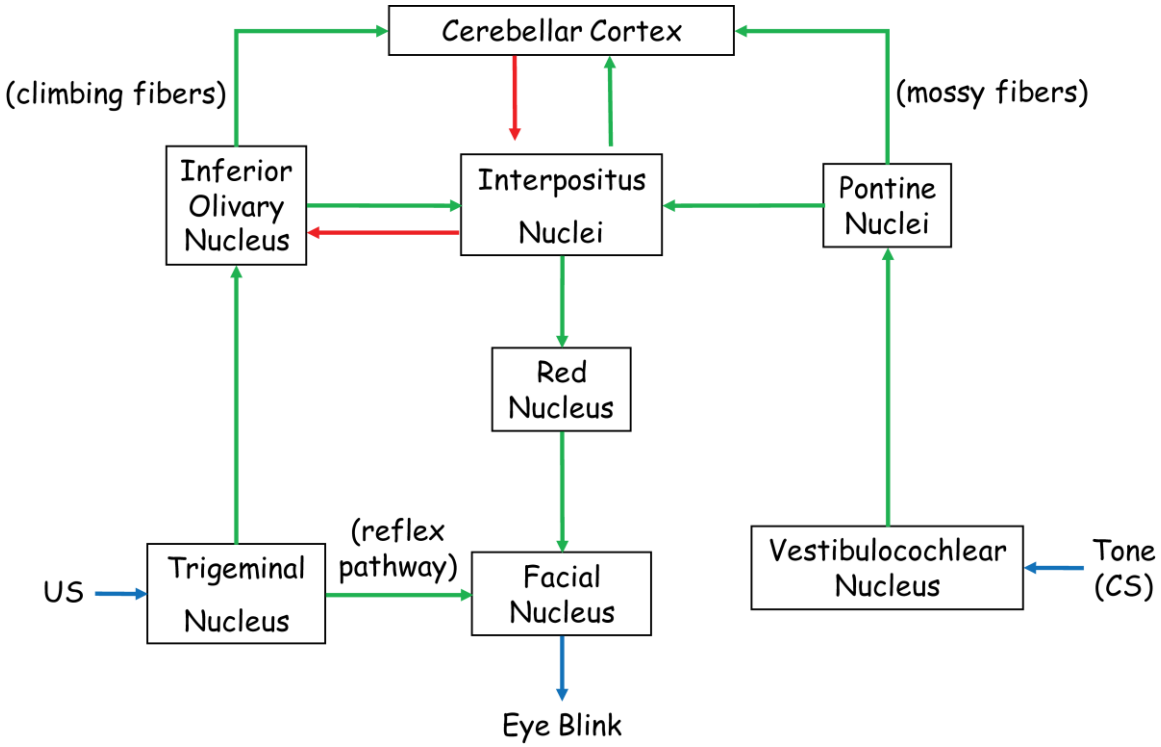

Figure 1.1: Simplified schematic of the essential circuitry for eyeblink conditioning. The tone CS pathway involves projections from auditory nuclei to the pontine nuclei and to the cerebellum. The US pathway includes projections from the trigeminal to the inferior olive and to the cerebellum. The CR pathway projects from the interpositus nucleus to the red nucleus and to the facial nucleus. Red lines indicate inhibitory synapses, and green lines indicate

nucleus (Maricich and Herrup, 1999; Czubayko et al., 2001; Uusisaari et al., 2007). The anterior interpositus is a critical site of learning-related plasticity. For example, anterior interpositus activity models acquisition of the CR (McCormick and Thompson, 1984a; McCormick and Thompson, 1984b) and temporary inactivation of the interpositus reversibly prevents learning, whereas temporary inactivation of the output structure (red nucleus) prevents expression of the CR but not learning (Krupa et al., 1993; Nordholm et al., 1993).

The Purkinje cells of the cerebellar cortex are an additional site of convergence for the CS and US (Yeo et al., 1985; Steinmetz and Sengelaub, 1992). Purkinje cells in the anterior lobe seem to be critical for a well-timed CR. For example, lesions that include the anterior lobe result in short latency (i.e., poorly timed) CRs (Garcia et al., 1999). Similarly, the activity pattern of Purkinje cells in the anterior lobe during eyeblink conditioning trials would serve to inhibit the interpositus early in the CS period and disinhibit the interpositus late in the CS period, thus providing the appropriate modulation for a well-timed CR (Green and Steinmetz, 2005). The role of lobule H-VI in eyeblink conditioning is less clear, but at least some of the Purkinje cells in H-VI seem to promote interpositus activity in response to the CS (Christian, 2004; Thompson and Steinmetz, 2009).

Finally, eyeblink conditioning has also been well characterized in humans, involves the same cerebellar structures (for review see; Woodruff-Pak and Steinmetz, 2000), and is altered in disorders such as schizophrenia (Sears et al., 2000; Hofer et al., 2001; Marenco et al., 2003; Brown et al., 2005; Winslow, 2005; López-Gallardo et al., 2008; Laviola et al., 2009) and autism (Sears and Finn, 1994). Further, a genetic predisposition for schizophrenia may interact with

early aversive experience to produce changes in the cerebellum that contribute to schizophrenia (Winslow, 2005; López-Gallardo et al., 2008; Laviola et al., 2009).

Glucocorticoid receptors have been shown to modulate several adult learning and memory tasks (Roozendaal and McGaugh, 1997; Roozendaal et al., 1997; Kim et al., 2006). Given that neonatal maternal separation increases plasma concentrations of the ligand for the glucocorticoid receptor, corticosterone (Huot et al., 2002; Wilber et al., 2007a), glucocorticoids may play a critical role in neonatal separation effects on adult learning and memory. Glucocorticoid receptors are distributed throughout the forebrain, cerebellum (including the deep nuclei), and the spinal cord (Ahima and Harlan, 1990). The glucocorticoid receptor is a homodimer present in the cytosol of neurons and many other cell types throughout the body. When activated, it translocates to the nucleus to produce a wide variety of genomic effects (Giguere et al., 1986; Evans, 1988). Therefore, I used eyeblink conditioning and its neural circuitry to explore the relationship between neonatal stress, glucocorticoid receptor expression, and adult learning and memory.

Background.

Characterization of neonatal separation effects on adult eyeblink conditioning and cerebellum.

To begin to characterize the effects of neonatal stress on adult learning and memory, I assessed adult eyeblink conditioning and adult glucocorticoid receptor expression in the posterior interpositus nucleus of the cerebellum

following brief (15 min/day) or long (1 h/day) neonatal maternal separation on postnatal days 2 to 14. Consistent with previous findings (Huot et al., 2002), brief and long neonatal maternal separation increased plasma corticosterone concentrations in male and female rats, suggesting that neonatal maternal separation is stressful (Figure 1.2). In order to assess potential brain and behavioral consequences of neonatal stress, eyeblink conditioning and glucocorticoid receptor expression were assessed in adult rats that had undergone neonatal separation. In male but not female rats, neonatal separation had long-lasting deleterious consequences on adult eyeblink conditioning and increased glucocorticoid receptor expression in a key component of the neural substrate for eyeblink conditioning, the posterior interpositus nucleus. Specifically, both brief and long neonatal maternal separation impaired eyeblink conditioned responding in males but not females (Figure 1.3). Maternally separated rats did not differ significantly from controls in early acquisition trials; however, they showed significantly fewer CRs during late acquisition (post asymptotic performance), suggesting that maternal separation impaired but did not prevent learning. In addition, I used immunohistochemistry to assess glucocorticoid receptor expression in the cerebellum and found that maternal separation produced changes in glucocorticoid receptor expression in the interpositus nucleus, a critical component in the eyeblink conditioning circuitry. Long neonatal maternal separation resulted in a 33% increase in glucocorticoid receptor expression in the posterior interpositus nucleus, while brief separation resulted in a non-significant 19% increase in glucocorticoid receptor expression in the posterior interpositus nucleus (Figure 1.4). Furthermore, eyeblink

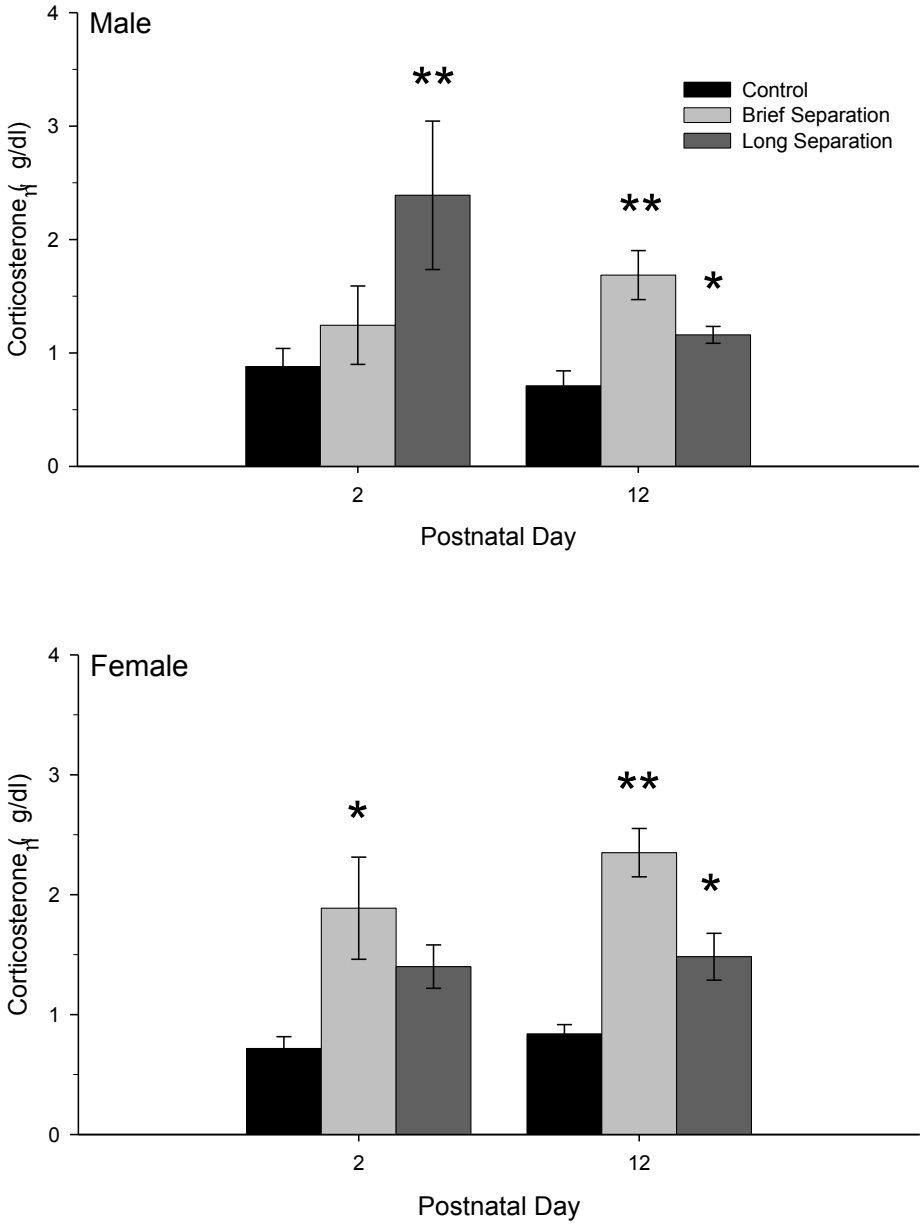

Figure 1.2: Mean (± SEM) corticosterone (μg/dl) sampled on PND 2 and 12 for animals that underwent either standard animal facilities rearing (Control), or maternal separation for either 15 min (Brief Separation) or 60 min (Long Separation) per day starting on PND 2 for male (above) and female pups (below).

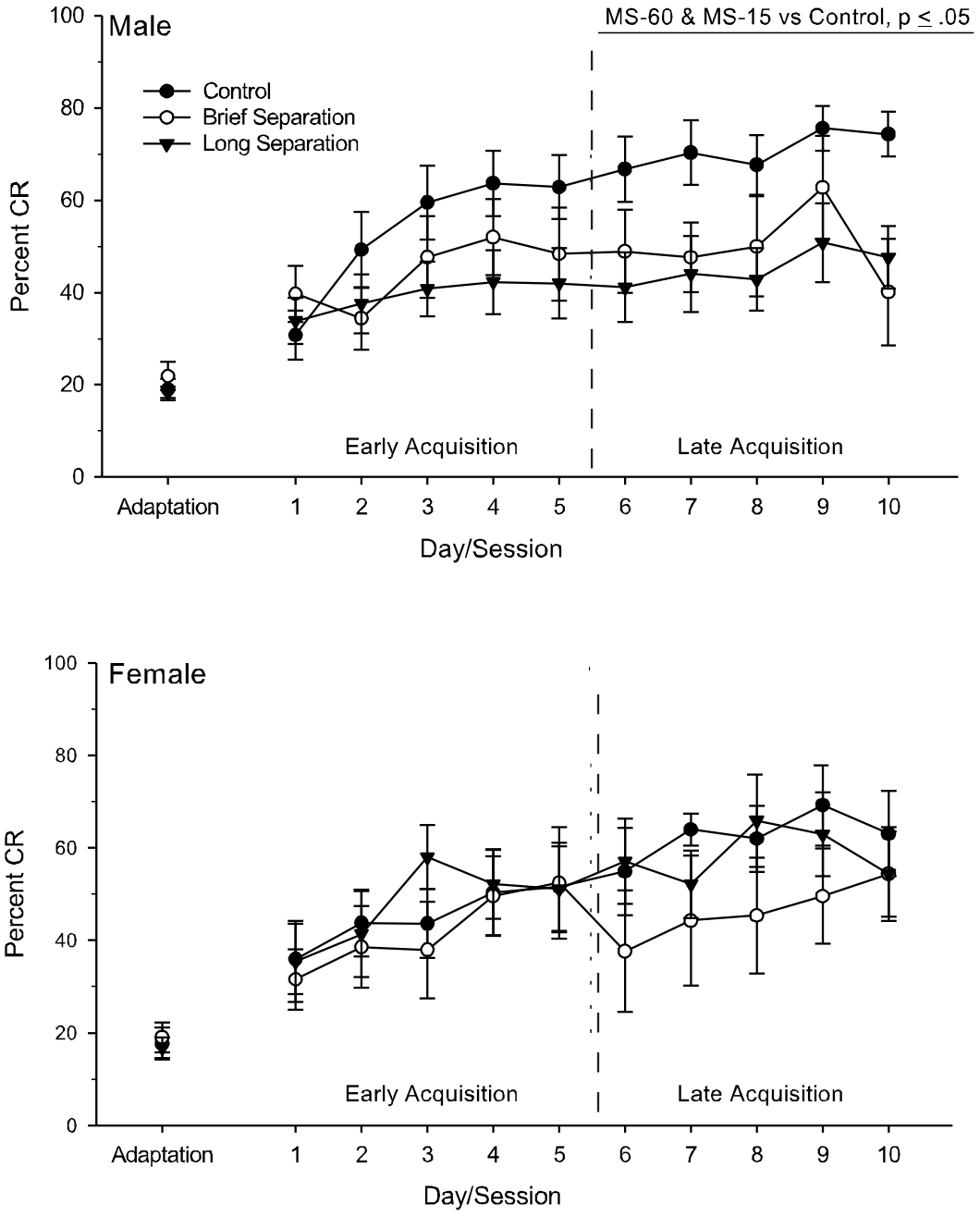

Figure 1.3: Mean (± SEM) percentage of conditioned responses (CR) for animals that underwent either standard animal facilities rearing (Control), or maternal separation for 15 min (Brief Separation) or 60 min (Long Separation) per day on PND 2 – 14 for male animals (above) and female animals (below).

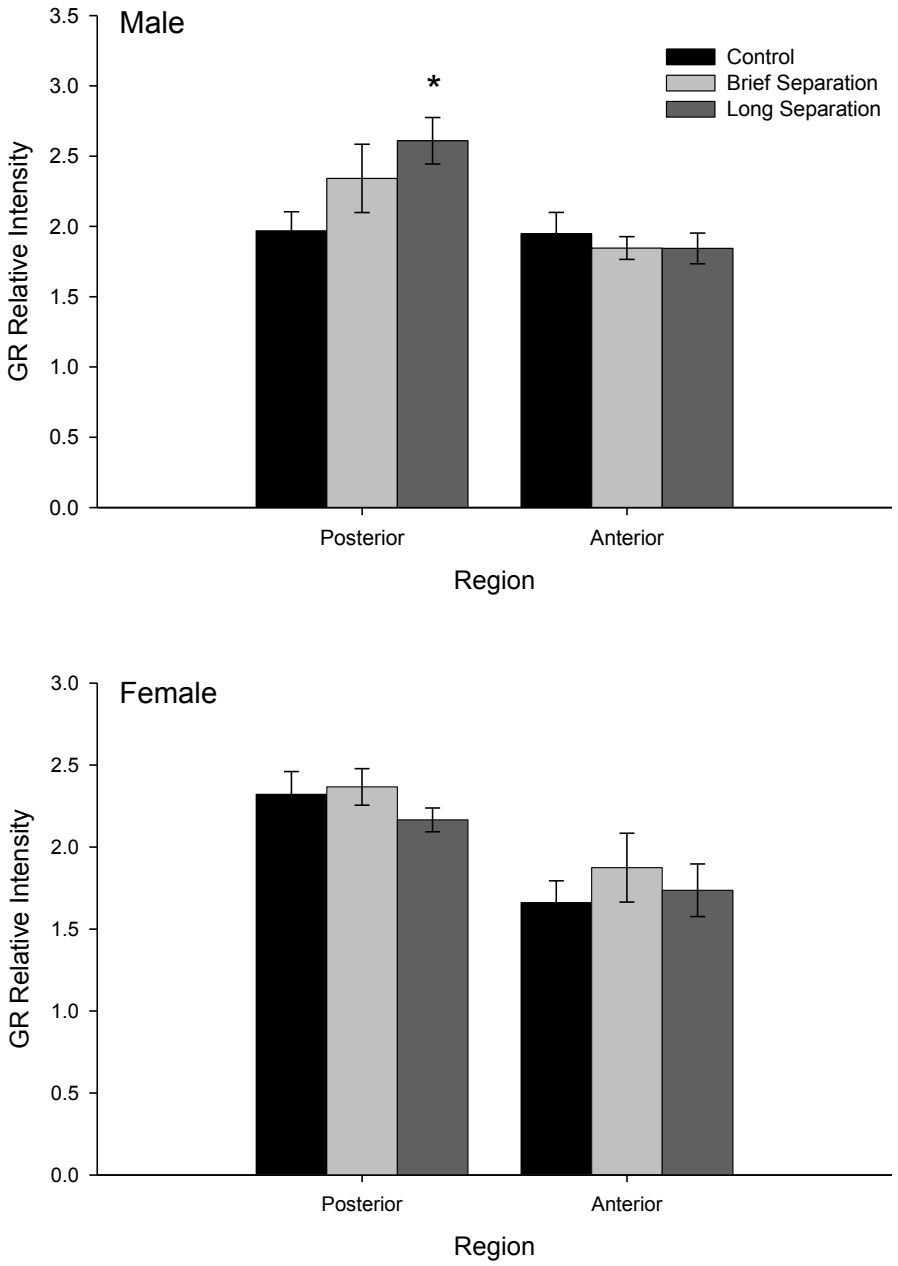

Figure 1.4: Mean (± SEM) GR relative to white matter intensity of immunostaining for animals that underwent either standard animal facilities rearing (Control), or maternal separation for either 15 min (Brief Separation) or 60 min (Long Separation) per day on PND 2 – 14 for male animals (above) and female animals (below).

conditioning was negatively correlated with posterior interpositus glucocorticoid receptor expression (Figure 1.5): Animals with increased glucocorticoid receptor staining in the posterior interpositus nucleus had fewer conditioned responses during late acquisition. Thus, consistent with previous findings that GRs modulate learning and memory tasks such as passive avoidance (Roozendaal and McGaugh, 1997; Roozendaal et al., 1997; Kim et al., 2006), it is possible that the increased glucocorticoid receptor expression reflects altered modulation of eyeblink conditioning.

The finding of an intermediate level of impairment with brief versus long separation is consistent with other studies showing greater adult learning impairments with longer neonatal maternal separation episodes (Huot et al., 2002; Uysal et al., 2005; Aisa et al., 2007; Wilber et al., 2007b; Aisa et al., 2009c; Stevenson et al., 2009; Wilber et al., 2009). Because the longer separation produced the most robust effects on adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus, the remainder of my background and thesis experiments utilize a long (1h) daily neonatal maternal separation paradigm.

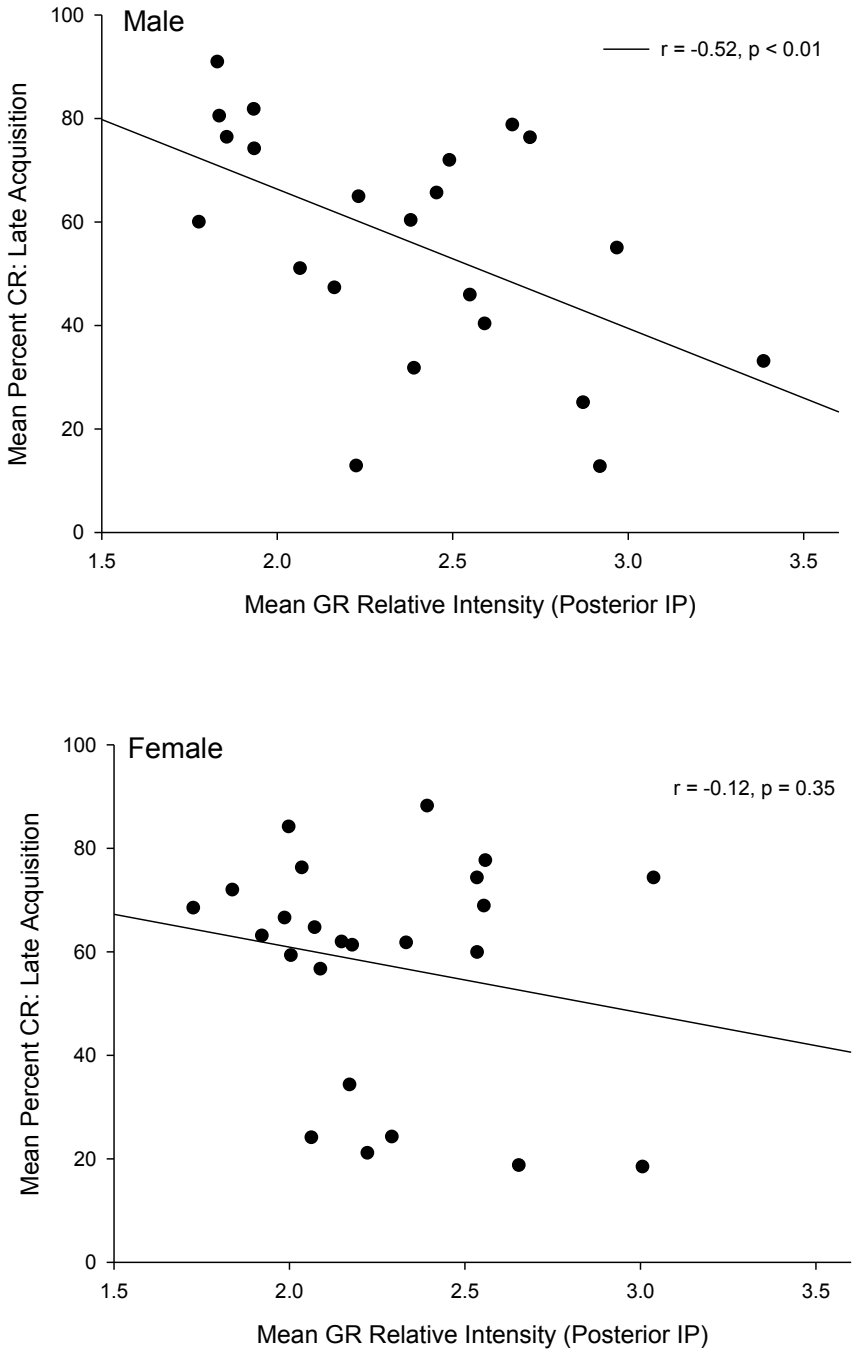

Figure 1.5: Linear regression analysis for mean (± SEM) glucocorticoid receptor (GR) intensity in the posterior interpositus (IP) and percent CR for eyeblink conditioning during late acquisition (days 6 – 10) for male (above) and female (below) rats.

Localization of maternal separation effects within the interpositus nucleus.

Evidence suggests a topographical (i.e., anterior to posterior) organization of the circuitry important for learning (Steinmetz et al., 1992a; Steinmetz et al., 1992b; Plakke et al., 2007) and modulating the CR (Delgado-Garcia and Gruart, 2006; Sanchez-Campusano et al., 2007). Thus, the altered glucocorticoid receptor expression localized to the posterior interpositus and impaired but not absent acquisition of eyeblink conditioned responding suggests that maternal separation may alter modulation of eyeblink conditioning. To further localize this effect, I assessed adult eyeblink conditioning and glucocorticoid receptor expression in projection versus interneurons in the interpositus of rats that had undergone standard rearing or maternal separation (1 h/day) on postnatal days 2-14. At three months of age, interpositus neurons were labeled with the retrograde tracer biotinylated dextran amine (BDA). After eyeblink conditioning, brains were processed immunohistochemically for glucocorticoid receptor and BDA labeling of interpositus neurons, and glucocorticoid receptor expression was quantified in BDA-labeled and unlabeled neurons (Figure 1.6). I found that maternal separation impaired adult eyeblink conditioning and preferentially increased glucocorticoid receptor expression in putative interneurons in the posterior interpositus (Figure 1.7; Wilber and Wellman, 2009a). Further, more intense glucocorticoid receptor staining in posterior interpositus interneurons was associated with poor learning (Figure 1.8; Wilber and Wellman, 2009a). Thus, neonatal maternal separation may alter interneuronal modulation of anterior interpositus output neurons, producing deficits in adult eyeblink conditioning.

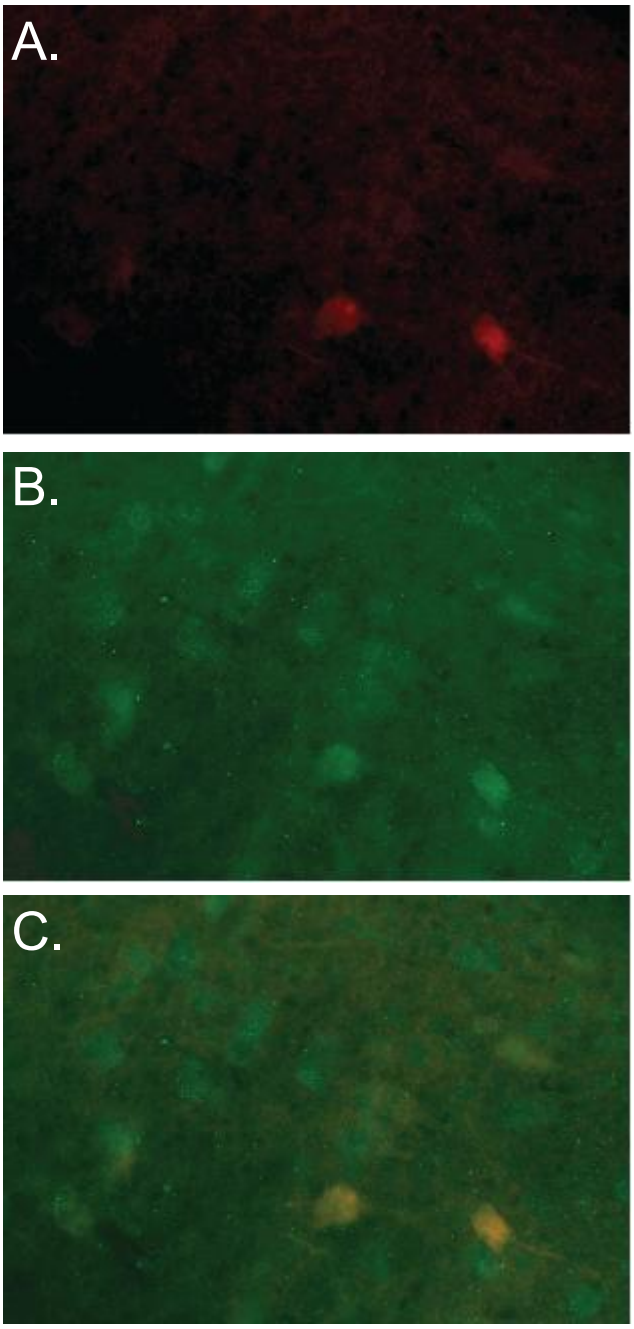

Figure 1.6: Digital light micrographs of BDA positive (red, A), glucocorticoid receptor-immunopositive (green, B) neurons in the interpositus nucleus. C. Merged image of A and B (yellow, double-labeled).

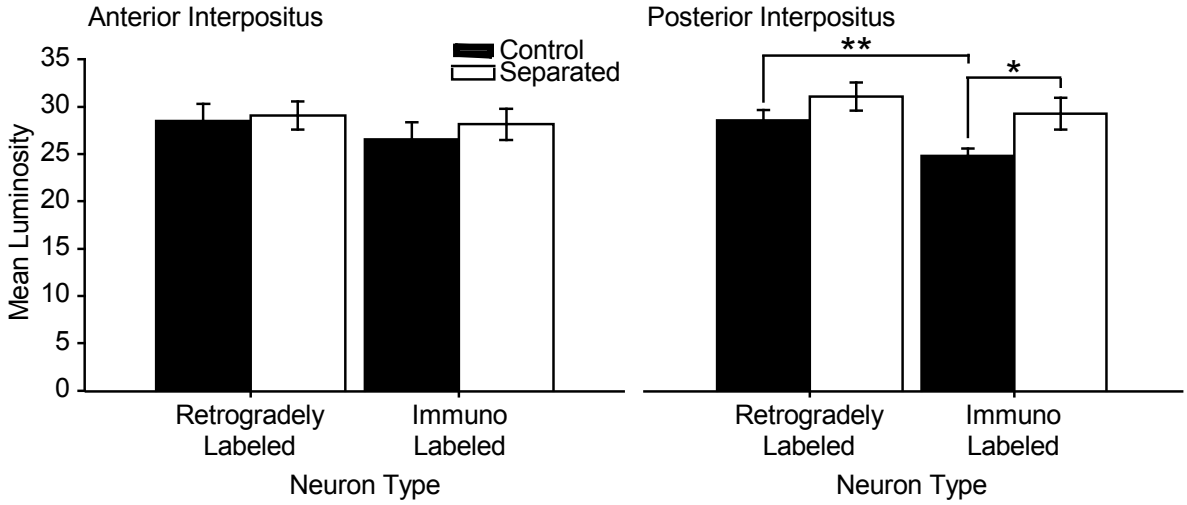

Figure 1.7: Mean (± SEM) GR luminosity in the anterior (left) and posterior (right) interpositus nucleus of adult control rats versus rats that underwent 1 h of daily maternal separation on PND 2–14 (Separated). * $p < 0.05$. ** $p \leq 0.01$.

Sex differences in neonatal separation effects on eyeblink conditioning and cerebellum.

My finding that neonatal stress results in impaired eyeblink conditioning in adult males but not females (Wilber et al., 2007a) was unexpected given what is known about adult stress and eyeblink conditioning. 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; Shors, 2006b). Interestingly, overall, these studies suggest that acute stress in adult rats facilitates eyeblink conditioning in males but not female rats, a finding that is opposite the effects of neonatal stress on male and female adult eyeblink conditioning (Figure 1.4). This apparent contradiction suggests that the organizational effects of neonatal stress may be markedly different from its activational effects in adulthood. In fact, there is a common component that could play a role in adult and neonatal stress effects, corticosterone; however, the site of action differs. Corticosterone mediates the adult stress effect through action on the hippocampus and amygdala (Beylin and Shors, 2003; Bangasser et al., 2007; Waddell et al., 2008), while separation-induced changes in glucocorticoid receptor expression in the posterior interpositus may underlie the neonatal stress effect on adult learning and memory.

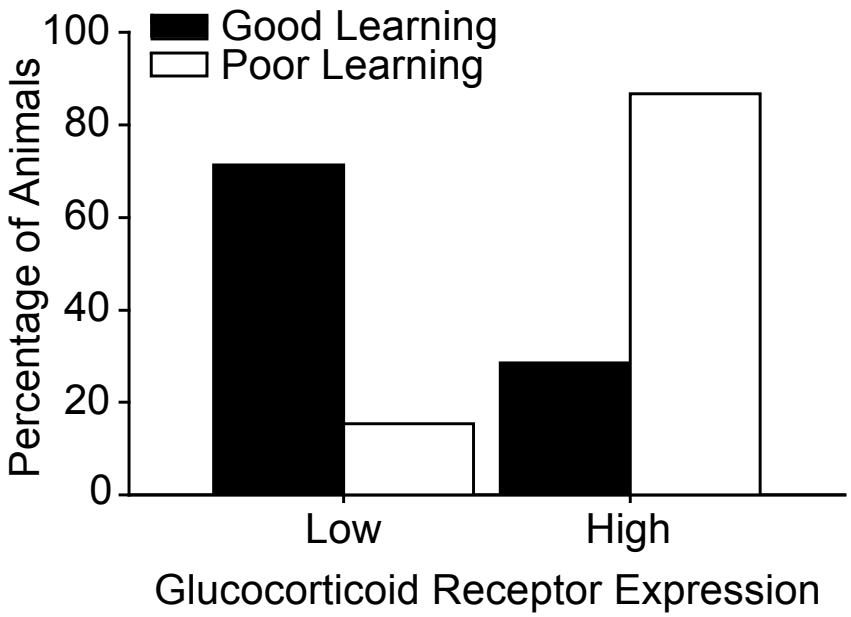

Figure 1.8: Percentage of rats with good and poor post-asymptotic learning and high versus low GR expression in immuno-labeled neurons in the posterior interpositus nucleus. Increased GR expression is significantly associated with poor learning, * $p \leq 0.01$.

Rationale for experiments.

My data demonstrate that neonatal maternal separation has consequences for the adult animal. However, the effects of neonatal maternal separation may be immediately evident early in development and may persist into adulthood, or the increased glucocorticoid receptor expression may appear later in development. In order to understand the mechanism by which neonatal maternal separation alters adult glucocorticoid receptor expression, it is important to first establish when glucocorticoid receptor expression is altered. For instance, increased glucocorticoid receptors early in development may result in altered glucocorticoid influence on brain development throughout the life span. Further, in order to begin to understand why males but not females have increased glucocorticoid receptor expression in the posterior interpositus it is important to explore the timing of this interaction of sex and rearing condition. Thus, we assessed the effects of neonatal maternal separation on glucocorticoid receptor expression in the interpositus nucleus in male and female rat pups early in development (Chapter 3).

Interestingly, males and females both showed neonatal increases in corticosterone following maternal separation; however, maternal separation only altered eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus in males (Wilber et al., 2007a). This is consistent with the impaired spatial learning in males and enhanced spatial learning in females following chronic stress (Bowman et al., 2002; Luine et al., 2007). Given that eyeblink conditioning and interpositus glucocorticoid receptor expression were not altered in females, the focus of the mechanism studies in this thesis is on

understanding the contributions of glucocorticoids to separation-induced alterations in eyeblink conditioning and glucocorticoid receptor expression in the posterior interpositus in males (Chapter 4-6).

The separation-induced increased adult glucocorticoid receptor expression in the posterior interpositus nucleus is correlated with adult eyeblink conditioning performance, and thus, may be responsible for the deficits in eyeblink conditioning. I tested this hypothesis by blocking GRs during eyeblink conditioning in adult animals that have undergone neonatal maternal separation (Chapter 4).

Finally, neonatal maternal separation increases neonatal corticosterone (Wilber et al., 2007). Given that elevations in corticosterone can alter aspects of development and adult brain function, neonatal elevations in corticosterone may be responsible for the adult increases in glucocorticoid receptors and deficits in eyeblink conditioning that result from neonatal maternal separation. To test this hypothesis, I assessed eyeblink conditioning and glucocorticoid receptor expression in adult rats that have received daily injections of corticosterone on postnatal days 2-14 (Chapter 5). In addition, I assessed whether neonatal glucocorticoid receptor blockade can prevent the effects of maternal separation on adult eyeblink conditioning and glucocorticoid receptor expression (Chapter 6).

Thus, to begin to explore the role of corticosterone in mediating neonatal maternal separation induced deficits in eyeblink conditioning, the experiments described in chapters 3-6 address several questions: 1) Does neonatal stress increase glucocorticoid receptor expression in the posterior interpositus nucleus

early in development? 2) Does glucocorticoid receptor blockade in adults ameliorate the eyeblink conditioning deficits induced by maternal separation? 3) Does neonatal elevation of corticosterone mediate the effects of maternal separation on adult eyeblink conditioning and glucocorticoid receptor expression in the posterior interpositus?

Organization of the Dissertation

This thesis is comprised of seven chapters. This first chapter serves as a review of the literature on neonatal stress effects on adult brain and behavior pertinent to the experiments in this thesis and reviews the background characterization experiments that contributed to the design of these thesis experiments. Chapter 2 contains a detailed description of the methods used in the dissertation that are common to multiple experiments. Minor variations in methodology and methodologies that are specific to a particular experiment, are noted in the subsequent individual chapters. Chapter 3 is the first experiment of the thesis, in which I characterize the effects of neonatal separation on cerebellar glucocorticoid receptor expression during development. Chapters 4-6 describe experiments in which I explore glucocorticoid mechanisms of neonatal separation effects. I explore an *adult* glucocorticoid mechanism of neonatal separation-induced impaired eyeblink conditioning (Chapter 4) and explore a *neonatal* glucocorticoid mechanism of neonatal stress effects on adult impaired eyeblink conditioning and increased glucocorticoid receptor expression (Chapter 5 and 6). In Chapter 4, I attempt to reverse neonatal separation effects on adult eyeblink conditioning by blocking GRs in the posterior interpositus during eyeblink

conditioning sessions. In Chapter 5, I attempt to mimic neonatal separation effects on adult eyeblink conditioning with neonatal corticosterone injections. Finally, in Chapter 6, I attempt to block neonatal separation effects on adult eyeblink conditioning and glucocorticoid receptor expression by combining neonatal separation and systemic injections of a glucocorticoid receptor antagonist. All experimental chapters of the thesis (Chapters 3-6) contain a specific introduction and rationale for the particular experiments performed, a description of the methods used that are specific to that experiment (i.e., not outlined in Chapter 2), a description of the results obtained in that experiment (with relevant illustrations), and a discussion of the results obtained in that experiment. In Chapter 7, I synthesize and interpret the major findings of the dissertation, and discuss the significance and implications for adverse early experiences in humans.

Chapter 2:

Techniques and Methodologies

Animals.

As in previous studies (Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009a), untimed pregnant Long Evans Blue Spruce rats (Harlan Indianapolis, IN) arrived approximately 1 week before giving birth. Dams were housed individually in standard laboratory cages (48 cm x 20 cm x 26 cm), with food and water available ad libitum and a 12:12 h light/dark cycle (lights on at 0700 h). On postnatal day (PND) 2 (day of birth = PND 0), pups were culled to litters of 7-10 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.

Rats from each litter were randomly assigned to one of two rearing conditions: standard animal facilities rearing (Control) or maternal separation for 1 h per day on PND 2 to 14 (Separated). Maternal separation was initiated between 0700 and 0900 hours each day. Maternal separation was carried out using procedures described previously (Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009a). 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 x 17 cm x 12 cm) lined with clean bedding. Pups in the separated group 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 ± 0.5 ˚C) for the duration of the separation period. For experiments that included adult experiments (Chapters 4-6), on PND 28, animals were weaned and housed in same sex/same litter groups of 2-3 until surgery.

Eyeblink Conditioning.

At 73-181 days of age, electromyographic (EMG) and stimulating electrodes 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. Finally, the connectors were secured to skull screws with dental acrylic and the skin was sutured around the headstage. Rats were housed individually after surgery and handled three times prior to eyeblink conditioning.

After recovery from surgery ($\geq$ 5 days), eyeblink conditioning 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×) 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. 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., 2007a; 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 repeated measures ANOVAs followed by appropriate planned comparisons.

Glucocorticoid Receptor Immunohistochemistry.

Rats were deeply anesthetized with urethane and transcardially perfused with cold 0.1 M phosphate buffered saline (pH 7.4), followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed, postfixed for 24 h, and cryoprotected in 30% sucrose in 0.1M phosphate buffer (pH 7.4). Frozen sections were cut coronally at 40 µm on a sliding microtome and collected in 0.01M phosphate buffered saline (PBS, pH 7.4). For each brain, a series of equally spaced sections were collected through the entire interpositus nucleus of the cerebellum. For immunohistochemical labeling of glucocorticoid receptors (GR)s, sections were incubated for 1 h in PBS containing 3% normal goat serum and 0.1% Triton X-100 to block nonspecific binding. Sections were then incubated for 1 h in 0.2% H_2O_2 in 50% methanol. After rinsing in PBS, sections were incubated overnight at 4˚ C in PBS containing 3% normal goat serum (NGS), 0.1% Triton X-100, and a polyclonal antibody to the rat GR (1:3000; Santa Cruz Biotechnology; Santa Cruz, CA). Sections were rinsed in PBS and then 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 PBS (PBST), sections were incubated for 1 h in PBST with ABC Complex (Vector Laboratories). Staining was visualized using a nickel-intensified DAB reaction. 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.

Quantification of GR Expression.

GR expression in the interpositus nucleus was quantified using a computer-based image analysis system (Neurolucida; MBF Bioscience; Willston, VT) interfaced via a video camera (Microfire; Optronics; Santa Barbara, CA) with a microscope (Nikon Eclipse 80i; Nikon Instruments; Melville, NY). Sampling frames were centered mediolaterally within the interpositus, and the sampling area size was chosen to yield approximately 15 neurons per frame. All labeled neurons contained within each sampling area were identified based on standard morphological criteria (large, multipolar soma) and the average luminosity per pixel of each soma was measured with values ranging from 0 (black) to 255 (white). Luminosity was measured in an area of white matter free of visible cell bodies directly above the interpositus. To control for spurious differences in staining and illumination across sections and animals: 1) each round of staining contained animals from each group, 2) care was taken to minimize differences in illumination across samples, and 3) luminosity measures within each section were expressed relative to white matter staining. Finally, to facilitate data interpretation, each measure was multiplied by 1/x so that larger ratios indicate darker staining.

I have previously shown that the separation-induced increase in GR staining is localized to the posterior portion of the interpositus nucleus (Wilber et al., 2007a; Wilber and Wellman, 2009a). Therefore, I analyzed data separately from the three most anterior sections (Anterior) and the three most posterior sections (Posterior) of the interpositus nucleus.

Data were analyzed using two different approaches. First, mean relative intensities for each region of the interpositus were compared across rearing condition and age using ANOVAs followed by appropriate planned comparisons. Second, to assess potential differences in the distribution of staining intensities, frequency distributions of staining intensities were constructed (see Chapters 3 & 5-6 for a detailed description of this method). The distribution of staining intensities data analysis method has been shown to be reliable for categorizing neurons by immunostaining intensity for subsequent frequency analyses and tends to be more sensitive to differences in protein expression assessed immunohistochemically than are simple means comparisons (Wood et al., 2005; Garrett et al., 2006; Osborne et al., 2007; Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009a).

Chapter 3:

Neonatal maternal separation alters the development of glucocorticoid receptor expression in the posterior interpositus nucleus of the cerebellum

Wilber AA, Wellman CL (2009) International Journal of Developmental Neuroscience 27:649-654.

Adverse early experiences such as neonatal maternal separation alter adult learning and memory (Meerlo et al., 1999; Huot et al., 2002; Pryce et al., 2003; Gibb and Kolb, 2005; Uysal et al., 2005; Aisa et al., 2007; Kosten et al., 2007; Wilber et al., 2007a; Aisa et al., 2009c; Wilber et al., 2009; Wilber and Wellman, 2009a). Previously, I showed that neonatal maternal separation produced adult deficits in eyeblink conditioning and increased GR expression in the posterior region of the interpositus nucleus (Wilber et al., 2007a; Wilber and Wellman, 2009a). Interestingly, although both male and female rats had increased plasma corticosterone concentrations following neonatal maternal separation, only male rats had impaired eyeblink conditioning, increased GR expression in the posterior interpositus, and a significant correlation between high GR expression and poor eyeblink conditioning (Wilber et al., 2007a).

In addition to the increased GR expression in the posterior interpositus nucleus of the cerebellum that I observed (Wilber et al., 2007a; Wilber and Wellman, 2009a), prolonged neonatal maternal separation also alters GR expression in the adult hippocampus and frontal cortex (Meaney et al., 1985a; Ladd et al., 2004; Aisa et al., 2007; Wilber et al., 2009). It is generally assumed that the neonatal manipulation results in an immediate alteration in GR

expression. In fact, a single episode of 24 h maternal deprivation on PND 9 or 11 will immediately alter GR expression in the hippocampus and hypothalamic-pituitary-adrenal (HPA) circuitry (van Oers et al., 1998; Avishai-Eliner et al., 1999). However, less is known about the effect of a less severe but repeated daily maternal separation on GR expression early in development, or the effect of maternal separation on early GR expression in non-HPA-axis structures. I previously assessed GR expression in the interpositus nucleus of adult rats only; thus, the timing of separation-induced changes in GR expression in this structure is unknown. To begin to characterize the developmental timecourse of altered GR expression after neonatal maternal separation, I assessed GR expression in the interpositus nucleus at PND 15 (immediately following the cessation of daily maternal separation), at PND 21 (chosen to allow sufficient time for recovery from any potential effects of receptor desensitization; Reid et al., 1994; Erdeljan et al., 2001; Erdeljan et al., 2005; Solt et al., 2007), and in adulthood in male and female rats that had undergone either standard animal facilities rearing or daily maternal separation (1 h per day) on PND 2-14.

Methods

Animals.

Offspring of untimed pregnant Long Evans Blue Spruce rats (Harlan Indianapolis, IN, N = 13) underwent either standard animal facilities rearing (Control) or 1h daily maternal separation (Separated) as described in Chapter 2. Each rearing condition consisted of 6-7 litters.

Glucocorticoid Receptor Immunohistochemistry.

On PND 15 (n=11 male control, n=10 female control, n=10 male separated, and n = 12 female separated), PND 21 (n=13 male control, n=9 female control, n=11 male separated, and n=12 female separated), or in adulthood (79-116 days of age; n=10 male control, n=7 female control, n=7 male separated, and n=8 female separated), rats were deeply anesthetized with urethane and transcardially perfused so that GR immunohistochemistry could be conducted as described in Chapter 2. Staining was visualized using a nickel-intensified DAB reaction (Figure 3.1). The adult data were collected from animals used in a previous study (Wilber et al., 2007a), and immunohistochemical procedures were identical for all age groups. For each brain, 12 to 14 equally spaced sections (approximately 40 μm apart) were collected through the entire interpositus nucleus of the cerebellum.

Quantification of Glucocorticoid Receptor Expression.

Neurons were sampled from a 65 μm by 65 μm sampling frame at a final magnification of 2200x (PND 15), and a 100 μm by 120 μm sampling frame at a final magnification of 1440x (PND 21 and adult). Luminosity was measured in an area of white matter free of visible cell bodies directly above the interpositus averaging 138 μm^2 on PND 15, 490 μm^2 on PND 21, and 1519 μm^2 in adult rats.

As in our previous study (Wilber et al., 2007a), data for males and females were analyzed separately for comparisons across rearing conditions (separated versus control). In addition, to examine the hypothesis that differences in the normal development of GR expression in male and female rats might explain

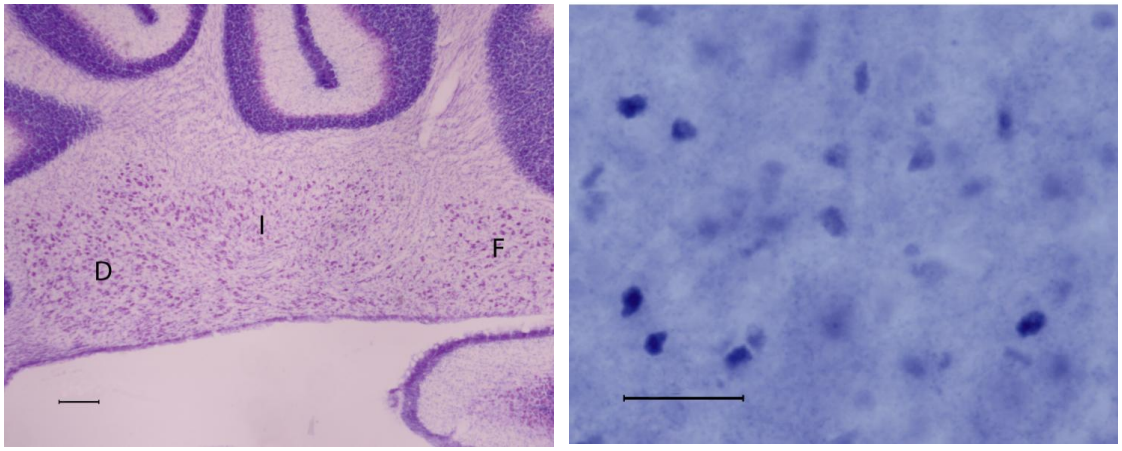

Figure 3.1: *Left*. Digital light micrographs of a thionin-stained section through the cerebellar deep nuclei dentate (D, left), interpositus (I, middle) and fastigial (F, right) in a postnatal day 15 control pup. Scale bar = 200 μm. *Right*. GR-immunopositive neurons in the interpositus nucleus of a postnatal day 15 control pup. Scale bar = 25 μm.

the sparing of adult females from the effects of neonatal maternal separation that I observed in our previous study (Wilber et al., 2007a), I also compared GR expression in male versus female control rats.

Data were analyzed using two different approaches. First, mean relative intensities for each region of the interpositus were compared across rearing condition and age using two-way ANOVAs (age x rearing condition) followed by appropriate planned comparisons. Second, to assess potential differences in the distribution of staining intensities, 2-bin histograms of the mean number of neurons (expressed as percent of total) categorized as having relative intensities greater than the mean of controls (high GR) versus neurons having relative intensities less than or equal to the mean of controls (low GR) were generated for each region. Because age differences in relative intensity were quite large relative to rearing condition differences, only rearing condition differences at each age were subjected to this second more sensitive analysis. Data were compared across rearing conditions using repeated measures ANOVAs (rearing condition x intensity bin) followed by appropriate planned comparisons.

Results

Overall, the mean (±SEM) number of neurons measured per section was 17.37 ± 0.66 for male rats and 15.46 ± 0.49 for female rats. The average total number of neurons measured per rat was 87.44 ± 4.25 for males and 71.86 ± 3.55 for females.

For male rats, mean relative intensity of GR staining in anterior interpositus (Figure 3.2) varied significantly across age (main effect of age, $F_{(2, 56)}$

= 31.39, $p < 0.001$), but there was not a significant effect of neonatal rearing (main effect of group, $F_{(1, 56)} = 0.32$, *ns*) or an age by rearing condition interaction ($F_{(2, 56)} = 0.64$, *ns*). Planned comparisons indicated that for both control and neonatally separated rats, mean relative intensity of GR staining on PND 21 was significantly lower than staining on PND 15 and in adults (control, $ts_{(21\&22)} > 4.7$, $ps < 0.001$; maternally separated, $ts_{(19\&16)} > 5.08$, $ps < 0.001$) while staining on PND 15 and in adulthood did not differ (control, $t_{(19)} = 0.63$, *ns*; maternally separated, $t_{(15)} = 0.55$, *ns*).

In the posterior interpositus, mean relative intensity of GR staining for male rats (Figure 3.2) varied significantly across age (main effect of age, $F_{(2, 56)} = 16.03$, $p < 0.001$). Unlike anterior interpositus, the effect of neonatal rearing condition approached significance (main effect of group, $F_{(1, 56)} = 3.81$, $p = 0.06$), and this effect varied significantly with age (age x rearing condition interaction, $F_{(2, 56)} = 7.13$, $p < 0.01$). Planned comparisons indicated that in control rats, as with the anterior interpositus, mean relative intensity of GR staining on PND 21 was significantly lower than staining on PND 15 and in adults (control, $ts_{(22\&21)} > 2.14$, $ps < 0.05$) while staining on PND 15 and in adulthood did not differ ($t_{(19)} = 1.63$, *ns*). Unlike anterior interpositus, the pattern of GR expression in posterior interpositus of male maternally separated rats varied relative to that of controls. Planned comparisons indicated that GR staining in posterior interpositus was initially decreased relative to controls (PND 15, $t_{(19)} = 2.14$, $p \leq 0.05$), but by PND 21 separated rats had significantly higher GR expression than controls ($t_{(22)} = 2.72$, $p \leq 0.01$) and this elevation was maintained into adulthood ($t_{(15)} = 2.18$, $p \leq 0.05$). Furthermore, neonatal maternal separation prevented the decrease in GR

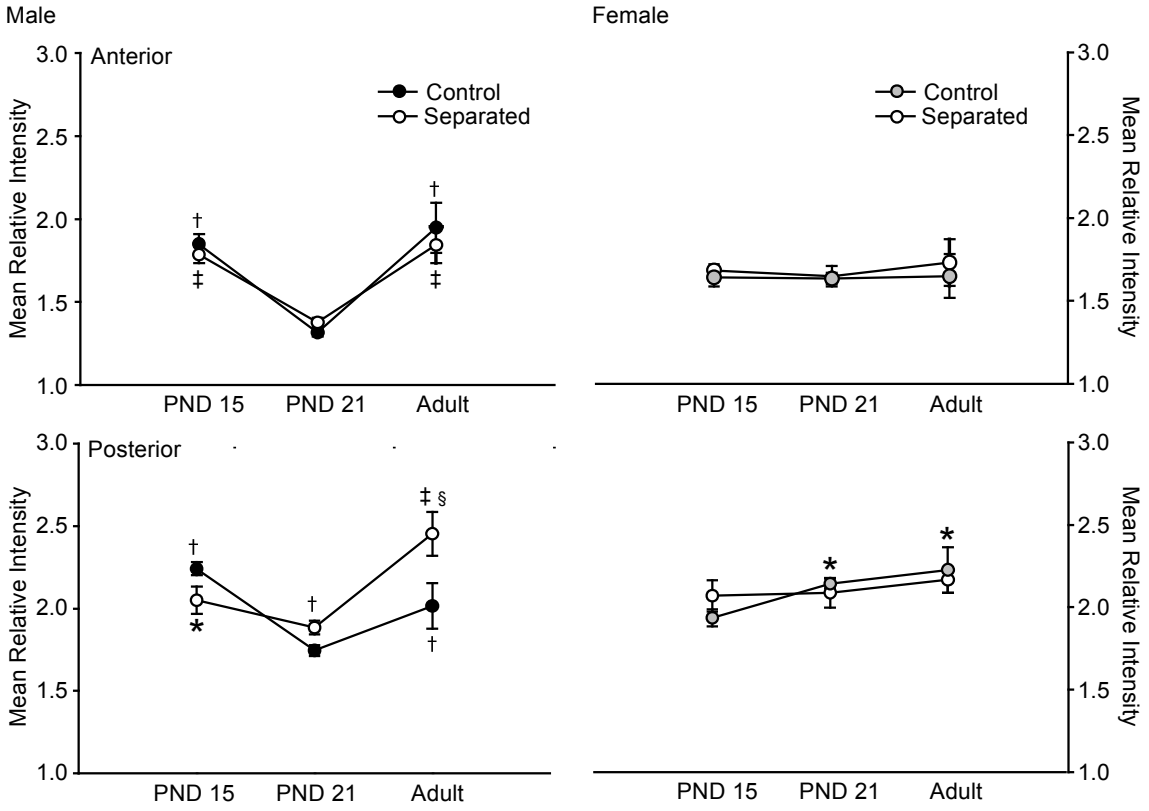

Figure 3.2: Mean (± SEM) relative intensity of glucocorticoid receptor staining in the anterior (Above) and posterior (Below) interpositus nucleus of male (Left) and female (Right) control rats versus rats that underwent 1 h of daily maternal separation on postnatal day (PND) 2–14 (Separated). Asterisks (*) indicate significant differences relative to PND 15 controls; daggers (†) indicate significant differences relative to PND 21 controls; section signs (§) indicate significant difference relative to adult controls; double daggers (‡) indicate significant difference relative to PND 21 separated rats.

staining normally seen from the second to third postnatal week (PND 15 versus PND 21, $t_{(19)} = 1.85$, *ns*) and significantly elevated GR expression in adulthood relative to both PND 15 ($t_{(15)} = 2.71$, $p < 0.05$) and PND 21 ($t_{(16)} = 4.91$, $p < 0.001$).

For female rats, mean relative intensity of GR staining in anterior interpositus did not vary significantly across age (Figure 3.2; main effect of age, $F_{(2, 52)} = 0.21$, *ns*) or neonatal rearing condition (main effect of group, $F_{(1, 52)} = 0.49$, *ns*), and there was not an age by rearing condition interaction ($F_{(2, 52)} = 0.14$, *ns*). On the other hand, mean relative intensity of GR staining in the posterior interpositus for female rats (Figure 3.2) varied significantly across age (main effect of age, $F_{(2, 52)} = 3.65$, $p < 0.05$), but not neonatal rearing condition (main effect of group, $F_{(1, 52)} = 0.72$, *ns*), and there was not an age by rearing condition interaction ($F_{(2, 52)} = 1.43$, *ns*). Planned comparisons indicated that for control females, GR staining on PND 21 and adulthood was not different ($t_{(14)} = 1.42$, *ns*), and staining at both ages was significantly greater than PND 15 ($ts_{(17\&19)} > 2.97$, $ps \leq 0.01$). GR staining was not significantly different between any of the ages for separated females ($ts_{(18\text{-}22)} < 0.72$, *ns*).

I have previously shown that corticosterone is significantly increased in male and female pups in response to maternal separation; however, as adults only males have impaired eyeblink conditioning and increased posterior interpositus GR expression (Wilber et al., 2007a). Therefore, to examine the hypothesis that differences in the normal development of GR expression in male and female rats might explain the sparing of adult females from the effects of neonatal maternal separation, I also compared GR expression in male versus

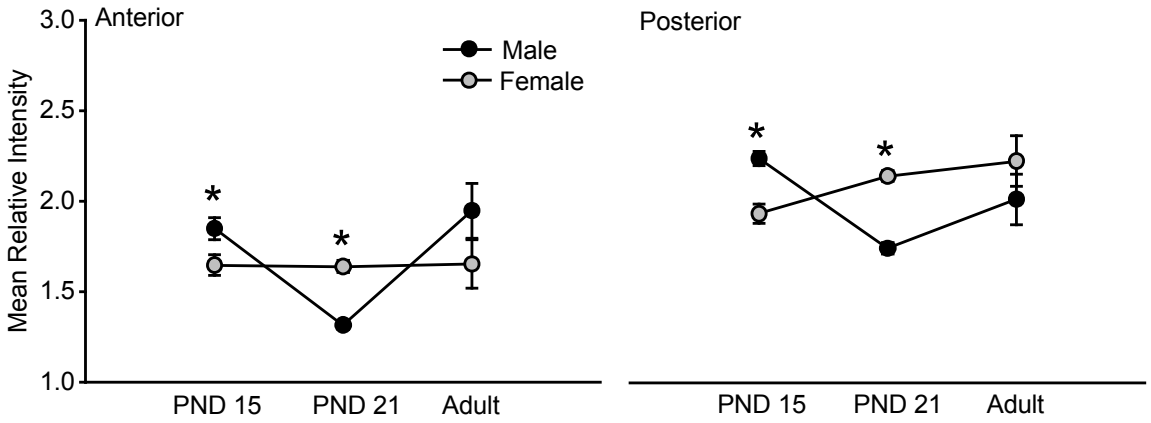

Figure 3.3: Mean (± SEM) relative intensity of glucocorticoid receptor staining in the anterior (Left) and posterior (Right) interpositus nucleus of male and female adult control rats. * $p < 0.05$ versus same age control males.

female control rats. Mean relative intensity of GR staining in anterior interpositus for control male and female rats (Figure 3.3) varied significantly across age (main effect of age, $F_{(2, 54)} = 8.91$, $p \leq 0.001$), but not sex (main effect of group, $F_{(1, 54)} = 0.66$, *ns*), and there was a significant age by sex interaction ($F_{(2, 54)} = 7.99$, $p \leq 0.001$). Planned comparisons indicated that GR staining in the anterior interpositus for male control pups was initially greater than female controls ($t_{(19)} = 2.39$, $p < 0.05$), but by PND 21 males had significantly lower GR staining than females ($t_{(20)} = 8.13$, $p < 0.001$). GR staining for male and female control rats was not significantly different in adult rats ($t_{(15)} = 1.35$, *ns*).

Similarly, mean relative intensity of GR staining in posterior interpositus for control male and female rats (Figure 3.3) varied significantly across age (main effect of age, $F_{(2, 54)} = 4.23$, $p < 0.05$) and sex (main effect of group, $F_{(1, 54)} = 4.35$, $p < 0.05$), and there was an age by sex interaction ($F_{(2, 54)} = 12.77$, $p < 0.001$). As in anterior interpositus, planned comparisons indicated that GR staining in posterior interpositus is initially significantly greater in male controls relative to female controls ($t_{(19)} = 4.77$, $p < 0.001$) at PND 15, but by PND 21 males have significantly lower GR staining than females ($t_{(20)} = 8.75$, $p < 0.001$). GR staining for male and female rats was not significantly different in adults ($t_{(15)} = 1.51$, *ns*). Thus, male GR expression declines between the second and third postnatal weeks, while female GR expression does not, and as a result male and female rats have different levels of GR expression early in development (Figure 3.3).

Differences in the distribution of GR staining intensities (Figure 3.4) paralleled those seen in the more gross measure of mean relative intensity. As with the mean GR analyses, the effect of rearing condition on distribution of GR

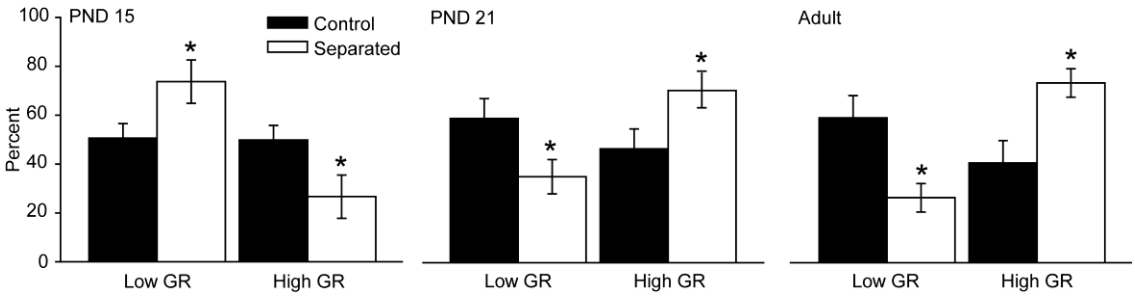

Figure 3.4: Frequency histograms of glucocorticoid receptor staining of neurons in posterior interpositus of male rats that underwent standard rearing (Control) versus 1 h of daily maternal separation on postnatal day (PND) 2–14 (Separated) at PND 15 (Left), PND 21 (Middle), and adulthood (Right). Graphs show the mean (± SEM) number of neurons (expressed as percent of total) categorized as having relative intensities less than or equal to the control group mean (Low GR) or greater than the control group mean (High GR). * $p < 0.05$ versus same staining intensity bin for controls.

staining intensity was confined to the posterior region of the interpositus of male rats, with no rearing condition differences in GR staining observed in the anterior region for male rats (rearing condition x bin interaction, $Fs_{(1, 15\text{-}22)} < 2.33$, *ns*; data not shown) or any region for female rats (rearing condition x bin interaction, $Fs_{(1, 13\text{-}20)} < 2.17$, *ns*; data not shown). In the posterior interpositus of male rats, the distribution of staining intensities was significantly altered by neonatal separation at PND 15 (rearing condition x bin interaction, $F_{(1, 19)} = 4.87$, $p < 0.05$), PND 21 (rearing condition x bin interaction, $F_{(1, 22)} = 4.73$, $p < 0.05$), and in adults (rearing condition x bin interaction, $F_{(1, 15)} = 7.47$, $p < 0.05$). Planned comparisons were conducted comparing separated to control animals. At PND 15, the frequency of intensely stained neurons was decreased in neonatally separated animals, with a 47% reduction in the frequency of neurons with high GR staining ($t_{(19)} = 2.21$, $p < 0.05$) and a 46% increase in the frequency of neurons with low GR staining ($t_{(19)} = 2.21$, $p < 0.05$). The opposite pattern was observed at PND 21, with an 52% increase in the frequency of neurons with high GR staining ($t_{(22)} = 2.17$, $p < 0.05$) and a 41% decrease in the frequency of neurons with low GR staining ($t_{(22)} = 2.17$, $p < 0.05$), and again in adult animals with an 80% increase in the frequency of neurons with high GR staining ($t_{(15)} = 2.73$, $p < 0.05$) and a 55% reduction in the frequency of neurons with low GR staining ($t_{(15)} = 2.73$, $p < 0.05$). Thus, although group differences appear modest when compared using the more gross measure of mean relative intensity, the distribution of GR staining intensities reveals that group differences are quite large.

Discussion

In control males, GR expression in the interpositus nucleus declined between PND 15 and 21 and then increased into adulthood. However, neonatal separation decreased GR expression at PND 15 and significantly attenuated the normal decline in the third postnatal week, resulting in significantly greater GR expression in the posterior interpositus relative to control rats at PND 21. As with control males, GR expression increased in separated male rats so that GR expression was significantly higher in adult separated rats than in PND 21 separated rats, as well as PND 21 and adult controls. Consistent with previous findings in adult rats (Wilber et al., 2007a; Wilber and Wellman, 2009a), rearing condition-related differences were not present in the anterior interpositus of male rats at any age examined.

Also consistent with previous findings (Wilber et al., 2007a), rearing condition-related differences at all ages examined in the present study were absent in females. Further, the developmental pattern of GR expression in the posterior interpositus of control females was opposite that seen in males (i.e., increasing GR expression in females versus decreasing GR expression in males). Perhaps the lack of effect in females is due to the absence of a normally-occurring decline in GR expression in the posterior interpositus during this postnatal period. Alternatively, the absence of altered GR expression in adult maternally separated females could be due to sex differences in gonadal hormones. Finally, it is possible that I did not observe changes in GR expression in adult females because they are dependent on estrous phase, which was not measured in the present study. However, it is unlikely that separation-induced

alterations in GR expression in adult females are masked by variations in GR expression across the estrous cycle, because neonatally separated males but not females have altered GR expression at PND 15 and 21—prior to the onset of female estrous cycles —suggesting that maternal separation does not alter GR expression in females.

GR desensitization via reduced GR expression has been described following exposure to increased concentrations of the receptor ligand (Erdeljan et al., 2001; Erdeljan et al., 2005; Chebotaev et al., 2007; Kozhevnikova et al., 2007), and I have shown that neonatal maternal separation increases corticosterone following both initial (measured at PND 2) and repeated daily maternal separation (measured at PND 12). Thus, the observed reduction in GR expression at PND 15 could reflect GR desensitization. Further, this is a period when GR expression in the posterior interpositus is normally fluctuating, as described in the present and previous studies (Pavlik and Buresova, 1984; Lawson et al., 1992), and the reduced GR expression might further dampen the reduction in GR expression normally seen from PND 15 to 21, ultimately producing increased GR expression relative to controls.

A mechanism has been identified for brief separation (i.e., handling) induced increase in hippocampal GR expression (Meaney et al., 1996; Meaney et al., 2000). However, brief maternal separation results in an initial increase in GR expression in the hippocampus (Mitchell et al., 1990a; Mitchell et al., 1990b; Meaney et al., 2000; Erdeljan et al., 2001; Erdeljan et al., 2005), whereas I observed an initial decrease in GR expression followed by a later increase in GR expression. Further, I have found that brief and prolonged maternal separation

both increase adult GR expression in the cerebellum (Wilber et al., 2007a; Wilber and Wellman, 2009a), while in the hippocampus these manipulations have opposite effects on GR expression (Meaney et al., 1985a; Ladd et al., 2004). Taken together, these findings suggest that a different mechanism is responsible for the changes I have observed in cerebellar GR expression.

In summary, I have shown that in male rats, prolonged neonatal maternal separation attenuates the normally occurring decrease in GR expression in posterior interpositus during the third postnatal week. While separated rats had significantly less GR expression than did controls at PND 15, they had significantly greater GR expression than control rats at PND 21 and in adulthood. Thus, normal developmental changes in male posterior interpositus GR expression may result in a vulnerability to early postnatal stress-induced changes in GR expression that impair adult learning and memory.

Chapter 4:

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

Wilber, A. A., Lin, G. L., & Wellman, C. L. (in press). Neurobiology of Learning & Memory.

Neonatal maternal separation produces deficits in eyeblink conditioning and increases GR expression in the posterior region of the interpositus nucleus in adult male rats (Wilber et al., 2007a). 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 (Roozendaal and McGaugh, 1997; Roozendaal et al., 1997; Kim et al., 2006), 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, I 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.

Methods

Animals.

Offspring of untimed pregnant Long Evans rats (Harlan Indianapolis, IN, N = 15) underwent either standard animal facilities rearing or maternal separation. Each rearing condition consisted of 8-9 litters.

Eyeblink Conditioning.

During the surgery in which EMG and stimulating electrode connectors (see Chapter 2 for methods) were implanted, 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.

After recovery from surgery ($\geq$ 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 the dummy cannula was removed and an infusion cannula (Bioanalytical Systems, West Lafayette, IN) was inserted into the guide cannula, through which 0.2 μl 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. The infusion cannula was left in place for 5 min to ensure sufficient time for diffusion. Eyeblink conditioning commenced immediately after the infusion cannula was withdrawn. Eyeblink conditioning data were analyzed using three-way repeated measures ANOVAs (rearing condition x drug x day) followed by appropriate planned comparisons.

Verification of Guide Cannula Placement.

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 µm, mounted on chrome alum-subbed slides, and stained with cresyl echt violet. Infusion cannula placement was verified based on standard cytoarchitectural criteria (Paxinos and Watson, 1998) and confined to the posterior third of the interpositus nucleus (Figure 4.1).

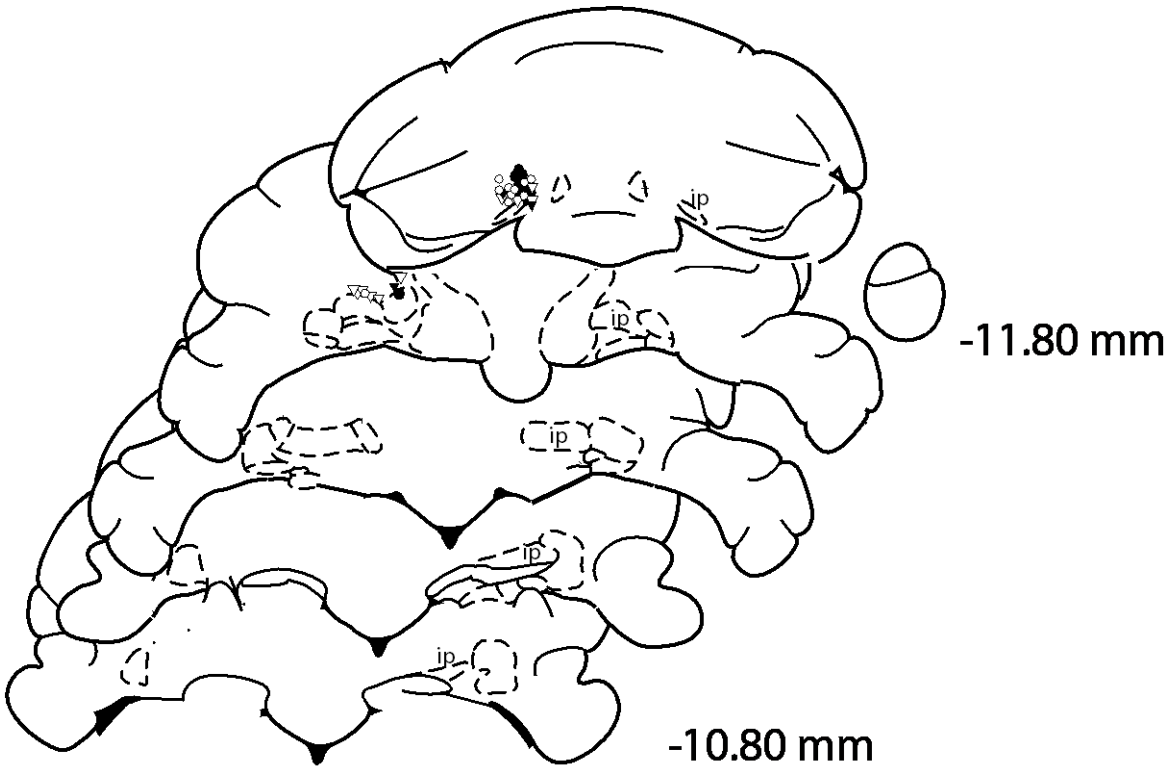

Figure 4.1: Schematic of coronal sections through the cerebellar deep nuclei showing the posterior interpositus nucleus (ip) cannula placement for adult rats that underwent standard animal facilities rearing (Control; circle) or maternal separation for 1 h per day on PND 2 – 14 (Separated; triangle) and received daily vehicle (White) or mifepristone (Black) infusions.

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 4.3; 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 4.3; 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., 2007a), there was no effect of rearing condition on responsiveness to the tone (Control Vehicle, mean = 0.11 V ± 0.03 SEM; Separated Vehicle 0.10 V ± 0.01; $F_{(1, 30)} = 3.35$, *ns*). However, there was a significant effect of mifepristone treatment on tone responsiveness (Control Mifepristone, 0.28 V ± 0.07; Separated Mifepristone, 0.16 V ± 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 4.2), and statistical analyses confirmed this (Figure 4.3). Performance improved during training, as indicated by increasing percent CR across day (Figure 4.3; 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*).

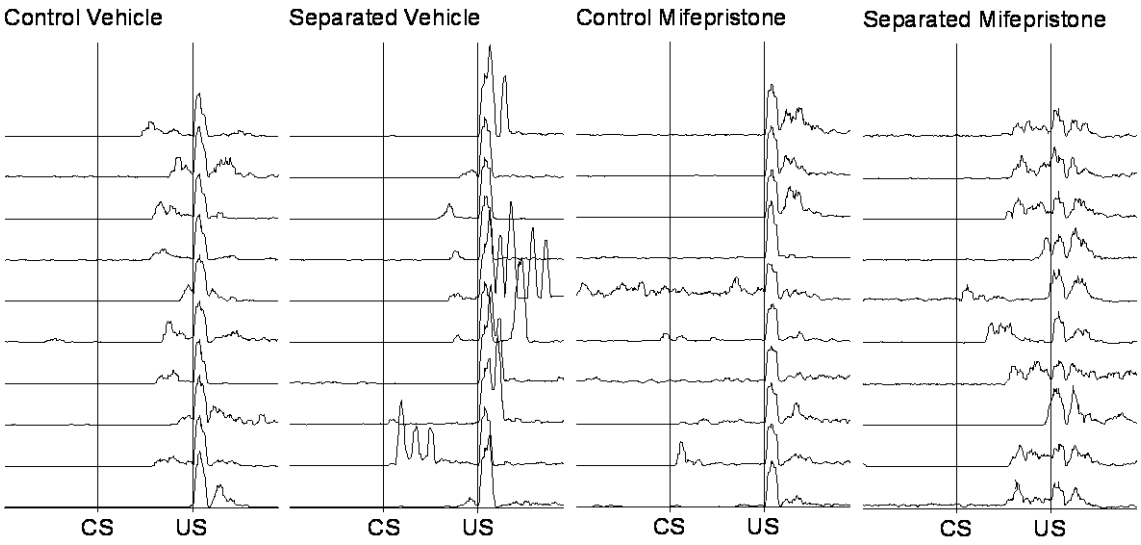

Figure 4.2: Raw EMG traces for the last 10 paired trials on the final acquisition day for representative rats that underwent either standard animal facilities rearing (Control) or maternal separation for 1 h per day on PND 2 – 14 (Separated) and received daily vehicle or mifepristone infusions just prior to the training session. Rats were chosen because their raw data were near their respective group means.

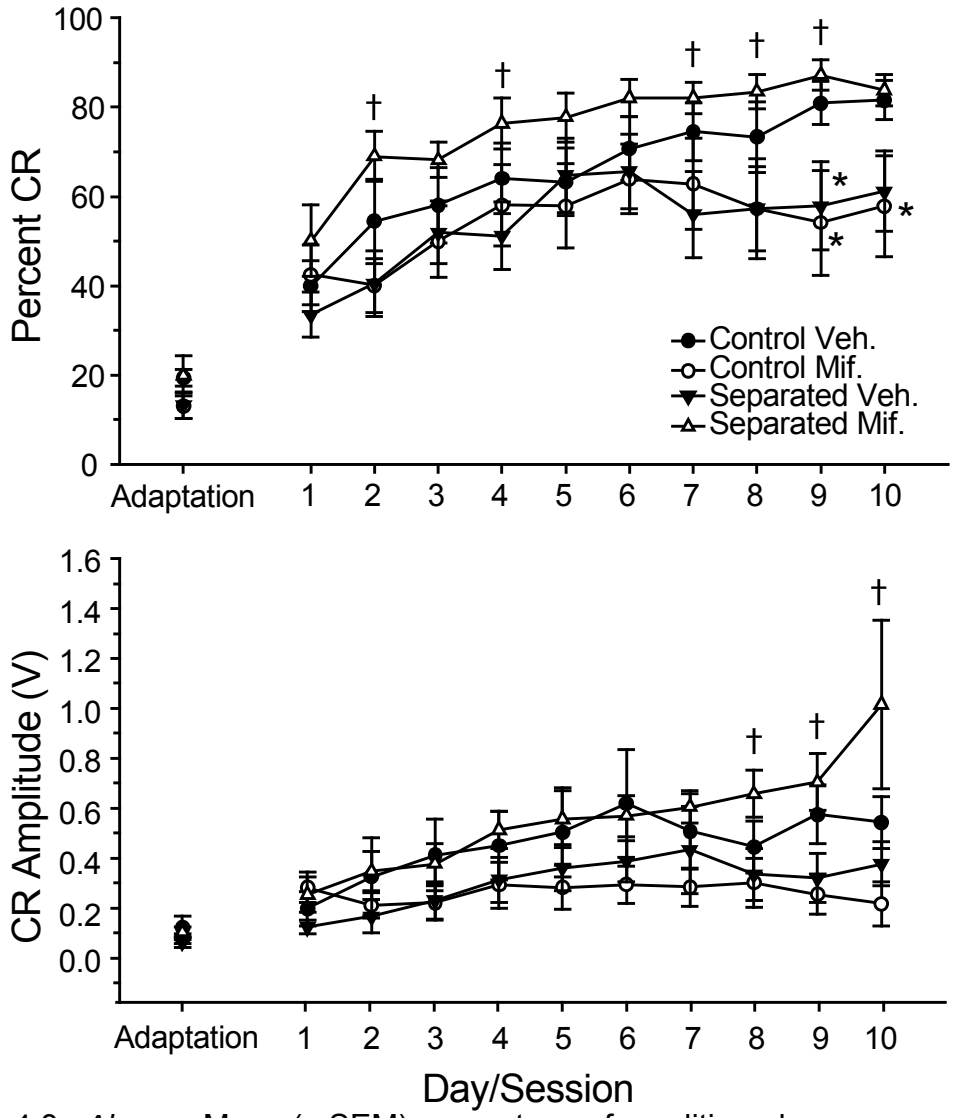

Figure 4.3: *Above*. Mean (± SEM) percentage of conditioned responses (CR) for rats that underwent either standard animal facilities rearing (Control) or maternal separation for 1 h per day on PND 2 – 14 (Separated) and received daily vehicle (n=10 control; n=10 separated) or mifepristone (Mif; n=7 control; n=7 separated) infusions just prior to each adaptation and paired training session. *Below*. Mean (± 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.

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, $Fs_{(1, 18)} < 3.78$, *ns*). Further, mifepristone significantly improved performance of separated rats compared to vehicle treated separated rats on acquisition days 2, 4, and 7-9 ($Fs_{(1, 15)} > 4.57$, $p \leq 0.05$; days 1, 3, 5, 6, and 10, $Fs_{(1, 15)} < 4.15$, *ns*). Finally, mifepristone infusions had the opposite effect on control rats. Mifepristone infusion significantly impaired performance in control rats compared to vehicle treated control rats at the end of acquisition (days 9 and 10, $Fs_{(1, 15)} > 4.85$, $p < 0.05$; days 1-8, $Fs_{(1, 15)} < 1.45$, *ns*).

CR amplitude increased during training (Figure 4.3; main effect of day, $F_{(9, 270)} = 7.99$, $p < 0.001$). There was not a significant main effect of drug (Figure 4.3; $F_{(1, 30)} = 0.12$, *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 ($Fs_{(1, 18)} < 2.18$, *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 ($Fs_{(1, 15)} > 4.76$, $p \leq 0.05$; days 1-7, $Fs_{(1, 15)} < 3.63$, *ns*). Finally, planned comparisons demonstrated that mifepristone did not alter CR amplitude for control rats (all $Fs_{(1, 15)} < 3.31$, *ns*).

Discussion

Consistent with our previous findings (Wilber et al., 2007a; 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., anterior to posterior) organization of the interpositus nucleus, with the anterior interpositus playing an important role in learning the CR (Steinmetz et al., 1992a; Steinmetz et al., 1992b) and the posterior interpositus modulating (Delgado-Garcia and Gruart, 2006; Sanchez-Campusano et al., 2007) the CR. There is evidence for a similar anterior localization of the critical circuitry for learning in the rat (Plakke et al., 2007). I previously demonstrated that maternal separation produces more robust changes in GR expression in posterior interpositus interneurons compared to 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 (for reviews see, Steinmetz, 2000; Medina et al., 2002; Christian, 2003). 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 (Pavlik and Buresova, 1984; Aden et al., 2008; Noguchi et al., 2008a; Noguchi et al., 2008b). Alternatively, neonatal separation produces changes the hippocampus (Vazquez et al., 1996; Ladd et al., 2004) and hippocampal 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); therefore, separation-induced changes in the hippocampus may also play a role.

Little is known about the normal function of GRs in the cerebellum. In fact, given that increased GR expression in the hippocampus is associated with facilitated learning (Liu et al., 1997; Liu et al., 2000), opposite the impaired learning following increased GR expression I described in our earlier studies (Wilber et al., 2007a; 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 (Huot et al., 2002; Aisa et al., 2007; Wilber et al., 2007a; Aisa et al., 2009b; Stevenson et al., 2009; 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 et al., 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., 2007a; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b), 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 (Roozendaal 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 et al., 1985a; Wilber et al., 2009) or hippocampus (Meaney et al., 1985a; Ladd et al., 2004; Aisa et al., 2007).

Alternatively, I 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 for at least two reasons: 1) increased alpha response amplitude indicates increased sensory responsivity, not necessarily increased amplitude of the conditioned blink responses, and 2) 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 (Guerra-Araiza et al., 2001; Curran-Rauhut and Petersen, 2002); therefore, I cannot rule out the possibility that mifepristone's action on progesterone receptors contributes to the effects I 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 et al., 2009). My 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 adult eyeblink conditioning seen in neonatally separated rats.

Chapter 5:

Neonatal corticosterone administration impairs adult eyeblink conditioning and decreases glucocorticoid receptor expression in the cerebellar interpositus nucleus

Wilber, A. A., Lin, G. L., & Wellman, C. L. (under review). Psychoneuroendocrinology.

Neonatal maternal separation produces deficits in eyeblink conditioning and increases GR expression in the posterior region of the interpositus nucleus in adult male rats (Wilber et al., 2007a; 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., in press), 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., 2007a), GR expression is altered by 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., 1988; Sousa et al., 1998; Roskoden et al., 2005), including the cerebellum (Velazquez and Romano, 1987). Thus, the effects of maternal separation on GR expression in the interpositus, and consequently adult eyeblink conditioning, may be mediated by neonatal increases in corticosterone. To begin to test this hypothesis, I attempted to mimic neonatal separation effects on adult eyeblink

conditioning and GR expression in the interpositus with daily (PND 2-14) corticosterone injections.

Methods

Animals.

Offspring of untimed pregnant Long Evans Blue Spruce rats (Harlan Indianapolis, IN, N = 11; 3-4 liters per group) were randomly assigned to either standard animal facilities rearing (n = 8; Control), corticosterone (n = 14; approximately 1mg/kg based on unpublished data from our laboratory on average weight for age; injection volume did not exceed 0.05 ml), or vehicle treatment groups (n = 10; equivalent volume). Pilot studies in our laboratory indicated that this dose produced plasma corticosterone values that others have shown are sufficient to produce changes in adult behavior when administered neonatally (Claflin et al., 2005). Weight data from a separate group of animals were used in place of daily weighing so that time away from the dam could be minimized. Corticosterone or vehicle was injected subcutaneously at approximately 1800h each day, the time of day when plasma corticosterone reaches asymptote following 1 h maternal separation (Walker, 1991; Wilber et al., 2007a) conducted as described in our previous studies (Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b). Prior to vehicle or corticosterone injections dams were removed from the home cage, after which pups were placed in a Plexiglas cage (28 x 17 x 12 cm) lined with bedding. 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 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., 2007a; Wilber and Wellman, 2009b).

Eyeblink Conditioning.

At 77-115 days of age, electromyographic (EMG) and stimulating electrodes were implanted. Eyeblink conditioning surgery and data analysis were conducted as described in the general methods section (Chapter 2). 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. 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 (days 1-5) versus late acquisition (days 6-10) followed by planned comparisons.

GR Immunohistochemistry.

After eyeblink conditioning, rats were deeply anesthetized with urethane and transcardially perfused with cold 0.1 M phosphate buffered saline (pH 7.4), followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For each brain, 8 to 9 equally spaced sections (approximately 120 μm apart) were collected through the entire interpositus nucleus of the cerebellum. Immunohistochemistry for the GR was conducted as described in the general methods section (Chapter 2) and staining was visualized using a nickel-intensified DAB reaction (Figure 5.2). For each brain, 9 equally spaced sections

(approximately 160 μm apart) were collected through the entire interpositus nucleus of the cerebellum.

Quantification of GR Expression.

GR expression in the interpositus nucleus was quantified as described in the general methods section (Chapter 2). Neurons were sampled from a 100 μm by 125 μm sampling frame at a final magnification of 1440x. Luminosity was measured in an area of white matter free of visible cell bodies directly above the interpositus averaging 931 μm^2.

Data were analyzed using two different approaches. First, mean relative intensities were compared across interpositus regions (posterior versus anterior) and neonatal treatment conditions using two-way ANOVAs followed by appropriate planned comparisons. Second, to assess potential differences in the distribution of relative staining intensities, 3-bin histograms of the mean number of neurons (expressed as percent of total) categorized as having relative intensities varying from more than 1.0 standard deviation below the mean of controls (low GR expression; light) to within 1.0 standard deviations of the mean of controls (average GR expression; moderate) to greater than 1.0 standard deviations above the mean of controls (high GR expression; dark) were generated. Data were compared across groups using repeated measures ANOVAs followed by planned comparisons (two-group F-tests).

Corticosterone Assay.

Plasma corticosterone was measured in separate groups of vehicle (n = 11), and corticosterone injected (n = 4) pups 1 h after injection on PND 2. These

data were compared to plasma corticosterone data taken from control (n = 10) and separated (1 h/day as described previously; n = 17; Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a) pups used in a previous study (Wilber et al., 2007a). During the first 2 postnatal weeks (stress hyporesponsive period) corticosterone levels peak approximately 12 h after 60 min of maternal separation (Walker, 1991). The timing of injections in the present study was chosen to coincide with this peak; therefore, blood samples were taken 12 h after the onset of separation and at an approximately equal time of day for control, vehicle, and corticosterone injection groups. Corticosterone assay procedures were identical for all groups. Pups were rapidly decapitated and trunk blood was collected in heparinized microcapillary tubes and centrifuged at 2000 g 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. Stress-induced changes in corticosterone concentrations were evaluated using one-way ANOVA (group), followed by planned comparisons (two-group F-tests).

Results

Eyeblink Conditioning.

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 (Figure 5.1; $F_{(2, 29)} = 0.32$, ns) and amplitude

(Figure 5.1; $F_{(2, 29)} = 0.14$, ns) did not vary across neonatal treatment. Similarly, there was no effect of neonatal treatment on responsiveness to the tone (Data not shown; Control, mean = 0.23 V ± 0.05 SEM; Vehicle 0.12 V ± 0.03; Cort, mean = 0.14 V ± 0.03 SEM; $F_{(2, 29)} = 2.06$, *ns*).

Consistent with our definition for early versus late (post asymptotic) acquisition phases of training, performance improved during early acquisition training, as indicated by increasing percent CR across day (Figure 5.1; main effect of day, $F_{(4, 116)} = 28.76$, $p < 0.001$), but not late acquisition (main effect of day, $F_{(4, 116)} = 0.42$, ns). There was a significant main effect of neonatal treatment on the percentage of CRs during both early ($F_{(2, 29)} = 6.83$, $p < 0.01$) and late acquisition ($F_{(2, 29)} = 4.13$, $p < 0.05$). However, there was no day by neonatal treatment interaction for either early or late acquisition ($Fs_{(8, 116)} < 0.70$, *ns*); thus, planned comparisons were conducted for early and late acquisition comparing neonatal treatments collapsed across days. During early acquisition, the percentage of conditioned responses did not differ for control and vehicle treated rats ($F_{(1, 16)} = 2.71$, ns), while rats that received neonatal corticosterone treatment performed significantly worse than both control ($F_{(1, 20)} = 11.02$, $p < 0.01$) and vehicle ($F_{(1, 22)} = 4.70$, $p < 0.05$) treated rats. During late acquisition, neonatal corticosterone treatment resulted in impaired performance compared to controls ($F_{(1, 20)} = 9.18$, $p \leq 0.01$). Conditioned responding for vehicle treated rats did not differ significantly from either control ($F_{(1, 16)} = 3.09$, ns) or corticosterone-treated rats ($F_{(1, 22)} = 1.34$, ns) during late acquisition. Thus, neonatal corticosterone treatment impaired conditioned responding across all phases of training, while performance in vehicle-treated rats was intermediate to that of

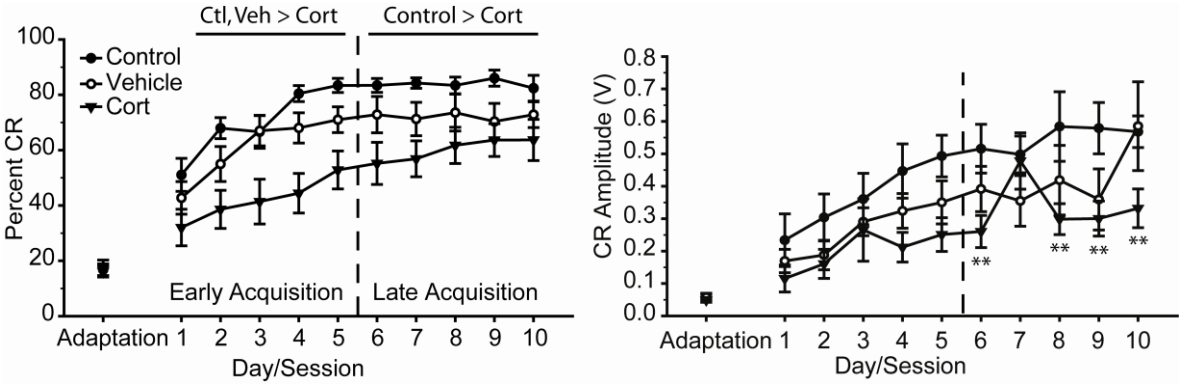

Figure 5.1: *Left*. Mean (± SEM) percentage of conditioned responses (CR) for rats that underwent standard animal facilities rearing (Control, n=8), or received daily vehicle (Vehicle, n=10) or corticosterone injections (Cort, n=14) on PND 2 – 14. Control and vehicle rats performed significantly better than corticosterone-injected rats during early acquisition (Days 1-5, ps < 0.05), and control rats performed significantly better than corticosterone-injected rats during late acquisition (Days 6-10, $p \leq 0.01$), while the performance of vehicle treated rats was intermediate (i.e., not significantly different from control or corticosterone-injected rats) during late acquisition. *Right*. Mean (± SEM) amplitude of CRs for control, vehicle, and corticosterone injected rats. ** $p \leq 0.01$ versus Control.

controls and corticosterone-treated rats.

CR amplitude increased during early acquisition, (Figure 5.1; main effect of day, $F_{(4, 116)} = 14.98$, $p < 0.001$), but not late acquisition (main effect of day, $F_{(4, 116)} = 2.13$, ns). There was not a significant main effect of neonatal treatment on CR amplitude for either early or late acquisition ($Fs_{(2, 29)} < 2.46$, ns). However, there was a day by neonatal treatment interaction for late ($F_{(8, 116)} = 3.25$, $p < 0.01$), but not early acquisition ($F_{(8, 116)} = 1.01$, *ns*). Therefore, planned comparisons were conducted comparing neonatal treatments for each day of late acquisition. Consistent with percent CR analyses, CR amplitude for vehicle-treated rats was intermediate, and not significantly different from control ($Fs_{(1, 16)} < 2.99$, ns) or corticosterone treated rats ($Fs_{(1, 22)} < 3.52$, ns). However, compared to control treatment, neonatal corticosterone treatment significantly decreased CR amplitude on acquisition days 6 and 8-10 ($Fs_{(1, 20)} > 7.26$, $p \leq 0.01$; day 7, $F_{(1, 20)} = 0.03$, ns). Thus, consistent with percent CR data, neonatal corticosterone treatment significantly reduced CR amplitude compared to controls, while vehicle treatment resulted in a mild but non-significant impairment.

GR Expression.

Overall, mean (±SEM) relative intensity of the white matter samples in the interpositus nucleus was $4.9 \times 10^{-3} \pm .0314 \times 10^{-3}$, and white matter relative intensities did not vary across neonatal treatment for the posterior (Data not shown; $F_{(2, 29)} = 0.46$, *ns*) or anterior interpositus (Data not shown; $F_{(2, 29)} = 0.28$, *ns*). The mean number of neurons measured per section was 15.33 ± 0.65 and did not differ across neonatal treatment for either posterior (Data not shown; $F_{(2,}$

$_{29)}$ = 2.85, *ns*) or anterior interpositus (Data not shown; $F_{(2, 29)}$ = 1.65, *ns*). The average number of neurons measured per animal was 78.31 ± 4.30 and did not differ across neonatal treatment for either posterior (Data not shown; $F_{(2, 29)}$ = 1.52, *ns*) or anterior interpositus (Data not shown; $F_{(2, 29)}$ = 0.72, *ns*).

Overall, the mean relative intensity of GR staining varied significantly across neonatal treatment (Figure 5.2; main effect of neonatal treatment, $F_{(2, 29)}$ = 5.67, $p \leq 0.01$) and brain region (posterior versus anterior interpositus; main effect of region, $F_{(1, 29)}$ = 21.81, $p < 0.001$), and there was a significant brain region by neonatal treatment interaction ($F_{(2, 29)}$ = 4.32, $p < 0.05$). The mean relative intensity of GR staining varied significantly across neonatal treatment within the posterior ($F_{(2, 29)}$ = 10.49, $p < 0.001$) but not anterior interpositus ($F_{(2, 29)}$ = 1.38, ns). Therefore, planned comparisons were conducted comparing mean GR expression in the posterior interpositus across neonatal treatments and indicated that both vehicle ($F_{(1, 16)}$ = 8.69, $p \leq 0.01$) and corticosterone ($F_{(1, 20)}$ = 23.12, $p < 0.001$) treatments had significantly reduced adult GR expression relative to control rats, while GR expression did not significantly differ between corticosterone and vehicle treated rats ($F_{(1, 22)}$ = 2.40, ns). Thus, changes in GR expression in the posterior interpositus nucleus paralleled eyeblink conditioning performance, with the largest change in GR expression in corticosterone-treated rats and a smaller change in GR expression in vehicle-treated rats.

Differences in the distribution of GR staining intensities (Figure 5.3) paralleled those seen in the more gross measure of mean relative intensity. As with the mean GR analyses, the effect of neonatal treatment on distribution of GR staining intensities was confined to the posterior region of the interpositus

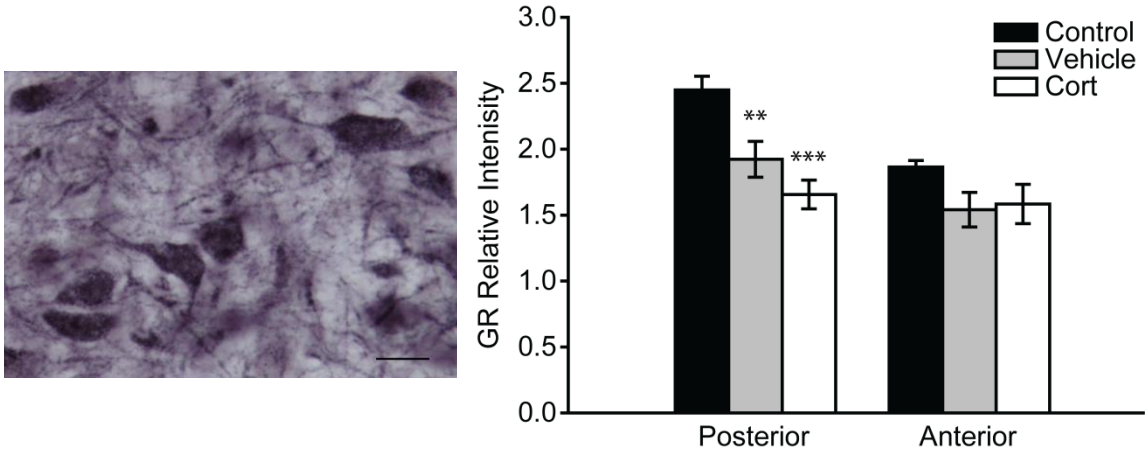

Figure 5.2: *Left*. GR-immunopositive neurons in the posterior interpositus nucleus of a control animal. Scale bar = 20 μm. Right. Mean (± SEM) relative intensity of glucocorticoid receptor staining in the anterior and posterior interpositus nucleus for animals that underwent either standard animal facilities rearing (Control), or received daily vehicle (Vehicle) or corticosterone (Cort) injections on PND 2 – 14.

(neonatal treatment x bin interaction, $F_{(4, 58)} = 18.35$, $p < 0.001$), with no neonatal treatment differences in GR staining observed in the anterior region (neonatal treatment x bin interaction, $F_{(4, 58)} = 1.29$, *ns*). Therefore, for the posterior interpositus, planned comparisons were conducted comparing across neonatal treatments for each intensity bin. Neonatal corticosterone treatment significantly decreased the frequency of darkly ($Fs_{(1, 20)} > 11.27$, $p < 0.01$) and moderately stained neurons, and significantly increased the frequency of lightly stained neurons ($F_{(1, 20)} = 6.02$, $p < 0.05$). A similar but smaller shift towards less intensely stained neurons was apparent in vehicle-treated rats, with a decrease in the frequency of darkly and moderately stained neurons ($Fs_{(1, 16)} > 4.37$, $p \leq 0.05$), but no change in the frequency of lightly stained neurons ($F_{(1, 16)} = 3.51$, ns). Vehicle- and corticosterone-treated groups did not significantly differ in the frequency of lightly, moderately, or darkly stained neurons ($Fs_{(1, 22)} < 1.71$, ns). Thus, consistent with the more gross measure of mean relative intensity, changes in GR expression in the posterior interpositus nucleus paralleled eyeblink conditioning performance with the largest reduction in intensely stained neurons in corticosterone-treated rats and a smaller reduction in intensely stained neurons in vehicle treated rats.

GR-Eyeblink Conditioning Correlation.

The relationship between average relative intensity of GR staining in the posterior and anterior interpositus and eyeblink conditioning averaged across late acquisition days (day 6 - 10) was examined. For the posterior interpositus, there was a strong and significant positive correlation between GR staining and percent CR (Figure 5.4; $r = 0.64$, $p < 0.001$). In addition, GR immunostaining in

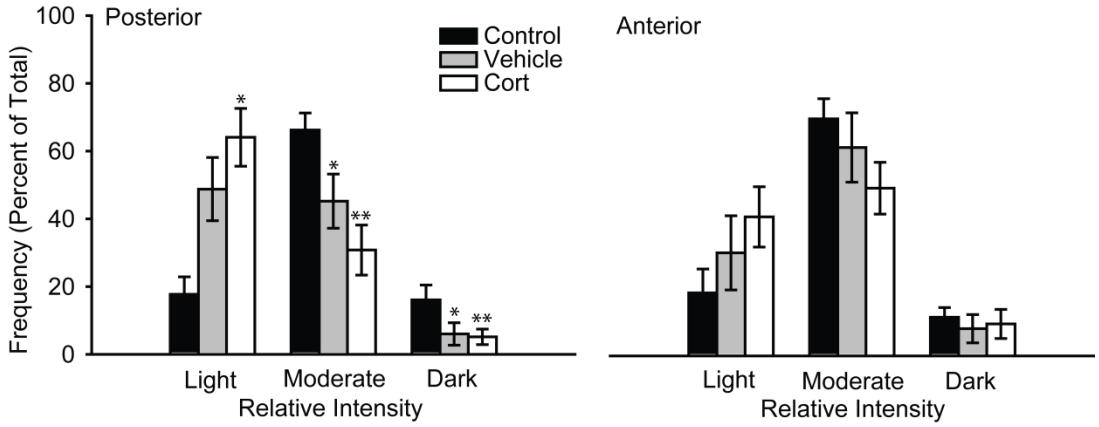

Figure 5.3: Immunostaining of neurons in the posterior (left) and anterior (right) interpositus categorized as light (having a relative intensity of more than one standard deviation below the mean of controls), moderate (within one standard deviation of the mean), or dark (greater than one standard deviation above the mean) for animals that underwent either standard animal facilities rearing (Control), or received daily vehicle (Vehicle) or corticosterone injections (Cort) on PND 2 – 14. * $p \leq 0.05$ versus control. ** $p < 0.01$ versus control. *** $p < 0.001$ versus control.

the anterior interpositus was also significantly correlated with average percent CR for late acquisition (Figure 5.4; $r = 0.42$, $p < 0.05$). Thus, following neonatal corticosterone or vehicle treatment, GR expression in both the anterior and posterior interpositus nucleus predicts eyeblink performance.

Effect of Neonatal Treatment on Neonatal Plasma Corticosterone Concentration.

Neonatal corticosterone treatment and vehicle injections decreased GR expression, an effect that is opposite the increased GR expression that occurs following maternal separation (Wilber et al., 2007a; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a). To test the hypothesis that this dissociation might be driven by a difference in the magnitude of the corticosterone response to neonatal injections compared to maternal separation, plasma corticosterone concentrations on PND 2 were compared for control pups and pups that had undergone a single separation, vehicle injection, or corticosterone injection treatment. There was a significant effect of treatment on plasma corticosterone concentration (Figure 5.5; $F_{(3, 28)} = 138.06$, $p < 0.001$). Planned comparisons revealed that corticosterone concentrations were elevated relative to controls for all treatments ($Fs_{(1, 12\text{-}33)} > 6.95$, $p < 0.05$). Further, vehicle injections increased plasma corticosterone concentrations significantly more than did neonatal separation ($F_{(1, 16)} = 10.64$, $p < 0.01$), and corticosterone injection produced plasma corticosterone concentratiron that were significantly higher than that seen after either neonatal separation or vehicle injection ($Fs_{(1, 9\text{-}13)} > 130.72$, $p < 0.001$).

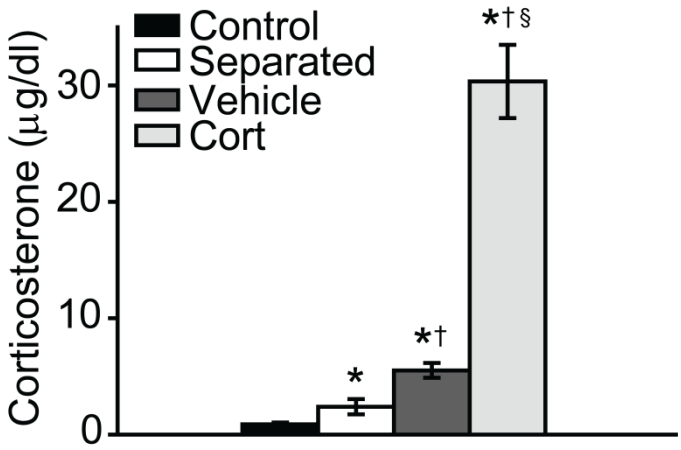

Figure 5.5: Mean (± SEM) corticosterone (μg/dl) sampled at approximately 1800h on PND 2 for animals that underwent either standard animal facilities rearing (Control), 1 h of maternal separation (Separated), received a vehicle (Vehicle), or corticosterone injection (Cort). Asterisks (*) indicate significant differences relative to controls; daggers (†) indicate significant differences relative to separated; section signs (§) indicate significant difference relative to vehicle injected.

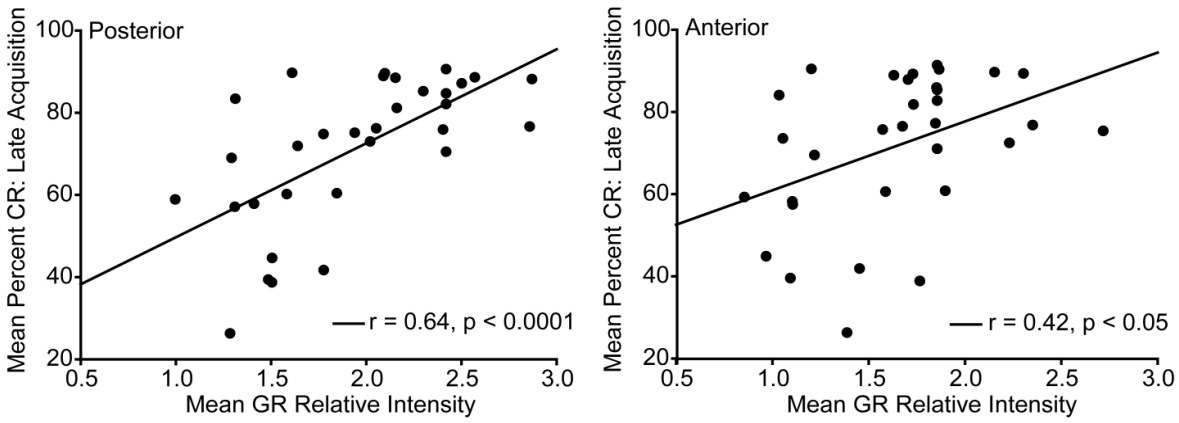

Figure 5.4: Linear regression analysis for mean (± SEM) GR relative intensity in the posterior (above) and anterior interpositus (below) and percent CR for eyeblink conditioning during late acquisition (day 6 – 10) for animals that underwent either standard animal facilities rearing, or received daily vehicle or corticosterone injections on PND 2 – 14.

Discussion

Neonatal vehicle injections increased plasma corticosterone concentrations more than neonatal separation, while the neonatal corticosterone dose used in the present study produced an even greater increase. A similar graded effect of vehicle and corticosterone treatments was apparent on adult eyeblink conditioning, in which neonatal corticosterone injections significantly impaired eyeblink conditioning performance throughout training, whereas rats that received daily vehicle injections had eyeblink conditioning performance intermediate to controls and corticosterone-treated rats. Similarly, daily neonatal vehicle and corticosterone injections both significantly decreased GR expression in the posterior interpositus nucleus, with the largest decrease in GR expression in the corticosterone-treated animals. Finally, there was a significant positive correlation between eyeblink conditioning performance and GR expression in the posterior interpositus. Surprisingly, there was also a significant, though less robust, correlation between eyeblink conditioning performance and GR expression in the anterior interpositus.

The differential effect of neonatal treatment across anterior versus posterior interpositus was consistent with neonatal separation effects on posterior but not anterior interpositus observed in my previous studies (Wilber et al., 2007a; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b), and provides further support for differential vulnerability of the posterior interpositus to neonatal stress and/or glucocorticoids. The finding of impaired adult learning following neonatal corticosterone exposure is consistent with other studies showing altered hippocampally mediated learning following neonatal exposure to

elevated corticosterone (Claflin et al., 2005; Roskoden et al., 2005). Similarly, neonatal separation-induced increases in plasma corticosterone concentration have been associated with impaired spatial learning and memory (Huot et al., 2002), eyeblink conditioning and extinction of conditioned fear (Wilber et al., 2007a; Wilber et al., 2009). However, the timing or route of administration for neonatal corticosterone exposure may be critical, because corticosterone administration at a later time point (PND 18-21) did not significantly impair delay eyeblink conditioning (Claflin et al., 2005).

I have previously shown that neonatal separation produces adult impairments in eyeblink conditioning that are correlated with *increased* GR expression (Wilber et al., 2007a). In the present study, I demonstrated that neonatal vehicle and corticosterone injections result in graded impairments in eyeblink conditioning and *decreases* in posterior interpositus GR expression. Thus, neonatal separation, vehicle injections, and corticosterone injections all impair eyeblink conditioning but have opposite effects on adult GR expression in the posterior interpositus. The pattern of results from these studies suggests that either too much or too little GR expression in the interpositus can impair eyeblink conditioning performance. Consistent with this hypothesis, GR antagonism in the posterior interpositus reverses separation-induced deficits but impairs performance in control rats (Wilber et al., in press). However, the opposite effects of neonatal separation and neonatal injections on adult GR expression are surprising given that neonatal separation, vehicle injections, and corticosterone injections produce successively larger increases in neonatal plasma corticosterone concentrations (i.e., linear relationship). Thus, an inverted

U-shaped relationship may exist between neonatal corticosterone levels and adult GR expression, in which modest increases in corticosterone (neonatal separation) increase adult GR expression but larger increases (neonatal injections) decrease adult GR expression. In order to test this hypothesis we examined the relationship between average relative intensity of GR staining in the posterior interpositus and eyeblink conditioning averaged across late acquisition days (day 6 - 10) for animals in the present study (control and injected) and our previous study (control and separated). Non-linear regression analysis revealed a significant inverted U-shaped relationship between posterior interpositus GR staining and eyeblink conditioning performance (Figure; 5.6; $F_{(2,51)} = 3.91$, $p < 0.05$). This inverted U-shaped function is consistent with previous studies showing an inverted U-shaped relationship between GR activation and consolidation of passive avoidance learning in adults (Roozendaal and McGaugh, 1997; Roozendaal et al., 2009). Alternatively, the effects I observed may be due to alterations in neonatal maternal care and not neonatal plasma corticosterone levels. Long neonatal maternal separation and brief separation may have opposite effects on maternal care (Pryce et al., 2001), and have opposite effects on adult GR expression (Meaney et al., 1989; Liu et al., 1997; Ladd et al., 2004). Thus, differential alterations in maternal care in separated versus injected pups could contribute to the opposite effect of maternal separation and neonatal injections on adult interpositus GR expression since administration of neonatal injections requires a brief separation from the dam. However, both long (1 h) and brief (15 min) separation increase GR expression in the posterior interpositus (Wilber et al., 2007a), so differences in

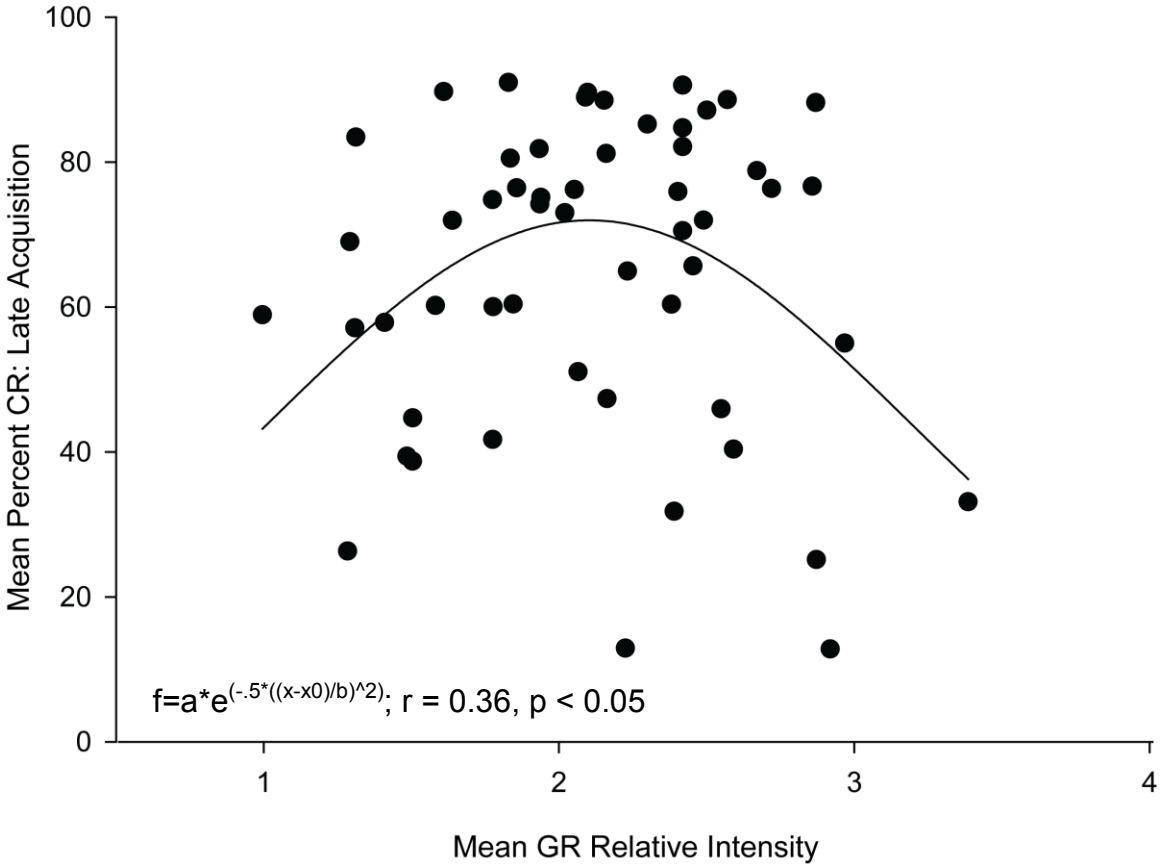

Figure 5.6: Non-linear regression analysis for mean (± SEM) GR relative intensity in the posterior interpositus (below) and percent CR for eyeblink conditioning during late acquisition (day 6 – 10) for animals that underwent either standard animal facilities rearing or daily maternal separation on PND 2-14, or received daily vehicle or corticosterone injections on PND 2 – 14.

maternal care between our separated and injected pups are not likely to be directly responsible for the effects I observed.

Previously, I reported that neonatal separation-induced increased GR expression and correlated deficits in eyeblink conditioning were confined to the posterior interpositus and absent in the anterior interpositus (Wilber et al., 2007a; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a). However, in the present study, in addition to the robust changes in posterior interpositus GR expression and correlated eyeblink conditioning performance, I also found a significant correlation between anterior interpositus GR expression and eyeblink conditioning performance. The significantly larger increase in neonatal plasma corticosterone following injections versus maternal separation observed in the present study may suggest that the anterior interpositus is less sensitive but not immune to the effects of neonatal stress. Further, there are developmental differences in GR receptor expression in anterior versus posterior interpositus (Wilber and Wellman, 2009b). Thus, the more robust effect on posterior interpositus may result from differences in the timing of the development of GR receptors in anterior versus posterior interpositus.

In summary, neonatal corticosterone injections resulted in impaired adult eyeblink conditioning and decreased GR expression in the posterior interpositus nucleus, and lower GR expression in both posterior and anterior interpositus was associated with impaired learning. Maternal separation, neonatal vehicle injections, and neonatal corticosterone injections produced successively larger increases in neonatal plasma corticosterone; however, while neonatal injections and maternal separation both produce adult impairments in learning and

memory, these manipulations produce opposite changes in GR expression. Thus, there may be an inverted U-shaped relationship between both neonatal corticosterone levels and adult GR expression in the interpositus nucleus, and adult GR expression in the interpositus and eyeblink conditioning.

Chapter 6:

Neonatal mifepristone administration attenuates the effect of neonatal separation on adult eyeblink conditioning and posterior interpositus glucocorticoid receptor expression

Neonatal maternal separation produces deficits in eyeblink conditioning and increases GR expression in the posterior region of the interpositus nucleus in adult male rats (Wilber et al., 2007a; 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., in press), suggesting that the increased GR expression in the adult 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., 2007a), GR expression is altered by PND 15 (Wilber and Wellman, 2009b), and neonatal corticosterone exposure mimimics neonatal separation-induced impairments in adult eyeblink conditioning (Chapter 5), suggesting that the effects of maternal separation on GR expression, and consequently adult learning and memory, may be mediated by neonatal increases in corticosterone. To test this hypothesis, I attempted to block neonatal separation effects on adult eyeblink conditioning and GR expression in the interpositus with maternal separation (PND 2-14) preceded by systemic daily mifepristone injections.

Methods

Animals.

Untimed pregnant Long Evans rats (Harlan Indianapolis, IN, N = 25) arrived approximately 1 week before giving birth. Maternal separation or control rearing was conducted as described in the general methods section (Chapter 2). Litters were randomly assigned to either standard animal facilities rearing or maternal separation for 1 h per day on PNDs 2 to 14. Each rearing condition consisted of 11-13 litters. Within each rearing condition, litters received either no injections (Control No Injection, n = 19; Separated No Injection, n = 13), daily vehicle injections (Separated+Vehicle, n = 15; Control+Vehicle, n = 10), or daily mifepristone injections (Control+Mifepristone, n = 7; Separated+Mifepristone, n = 9). Each group consisted of 3-8 litters.

Mifepristone injections were approximately 40 mg/kg in 4% ethanol in sesame oil, based on average weight for age unpublished data collected from a separate group of animals; injection volume did not exceed 0.05 ml. Weight data from a separate group of animals was used in place of daily weighing so that time away from the dam could be minimized. This dose of mifepristone administered on PND 1 to 10 significantly altered adult fear behavior (Lonstein et al., 2001) and repeated administration on PNDs 3 to 13 significantly altered CRH mRNA in the hypothalamus (Yi et al., 1993). Injections occurred just prior to each daily maternal separation. Volumes of vehicle injections were matched to volumes used in mifepristone-injected pups. Mifepristone or vehicle was injected subcutaneously just prior to each daily separation or at an equivalent time of day for control reared pups.

Eyeblink Conditioning.

Eyeblink conditioning was conducted as described in the general methods section (Chapter 2). To capture potential differences in CR production during early versus late training (i.e., acquisition versus asymptotic CR performance), separate three-way repeated measures ANOVAs (rearing condition x neonatal drug treatment x day) were performed for early acquisition (days 1-5) versus late acquisition (days 6-10) followed by planned comparisons.

Glucocorticoid Receptor Immunohistochemistry.

Immediately following completion of eyeblink conditioning, rats were deeply anesthetized with urethane and transcardially perfused so that GR immunohistochemistry could be conducted as described in the general methods section (Chapter 2). Staining was visualized using a nickel-intensified DAB reaction (Figure 6.2). For each brain, 8-9 equally spaced sections (approximately 160 μm apart) were collected through the entire interpositus nucleus of the cerebellum.

Quantification of Glucocorticoid Receptor Expression.

Neurons were sampled from a 100 μm by 125 μm sampling frame at a final magnification of 1440x. Luminosity was measured in an area of white matter free of visible cell bodies directly above the interpositus averaging 578.3 μm^2.

Data were analyzed using two different approaches. First, mean relative intensities were compared for each interpositus regions (posterior and anterior) across rearing condition (separated versus control) and neonatal drug treatment

conditions (mifepristone, vehicle, or no injection) using two-way ANOVAs followed by appropriate planned comparisons. Second, to assess potential differences in the distribution of staining intensities, 4-bin histograms of the mean number of neurons (expressed as percent of total) categorized as having relative intensities varying from more than 1.0 standard deviation below the mean of controls (very low GR; very light) to within 1.0 standard deviations below the mean of controls (low GR; light) to within 1.0 standard deviations above the mean of controls (high GR; intense) to greater than 1.0 standard deviations above the mean of controls (very high GR; very intense) were generated for each region. Data were compared across intensity bins, and rearing and neonatal drug treatment conditions using three-way repeated measures ANOVAs (rearing condition x drug treatment x intensity bin) followed by appropriate planned comparisons.

Corticosterone Assay.

Plasma corticosterone was measured in separate groups of vehicle injected pups 12 h after the morning vehicle injection used in this experiment (n = 14) and 12 h after the afternoon vehicle injection used in the experiment described in Chapter 5 (n = 11) on PND 2. These data were compared to plasma corticosterone data taken from control (n = 10) and separated (1 h/day as described previously; n = 17; Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a) pups used in a previous study (Wilber et al., 2007a). During the first 2 postnatal weeks (stress hyporesponsive period) corticosterone levels peak approximately 12 h after 60 min of maternal

separation (Walker, 1991). The 12-hour time period was chosen to coincide with separation-induced peak plasma corticosterone values. Corticosterone assay procedures were identical for all groups, and described in Chapter 5. Differences in corticosterone concentrations were evaluated using one-way ANOVA (group), followed by planned comparisons (two-group F-tests).

Results

Eyeblink Conditioning.

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 neonatal rearing condition (Figure 6.1; $F_{(1, 67)} = 0.00$, *ns*) or drug treatment ($F_{(2, 67)} = 1.31$, *ns*), but there was a significant rearing condition x drug treatment interaction ($F_{(2, 67)} = 4.66$, $p < 0.05$). Therefore, planned comparisons were conducted comparing each neonatal condition to control no injection treatment and indicated that separated non-injected rats had a significantly lower baseline blink rate ($F_{(1, 30)} = 5.74$, $p < 0.05$), while all other neonatal treatments did not alter baseline blink rate ($Fs_{(1,24-32)} < 1.60$, *ns*). However, spontaneous blink amplitude did not vary across neonatal rearing condition (Figure 6.1; $F_{(1, 67)} = 2.21$, *ns*) or drug treatment ($F_{(2, 67)} = 0.65$, *ns*), nor was there a significant rearing condition x drug treatment interaction ($F_{(2, 67)} = 2.58$, *ns*). Finally, there was no effect of neonatal treatment ($F_{(1, 67)} = 0.07$, *ns*), drug treatment ($F_{(2, 67)} = 2.96$, *ns*), or a rearing condition x drug treatment interaction ($F_{(2, 67)} = 0.40$, *ns*) on responsiveness to the tone (Control No Injection, mean = 0.18 V ± 0.03 SEM; Separated No Injection, mean

= 0.16 V ± 0.03 SEM; Control Vehicle 0.14 V ± 0.02; Separated Vehicle 0.12 V ± 0.01; Control Mifepristone, mean = 0.10 V ± 0.02 SEM; Separated Mifepristone, mean = 0.12 V ± 0.02 SEM).

Percentage of Conditioned Responses. For early acquisition, performance improved during training, as indicated by increasing percent CR across days (Figure 6.1; main effect of day, $F_{(4, 268)} = 45.81$, $p < 0.001$). In addition, there was a significant main effect of rearing condition on the percentage of CRs ($F_{(1, 67)} = 6.83$, $p \leq 0.05$) but not a main effect of neonatal drug treatment ($F_{(2, 67)} = 1.91$, *ns*) and no significant interactions ($Fs_{(4\text{-}8,268)} < 1.64$, *ns*). Thus, planned comparisons were conducted for early acquisition comparing neonatal treatments collapsed across days. During early acquisition, non-injected control rats had a significantly higher percentage of conditioned responses than separated rats that were non-injected ($F_{(1, 30)} = 4.24$, $p \leq 0.05$), vehicle injected ($F_{(1, 32)} = 15.09$, $p \leq 0.001$), and mifepristone injected ($F_{(1, 26)} = 7.85$, $p \leq 0.01$). Separated rats that were mifepristone injected did not significantly differ from non-injected ($F_{(1, 20)} = 0.22$, *ns*) or vehicle injected separated rats ($F_{(1, 22)} = 0.33$, *ns*). Non-injected control rats also performed significantly better than control rats that were mifepristone injected ($F_{(1, 24)} = 7.40$, $p < 0.05$) but not vehicle injected ($F_{(1, 27)} = 0.93$, *ns*). Finally, there was not a significant difference in the percentage of conditioned responses for vehicle- and mifepristone-treated separated rats ($F_{(1, 22)} = 0.33$, *ns*).

For late acquisition, performance also improved during training, as indicated by increasing percent CR across day (Figure 6.1; main effect of day, $F_{(4, 268)} = 4.30$, $p < 0.01$). During late acquisition, there was not a significant main

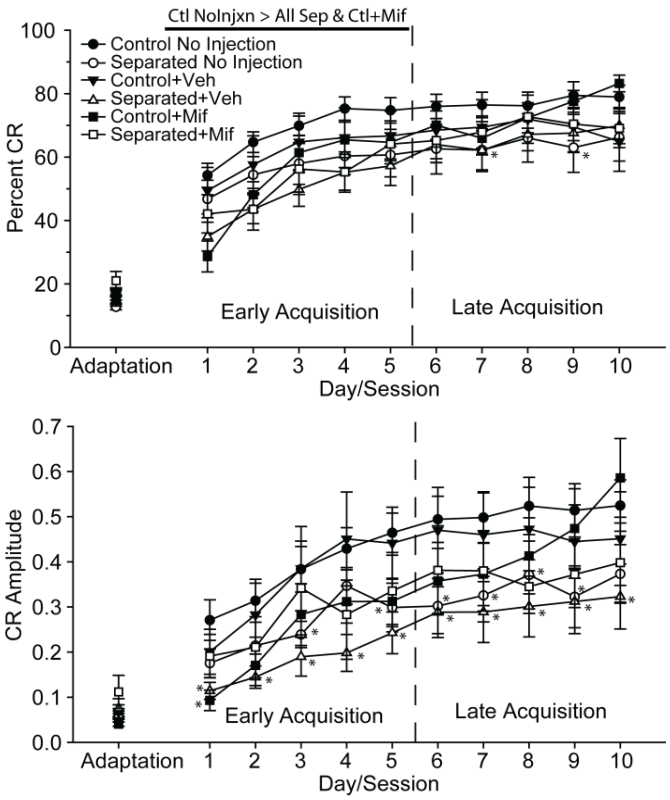

Figure 6.1: *Above*. Mean (± SEM) percentage of conditioned responses (CR) for rats that underwent standard animal facilities rearing (Control), or 1 h daily maternal separation on PND 2 – 14 (Separated) and were not injected (No Injection) or injected daily with vehicle (Veh), or the glucocorticoid receptor antagonist mifepristone (Mif). Control no injection rats performed significantly better than control mifepristone injected rats and separated rats that received no injection, vehicle injection or mifepristone injection during early acquisition (Days 1-5, ps < 0.05), and control no injection rats performed significantly better than corticosterone-injected rats during late acquisition (Days 6-10, $p \leq 0.01$), while the performance of vehicle treated rats was intermediate (i.e., not significantly different from control or corticosterone-injected rats) during late acquisition. *Below*. Mean (± SEM) amplitude of CRs for control, vehicle, and corticosterone injected rats. ** $p \leq 0.01$ versus Control.

effect of rearing condition ($F_{(1, 67)}$ = 1.98, *ns*) or neonatal drug treatment ($F_{(2, 67)}$ = 0.24, *ns*) on the percentage of CRs, and no significant two-way interactions ($Fs_{(4-8,268)}$ < 0.83, *ns*). However, there was a significant day x rearing condition x neonatal drug treatment interaction ($F_{(8,268)}$ = 2.09, $p < 0.05$), suggesting a differential effect of drug treatment and neonatal separation on learning during late acquisition. Thus, planned comparisons were conducted for late acquisition comparing neonatal treatments for each day. During late acquisition, non-injected control rats had a significantly higher percentage of conditioned responses than separated rats that were non-injected (day 9: $F_{(1, 30)}$ = 3.99, $p \leq 0.05$; day 6-8 & 10: $Fs_{(1, 30)}$ < 3.72, *ns*) or vehicle injected (day 7: $F_{(1, 32)}$ = 4.10, $p \leq 0.05$; day 6, 8-10: $Fs_{(1, 32)}$ < 3.26, *ns*). However, separated rats that were mifepristone injected did not significantly differ from non-injected (days 6-10: $Fs_{(1, 20)}$ < 0.51, *ns*) or vehicle injected separated rats (days 6-10: $Fs_{(1, 22)}$ < 0.59, *ns*). Further, non-injected control rats did not differ significantly from mifepristone-injected separated rats (days 6-10: $Fs_{(1, 26)}$ < 2.16, *ns*). Non-injected control rats were also not significantly different from control rats that were vehicle injected (days 6-10: $Fs_{(1, 27)}$ < 2.74, *ns*) or mifepristone injected (days 6-10: $Fs_{(1, 24)}$ < 2.03, *ns*). Thus, neonatal separation impaired performance during both early and late acquisition of eyeblink conditioning and neonatal mifepristone attenuated the effect of neonatal separation on the percentage of conditioned responses during late but not early acquisition eyeblink conditioning.

Conditioned Response Amplitude. CR amplitude increased during early acquisition, (Figure 6.1; main effect of day, $F_{(4, 268)}$ = 36.25, $p < 0.001$) and late acquisition (main effect of day, $F_{(4, 268)}$ = 4.62, $p < 0.01$). In addition, there was a

significant main effect of rearing condition on CR amplitude during both early acquisition ($F_{(1, 67)} = 4.30$, $p < 0.05$) and late acquisition ($F_{(1, 67)} = 4.91$, $p < 0.05$), but not a main effect of neonatal drug treatment during early ($F_{(2, 67)} = 0.90$, *ns*) or late acquisition ($F_{(2, 67)} = 0.80$, *ns*). For early acquisition, there was a significant day x rearing condition interaction ($F_{(4, 268)} = 2.37$, $p \leq 0.05$), suggesting that neonatal separation significantly reduced CR amplitude during early acquisition; however, there was not a significant rearing condition x neonatal drug treatment ($F_{(2, 67)} = 1.93$, *ns*), day x neonatal drug treatment ($F_{(8, 268)} = 0.82$, *ns*), or day x rearing condition x neonatal drug treatment interaction ($F_{(8, 268)} = 0.82$, *ns*). For late acquisition, there was a significant day x rearing condition x neonatal drug treatment interaction ($F_{(8, 268)} = 2.14$, $p < 0.05$), suggesting a differential effect of drug treatment and neonatal separation on CR amplitude during late acquisition; however, there was not a significant rearing condition x neonatal drug treatment ($F_{(2, 67)} = 0.27$, *ns*), day x rearing condition ($F_{(4, 268)} = 0.61$, *ns*), or day x neonatal drug treatment interaction ($F_{(8, 268)} = 1.73$, *ns*).

Thus, planned comparisons were conducted for early and late acquisition comparing neonatal treatments for each acquisition day. Non-injected control rats had a significantly higher percentage of conditioned responses than separated rats that were non-injected (days 3, 5-9: $Fs_{(1, 30)} > 4.46$, $p \leq 0.05$; days 1, 2, 4, & 10: $Fs_{(1, 30)} < 2.87$, *ns*), vehicle-injected (days 1-10: $Fs_{(1, 32)} > 4.97$, $p < 0.05$), but not mifepristone-injected ($Fs_{(1, 26)} < 3.11$, *ns*). Non-injected control rats also performed significantly better than control rats that were mifepristone-injected (day 1: $F_{(1, 24)} = 5.45$, $p < 0.05$; days 2-10: $F_{(1, 24)} < 3.71$, *ns*), but not vehicle-injected (days 1-10: $Fs_{(1, 27)} < 0.99$, *ns*). Finally, separated rats that were

mifepristone injected did not significantly differ from non-injected (days 1-10: $Fs_{(1, 20)} < 1.20$, *ns*) or vehicle injected separated rats (days 1-10: $Fs_{(1, 22)} < 3.04$, *ns*). Thus, neonatal separation impaired performance and mifepristone attenuated the effect of neonatal separation on conditioned response amplitude during both early and late acquisition phases of eyeblink conditioning.

GR Expression.

Overall, mean (±SEM) relative intensity of the white matter samples in the interpositus nucleus was $5.3 \times 10^{-3} \pm 0.03 \times 10^{-3}$, and white matter relative intensities did not vary across rearing condition or neonatal drug treatment for the posterior (Data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.25$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 1.96$, *ns*; rearing condition x drug treatment interaction; $F_{(2, 67)} = 0.72$) or anterior interpositus (Data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.04$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 1.14$, *ns*; rearing condition x drug treatment interaction ; $F_{(2, 67)} = 0.25$). The mean number of neurons measured per section was 10.76 ± 0.26 and did not differ across rearing condition or neonatal drug treatment for either posterior (data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.27$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 0.50$, *ns*; rearing condition x drug treatment interaction ; $F_{(2, 67)} = 0.46$) or anterior interpositus (data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.18$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 0.80$, *ns*; rearing condition x drug treatment interaction ; $F_{(2, 67)} = 0.19$). The average number of neurons measured per animal was 63.12 ± 1.70 and did not differ across rearing condition or neonatal drug treatment for either

posterior (Data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.41$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 1.05$, *ns*; rearing condition x drug treatment interaction ; $F_{(2, 67)} = 0.15$) or anterior interpositus (Data not shown; main effect of rearing condition; $F_{(1, 67)} = 0.62$, *ns*; main effect of neonatal drug treatment; $F_{(2, 67)} = 0.85$, *ns*; rearing condition x drug treatment interaction ; $F_{(2, 67)} = 0.12$).

The mean relative intensity of GR staining did not vary significantly across neonatal rearing condition within the posterior (Figure 6.2; $F_{(1, 67)} = 0.93$, *ns*) or anterior interpositus (Figure 6.2; $F_{(1, 67)} = 1.23$, *ns*), and did not vary across neonatal drug treatment within the posterior ($F_{(2, 67)} = 0.03$, *ns*) or anterior interpositus ($F_{(2, 67)} = 1.37$, *ns*). Further, there was no rearing condition x neonatal drug treatment interaction within the posterior ($F_{(2, 67)} = 1.73$, *ns*) or anterior interpositus ($F_{(2, 67)} = 0.01$, *ns*). However, visual inspection of the data suggested that, consistent with our previous studies (Wilber et al., 2007a; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b), non-injected neonatally separated animals had significantly increased GR staining in the posterior interpositus compared to non-injected controls. Therefore, a one-way ANOVA was conducted comparing posterior and anterior interpositus GR staining for non-injected controls and non-injected separated animals, and revealed a neonatal separation-induced increase in GR expression in the posterior ($F_{(1, 30)} = 4.52$, $p < 0.05$) but not anterior interpositus ($F_{(1, 30)} = 0.62$, *ns*). Thus, neonatal separation increased GR expression in the posterior interpositus nucleus.

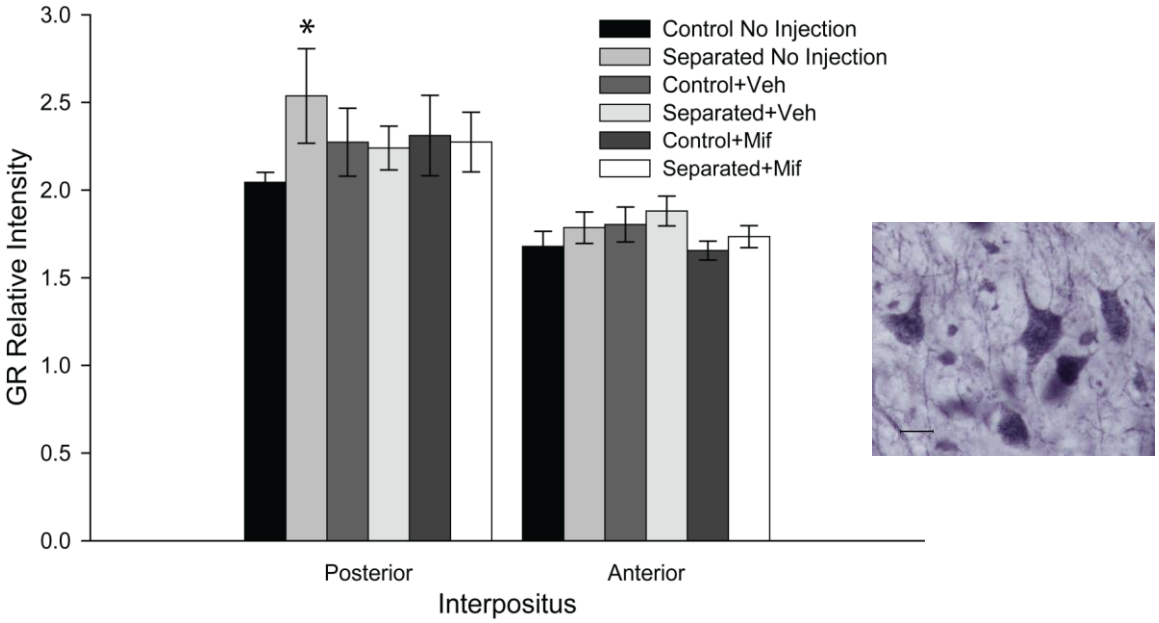

Figure 6.2: *Left.* Mean (± SEM) relative intensity of glucocorticoid receptor staining in the posterior (left), and anterior (right) interpositus nucleus for rats that underwent standard animal facilities rearing (Control), or 1 h daily maternal separation on PND 2 – 14 (Separated) and were not injected (No Injection), injected daily with vehicle (Veh), or the glucocorticoid receptor antagonist mifepristone (Mif). * $p < 0.05$ versus control. *Right.* GR-immunopositive neurons in the posterior interpositus nucleus of a control animal. Scale bar = 20 μm.

Differences in the distribution of GR staining intensities (Figure 6.3) revealed changes in GR staining that were not apparent in the more gross measure of mean relative intensity (above). The effect of neonatal treatment on distribution of GR staining intensity was confined to the posterior region of the interpositus (main effect of rearing condition $F_{(1, 67)} = 4.44$, $p < 0.05$; neonatal treatment x bin interaction, $F_{(2, 67)} = 3.74$, $p < 0.05$ but no main effect of neonatal drug treatment; $F_{(2, 67)} = 0.72$, *ns*; or other interaction; $Fs_{(3\text{-}6, 201)} < 1.31$, *ns*), with no rearing condition effect ($F_{(1, 67)} = 1.31$, *ns*), neonatal drug treatment effect ($F_{(2, 67)} = 0.77$, *ns)*, or interactions ($Fs_{(2\text{-}6, 67 \& 201)} < 0.78$, *ns*) observed in the anterior region of the interpositus nucleus. Therefore, for the posterior interpositus, planned comparisons were conducted comparing across neonatal treatments for each intensity bin. Neonatal maternal separation treatment alone (no-injection) shifted the distribution of staining intensities towards more intensely stained neurons as indicated by a significantly decreased frequency of very lightly stained neurons ($F_{(1, 30)} = 4.49$, $p < 0.05$), without significantly changing the frequency of light, intense, or very intensely stained neurons ($Fs_{(1, 30)} < 0.68$, *ns*). A similar shift towards more intensely stained neurons was apparent in neonatally separated, vehicle-treated animals, with a decrease in the frequency of lightly stained neurons ($F_{(1, 32)} = 4.39$, $p < 0.05$), but no change in the frequency of very light, intense, or very intensely stained neurons ($Fs_{(1, 32)} < 3.43$, *ns*). However, neonatal mifepristone injections prevented a significant shift in the distribution of staining intensities towards more intensely stained neurons ($Fs_{(1, 26)} < 3.90$, *ns*). There were no significant differences between control no-injection and either control vehicle-injection ($Fs_{(1, 27)} < 1.62$, *ns*) or control mifepristone-injection

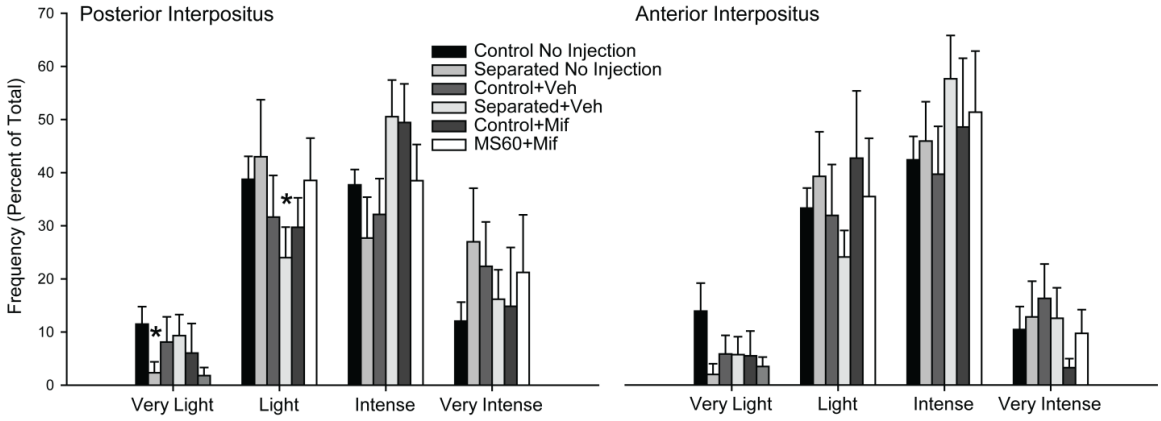

Figure 6.3: Immunostaining of neurons in the posterior (left) and anterior (right) interpositus categorized as very light (having a relative intensity more than 1.0 standard deviation below the mean of controls), light (within 1.0 standard deviation below the mean of controls), intense (within 1.0 standard deviations above the mean of controls), or very intense (greater than 1.0 standard deviations above the mean of controls) for rats that underwent standard animal facilities rearing (Control), or 1 h daily maternal separation on PND 2 – 14 (Separated) and were not injected (No Injection), injected daily with vehicle (Veh), or the glucocorticoid receptor antagonist mifepristone (Mif). * $p < 0.05$ versus control.

groups ($Fs_{(1, 24)} < 3.31$, *ns*) in the frequency of intensely or lightly stained neurons. Finally, consistent with eyeblink conditioning performance, there were no significant differences between separated rats that were mifepristone injected and non-injected ($Fs_{(1, 20)} < 0.84$, *ns*) or vehicle injected separated rats ($Fs_{(1, 22)} < 2.27$, *ns*) in the frequency of intensely or lightly stained neurons. Thus, changes in GR expression in the posterior interpositus nucleus paralleled eyeblink conditioning performance with increases in intensely stained neurons in neonatally separated rats, and mifepristone treatment attenuated both effects.

Effect of Neonatal Treatment on Neonatal Plasma Corticosterone Concentration.

Neonatal vehicle injections administered in the afternoon decreased GR expression, an effect that is opposite the increased GR expression that occurs following maternal separation (Wilber et al., 2007a; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a); however, neonatal vehicle injections administered in the morning did not change GR expression. To assess whether differential effects of morning and evening injections on corticosterone concentrations could account for the differential adult effects, plasma corticosterone concentrations on PND 2 were compared for control pups and pups that had undergone a single, separation, evening vehicle injection (as in Chapter 5), or morning vehicle injection (as in the current experiment). There was a significant effect of neonatal treatment on plasma corticosterone concentration (Figure 6.4; $F_{(3, 38)} = 23.48$, $p < 0.001$). Planned comparisons revealed that corticosterone concentrations were elevated relative to controls for all neonatal treatments ($Fs_{(1, 15\text{-}22)} > 6.95$, $p < 0.05$). Further, both evening and morning vehicle injections

increased plasma corticosterone concentrations significantly more than neonatal separation ($Fs_{(1, 16\text{-}19)} > 9.72$, $p \leq 0.01$), and evening vehicle injection increased plasma corticosterone significantly more than morning vehicle injection ($F_{(1,23)} = 16.61$, $p \leq 0.001$).

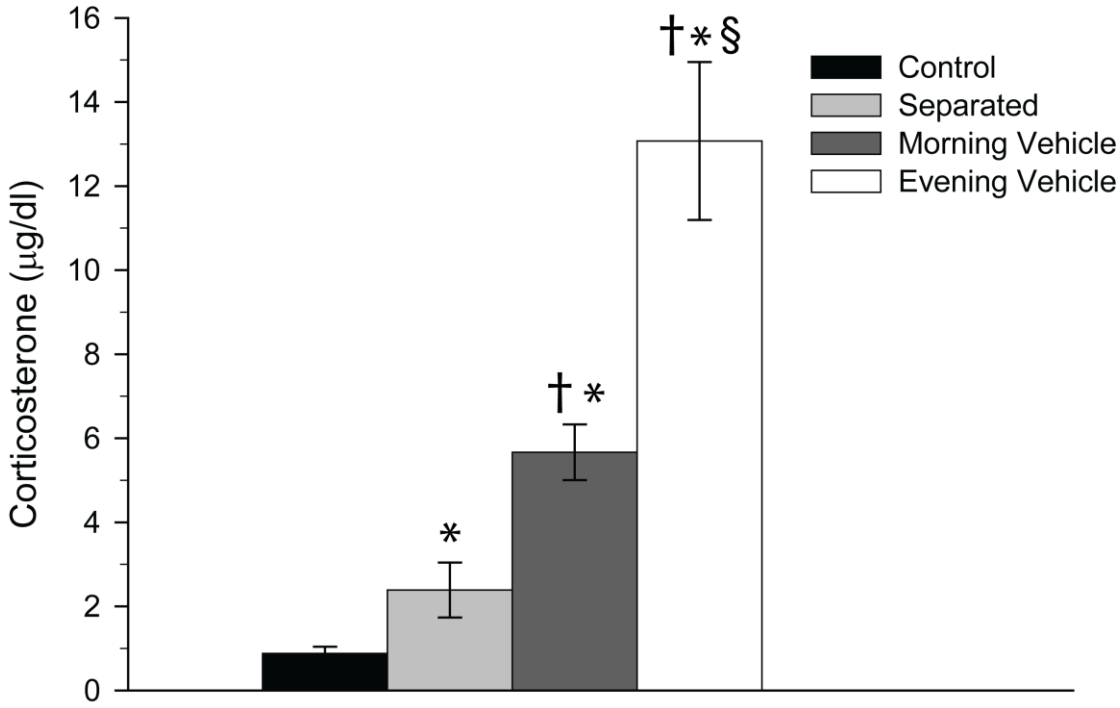

Figure 6.4: Mean (± SEM) corticosterone (μg/dl) sampled at approximately 1800h on PND 2 for animals that underwent standard animal facilities rearing (Control) or 1 h of maternal separation (Separated), or sampled 12 h after vehicle injections administered between 0700 and 0900h (Morning Veh) or between 1600 and 1900h (Evening Veh). Asterisks (*) indicate significant differences relative to controls; daggers (†) indicate significant differences relative to separated; section signs (§) indicate significant difference relative to morning vehicle injected.

Discussion

Neonatally separated animals receiving either vehicle or no injection had impaired adult eyeblink conditioning, while mifepristone treatment just prior to separation attenuated this impairment. Similarly, neonatally separated animals that received either vehicle or no injection had increased adult GR expression in the posterior interpositus nucleus, while neonatal mifepristone treatment attenuated the significant increase in GR expression. Control vehicle-injected rats did not have increased adult GR expression or impaired eyeblink conditioning, while neonatal mifepristone treatment produced subtle impairments in adult eyeblink conditioning but did not significantly increase GR expression. Neonatal mifepristone vehicle injections increased plasma corticosterone concentrations more than neonatal separation, while neonatal corticosterone vehicle injections (Chapter 5) produced even greater increases.

The differential effect of neonatal treatment across anterior versus posterior interpositus was consistent with neonatal separation effects on posterior but not anterior interpositus observed in my previous studies, and provides further support for differential vulnerability of the posterior interpositus to neonatal stress and glucocorticoids (Chapter 5; Wilber et al., 2007a; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b). The finding that mifepristone administration during neonatal separation attenuated the increased GR expression and impaired eyeblink conditioning is consistent with our finding that neonatal corticosterone exposure produces adult impairments in eyeblink conditioning (Chapter 5), and suggests that corticosterone's activation of

glucocorticoid receptors during maternal separation may contribute to the separation–induced alterations in adult eyeblink conditioning and its neural circuitry.

Previously, I found that neonatal vehicle injections administered in the afternoon produce large increases in plasma corticosterone, and result in adult *decreases* in GR expression and mild impairments in eyeblink conditioning (Chapter 5); however, in this experiment neonatal vehicle injections administered in the morning produced very small (non-significant) *increases* in GR expression and did not impair eyeblink conditioning. This is surprising given that both morning and evening neonatal injections produced increases in corticosterone values that are significantly greater than neonatal separation-induced increases in corticosterone; however, evening injections produce very large increases in plasma corticosterone that are significantly greater than those induced by morning injections. Thus, while neonatal separation produces adult impairments in eyeblink conditioning that are correlated with *increased* GR expression (Wilber et al., 2007a), neonatal evening vehicle and corticosterone injections result in graded impairments in eyeblink conditioning and *decreases* in posterior interpositus GR expression. The pattern of results from these studies suggests that an inverted U-shaped relationship may exist between neonatal corticosterone levels and adult GR expression, in which modest increases in corticosterone (neonatal separation) increase adult GR expression while much larger increases (neonatal evening injections) decrease adult GR expression, consistent with previous studies showing an inverted U-shaped relationship between GR activation and consolidation of passive avoidance learning

(Roozendaal and McGaugh, 1997; Roozendaal et al., 2009) or eyeblink conditioning learning and memory in adult rats (Wilber et al., in press). The corticosterone concentrations produced by morning injections are intermediate to separation-induced concentrations, which increase adult GR expression, and evening injection values, which decrease adult GR expression, and as a result do not alter adult GR expression or eyeblink conditioning (Figure 6.5).

The finding that an identical neonatal manipulation performed at different times of day (morning versus afternoon) produced different patterns of neonatal corticosterone values, adult GR expression, and adult eyeblink conditioning performance is unexpected. There are at least two possible explanations: the internal state of the pup varies with time of day and leaves the pup especially susceptible to perturbations in the afternoon, or the normal pattern of maternal care and/or response of the dam to a perturbation varies with time of day and leaves the pups more susceptible to afternoon perturbations. Internal factors could certainly play a role, given that blind rat pups may have a circadian rhythm for plasma corticosterone levels as early as postnatal day 1 (Hiroshige et al., 1982; Yamazaki and Takahashi, 1983), but this rhythm may not be apparent in sighted rats until PND 14-18 (Hiroshige and Ssto, 1970; Poland et al., 1981). Maternal care could also play a role in the differential effect of morning and evening vehicle injections, because there is a circadian rhythm to maternal care (Schelstraete et al., 1992). Maternal care is triggered by neonatal stressors like maternal separation, perhaps to act in opposition to the adverse consequences of neonatal stressors (Pryce et al., 2001; Macri et al., 2004; Rüedi-Bettschen et al., 2004), and different maternal behaviors (e.g., arched back nursing, supine

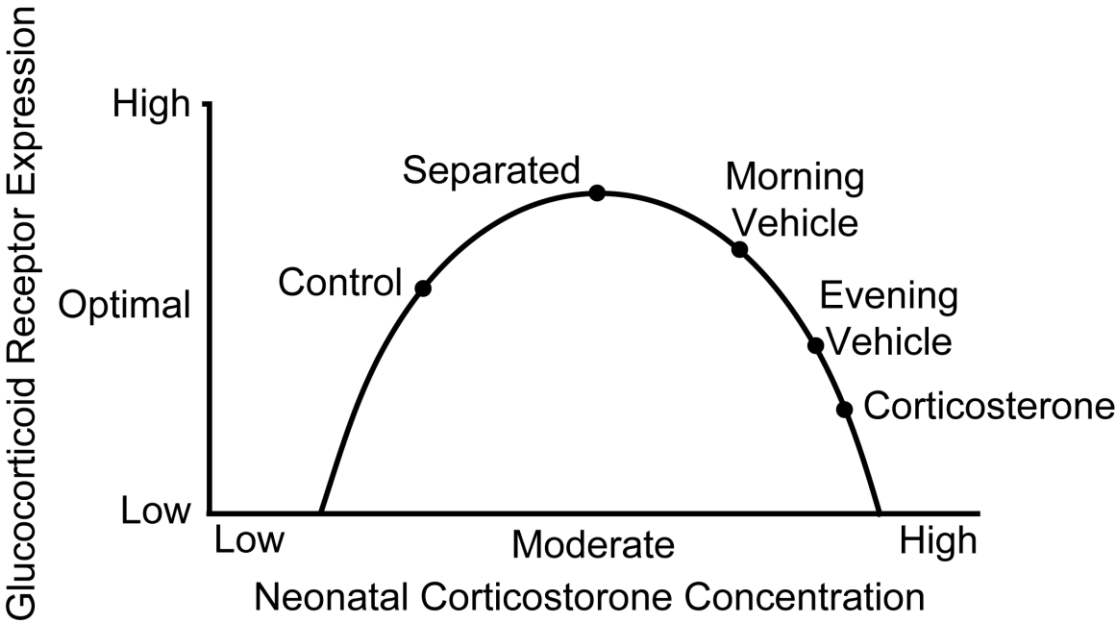

Figure 6.5: For many learning related behaviors there is an inverted U-shaped relationship between corticosterone levels and performance. A similar relationship may exist between neonatal levels of corticosterone and adult glucocorticoid receptor expression. Neonatal maternal separation increases glucocorticoid receptor expression, morning vehicle injections do not significantly increase adult glucocorticoid receptor expression, and evening vehicle and corticosterone injections significantly *decrease* adult glucocorticoid receptor expression.

nursing, and licking) increase in response to 4h maternal separation depending on whether the separation is carried out in the light or dark cycle (Rüedi-Bettschen et al., 2004). In fact, circadian factors in the pup and dam can interact and could both contribute. For instance, some but not all blind pups will shift their circadian rhythm to those of a foster mother, suggesting that dam cues may help set the pups' circadian rhythm (Hiroshige et al., 1982; Yamazaki and Takahashi, 1983).

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 neonatal cerebellum (Sakamoto et al., 2003), and in the deep nuclei of adult rats (Guerra-Araiza et al., 2001; Curran-Rauhut and Petersen, 2002); therefore, I cannot rule out the possibility that mifepristone's action on progesterone receptors contributes to the effects I observed. Nonetheless, given that I know maternal separation increases GR expression in the posterior interpositus and mifepristone has a higher affinity for GRs (Van Look and Bygdeman, 1989), and given that neonatal corticosterone exposure produced adult deficits in eyeblink conditioning (Chapter 5), it is likely that mifepristone's ability to reverse the separation effect is due to its blockade of GRs.

In summary, neonatal maternal separation resulted in impaired adult eyeblink conditioning and increased GR expression in the posterior interpositus nucleus and neonatal injections of mifepristone, but not vehicle, ameliorated neonatal separation effects on GR expression and eyeblink conditioning. Thus,

the neonatal separation-induced increase in corticosterone may be a critical component of neonatal separation effects on adult learning and memory.

Chapter 7:

General Discussion

This dissertation has described the role of neonatal and adult glucocorticoids in mediating neonatal maternal separation induced deficits in adult eyeblink conditioning. In Chapter 3, I found that neonatal separation reorganized the development of glucocorticoid receptor expression to ultimately produce increases in adult glucocorticoid receptor expression in the posterior interpositus nucleus. In Chapter 4, I found that glucocorticoid receptor blockade in adults ameliorates the eyeblink conditioning deficits induced by maternal separation, suggesting that the increased glucocorticoid receptor expression in the posterior interpositus is responsible for the separation-induced impairment in eyeblink conditioning. In Chapter 5, I demonstrated that neonatal corticosterone administration produces adult impairments in eyeblink conditioning but decreases glucocorticoid receptor expression in the interpositus nucleus. Finally, in Chapter 6, I found that glucocorticoid receptor blockade during separation attenuates the separation-induced impaired eyeblink conditioning and increased glucocorticoid receptor expression.

Characterization of neonatal separation effects on adult eyeblink conditioning and glucocorticoid receptor expression

Eyeblink conditioning

The studies described in this thesis provide strong evidence that neonatal maternal separation (15 min or 1h/day on postnatal day 2-14) produces adult impairments in eyeblink conditioning (Chapter 5; Chapter 6; Chapter 7; Wilber et

al., 2007a; Wilber and Wellman, 2009a). This finding is consistent with reports of neonatal maternal separation-induced impaired spatial learning and memory (3 – 6 h/day on postnatal days 2-14 or 2-21; Huot et al., 2002; Uysal et al., 2005; Aisa et al., 2007; Aisa et al., 2009b), object recognition (Aisa et al., 2007), fear conditioning (6 h/day on postnatal days 2-14; Stevenson et al., 2009), and extinction of conditioned fear (15 min/day on postnatal days 2-14; Stevenson et al., 2009; Wilber et al., 2009). There is a graded effect of duration of maternal separation, with larger adult learning impairments with longer neonatal maternal separation episodes (Huot et al., 2002; Uysal et al., 2005; Aisa et al., 2007; Wilber et al., 2007b; Aisa et al., 2009c; Stevenson et al., 2009; Wilber et al., 2009). However, it is not clear if the number of daily maternal separation episodes (e.g., 2 versus 3 postnatal weeks) also has a graded effect on adult learning and memory. It is possible that maternal separation during the first or second postnatal week alone is critical for impaired adult learning and that separation during the additional week has little additional impact on adult learning.

Regardless, the effect of multiple stress episodes at different time points is not graded. In fact there is evidence that repeated stressors at different points in development do not sum to produce larger impairments in adult learning and memory when compared to stressors confined to a single developmental period. For example, prenatal stress may block the effects of postnatal maternal separation on adult latent inhibition learning (Lehmann et al., 2000) and postnatal maternal separation followed by juvenile isolation rearing may result in facilitated adult avoidance learning (Schäble et al., 2007). Thus, while we are beginning to

characterize some of the critical variables for maternal separation effects on adult learning, much less is known about the precise postnatal period that is critical for these effects and how stressors at different time points during development interact. Future experiments could begin to address this question by directly comparing the effects of different timings and durations of daily maternal separation on eyeblink conditioning.

Glucocorticoid receptor expression

Prenatal glucocorticoid exposure produces cell death in the cerebellum of mice and chickens (Aden et al., 2008; Noguchi et al., 2008a; Noguchi et al., 2008b), suggesting that in addition to the changes in adult glucocorticoid receptor expression, changes in neuron number could play a role in the effects I observed. However, postnatal maternal separation does not reduce interpositus nucleus neuron number or volume (Wilber et al., 2007a), suggesting that the effects of pre-and postnatal stress on the cerebellum may differ. Future studies could directly compare the effects of pre- and postnatal stress to elucidate whether or not there is a relationship between pre- and post-natal stress effects on the adult cerebellum.

In general, little is known about changes in the brain that might underlie postnatal stress effects on adult learning. Possible candidates include neonatal maternal separation-induced changes in mossy fibers, plasticity, or neurogenesis in the hippocampus linked to impaired spatial learning and memory (Huot et al., 2002; Aisa et al., 2009a), changes in NMDA receptor expression linked to impaired extinction of conditioned fear (Wilber et al., 2009), and changes in

glucocorticoid receptor expression linked to impaired eyeblink conditioning and extinction of conditioned fear (Chapter 5; Chapter 6; Chapter 7; Wilber et al., 2007a; Wilber et al., 2009; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b). This dissertation has focused on exploring one of the potential neural changes that may contribute to separation-induced changes in adult learning and memory and glucocorticoid receptor expression in the interpositus nucleus. The increased glucocorticoid receptor expression in the interpositus nucleus following neonatal maternal separation is consistent with previous demonstrations of altered glucocorticoid receptor expression in forebrain structures (Meaney and Aitken, 1985; Meaney et al., 1985c; Meaney et al., 1985b; Sapolsky et al., 1985; Dallman et al., 1991; Buijs et al., 1993; Abdulla et al., 1995; Vazquez, 1998). Alternatively, prolonged maternal separation (3 h/day on postnatal days 2-14) decreases adult hippocampal glucocorticoid receptor expression compared to brief separation (15 min/day on postnatal days 2-14; Ladd et al., 2004). However, I have shown that brief and prolonged maternal separation both increased adult glucocorticoid receptor expression (Wilber et al., 2007a), suggesting that the mechanisms underlying separation-induced changes in glucocorticoid receptor expression in hypothalamic-pituitary-adrenal (HPA) axis structures such as the hippocampus may be different from those underlying changes in the interpositus. Alternatively, a similar mechanism may drive changes in glucocorticoid receptor expression in the hippocampus and cerebellum: Maternal separation for 3h/day produces dramatic increases in neonatal plasma corticosterone comparable to the dramatic increases in neonatal plasma corticosterone I observed following evening vehicle injections

(Figure 5.5; Huot et al., 2002). Thus, it is possible that the inverted U-shaped relationship in the cerebellum between neonatal corticosterone levels and adult glucocorticoid receptor expression, in which modest increases in corticosterone (neonatal separation) increase adult glucocorticoid receptor expression but larger increases (corticosterone injections) decrease adult glucocorticoid receptor expression (Figure 5.6), may also exist in the hippocampus.

In the cerebellum, the increased glucocorticoid receptor expression seen in adult, maternally separated rats is not established immediately. Instead, it is the result of maternal separation-induced attenuation of the normally occurring decrease in glucocorticoid receptor expression in posterior interpositus during the third postnatal week. Separated rats had significantly less glucocorticoid receptor expression than did controls at postnatal day 15, but significantly greater glucocorticoid receptor expression than control rats at postnatal day 21 and in adulthood (Wilber and Wellman, 2009b). This was surprising given that a single episode of 24 h maternal deprivation on postnatal day 9 or 11 will immediately alter glucocorticoid receptor expression in the hippocampus and HPA circuitry (van Oers et al., 1998; Avishai-Eliner et al., 1999). However, recent evidence suggests that changes in glucocorticoid receptor expression following a less severe but repeated daily maternal separation might also produce changes in hippocampal glucocorticoid receptor expression that occur later in development: changes in deoxyribonucleic acid (DNA) methylation of the exon 1_7 glucocorticoid receptor promoter, which has been suggested to underlie separation-induced changes in adult hippocampal glucocorticoid receptor expression (Szyf et al., 2005), are absent on postnatal day 21 following maternal separation for 3h/day

on postnatal day 2-14 (Daniels et al., 2009). Therefore, maternal separation reorganizes the development of glucocorticoid receptor expression in the cerebellum and may work in a similar fashion in other brain regions such as the hippocampus.

Mechanism of neonatal separation effects on adult eyeblink conditioning

Adult mechanism

For this dissertation I have explored a) whether glucocorticoids mediate the effect of neonatal maternal separation on adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus; and b) whether elevated glucocorticoid receptor expression in the interpositus nucleus is responsible for the separation-induced deficit in adult eyeblink conditioning. I will begin by discussing the adult glucocorticoid receptor mechanism. In Chapter 4, I found that blocking glucocorticoid receptors in adult neonatally separated rats reverses separation-induced deficits in eyeblink conditioning, suggesting that the separation-induced increase in glucocorticoid receptor expression I observed is responsible for separation induced deficits in eyeblink conditioning. Further, there appears to be a topographical organization to the interpositus nucleus, with the anterior interpositus playing an important role in learning the CR (Steinmetz et al., 1992a; Steinmetz et al., 1992b; Plakke et al., 2007) and the posterior interpositus modulating the CR (Delgado-Garcia and Gruart, 2006; Sanchez-Campusano et al., 2007). Previously I found that posterior interpositus interneurons show more robust changes in glucocorticoid receptor expression than projection neurons, and only glucocorticoid receptor expression in

interneurons is associated with eyeblink conditioning performance (Wilber and Wellman, 2009a), suggesting that interneurons in the posterior interpositus may modulate the activity of projection neurons in the anterior interpositus. Together with the finding that blocking glucocorticoid receptors in the posterior interpositus reverses separation-induced impaired eyeblink conditioning (Wilber et al., in press) these studies suggests that neonatal separation alters interneuron modulation of eyeblink conditioning via changes in glucocorticoid receptor expression (Wilber et al., 2007a; Wilber and Wellman, 2009a; Wilber and Wellman, 2009b). Further, blocking posterior interpositus glucocorticoid receptors in adult rats impaired performance in control rats. This suggests that glucocorticoid receptors in the interpositus may normally play an important role in eyeblink conditioning. Blocking glucocorticoid receptors in control rats may reduce glucocorticoid receptor activation below normal, optimal levels and impair performance; while blocking glucocorticoid receptors in separated rats may reduce glucocorticoid receptor activation to an optimal level, thus improving performance (Wilber et al., in press). Together this interaction between neonatal rearing and drug treatment suggests an inverted U-shaped relationship between glucocorticoid receptor activation and performance, as has been described for other learning tasks (Roozendaal and McGaugh, 1997; Roozendaal et al., 2009). The data in this thesis suggest a similar relationship for cerebellar-dependent forms of learning and memory.

The precise cellular mechanism by which increased interneuron glucocorticoid receptor expression in the posterior interpositus dampens the conditioned response signal in the anterior interpositus principal neurons is

unknown, but could involve changes in morphology and glucocorticoid receptor/NMDA receptor interactions. For example, changes in neuronal morphology in the hippocampus have been reported following neonatal separation (Huot et al., 2002) and GABAergic interneurons are one of the two major cell types in the interpositus nucleus (Maricich and Herrup, 1999; Czubayko et al., 2001; Uusisaari et al., 2007); therefore, increased glucocorticoid receptor activation in posterior interpositus interneurons could lead to transcriptional changes that produce an increased number of inhibitory synapses on principal cells in the anterior interpositus, thus leading to a dampened conditioned response signal. Additionally, many adult stress effects are mediated by NMDA-GR interactions (Marginos and McEwen, 1995; Kim et al., 1996; Shors and Mathew, 1998; Roozendaal et al., 2003). Specifically, glucocorticoids modulate NMDA receptor-mediated Ca^{2+} influx in cultured hippocampal neurons (Takahashi et al., 2002) and NMDA-dependent LTP (Shors et al., 1989), these effects are mediated by glucocorticoid receptors (Xu et al., 1998; Yang et al., 2004). Further, neurosteroid modulation of GABAergic transmission can be NMDA-dependent (Wang et al., 2007). Therefore, increased glucocorticoid receptor expression on posterior interpositus interneurons could lead to increased GABAergic inhibition of anterior interpositus principal neurons, thus dampening CR transmission.

Previously, I reported that neonatal separation-induced increased glucocorticoid receptor expression and correlated deficits in eyeblink conditioning were confined to the posterior interpositus and absent in the anterior interpositus (Wilber et al., 2007a; Wilber and Wellman, 2009b; Wilber and Wellman, 2009a).

There is evidence for a topographical organization of anterior versus posterior interpositus nucleus for learning eyeblink conditioning CR (Steinmetz et al., 1992a; Steinmetz et al., 1992b; Plakke et al., 2007) and modulating the CR (Delgado-Garcia and Gruart, 2006; Sanchez-Campusano et al., 2007), and there are developmental differences in glucocorticoid receptor expression in anterior versus posterior interpositus (Wilber and Wellman, 2009b). However, neonatal injections that produce large increases in plasma corticosterone concentrations are capable of producing subtle but significant changes in glucocorticoid receptor expression in the anterior interpositus and less robust but significant correlations with eyeblink conditioning performance. This may suggest that the anterior interpositus is less sensitive but not immune to the effects of neonatal stress. Further, the modulatory system in the posterior interpositus may not act alone but together with an anterior interpositus modulatory system. It may be that the modulatory system in the posterior interpositus is more sensitive to neonatal perturbations during the first two postnatal weeks. Thus, the more robust effect on posterior interpositus may result from differences in the timing of the development of glucocorticoid receptors in anterior versus posterior interpositus.

In adult rats, blocking glucocorticoid receptors in separated animals results in eyeblink conditioning performance that is enhanced relative to controls (though not to the point of statistical significance; Figure 4.3), suggesting that it might be possible to enhance adult learning via modulation of glucocorticoid receptors in the interpositus nucleus. In Chapters 5 and 6, I demonstrated that neonatal manipulations that produce low (neonatal separation) and high (afternoon vehicle or corticosterone injections) levels of plasma corticosterone

both produced adult impairments in eyeblink conditioning but have opposite effects on adult glucocorticoid receptor expression. However, a neonatal manipulation that produced intermediate levels of neonatal corticosterone (morning injection) did not significantly alter adult glucocorticoid receptor expression and did not impair eyeblink conditioning. Therefore, an animal reared in an environment that produced a —just right" moderate elevation in neonatal corticosterone (e.g., natural environment) might produce adult animals with slightly reduced glucocorticoid receptor expression and consequently activation during eyeblink conditioning. This just right level of glucocorticoid receptor expression and activation could produce significantly better learning than that seen in standard animal facilities-reared control rats.

For this thesis I have focused on one possible adult mechanism of the impaired eyeblink conditioning that I observed (i.e., glucocorticoid receptor expression), and while the ability of adult mifepristone infusion into the posterior interpositus to reverse neonatal separation effects on adult eyeblink conditioning provides strong evidence that glucocorticoid receptors are playing a role, this does not preclude the possibility of other factors contributing to the adult impairment in eyeblink conditioning. For example, given that maternal separation alters morphology of neurons in hippocampus and prefrontal cortex, (Norrholm and Ouimet, 2001; Huot et al., 2002; Poeggel et al., 2003; Andersen and Teicher, 2004), neonatal separation could also alter dendritic morphology of interpositus neurons. Thus, changes in neuronal morphology and glucocorticoid receptor expression could act together or in opposition to alter the physiology of

interpositus neurons that convey the CR signal to the red nucleus to ultimately produce the deficit in eyeblink conditioning that I observed.

Neonatal mechanism

The glucocorticoid receptor-based modulatory system for adult eyeblink conditioning can be adjusted via a neonatal manipulation that produces long-lasting changes in glucocorticoid receptor expression. In Chapters 5 and 6, I tested the hypothesis that separation-induced elevations in neonatal glucocorticoids are responsible for the adult change in glucocorticoid receptor expression and consequently the separation-induced impairment in eyeblink conditioning. In support of this hypothesis, neonatal corticosterone injections impaired adult eyeblink conditioning, while glucocorticoid receptor blockade during neonatal separation attenuated the separation-induced impairment in eyeblink conditioning. However, while neonatal glucocorticoid receptor blockade ameliorated the separation-induced increase in glucocorticoid receptor expression, neonatal corticosterone injections *decreased* glucocorticoid receptor expression. This pattern of results suggests that different levels of neonatal corticosterone have opposite effects on adult glucocorticoid receptor expression. The opposite effects of neonatal separation and neonatal injections on adult glucocorticoid receptor expression are surprising given that neonatal separation and neonatal corticosterone injections produce successively larger increases in neonatal plasma corticosterone concentrations (i.e., linear relationship). Thus, an inverted U-shaped relationship may exist between neonatal corticosterone levels and adult glucocorticoid receptor expression, in which modest increases in

corticosterone (neonatal separation) increase adult glucocorticoid receptor expression but larger increases (neonatal injections) decrease adult glucocorticoid receptor expression.

Alternatively, the effects I observed may be due to alterations in neonatal maternal care and not neonatal plasma corticosterone levels. Long neonatal maternal separation and brief separation *may* have opposite effects on maternal care (Pryce et al., 2001), and *may* have opposite effects on adult glucocorticoid receptor expression (Meaney et al., 1989; Liu et al., 1997; Ladd et al., 2004). Thus, differential alterations in maternal care in separated versus injected pups could contribute to the opposite effect of maternal separation versus control rearing (Chapters 3-6). Further, neonatal injections may also alter maternal care and could contribute to the effects that I observed (Chapter 5), since administration of neonatal injections requires a brief separation from the dam. However, both long (1 h) and brief (15 min) separation increase glucocorticoid receptor expression in the posterior interpositus (Wilber et al., 2007a), so differences in maternal care between separated and injected pups (or control) are not likely to be directly responsible for the effects I observed. Further, neonatal injection of corticosterone and vehicle produced a different magnitude of effect on adult eyeblink conditioning and *decreased* glucocorticoid receptor expression. Therefore, differences in maternal care are not likely to be directly responsible for the effects I observed.

Additionally, mifepristone also binds to progesterone receptors, albeit with markedly less affinity than glucocorticoid receptors (Van Look and Bygdeman, 1989), and progesterone receptors are present in the neonatal cerebellum

(Sakamoto et al., 2003), and in the deep nuclei of adult rats (Guerra-Araiza et al., 2001; Curran-Rauhut and Petersen, 2002); therefore, I cannot rule out the possibility that mifepristone's action on progesterone receptors contributes to the neonatal and/or adult effects of mifepristone injection and infusion that I observed. Future experiments could address this issue by measuring adult progesterone receptor expression following neonatal maternal separation. Nonetheless, given that maternal separation increases glucocorticoid receptor expression in the posterior interpositus, mifepristone has a higher affinity for glucocorticoid receptors (Van Look and Bygdeman, 1989) and neonatal corticosterone exposure produced adult deficits in eyeblink conditioning (Chapter 5), it is likely that mifepristone's ability to reverse the separation effect is due to its blockade of glucocorticoid receptors.

Finally, neonatal mifepristone injections only attenuated and did not reverse the effect of maternal separation on adult eyeblink conditioning. This could be due to incomplete blockade of glucocorticoid receptors by the dose of mifepristone, or inadequate duration of blockade. Thus, it is possible that a more ideal dose of mifepristone or better delivery of the drug could fully prevent neonatal separation effects on adult eyeblink conditioning; however, it is also possible that other stress hormones or receptor systems could be involved. Corticosterone is only one of several hormones that are released in response to stress. For example, norepinephrine (Ward et al., 1976; Aston-Jones et al., 1986; Aston-Jones et al., 1991; Valentino et al., 1998; Sved et al., 2002) and β-endorphins (Chrousos and Gold, 1992) could contribute directly, or by interacting with the HPA system (Lavicky and Dunn, 1993), to the effects I observed. In fact,

other stress response systems are likely to be involved, because both glucocorticoid receptor and β-endorphins antagonists can reverse the effects of neonatal separation on adult object recognition learning and memory (Aisa et al., 2007; Aisa et al., 2008). Alternatively, corticosterone also acts on mineralocorticoid receptors (Reul and de Kloet, 1985; Funder and Sheppard, 1987) and activation of these receptors in addition to glucocorticoid receptors might contribute to the effects I observed.

Implications for adverse early experience in humans

The ability to learn and remember is fundamental to everything that human animals do, including our ability to successfully integrate into society, and problems with learning and memory systems are apparent in psychiatric disorders such as post-traumatic stress disorder (De Bellis et al., 2009; Milad et al., 2009; Xiao et al., 2009) and schizophrenia (Bora et al., 2010; Grillon et al., 2010; Jahshan et al., 2010; Lefebvre et al., 2010; Radek et al., 2010; Seeck-Hirschner et al., 2010) and neurological disorder such as Alzheimer's disease (Machado et al., 2009; Berridge, 2010; Li et al., 2010; Lublin and Gandy, 2010; Ondrejcak et al., 2010; Palop, 2010). This thesis has explored the impact of adverse early experience on adult learning and memory using a simple and well-characterized model system for learning and memory, eyeblink conditioning. The findings of these experiments have begun to describe a glucocorticoid-dependent neonatal mechanism that reorganizes glucocorticoid receptor development to produce changes in adult learning and memory modulation. If the findings from this model system translate into other more complex and less well understood

learning and memory systems (e.g., spatial learning, relational learning, fear conditioning and extinction), then these findings could be used to guide human treatments of adult disorders resulting from early life trauma. Regardless, the findings from this thesis are important for understanding the causes of complex mental disorders such as schizophrenia which are likely caused at least in part by perturbations during development. Indeed, dysfunction of cerebellar circuitry has been implicated in schizophrenia (Sears et al., 2000; Hofer et al., 2001; Marenco et al., 2003; Brown et al., 2005; López-Gallardo et al., 2008), making the findings of this thesis particularly relevant for understanding the disorder and seeking out more effective treatment.

Conclusions

I have explored the role of glucocorticoid receptors in modulation of adult learning and memory, and neonatal stress-induced elevations of glucocorticoids as a mechanism for reorganizing glucocorticoid receptor development to produce changes in this adult glucocorticoid based memory modulating system. The finding of an inverted U-shaped function between neonatal glucocorticoid levels and adult glucocorticoid receptor expression and leaning, where mild increases in the levels of neonatal corticosteroids (maternal separation) produce impaired learning through adult *increases* in glucocorticoid receptor expression, moderate increases (morning injections) *do not alter* glucocorticoid receptors or impair learning, and large increases (evening injections or administration of a large dose of corticosterone) *decrease* glucocorticoid receptor expression and also impair adult learning, has implications for many studies that involve neonatal

manipulations. These findings will further our understanding of the consequences of neonatal stress effects on adult learning and memory, and could further our understanding of the role of neonatal stressors in adult onset disorders such as schizophrenia that appear to have developmental underpinnings.

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Ziabreva I, Schnabel R, Braun K. 2000. Parental deprivation induces N-methyl-D-aspartate-receptor upregulation in limbic brain areas of Octodon degus: protective role of the maternal call. Neural Plasticity 7:233-244.

Curriculum Vitae

1. CITIZENSHIP

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2. ADDRESS AND TELEPHONE NUMBERS

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3. PRESENT POSITION

Doctoral Candidate
Indiana University
Department of Psychological and Brain Sciences
Program in Neural Science

4. EDUCATION AND TRAINING

a. undergraduate

1995 – 1998 Indiana University - Purdue University, Indianapolis, IN

1994 – 1995 William Jewell College, Liberty, MO

b. graduate

06/2006 – present	Doctoral Student Laboratory of Cara Wellman, Ph.D. Department of Psychological and Brain Sciences Program in Neural Science Indiana University, Bloomington, IN
08/2005 - 05/2006	Doctoral Student Laboratory of Joseph E. Steinmetz, Ph.D.
1998 – 2000	Masters of Science in Occupational Therapy Washington University in Saint Louis, School of Medicine, Program in Occupational Therapy, St. Louis, MO

c. training

11/2001 – 08/2005 & 08/1999-06/2000	Research Coordinator: Developmental Neuropsychobiology Laboratory, Director: C. R. Almli, Ph.D. Program in Occupational Therapy Department of Radiology Washington University in Saint Louis, School of Medicine, St. Louis, MO
03/2001-10/2001	Occupational Therapist Evanston Northwestern Health Care, Skokie, IL

03/2001-10/2001 Occupational Therapist
Easter Seals Therapeutic Day School, Tinley Park, IL

09/2000-12/2000 Occupational Therapy Student (Level II Clinical Work)
The Rehabilitation Institute of St. Louis, St. Louis, MO

05/2000-08/2000 Occupational Therapy Student (Level II Clinical Work)
PRIME/CARE Early Intervention, Belleville, IL

08/1997-08/1998 & 08/1999 Research Assistant
James Murphy, Ph.D. and Eric Englemann, Ph.D.

06/1999-07/1999 Research Assistant
David Balota, Ph.D.
Psychology Department
Washington University in Satin Louis, St. Louis, MO

5. LICENSURE AND BOARD CERTIFICATION

Nationally Registered Occupational Therapist, Certification Number: 1048960.
Licensed Occupational Therapist in the state of Illinois (Inactive Status as of 01/2004), License Number: 056-006086.

6. HONORS AND AWARDS

1995 Psi Chi, National Psychology Honor Society (Purdue University-Indianapolis)
1999 Pi Theta Epsilon Occupational Therapy Honor Society (Washington U.)
1999 President, Washington University Student Occupational Therapy Association
2000 Travel Award, Student Delegate to American Student Committee of the Occupational Therapy Association (Washington U.)
2000 Clinical Work Community Assistantship Scholarship (Washington U.)
2000 Collaborative fieldwork program (The Rehabilitation Institute of St. Louis)
2006 NIH Travel Award, annual meeting of the International Society for Developmental Psychobiology
2006 Travel Award, CISAB (Indiana U.)
2007 Honorable Mention, National Science Foundation (NSF) Graduate Research Fellowship
2007 Commendations for exemplary qualifying exam performance (Indiana U.)
2007 Travel Award, CISAB (Indiana U.)
2008 NIH Travel Award, annual meeting of the International Society for Developmental Psychobiology

2008 CISAB Scholars Fellowship CISAB (Indiana U.)
& 2009
2008 Clinical Training Grant Fellowship, Indiana U. (NIMH)
& 2009
2010 Ruth L. Kirschstein National Research Service Award (predoctoral NRSA; NIMH)
2010 J. R. Kantor Graduate Award (Indiana University)

7. PROFESSIONAL SOCIETIES/ORGANIZATIONS

Society for Neurosciences
The International Society for Developmental Psychobiology
Indiana University Center for Integrative Study of Animal Behavior

8. GRANT SUPPORT

a. active grants

1/01/10- (PI; 100%)
1/01/12 *Glucocorticoid mechanisms of neonatal separation effects on adult learning*
NIH/NIMH F31 MH085415

9. BIBLIOGRAPHY

a. peer reviewed manuscripts

1. Engleman, E. A., McBride, W. J., Wilber, A. A., Shaikh, S. R., Eha, R. D., Lumeng, L., Li, T. K., & Murphy, J. M. (2000). Reverse microdialysis of a dopamine uptake inhibitor in the nucleus accumbens of alcohol-preferring rats: effects on dialysate dopamine levels and ethanol intake. *Alcoholism: Clinical & Experimental Research*, **24**, 795-801.

2. Evans, A. C., & The Brain Development Cooperative Group*. (2005). The NIH MRI study of normal brain development. *Neuroimage*, **20**, 184-202.

3. Almli, C. R., Rivkin, M. J., McKinstry, R. C., & The Brain Development Cooperative Group* (2007). The NIH MRI Study of Normal Brain Development: Objective-2—Newborns, infants, toddlers, and preschoolers. *Neuroimage,* **35**, 308-325.

4. Waber, C.P., deMoor, C., Forbes, P.W., Almli, C.R., Botteron, K.N, Leonard, G., Milovan, D., Paus, T., Rumsey, J., & the Brain Development Cooperative Group (2007). The NIH MRI Study of Normal Brain Development: performance of a population based sample of healthy children aged 6 to 18 years on a neuropsychological battery. *Journal of the International Neuropsychological Society, **13***, 1-18.

5. Waber, D., De Moor, C., Forbes, P. W., Almli, C. R., Botteron, K. N., Leonard, G., Milovan, D., Paus, T, Rumsey, J. & The Brain Development Cooperative Group* (2007). The NIH MRI study of normal brain development: performance of a population based sample of healthy children aged 6 to 18 years on a neuropsychological battery. *Journal of the International Neuropsychological Society*, **13**, 729-746.

6. Wilber, A. A., Southwood, C. J., Sokoloff, G., Steinmetz, J. E., & Wellman, C. L. (2007). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. *Developmental Neurobiology*, **67**, 1751-1764.

7. Wilber, A. A., Southwood, C. J., & Wellman, C. L. (2009). Brief neonatal maternal separation alters extinction of conditioned fear and prefrontal NMDA receptor expression in adult rats. *Developmental Neurobiology*, **69**, 73-87.

8. Wilber, A. A., & Wellman, C. L. (2009). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in cerebellar interpositus nucleus interneurons. *Neuroscience Letters*, **460**, 214-218.

9. Wilber, A. A., & Wellman, C. L. (2009). Neonatal maternal separation alters development of glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. *International Journal of Developmental Neuroscience*, **27**, 649-654

10. Wilber, A. A., Lin, G.L., & Wellman, C. L. (**in press**). Glucocorticoid receptor blockade in the posterior interpositus nucleus reverses maternal separation-induced deficits in adult eyeblink conditioning. *Neurobiology of Learning and Memory*.

11. Wilber, A. A., Walker, A.G., Southwood, C. J., Rebec, G. V., & Wellman, C. L. (Submitted for Publication). Chronic stress alters neural activity in infralimbic and prelimbic cortex during recall of extinction*. Neuroscience.*

12. Wilber, A. A., Lin, G.L., & Wellman, C. L. (Submitted for Publication). Neonatal corticosterone administration impairs adult eyeblink conditioning and decreases glucocorticoid receptor expression in the cerebellar interpositus nucleus. *Psychoneuroendocrinology*.

* Aaron Wilber is listed in the author list

13. Wilber, A. A., Lin, G.L., & Wellman, C. L. (Manuscript in preparation). Neonatal mifepristone administration ameliorates the effect of neonatal separation on adult eyeblink conditioning and posterior interpositus glucocorticoid receptor expression.

b. invited publications (books, chapters)

1. Wilber, A. A., ADL Therapies. (2001). In *Angelman Syndrome Foundation Conference Book* (pp. 215-226). Westmont, IL: Angelman Syndrome Foundation.

c. research abstracts

1. Engleman, E. A., Wilber, A. A., Shaikh, S. R., Lumeng, L., Li, T. K., & Murphy, J. M. (1998). Reverse microdialysis of a dopamine uptake inhibitor in the nucleus accumbens of alcohol-preferring rats: effects on dialysate dopamine levels and ethanol intake [Abstract]. *Society for Neuroscience Abstracts*, 28th Annual Meeting.

2. Engleman, E. A., McBride, W. J., Wilber, A. A., Shaikh, S. R., Eha, R. D., Lumeng, L., Li, T. K., & Murphy, J. M. (1999). Ethanol drinking experience potentiates the increase in extracellular dopamine (DA) in alcohol-preferring (P) rats [Abstract]. Abstract in *Alcoholism: Clinical and Experimental Research*, **23**(5), 48A.

3. Wilber, A. A, & Almli, C. R. (2003). Simple visual associative memory in three year old children born premature or fullterm [Abstract]. *Developmental Psychobiology*, **43**, 277.

4. Wilber, A. A., Southwood, C. J., Sokoloff, G., Steinmetz, J. E., & Wellman, C. L. (2006). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. [Abstract]. *Developmental Psychobiology*, **48**, 629.

5. Wilber, A. A., Southwood, C. J., & Wellman, C. L. (2007). Neonatal maternal separation alters adult fear conditioning, extinction, and corticolimbic NMDA receptor expression. [Abstract]. *Developmental Psychobiology*, **49**, 744.

6. Wilber, A.A., & Wellman, C.L. (2008). Neonatal maternal separation alters adult eyeblink conditioning and GR expression in posterior interpositus interneurons [Abstract]. *Developmental Psychobiology*, **50**, 749.

10. PRESENTATIONS

a. Invited Presentations

1. Wilber, A. A. (2000, May). Neonatal white matter brain injury and visual perceptual outcome in preterm infants. Master's Project presented to the Program in Occupational Therapy, St. Louis, MO.

2. Wilber, A. A. (2001, July). ADL Therapies (Activities of Daily Living). Presented for International Annual Angelman's Syndrome Conference, Chicago, IL.

3. Wilber, A. A. (2001, September). Occupational Therapy for the Autistic Child. Presented to the Easter Seals Therapeutic Day School, Tinley Park, IL.

4. Almli, C. R., & Wilber, A. A. (2003, November). Development of relational memory abilities in four to seven year old children that were born fullterm or preterm. Poster presentation at the Society for Neuroscience 33rd Annual Meeting, New Orleans, LA.

5. Wilber, A. A. (2004, January). Differential Ability Scales (DAS) Hints and Clarifications. Presented for the Clinical Coordinating Center Workshop for The MRI study of Normal Brain Development (NIH), St. Louis, MO.

6. Wilber, A. A., (2006, October). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in the cerebellar interpositus nucleus. Presented to the 2006 meeting of the International Society for Developmental Psychobiology, Atlanta, GA.

7. Wilber, A. A., Southwood, C. J., Sokoloff, G., Steinmetz, J. E., & Wellman, C. L. (2007, April). Neonatal maternal separation alters adult eyeblink conditioning and cerebellar glucocorticoid receptor expression. 14th Annual Indiana University Animal Behavior Conference. Bloomington, IN.

8. Wilber, A. A. (2009, December). Glucocorticoid mechanisms of neonatal separation effects on adult learning and memory. Neuroscience Colloquium. Indiana University. Bloomington, IN.

9. Wilber, A. A. (2010, March). Glucocorticoid mechanisms of neonatal separation effects on adult learning and memory. Department of Psychology. Northwestern University. Chicago, IL.

10. Wilber, A. A. (2010, March). Glucocorticoid mechanisms of neonatal separation effects on adult learning and memory. Department of Psychology. Brown University. Providence, RI.

11. Wilber, A. A. (2010, April). Absence may make the heart grow fonder, but it doesn't do wonders for learning and memory. Department of Psychology. University of Illinois. Urbana, IL.

12. Wilber, A. A. (2010, April). Glucocorticoid mechanisms of neonatal separation effects on adult learning and memory. Center for Behavioral Neuroscience. University of Lethbridge. Lethbridge, Alberta, Canada.

13. Wilber, A. A. (2010, June). Absence may make the heart grow fonder, but it doesn't do wonders for learning and memory. Washington University in St. Louis, School of Medicine. St. Louis, MO.

b. Poster Presentations

1. Wilber, A. A., & Almli, C. R. (2003, October). Simple visual associative memory ability and developmental ability in three year old children that were born preterm or fullterm. Poster presented at the Washington University Neuroscience Retreat, Pitosi, MO.

2. Wilber, A. A., & Almli, C. R. (2004 October). Development of relational memory abilities in children born fullterm. Poster presented at the Washington University Neuroscience Retreat, Potosi, MO.

3. Wilber, A. A., Southwood, C. J., Sokoloff, G., Steinmetz, J. E., & Wellman, C. L. (2006, May) Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. Poster presented at the Gill Symposium, Bloomington, IN.

4. Southwood, C. J., Wilber, A. A., Sokoloff, G., Steinmetz, J. E., & Wellman, C. L. (2006, September). Effects of neonatal maternal separation on fear conditioning and forebrain glucocorticoid receptor expression. Poster presented at the Neuroscience Retreat, Martinsville, IN.

5. Wilber, A. A., Southwood, C. J., Sokoloff, G., Wellman, C. L., & Steinmetz, J. E. (2006, October). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. Poster presented at the Society for Neuroscience 36th annual meeting, Atlanta, GA.

6. Wilber, A. A., Southwood, C. J., Sokoloff, G., Steinmetz, J. E. & Wellman, C. L. (2007, November). Neonatal maternal separation alters eyeblink conditioning and glucocorticoid receptor expression in cerebellar interpositus and cortex in male but not female rats. Poster presented at the Society for Neuroscience 37th annual meeting, San Diego, CA.

7. Wilber, A. A., Southwood, C. J., & Wellman, C. L. (2007, November). Neonatal maternal separation alters adult fear conditioning, extinction, and corticolimbic NMDA receptor expression. Poster presented at the Society for Neuroscience 37th annual meeting, San Diego, CA.

8. Wilber, A. A., & Wellman, C. L. (2008, April). Brief neonatal maternal separation alters extinction of conditioned fear and corticolimbic glucocorticoid and NMDA receptor expression in adults. The 15th Annual Indiana University Animal Behavior Conference. Bloomington, IN.

9. Wilber, A. A., & Wellman, C. L. (2008, November). Neonatal maternal separation alters adult eyeblink conditioning and glucocorticoid receptor expression in cerebellar interpositus nucleus interneurons. Poster presented at the Society for Neuroscience 38th annual meeting, Washington, DC.

10. Wilber, A. A., Walker, A. G., Southwood, C. J., & Wellman, C. L. (2008, November). Chronic stress alters neural activity in infralimbic cortex during recall of extinction. Poster presented at the Society for Neuroscience 38th annual meeting, Washington, DC.

11. Wilber, A. A., & Wellman, C. L. (2009, April). Neonatal maternal separation alters development of glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. Poster presented at the 16th Annual Indiana University Animal Behavior Conference. Bloomington, IN.

12. Wilber, A. A., Lin, G. L. & Wellman, C. L. (2009, October). Mifepristone infusion into the interpositus nucleus reverses the effect of neonatal maternal separation on adult eyeblink conditioning. Poster presented at the Society for Neuroscience 39th annual meeting, Chicago, IL.

11. TEACHING EXPERIENCE

a. Courses Taught
- Methods in Experimental Psychology Laboratory

b. Assisted Teaching
- Introductory Psychology
- Biological Psychology
- Abnormal Psychology
- Health Psychology

12. CONTINUING EDUCATION
- Departmental/Program Seminars and Colloquium (e.g., Neuroscience, Psychology, Center for Integrative Study of Animal Behavior, Clinical Psychology)
- State/National/International scientific meetings (e.g., International Society for Developmental Psychobiology, Society for Neuroscience, Indiana University Animal Behavior Conference, Indiana University Neuroscience Retreat).

13. SERVICE
- Animal Behavior Conference Planning Committee (2007-2010), Bloomington, IN.
- Special Olympics, St. Louis, MO, Adaptive Skiing program, basketball, softball and floor hockey for school age children.
- Brain Injury Association of Missouri, St. Louis, MO, Student recruiting and redevelopment of the peer support partner program.
- National Sports Center for the Disabled, Winter Park, CO,
- Saint Vincent Hospice Program, Indianapolis, IN.