



Differential effects of two early life stress paradigms on cerebellar-dependent delay eyeblink conditioning

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ABSTRACT

Early life stress paradigms have become prominent in the animal literature to model atypical development. Currently, two models have prevailed within the literature: (1) limited bedding or nesting and (2) maternal separation or deprivation. Both models have produced aberrations spanning behavior and neural circuitry. Surprisingly, these two models have yet to be directly compared. The current study utilized delay eyeblink conditioning, an associative learning task with a well-defined cerebellar circuit, to compare the behavioral effects of standard limited bedding (postnatal day 2–9, $n = 15$) and maternal separation (60 min per day during postnatal day 2–14, $n = 13$) early life stress paradigms. Animals in all groups exhibited robust learning curves. Surprisingly, facilitated conditioning was observed in the maternal separation group. Rats that underwent limited bedding did not differ from the control or maternal separation groups on any conditioning measures. This study contributes to a clearer understanding of early life stress paradigms and the claims made about their mechanisms, which if better clarified can be properly leveraged to increase translational value.

1. Introduction

Atypical development of neural and psychological systems is a topic of great interest. One particular risk factor known to skew developmental trajectories is early life stress. Accordingly, paradigms that model early life stress in animals to produce the associated developmental aberrations have become widely utilized in the psychological and neuroscience literatures. In particular, two models have been adopted. One such model is limited bedding, variations of which are also referred to as limited nesting or scarcity. Another prominent model is maternal separation or maternal deprivation. Though both models have become widely used and characterized independently, we are not aware of any studies directly comparing these two paradigms.

1.1. Aberrant maternal care as an early life stressor

Different levels of maternal care result in diverse outcomes for children that can persist into adulthood, including varied psychological processing spanning learning and memory, self-regulation, emotion

perception and regulation, executive functioning, sleep, academic attainment, and clinically significant psychopathology (Davis et al., 2019; Lewin et al., 2019; Short and Baram, 2019; Lapp and Hunter, 2019; Palacios-Barrios and Hanson, 2018). Animal models are invaluable for understanding the neural mechanisms by which these aberrations may occur; they allow us to interrogate specific, anatomical and cellular circuits at the macro and micro scales as well as perform highly controlled experiments that better link such circuits to functional outcomes like behaviors (Joels et al., 2018). Moreover, better characterizing our current models may enhance translatability by clarifying which functions each paradigm best models (Glynn and Baram, 2019; Murthy and Gould, 2018).

To date, limited bedding and maternal separation models have been heavily investigated, with some shared outcomes (Fareri and Tottenham, 2016). Limited bedding, as first developed (Ivy et al., 2008), involves restricting the amount of nesting material and access to bedding provided to the dam to induce fragmented maternal care behaviors (Molet et al., 2014). Generally, the limited bedding model has been associated with aberrations of hypothalamic-pituitary-adrenal axis

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activity and regulation in the pups, suggesting it does produce stress (Molet et al., 2014). Moreover, impaired attachment learning and social behavior, heightened fear response and conditioning, increased depressive-like behaviors in forced swim and sucrose preference assays, and impairments in a variety of cognitive processes including learning and memory have been reported (for extensive review, cf. (Walker et al., 2017)). Similarly to limited bedding, maternal separation has been used to alter maternal care and behavior consistent with neglect and abuse (Alves et al., 2019). Most consistently, studies have found impaired memory and increased depressive-like behaviors in mice (Tractenberg et al., 2016) and impaired spatial and recognition learning and memory and increased depressive-like and fear behaviors in rats (Aisa et al., 2007; Lehmann and Feldon, 2000).

Though impairments are traditionally noted, many studies find no differences or even note improvements in behaviors following early life stress. In fact, limited bedding and maternal separation have more recently garnered interest as models of resilience (Murthy and Gould, 2018; Singh-Taylor et al., 2015; Brenhouse and Bath, 2019). The variable effects of these models can be diffuse and difficult to understand. Moreover, the inability to compare two stress paradigms across labs due to a host of confounding factors (e.g., time and duration of the stressor, time (age and time of day) of assessment, light-dark cycle, laboratory-specific environmental features, rodent strain, individual differences in maternal care, etc.) further blurs our understanding of what these paradigms are modeling (Murthy and Gould, 2018). Accordingly, it is critical and timely to compare the specific effects of these models. An especially useful task for doing so is delay eyeblink conditioning.

1.2. Delay eyeblink conditioning

Delay eyeblink conditioning (dEBC) is a classical conditioning paradigm in which a neutral stimulus, typically a tone or light, is followed by and co-terminates with eye stimulation that elicits a reflexive eye blink. Pairing of these two stimuli will result in the tone eliciting an anticipatory (learned) eyeblink response. The individual, or animal, has learned an association between the tone and the eye stimulation.

Methodologically, dEBC has many built in control measures that make it an excellent way to assess long-term deficits in learning and memory induced by early life stress (Steinmetz et al., 2001). First, the unlearned, reflexive responses to both the tone and the eye stimulation are easily measured and quantified, and serve as sensorimotor control measures. Second, because the learned eye blink takes a number of trials to emerge, subtle differences in learning rate can be identified. Third, much is known about the associative learning mechanisms of dEBC. Finally, the neural circuitry that supports dEBC is well-characterized (Freeman, 2015).

Unsurprisingly, dEBC deficits have been identified across the stress literature broadly (Shors, 2001; Shors et al., 2000). A series of studies by Wilbur and colleagues (Wilbur and Wellman, 2009a; Wilbur et al., 2007, 2010) have investigated how an early life stressor, maternal separation across postnatal day (PND) 2–14, influenced dEBC performance of adult rats. Compared to a standard rearing control group and a 15-min handling control group that did not differ in performance, maternal separation of 60 or 15 min significantly decreased conditioning in adult (PND70–114) males only (Wilbur et al., 2007). Moreover, these effects were specific to the late acquisition period, resulting in 37% (60-min separation) and 30% (15-min separation) fewer eyeblink conditioned responses compared to the control group (Wilbur et al., 2007). These conditioning rates were comparable to findings from this group in later studies using the same, optimal 60-min maternal separation (Wilbur et al., 2010).

Understanding how early stressors impact brain-behavior relationships is critical for optimizing our understanding and use of these paradigms and for streamlining the translatability of these models to human conditions. Thus, the current study leveraged the well-defined and

controlled dEBC paradigm to compare the behavioral effects of two, prominent early life stress models: standard limited bedding and maternal separation.

2. Methods

All experimental protocols were approved by the University of Vermont IACUC Board and complied with ethical standards for the care and treatment of animals.

2.1. Animals

Long-Evans rats (Envigo, Indianapolis, IN) arrived timed-pregnant (between E10 and E15) and were individually housed in polypropylene cages (26.67 × 48.26 × 20.32 cm) in a 12:12 h light-dark cycle (6:00 lights on, 18:00 lights off) and temperature-controlled (approximately 22.8 °C) vivarium. Food and water were provided ad libitum. Bedding was changed once per week. Rats were checked every 12 h surrounding the expected date of birth. Birth was labeled P0.

2.2. Early life stressors

2.2.1. Limited bedding

At P2, rats were randomly cross-fostered, cages were sex-balanced, and rats were randomly assigned to experience limited bedding or typical rearing. Limited bedding was performed as outlined previously by this group (cf. Moussa-Tooks et al., 2020). Limited bedding cages consisted of a wire mesh insert (Plastic-coated aluminum mesh, 0.4 × 0.9 cm, McNichols Co., Tampa, FL) that was fitted 2.5 cm above the cage floor (cf. Molet et al., 2014). The mesh allowed the passage of excrement to the bedding material below the mesh. Additionally, limited bedding cages were given half of a paper towel square (13.97 × 27.94 cm) for the dam to use as nesting material. Normal rearing cages were given a full paper towel square and standard access to bedding material. All cages were left undisturbed from P2–9. On P10, all cages were changed and limited bedding inserts were removed. All animals were reared normally from the end of the stressor forward. At P21 pups were weaned into treatment- and sex-matched cages of 3–4 animals.

2.2.2. Maternal separation

The maternal separation paradigm was performed in accordance with Wilber and colleagues (cf. 60 min separation condition in Wilber et al., 2007). From P2–P14, dams and pups were separated for 60 min each day (initiated between 7:30 and 9:00 am each day). During this time, dams were removed from the home cage and placed in a novel cage. Additionally, pups were removed from the home cage and placed in a small cage with clean bedding that sat within an incubator (Thermocare W-1 Ten/Care Warmer) set to 22.2–22.8 °C (approximately 45–55% humidity) to maintain body temperature. After the 60 min, pups and dams were returned to their home cage. On P15, all cages were changed. All animals were reared normally from the end of the stressor forward. At P28 (cf. Wilber et al., 2007) pups were weaned into treatment- and sex-matched cages of 3–4 animals.

2.3. Eyeblink conditioning surgery

Only male rats were used, since previous studies suggested limited or no effect of maternal separation on EBC in females (cf. Wilber et al., 2007). At PND41–48 rats underwent eyeblink conditioning surgery to implant stimulation and recording electrodes. Rats were anesthetized with isoflurane (3% for induction and then 1.5–2% for surgery). Anesthetic depth was assessed by monitoring lack of withdrawal from tail and/or toe pinches to ensure the animal was fully anesthetized. Upon confirmation of anesthetization, the animal's head was shaven and the animal was placed in a stereotaxic head-stage secured with bite and ear bars and kept warm by using a commercial rat heating pad with

temperature controller. Eye ointment was applied to prevent the animal's eyes from drying out. An injection of bupivacaine (0.1 mL) at the incision site was administered as a local analgesic and the surgical site was covered with chlorhexidine gluconate. A single intraperitoneal injection of a nonsteroidal anti-inflammatory drug (NSAID; 5.0 mg/kg carprofen) was administered in addition to Ringer solution (1 mL) for hydration.

The surgical site was cleaned with an aseptic scrub and solution. After cutting open the scalp over the top of the skull, 4 holes were drilled through the skull with a micro-drill and 4 skull screws implanted. The ground electrode of a plastic connector (Plastics One, Roanoke, VA) was attached to the skull screws. The two eyelid electrodes also attached to this connector were threaded through the upper eyelid. A bipolar electrode affixed to a separate plastic connector (Plastics One, Roanoke, VA) was placed subcutaneously dorsocaudal to the left eye. The electrodes and skull screws were fixed in place with dental cement.

After surgical procedures, rats were kept under observation on an electric heating pad until recovered from the anesthesia, then were placed back in the cage and returned to the rat colony. Twenty-four hours following surgery, rats were given another injection of carprofen (5.0 mg/kg). Post-operative monitoring and assessment (alert and freely moves around cage; smooth coat and not hunched; clean and not swollen; no more than 10% weight loss compared to pre-surgical weight; breathing normally) was conducted daily until five consecutive days of an assessment score of 0. No rats required euthanasia due to poor recovery. Following recovery, rats underwent delay eyeblink conditioning.

2.4. Apparatus

Eyeblink conditioning took place in one of four identical testing chambers (30.5 x 24.1 x 29.2 cm; Med-Associates, St. Albans, VT), each with a grid floor. The top of the chamber was altered so that a 25-channel tether/commutator could be mounted to it. Each testing chamber was kept within a separate electrically-shielded, sound attenuating chamber (45.7 x 91.4 x 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB sound pressure level. A speaker was mounted in each corner of the rear wall and a light (off during testing) was mounted in the center of the rear wall of each chamber. The sound-attenuating chambers were housed within a walk-in sound-proof chamber.

Stimulus delivery was controlled by a computer running Spike2 software (CED, Cambridge, UK). A 2.8 kHz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the conditioned stimulus (CS). The CS was 295-ms in duration. A 15-ms, 4.0 mA uniphasic periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments, Sarasota, FL), served as the unconditioned stimulus (US) during conditioning. Recording of the eyelid EMG activity was controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED, Cambridge, UK). Eyelid EMG signals were amplified (10k) and bandpass filtered (100–1000 Hz) prior to being passed to the Power 1401 and from there to the computer running Spike2. Sampling rate was 2 kHz for EMG activity. The Spike2 software was used to full-wave rectify, smooth (10 ms time constant), and time shift (10 ms, to compensate for smoothing) the amplified EMG signal to facilitate behavioral data analysis.

2.5. Eyeblink conditioning paradigm

Delay eyeblink conditioning (dEBC) began at PND49–55, a minimum of 5 days after surgery. Each session lasted approximately 50 min. On day 1, rats were plugged into tether/commutators and spontaneous eyelid activity was recorded, but no stimuli were delivered. On days 2–7, rats were plugged into the tether/commutator system and underwent dEBC: On each trial, a 365-ms, 80-dB, 2.8 kHz tone was delivered.

During the final 15-ms of the tone, a 4-mA eyelid stimulation was also delivered (350-ms dEBC). Rats received 100 paired tone CS-eyelid stimulation US trials per day, with trials separated by 20–40 s. Only paired trials were used in order to ensure robust conditioning in the control group. Beginning 9 days after the final session of acquisition, rats underwent two sessions of extinction, one per day. These sessions were identical to acquisition sessions except that the eyelid stimulation US was omitted. The day after the final session of extinction, rats underwent one session of reacquisition that was identical to acquisition.

2.6. Behavior analysis

Trials were subdivided into four time periods: (1) a “baseline” period, 280-ms prior to CS onset; (2) a non-associative “startle” period, 0–80 ms after CS onset; (3) a “CR” period, 81–350 ms after CS onset; and (4) a “UR period,” 65–165 ms after US onset (the first 65 ms is obscured by the stimulation artifact). In order for a response to be scored as a CR, eyeblinks had to exceed the mean baseline activity for that trial by 0.5 arbitrary units (where these units had a range of 0.0–5.0) during the CR period. Eyeblinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. Trials in which eyeblinks exceeded 1.0 arbitrary unit during the baseline period were discarded. Comparable scoring intervals and criteria were used to evaluate spontaneous blink rate during the initial adaptation day when no stimuli were administered. The primary dependent measure was the percentage of CRs across all CS-US (acquisition; reacquisition) or CS-alone (extinction) trials of each session.

For the percentage of CRs, data were analyzed using repeated measures ANOVAs. We computed all statistical analyses using SPSS 26.0. Significant interaction effects were followed-up with post-hoc, independent-samples t-tests to determine the source of the interaction. An alpha level of 0.05 was set as the rejection criterion for all statistical tests. To minimize the chance of Type II errors, we did not adjust the alpha level when analyzing the source of a significant interaction effect.

3. Results

3.1. Limited bedding

A total of 5 rats were removed prior to data analysis: 3 rats had a noisy eyelid EMG, 1 rat had an attenuated response to the eyelid stimulation, and 1 rat lost its headcap. Final group totals were 15 rats in Group Limited Bedding and 14 rats in Group No Limited Bedding. There was no significant difference between groups in pre-surgery weights, $F < 1$. The mean weight (in grams) of rats in Group Limited Bedding was 205.33 ± 5.23 and the mean weight (in grams) of rats in Group No Limited Bedding was 212.00 ± 5.21 .

3.1.1. Acquisition

Limited bedding had no effect on acquisition (Fig. 1). A 2 (Group: Limited Bedding, No Limited Bedding) x 6 (Acquisition Session) repeated-measures ANOVA on percentage of CRs revealed a significant Session effect, $F(5,135) = 100.43$, $p < 0.01$. Neither the Group main effect nor Group x Session interaction effect was significant, p 's > 0.69 .

Limited bedding had no effect on reflexive responding to either the CS (percentage of startle responses) or to the US (UR amplitude) during acquisition. A 2 (Group: Limited Bedding, No Limited Bedding) x 6 (Acquisition Session) repeated-measures ANOVA on percentage of startle responses (Group Limited Bedding: Percentage of startle response range of 2.3%–5.6% \pm 0.9–1.6; Group No Limited Bedding: Percentage of startle response range of 2.4%–8.3% \pm 0.9–2.5) revealed a significant Session effect, $F(5,135) = 5.55$, $p < 0.01$. Neither the Group main effect nor Group x Session interaction effect was significant, p 's > 0.38 . The same analysis on UR amplitude (Group Limited Bedding: UR amplitude range of 1.6–1.8 units \pm 0.2; Group No Limited Bedding: UR amplitude range of 1.7–2.3 units \pm 0.2–0.3) revealed a Session effect that

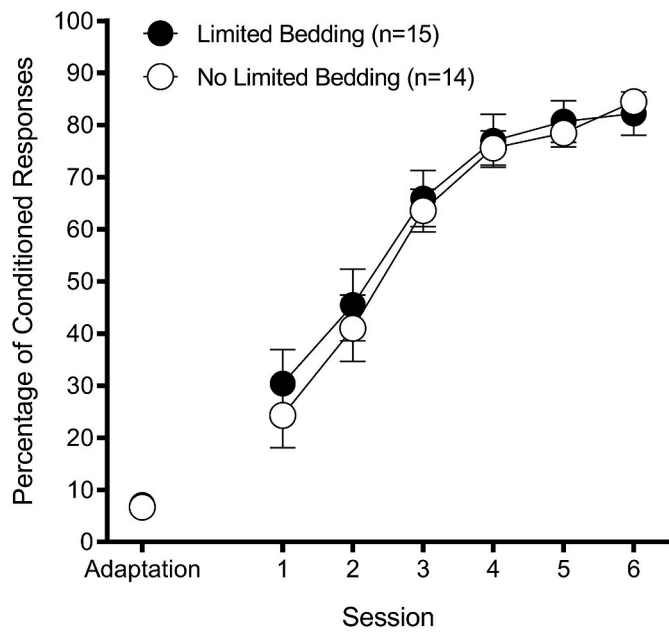


Fig. 1. Limited bedding acquisition. Percentage of eyeblink conditioned responses as a function of conditioning session (mean ± SEM). There was no difference between Group Limited Bedding and Group No Limited Bedding, $p > 0.05$.

approached significance, $F(5,135) = 2.24, p = 0.054$. Neither the Group main effect nor Group × Session interaction effect was significant, p 's > 0.22 .

3.1.2. Extinction

Limited bedding had no effect on retention or extinction (Fig. 2). Each extinction session was analyzed separately. Analysis was divided into 10 blocks of 10 trials each. A 2 (Group: Limited Bedding, No Limited Bedding) × 10 (Extinction Session Block) repeated-measures ANOVA on percentage of CRs in extinction session 1 revealed a significant Block effect, $F(9,243) = 3.62, p < 0.01$. Neither the Group main effect nor Group × Session interaction effect was significant, p 's > 0.45 . The same

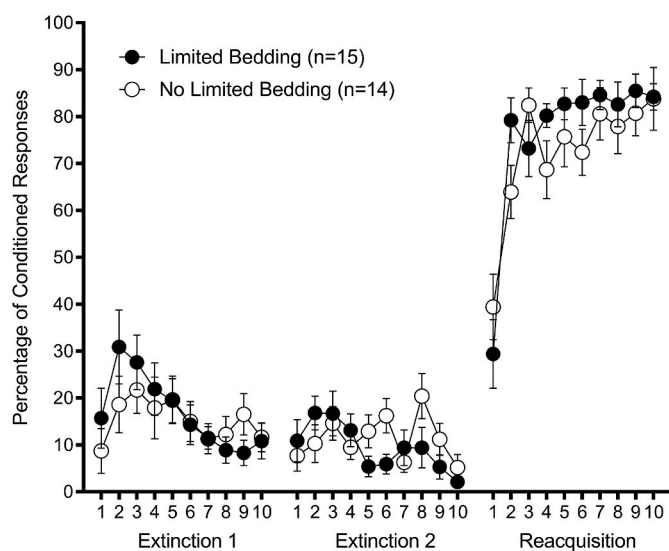


Fig. 2. Limited bedding extinction and re-acquisition. Percentage of eyeblink conditioning responses as a function of 10-trial block in extinction sessions 1 and 2, and re-acquisition (mean ± SEM). Extinction session 1 began 9 days after the last session of conditioning. There was no difference between Group Limited Bedding and Group No Limited Bedding in any of these sessions, $p > 0.05$.

analysis on percentage of CRs in extinction session 2 also revealed a significant Block effect, $F(9,243) = 2.55, p < 0.01$. The Group × Block effect approached but did not attain statistical significance, $F(9,243) = 1.80, p = 0.068$. There was a trend for Group Limited Bedding to show better extinction. The Group effect was not significant, $p = 0.41$.

3.1.3. Reacquisition

Limited bedding very slightly facilitated reacquisition (Fig. 2). Analysis was divided into 10 blocks of 10 trials each. A 2 (Group: Limited Bedding, No Limited Bedding) × 10 (Reacquisition Session Block) repeated-measures ANOVA on percentage of CRs revealed a significant Block effect, $F(9,243) = 24.07, p < 0.001$ and significant Block × Group effect, $F(9,243) = 1.94, p < 0.05$. The Group effect was not significant, $p > 0.40$.

The significant interaction effect was analyzed with a series of 10 independent samples t-tests comparing groups within each block. This analysis revealed a significantly greater percentage of CRs in Group Limited Bedding in block 2 ($p < 0.05$) of reacquisition.

3.2. Maternal separation

A total of 1 rat was removed prior to data analysis of acquisition for a noisy EMG. A total of 5 additional rats were removed prior to data analysis of extinction and reacquisition: 4 rats had developed a noisy eyelid EMG, and 1 rat lost its headcap immediately after the first session of extinction. Final group totals for acquisition were 15 rats in Group Maternal Separation and 16 rats in Group No Maternal Separation. Final group totals for extinction and reacquisition were 13 rats in Group Maternal Separation and 13 rats in Group No Maternal Separation. There was no significant difference between groups in pre-surgery weights, $F < 1$. The mean weight (in grams) of rats in Group Maternal Separation was 212.93 ± 6.83 and the mean weight (in grams) of rats in Group No Maternal Separation was 218.00 ± 4.08 .

3.2.1. Acquisition

Maternal separation enhanced acquisition (Fig. 3). A 2 (Group: Maternal Separation, No Maternal Separation) × 6 (Acquisition Session) repeated-measures ANOVA on percentage of CRs revealed a significant Group effect, $F(1,29) = 9.84, p < 0.01$, and a significant Session effect, F

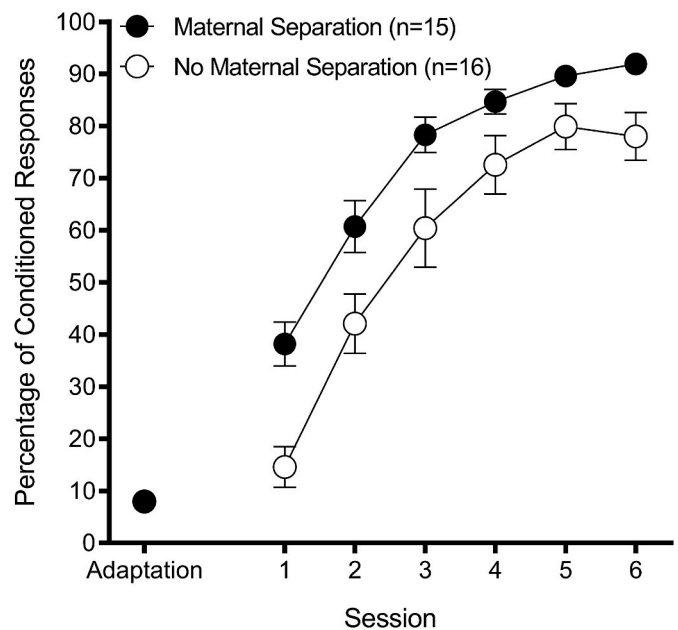


Fig. 3. Maternal separation acquisition. Percentage of eyeblink conditioned responses as a function of conditioning session (mean ± SEM). Group Maternal Separation outperformed Group No Maternal Separation, $p < 0.01$.

(5,145) = 117.65, $p < 0.01$. The Group \times Session interaction effect was not significant, $p = 0.22$.

Maternal separation had no effect on reflexive responding to either the CS (percentage of startle responses) or to the US (UR amplitude) during acquisition. A 2 (Group: Maternal Separation, No Maternal Separation) \times 6 (Acquisition Session) repeated-measures ANOVA on percentage of startle responses (Group Maternal Separation: Percentage of startle response range of 2.8%–5.4% \pm 0.7–1.0; Group No Maternal Separation: Percentage of startle response range of 3.1%–5.2% \pm 0.8–1.1) revealed a significant Session effect, $F(5,145) = 3.26$, $p < 0.01$. Neither the Group main effect nor Group \times Session interaction effect was significant, p 's > 0.62 . The same analysis on UR amplitude (Group Maternal Separation: UR amplitude range of 1.7–2.0 units \pm 0.2; Group No Maternal Separation: UR amplitude range of 1.8–2.0 units \pm 0.2–0.3) revealed a significant Session effect, $F(5,145) = 2.70$, $p < 0.03$. Neither the Group main effect nor Group \times Session interaction effect was significant, p 's > 0.65 .

3.2.2. Extinction

Maternal separation enhanced retention but did not affect extinction (Fig. 4). Each extinction session was analyzed separately. Analysis was divided into 10 blocks of 10 trials each. A 2 (Group: Maternal Separation, No Maternal Separation) \times 10 (Extinction Session Block) repeated-measures ANOVA on percentage of CRs in extinction session 1 revealed a significant Block effect, $F(9,216) = 4.64$, $p < 0.01$ that was qualified by a significant Block \times Group effect, $F(9,216) = 3.11$, $p < 0.01$. The Group main effect was not significant, $p > 0.38$. The significant interaction effect was analyzed with a series of 10 independent samples t-tests comparing groups within each block. This analysis revealed a significantly greater percentage of CRs in Group Maternal Separation in blocks 1 and 2 (p 's < 0.05) of extinction session 1, suggesting greater retention of conditioning across the 9 day interval between the last session of EBC and the first session of extinction.

The same analysis on percentage of CRs in extinction session 2 also revealed a significant Block effect, $F(9,216) = 2.69$, $p < 0.01$. Neither the Group main effect nor Group \times Session interaction effect was significant, p 's > 0.38 .

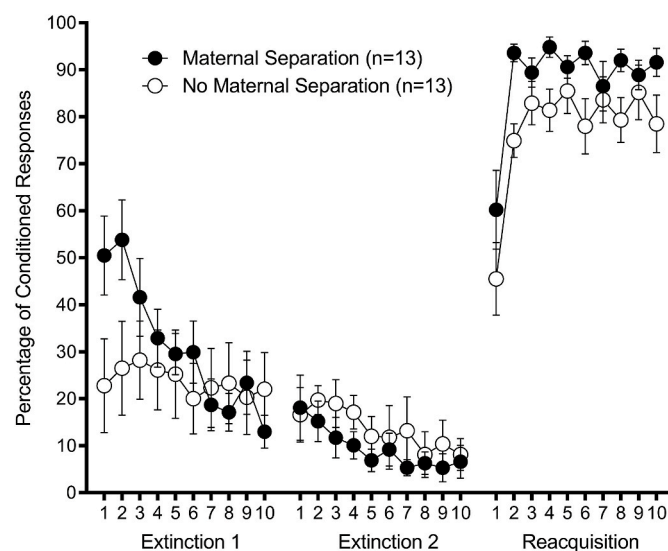


Fig. 4. Maternal separation extinction and re-acquisition. Percentage of eyeblink conditioning responses as a function of 10-trial block in extinction sessions 1 and 2, and reacquisition (mean \pm SEM). Extinction session 1 began 9 days after the last session of conditioning. Group Maternal Separation showed greater retention (greater percentage of conditioned responses in blocks 1 and 2 of extinction session 1) and greater reacquisition than Group No Maternal Separation, p 's < 0.05 .

3.2.3. Reacquisition

Maternal separation enhanced reacquisition (Fig. 4). Analysis was divided into 10 blocks of 10 trials each. A 2 (Group: Maternal Separation, No Maternal Separation) \times 10 (Reacquisition Session Block) repeated-measures ANOVA on percentage of CRs revealed a significant Block effect, $F(9,216) = 14.20$, $p < 0.01$ and a significant Group effect, $F(1,24) = 7.19$, $p < 0.02$. The Group \times Block interaction effect was not significant, $p > 0.45$.

3.3. Comparison of control groups

The performance of the two control groups was also compared, since one control group (Group No Limited Bedding) was cross-fostered and the other (Group No Maternal Separation) was not. These slightly different procedures were used in order to match the typical limited bedding and maternal deprivation procedures in the literature. There was no significant difference between groups in pre-surgery weights, $F < 1$.

These comparisons revealed no differences between control groups in acquisition (percentage of CRs, percentage of startle responses, UR amplitude), extinction session 1, or reacquisition, p 's > 0.10 for Group main effects and Session/Block \times Group interaction effects. The analysis of extinction session 2 revealed a significant Block \times Group effect, $F(9,225) = 1.94$, $p < 0.05$.

However, follow-up independent samples t-tests comparing groups within each block failed to reveal a statistically significant difference between control groups in any block of extinction session 2, p 's > 0.08 . Notably, in 3 blocks, Group No Limited Bedding showed a higher percentage of CRs while in the other 7 blocks it was Group No Maternal Deprivation.

4. Discussion

In the current study, the effects of two prominent rodent models of early life stress were compared using dEBC to better understand how such stressors at a critical developmental stage may confer behavioral risk. Interestingly, young adult male rats that experienced limited bedding (administered PND2-9) did not exhibit any differences from a normal rearing (cross-fostered) group in acquisition, retention, or reacquisition of the conditioned response. In contrast, male rats that underwent maternal separation (administered 60 min daily, PND2-14) showed facilitated conditioning, retention, and reacquisition compared to a normal rearing (not cross-fostered) control group.

dEBC has never, to our knowledge, been investigated following a limited bedding stress manipulation. However, maternal separation at PND2-14 has been previously shown to impair dEBC performance in male rats (Wilber et al., 2007, 2010; Wilber and Wellman, 2009b). The impairment in acquisition using a 60-min separation paradigm was further associated with upregulation of glucocorticoid receptors in the interpositus nucleus of the cerebellum, a key node in the dEBC circuit (Wilber and Wellman, 2009a, 2009b; Wilber et al., 2007). Some key differences between Wilber and colleagues' studies and the current study may help to explain the facilitation, rather than impairment, found here. First, the current study used a shorter CS onset-to-US onset interval of 350 ms, compared to the longer 580 ms interval used by Wilber and colleagues in two of their three studies (Wilber and Wellman, 2009a; Wilber et al., 2007). However, Wilber and colleagues used the same interval employed here in their third dEBC study (Wilber et al., 2010). Second, Wilber and colleagues used a mixture of 80% CS-US and 20% CS-alone trials in dEBC while the current study used 100% CS-US trials. Third, the maternal separation dEBC impairment reported by Wilber and colleagues typically emerged only after 5–6 sessions of dEBC; the maternal separation-associated facilitation reported here was present from the first session of dEBC, persisted through 6 sessions of dEBC into the beginning of extinction nine days later, and was still present during reacquisition. Fourth, the current study evaluated rats on dEBC at

young adulthood (PND49-55 at the start of dEBC), while Wilber and colleagues' rats were older (at least PND70-176 at the start of dEBC). Taken together, the inconsistency between the current findings and those from Wilber and colleagues indicates a limitation in the generalizability of previously reported impairments and warrants further investigation.

Age at assessment does appear to influence the neural alterations observed following stress. In a follow-up study by Wilber and colleagues, at PND15, immediately after the 60-min separation stressor that occurred from PND2-14, male rats exhibited fewer total glucocorticoid receptors and a lower percentage of glucocorticoid-rich neurons in the posterior interpositus nucleus of the cerebellum compared to non-stressed controls of the same age (Wilber and Wellman, 2009a). Although no significant difference was present at PND 21, glucocorticoid receptor expression was higher in maternal-stressed males in adulthood (PND79-116) compared to same age controls (Wilber and Wellman, 2009a; Wilber et al., 2007). Moreover, adult acquisition learning deficits following separation stress were likely mediated by these glucocorticoid changes, as injections of mifepristone impaired the performance of non-stressed rodents while normalizing stress-induced impairments in performance (Wilber et al., 2010).

Assessment in the current study (PND49-72) occurred between the developmental timepoints evaluated by Wilber and colleagues (Wilber and Wellman, 2009a), although this is likely too small a window during adulthood for large neural differences to emerge. An alternative explanation may be the role of maternal behavior beyond the duration of the stressor. Similar changes in glucocorticoid receptors have been reported following limited bedding, including fewer total glucocorticoid receptors in male rats immediately following the stressor (assessed at PND9, stressor occurring PND2-9) (Avishai-Eliner et al., 2001). In contrast to the adulthood increase observed for separation-stressed rats, this decrease in glucocorticoid receptors persists into adulthood (12 weeks) for limited bedding-stressed rats (Maniam et al., 2016). A significant discrepancy between the two early life stress paradigms that may have resulted in the different dEBC outcomes within the current study is maternal behavior.

Limited bedding as a stressor is chronic and consistent (i.e., the resources are consistently limited during the manipulation), which may help the dam adapt to the stressor, developing coping strategies (Walker et al., 2017). In contrast, the lack of consistency in maternal separation (i.e., the separation occurs daily, but only for a short period of time) may result in more variable changes in maternal behavior, including neglect and aggression that make this manipulation more severe (Murthy and Gould, 2018). Theoretically, these changes to dam behavior could extend until the pups are weaned, causing additional effects as the pups continue to mature. Likewise, various extraneous factors (e.g., staff, lighting, cage disruptions, etc.) may have altered maternal behavior between the Wilber studies and the current study, resulting in the differences in maternal separation conditions alone.

In addition to these indirect effects on the pup, some key differences in direct effects have previously been reported. For example, the wire mesh insert used in limited bedding paradigms can result in a loss of heat to the pups, perhaps contributing to weight loss and, accordingly, a host of other physiological and behavioral outcomes (Walker et al., 2017). Maternal separation rat pups do not have this metal platform and, in fact, are incubated during separation to maintain thermoregulation. It should be noted that in the current study there were no pre-surgery weight differences between treatment and control groups and, thus, findings are not likely better explained by developmental complications due to poor nutrition or insufficient weight.

Facilitated dEBC following stress is not novel to the animal literature. A series of studies by Shors and colleagues investigated the effects of long-term and acute adult stress on dEBC. In a 4-day restraint and inescapable shock stress paradigm, male rats exhibited facilitated conditioning (increased rate of acquisition and total percentage of CRs) (Shors et al., 1992). Interestingly, the delay paradigm used a CS

onset-to-US onset interval of 250 ms, which is closer to the interval employed in the current study. These effects were not specific to long-term or restraint/shock stress. Male rats exhibited facilitation of CR acquisition following tail shock alone or forced swim stress (Shors, 2001) and such effects were observed immediately (30 min) following the administration of the stressor as well as 24-h later (Shors, 2001). A single administration of restraint and inescapable shock stress elicited facilitated conditioning in male rats compared to unstressed male rats (Wood and Shors, 1998). Female rats that underwent this paradigm exhibited impaired conditioning relative to unstressed female rats, a finding which was prevented by ovariectomy or the pharmacological blockade of estrogen receptors (Wood and Shors, 1998). Perhaps unsurprising, increasing cerebellar estrogen alone has been shown to facilitate conditioning, likely by increasing the arborization of the primary inhibitory neurons in the cerebellum and dEBC circuit, Purkinje cells (Hoffman et al., 2016; Leuner et al., 2004).

4.1. Limitations and future directions

While the current study serves as a positive step in the differentiation of early life stress models, particularly regarding foundational processes, limitations must be considered. First, since this is the only independent replication of Wilber and colleagues' work, it remains unclear if the differences in findings are due to the variability in task parameters (e.g., ISL, age at evaluation, etc.), or other study factors. Going forward, it will be critical to match the two different paradigms, limited bedding and maternal separation, as closely as possible. One first step may be to extend the length of limited bedding to PND2-14 to match the duration of maternal separation. Alternatively, a scarcity model, in which bedding resources are limited but access to the bedding is not impeded may eliminate the confound of thermoregulation differences due to the metal insert.

Second, the impact on the cerebellum of these early life stress models remains to be fully characterized, including the measurement and quantification of key cells, proteins, and neurotransmitters (e.g., estrogen, glucocorticoid receptors). Maternal separation stress (4 h daily, PND1-21) has been shown to reduce c-Fos expression in cerebellar deep nuclei and decrease oxidative activity in the cerebellar medial zone in adult males and females (Gutierrez-Menendez et al., 2019). One potential explanation for these effects is stress-related changes in the modulatory role of the cerebellar endogenous cannabinoid (endocannabinoid) system. A study from our group identified long-term changes (at PND70) in the cerebellar endocannabinoid system in adult rats that had experienced limited bedding (PND2-9) (Moussa-Tooks et al., 2020). These findings showed that, in males, limited bedding decreased cerebellar 2-AG in tissue from the interpositus nucleus, but not tissue from cerebellar cortex. Relatedly, cannabinoid receptor type 1 (CB1R) knockout mice exhibit poor dEBC acquisition and retention of the conditioned response (Kishimoto and Kano, 2006). In a series of studies, Steinmetz and Freeman have shown that CB1R agonists injected peripherally or infused into cerebellar cortex prior to dEBC impair conditioning (Steinmetz and Freeman, 2010, 2011, 2013, 2016, 2020). CB1R agonists facilitate dEBC if administered 1 h after dEBC sessions (Steinmetz and Freeman, 2016). In the human literature, Skosnik and colleagues (Skosnik et al., 2008) have shown a similar modulatory role in humans by showing that dEBC acquisition is impaired in cannabis users (exogenous cannabinoids). Given these system changes and the broad deficits following early life stressors, it will be useful to the future of the field to integrate behavioral and neurobiochemical assays following early life stress, with a focus on changes to large regulatory networks including the endogenous cannabinoid system and cerebellum.

Third, sex differences were not evaluated in the current study, primarily because past studies of dEBC and maternal separation did not identify effects in females (Wilber et al., 2007). Substantial work on the sex-specific cerebellar effects following stress (Moussa-Tooks et al.,

2020; Wood and Shors, 1998), as well as the role of sex hormones in cerebellar development (Hoffman et al., 2016; Leuner et al., 2004) suggest that this is an important line of inquiry. Finally, although the task used in this study was highly controlled and well-defined, the questions in this study could be extended to additional cerebellar-related tasks (e.g., spatial navigation, working memory, cognitive flexibility, decision making, and social function) to assess the role of stress across cerebellar and cerebellar-cortical circuits (Shipman and Green, 2020; Stoodley et al., 2017; Deverett et al., 2018).

4.2. Conclusion

Taken together, it is critical that early life stress paradigms themselves and the claims made about their mechanisms be better specified to properly leverage these paradigms and increase their translational value. The current study provides a helpful first step in directly comparing two prominent models, limited bedding and maternal separation. Understanding how these behavioral stress models induce behavioral performance differences, specifically in a well-characterized behavioral task such as dEBC used here, will help investigate and pinpoint circuit alterations that promote resilience or risk.

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CRedit authorship contribution statement

Alexandra B. Moussa-Tooks: Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **William P. Hetrick:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **John T. Green:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition.

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References

- Aisa, B., Tordera, R., Lasheras, B., Del Rio, J., Ramirez, M.J., 2007. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology* 32, 256–266. <https://doi.org/10.1016/j.psyneuen.2006.12.013>.
- Alves, R.L., Portugal, C.C., Summavielle, T., Barbosa, F., Magalhaes, A., 2019. Maternal separation effects on mother rodents' behaviour: a systematic review. *Neurosci. Biobehav. Rev.* <https://doi.org/10.1016/j.neubiorev.2019.09.008>.
- Avishai-Eliner, S., Gilles, E.E., Eghbal-Ahmadi, M., Bar-El, Y., Baram, T.Z., 2001. Altered regulation of gene and protein expression of hypothalamic-pituitary-adrenal axis components in an immature rat model of chronic stress. *J. Neuroendocrinol.* 13, 799–807.
- Brenhouse, H.C., Bath, K.G., 2019. Bundling the haystack to find the needle: challenges and opportunities in modeling risk and resilience following early life stress. *Front. Neuroendocrinol.* <https://doi.org/10.1016/j.yfrne.2019.100768>.
- Davis, E.P., et al., 2019. Across continents and demographics, unpredictable maternal signals are associated with children's cognitive function. *EBioMedicine.* <https://doi.org/10.1016/j.ebiom.2019.07.025>.
- Deverett, B., Koay, S.A., Oostland, M., Wang, S.S., 2018. Cerebellar involvement in an evidence-accumulation decision-making task. *Elife* 7. <https://doi.org/10.7554/eLife.36781>.
- Fareri, D.S., Tottenham, N., 2016. Effects of early life stress on amygdala and striatal development. *Dev Cogn Neurosci* 19, 233–247. <https://doi.org/10.1016/j.dcn.2016.04.005>.

- Freeman, J.H., 2015. Cerebellar learning mechanisms. *Brain Res.* 1621, 260–269. <https://doi.org/10.1016/j.brainres.2014.09.062>.
- Glynn, L.M., Baram, T.Z., 2019. The influence of unpredictable, fragmented parental signals on the developing brain. *Front. Neuroendocrinol.* <https://doi.org/10.1016/j.yfrne.2019.01.002>.
- Gutierrez-Menendez, A., Banqueri, M., Mendez, M., Arias, J.L., 2019. How does maternal separation affect the cerebellum? Assessment of the oxidative metabolic activity and expression of the c-fos protein in male and female rats. *Cerebellum.* <https://doi.org/10.1007/s12311-019-01087-5>.
- Hoffman, J.F., Wright, C.L., McCarthy, M.M., 2016. A critical period in Purkinje cell development is mediated by local estradiol synthesis, disrupted by inflammation, and has enduring consequences only for males. *J. Neurosci.* 36, 10039–10049. <https://doi.org/10.1523/JNEUROSCI.1262-16.2016>.
- Ivy, A.S., Brunson, K.L., Sandman, C., Baram, T.Z., 2008. Dysfunctional nurturing behavior in rat dams with limited access to nesting material: a clinically relevant model for early-life stress. *Neuroscience* 154, 1132–1142. <https://doi.org/10.1016/j.neuroscience.2008.04.019>.
- Joels, M., Karst, H., Sarabdjitsingh, R.A., 2018. The stressed brain of humans and rodents. *Acta Physiol (Oxf)* 223, e13066. <https://doi.org/10.1111/apha.13066>.
- Kishimoto, Y., Kano, M., 2006. Endogenous cannabinoid signaling through the CB1 receptor is essential for cerebellum-dependent discrete motor learning. *J. Neurosci.* 26, 8829–8837. <https://doi.org/10.1523/JNEUROSCI.1236-06.2006>.
- Lapp, H.E., Hunter, R.G., 2019. Early life exposures, neurodevelopmental disorders, and transposable elements. *Neurobiology of Stress* 11. <https://doi.org/10.1016/j.ynstr.2019.100174>.
- Lehmann, J., Feldon, J., 2000. Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? *Rev. Neurosci.* 11, 383–408. <https://doi.org/10.1515/revneuro.2000.11.4.383>.
- Leuner, B., Mendolia-Loffredo, S., Shors, T.J., 2004. High levels of estrogen enhance associative memory formation in ovariectomized females. *Psychoneuroendocrinology* 29, 883–890. <https://doi.org/10.1016/j.psyneuen.2003.08.001>.
- Lewin, M., et al., 2019. Early life trauma has lifelong consequences for sleep and behavior. *Sci. Rep.* 9, 16701. <https://doi.org/10.1038/s41598-019-53241-y>.
- Maniam, J., Antoniadis, C.P., Le, V., Morris, M.J., 2016. A diet high in fat and sugar reverses anxiety-like behaviour induced by limited nesting in male rats: impacts on hippocampal markers. *Psychoneuroendocrinology* 68, 202–209. <https://doi.org/10.1016/j.psyneuen.2016.03.007>.
- Molet, J., Maras, P.M., Avishai-Eliner, S., Baram, T.Z., 2014. Naturalistic rodent models of chronic early-life stress. *Dev. Psychobiol.* 56, 1675–1688. <https://doi.org/10.1002/dev.21230>.
- Moussa-Tooks, A.B., et al., 2020. Long-term aberrations to cerebellar endocannabinoids induced by early-life stress. *Sci. Rep.* 10 <https://doi.org/10.1038/s41598-020-64075-4>.
- Murthy, S., Gould, E., 2018. Early life stress in rodents: animal models of illness or resilience? *Front. Behav. Neurosci.* 12 <https://doi.org/10.3389/fnbeh.2018.00157>.
- Palacios-Barrios, E.E., Hanson, J.L., 2018. Poverty and self-regulation: connecting psychosocial processes, neurobiology, and the risk for psychopathology. *Compr. Psychiatr.* <https://doi.org/10.1016/j.comppsy.2018.12.012>.
- Shipman, M.L., Green, J.T., 2020. Cerebellum and cognition: does the rodent cerebellum participate in cognitive functions? *Neurobiol. Learn. Mem.* 170, 106996. <https://doi.org/10.1016/j.nlm.2019.02.006>.
- Shors, T.J., 2001. Acute stress rapidly and persistently enhances memory formation in the male rat. *Neurobiol. Learn. Mem.* 75, 10–29. <https://doi.org/10.1006/nlme.1999.3956>.
- Shors, T.J., Weiss, C., Thompson, R.F., 1992. Stress-induced facilitation of classical conditioning. *Science* 257, 537–539.
- Shors, T.J., Beylin, A.V., Wood, G.E., Gould, E., 2000. The modulation of Pavlovian memory. *Behav. Brain Res.* 110, 39–52.
- Short, A.K., Baram, T.Z., 2019. Early-life adversity and neurological disease: age-old questions and novel answers. *Nat. Rev. Neurol.* <https://doi.org/10.1038/s41582-019-0246-5>.
- Singh-Taylor, A., Korosi, A., Molet, J., Gunn, B.G., Baram, T.Z., 2015. Synaptic rewiring of stress-sensitive neurons by early-life experience: a mechanism for resilience? *Neurobiol Stress* 1, 109–115. <https://doi.org/10.1016/j.ynstr.2014.10.007>.
- Skosnik, P.D., et al., 2008. Cannabis use disrupts eyeblink conditioning: evidence for cannabinoid modulation of cerebellar-dependent learning. *Neuropsychopharmacology* 33, 1432–1440. <https://doi.org/10.1038/sj.npp.1301506>.
- Steinmetz, A.B., Freeman, J.H., 2010. Central cannabinoid receptors modulate acquisition of eyeblink conditioning. *Learn. Mem.* 17, 571–576. <https://doi.org/10.1101/lm.1954710>.
- Steinmetz, A.B., Freeman, J.H., 2011. Retention and extinction of delay eyeblink conditioning are modulated by central cannabinoids. *Learn. Mem.* 18, 634–638. <https://doi.org/10.1101/lm.2254111>.
- Steinmetz, A.B., Freeman, J.H., 2013. Differential effects of the cannabinoid agonist WIN55,212-2 on delay and trace eyeblink conditioning. *Behav. Neurosci.* 127, 694–702. <https://doi.org/10.1037/a0034210>.
- Steinmetz, A.B., Freeman, J.H., 2016. Cannabinoid modulation of memory consolidation within the cerebellum. *Neurobiol. Learn. Mem.* 136, 228–235. <https://doi.org/10.1016/j.nlm.2016.11.002>.
- Steinmetz, A.B., Freeman, J.H., 2020. Intracerebellar cannabinoid administration impairs delay but not trace eyeblink conditioning. *Behav. Brain Res.* 378, 112258. <https://doi.org/10.1016/j.bbr.2019.112258>.
- Steinmetz, J.E., Tracy, J.A., Green, J.T., 2001. Classical eyeblink conditioning: clinical models and applications. *Integr. Physiol. Behav. Sci.* 36, 220–238.

- Stoodley, C.J., et al., 2017. Altered cerebellar connectivity in autism and cerebellar-mediated rescue of autism-related behaviors in mice. *Nat. Neurosci.* 20, 1744–1751. <https://doi.org/10.1038/s41593-017-0004-1>.
- Tractenberg, S.G., et al., 2016. An overview of maternal separation effects on behavioural outcomes in mice: evidence from a four-stage methodological systematic review. *Neurosci. Biobehav. Rev.* 68, 489–503. <https://doi.org/10.1016/j.neubiorev.2016.06.021>.
- Walker, C.D., et al., 2017. Chronic early life stress induced by limited bedding and nesting (LBN) material in rodents: critical considerations of methodology, outcomes and translational potential. *Stress* 20, 421–448. <https://doi.org/10.1080/10253890.2017.1343296>.
- Wilber, A.A., Wellman, C.L., 2009a. Neonatal maternal separation alters the development of glucocorticoid receptor expression in the interpositus nucleus of the cerebellum. *Int. J. Dev. Neurosci.* 27, 649–654. <https://doi.org/10.1016/j.ijdevneu.2009.08.001>.
- Wilber, A.A., Wellman, C.L., 2009b. Neonatal maternal separation-induced changes in glucocorticoid receptor expression in posterior interpositus interneurons but not projection neurons predict deficits in adult eyeblink conditioning. *Neurosci. Lett.* 460, 214–218. <https://doi.org/10.1016/j.neulet.2009.05.076>.
- 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. *Dev Neurobiol* 67, 1751–1764. <https://doi.org/10.1002/dneu.20549>.
- Wilber, A.A., Lin, G.L., Wellman, C.L., 2010. Glucocorticoid receptor blockade in the posterior interpositus nucleus reverses maternal separation-induced deficits in adult eyeblink conditioning. *Neurobiol. Learn. Mem.* 94, 263–268. <https://doi.org/10.1016/j.nlm.2010.06.004>.
- Wood, G.E., Shors, T.J., 1998. Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activation effects of ovarian hormones. *Proc. Natl. Acad. Sci. Unit. States Am.* 95, 4066–4071.