Stress and Aggression in Adolescent Female Mice:

A Multimodal Lifetime Model

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Significance

Research on aggression in adolescents focuses primarily on males, leaving crucial gaps in the understanding of female aggression. While the absolute number of person offences is higher in male juvenile offenders, person offences make up a higher percentage of total offences in female juvenile offenders[1]. Research links early life adversity, bullying, and sleep deprivation to aggression, but the interaction between these factors and its relation to lifetime gender and sex differences in agression remains to be investigated. In humans, early life adversity predicts higher levels of aggression during adolescence[2], and this effect may be mediated by gender[3]. Bullying has short and long term implications for aggression: both perpetration and victimization significantly predict aggression later in life[4]. The role of gender in this effect is unclear, but bullying victimization rates are higher in female versus male students[5]. This relationship between bullying and aggression may be impacted by sleep deprivation. A cross-sectional survey found that bullies had significantly higher rates of sleep deprivation than their peers[6]. The effect of sleep deprivation alone on aggression is unclear. In women, acute sleep deprivation either has no effect or increases aggression, while in men, it either has no effect or decreases aggression[7,8]. The risk for sleep deprivation associated effects, however, is elevated in female high school students, who report higher rates of sleep deprivation than their male peers[9].

These three factors have been studied in animal models, allowing the identification of possible neurobiological mechanisms. GABAergic basal forebrain (BF) input to the lateral habenula (lHb) assigns valence to aggressive interactions, modulating reward processing[10]. Post-weaning social isolation (PWSI), a model of early life adversity, has been shown to increase aggression in both male and female rats and change neuropeptide signaling in the nucleus accumbens (NAc)[11]. Aggressive experiences also increase extracellular dopamine in the NAc of

dominant rats, while injecting dopamine antagonists into the NAc decreases aggression self-administration[12].

Chronic social stress (CSS) is linked to sex-specific changes in aggression that may be mediated by activity shifts in the NAc and lHb. Males tend to demonstrate increased aggression following social defeat, while females tend to demonstrate decreased aggression[13]. Brain circuitry underlying post-CSS behavioral changes has been studied in males, where CSS was linked to reduced GABA signaling in the NAc[14] and lHb[15], but these effects remain to be investigated in females, as does their connection to aggression. CSS-induced changes in aggression may also be impacted by corticosterone levels. After three episodes of CSS, corticosterone levels in defeated females were higher than those in controls but lower compared to controls in males[13].

Sleep deprivation has been linked to aggression and changes in the BF and lHb in animals. In mice, chronic partial sleep deprivation (CPSD) increases various hyperactive behaviors, including aggression[16]. The BF cholinergic system contributes to the regulation of sleep, and prolonged wakefulness decreases BF activity[17]. The lHb has a similarly bidirectional relationship with sleep. LHb gene expression follows a circadian rhythm, and plays an important role in maintaining sleep homeostasis[18]. Considering the roles of the BF and the lHb in both sleep and aggression, it is plausible that altered activity in these projections increases aggression following chronic sleep deprivation. The lHb also plays an important role in processing aversive stimuli[19], so disrupted sleep homeostasis associated with CSS[20] could be due to chronic lHb activation by the aversive stimuli of social stress .

We propose a model that integrates PWSI, CSS, and CPSD to allow multimodal lifetime study of aggression (Fig.1), with the goal of determining which factor has the greatest impact on

aggression in female adolescents. We will use California mice, where both males and females exhibit territorial aggression[13]. We will use PWSI[11] to study the effects of early life adversity. Inter-adolescent aggression can be difficult to study, but the reverse-resident-intruder paradigm (rRIP) has successfully induced social stress in adolescent rodents[21], although it has not previously been applied to females. Sleep deprivation will be evaluated via the Modified Multiple Platform (MMP) paradigm[16]. We will measure behavioral aggression (Aim 1) and neural activity in the BF and lHb (Aim 2) during the first, middle, and last rRIP sessions in adolescence and during a traditional resident-intruder test (RIT)[11,12] test of aggression in adulthood; corticosterone will be assayed (Aim 3) 24 hours after RIT (Fig. 2).

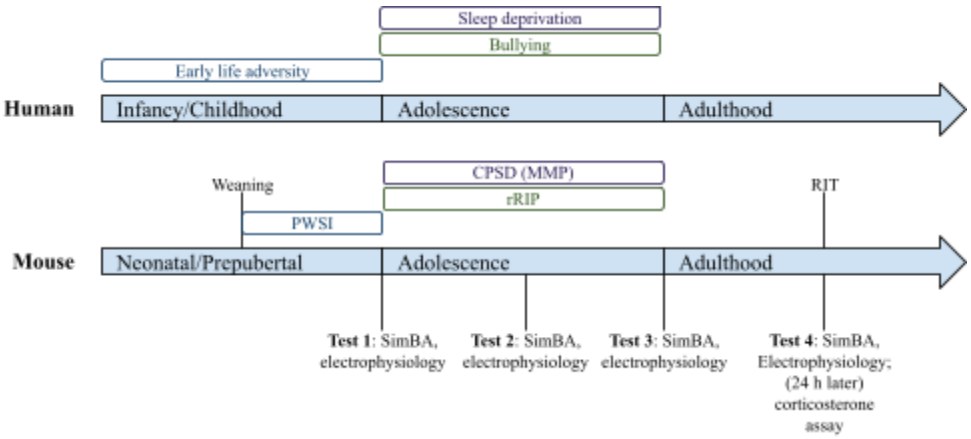

Figure 1. Multimodal lifetime model of aggression. The effects of early life adversity, sleep deprivation, and bullying will be modeled by PWSI, MPP, and rRIP, respectively. In adulthood, measures of aggression will be conducted during an RIT. Aggression throughout adolescence and adulthood will be assessed by behavioral analysis using SimBA and correlated with electrophysiological activity. A corticosterone assay will be conducted 24 hours after final assessment during adulthood.

Innovation

While the effects of PWSI[11], CSS[13], and CPSD[16] on aggression have been examined individually, the interaction between the three remains unclear. Furthermore, research on CSS and aggression is sparse in adolescent males[21] and adult females[13] and appears to be absent in adolescent females, a crucial gap that must be addressed. By comparing various potential

influences on aggression, we will identify important components for future study of aggression in female adolescents.

Approach

The three Aims of this study are hypothesized to be correlated, but also retain meaning independently. **Aim 1** tests the impact of the three stressors of interest on aggressive behavior throughout life in female versus male mice. **Aim 2** examines BF-lHb projections, which have a known role in modulating agression[10], for sex, stressor, and timepoint differences to identify possible neurobiological explanations for changes in aggressive behavior. **Aim 3** tracks corticosterone to compare stressors and establish a possible correlation between stress level and aggression. These results will provide insight into the behavioral and neurobiological etiology of aggression in adolescent females.

Aim 1: Establish impacts of PWSI, CSS, and CPSD on behavioral aggression throughout adolescence and adulthood in female and male mice.

Rationale

PWSI[11], CSS[13], and CPSD[16] impact behavioral aggression individually, and may amplify or counteract each others' effects. The potential interaction between PWSI and CSS in particular is interesting, because while PWSI increases aggression in both females and males[11], the California mouse model showed defeat during CSS increased aggression in males but decreased aggression in females[13], suggesting sex differences in stress responses during different development stages and/or to different stressors. Classifying behaviors will help determine the

complementary or competing roles of stage of development and stressor type on aggressive behavior in females and males.

Experimental Plan

These experiments will use the Simple Behavioral Analysis (SimBA) toolkit[22] to classify aggressive behavior using machine learning. Behavior will be video recorded during the first, middle, and last sessions of rRIP throughout adolescence (Fig. 2). The paradigm will be applied as described by Manz et al.[21] but with California mice instead of rats, to allow study of both females and males[13]. Both resident and intruder behavior will be analyzed for aggressive behavior patterns, using the mouse resident intruder protocol terms defined by Nilsson et al.[22]. Once mice reach adulthood, we will conduct a single RIT session, using the rRIP method[21] but with the expectation that the resident will be the aggressor and the intruder will be defeated, to assess

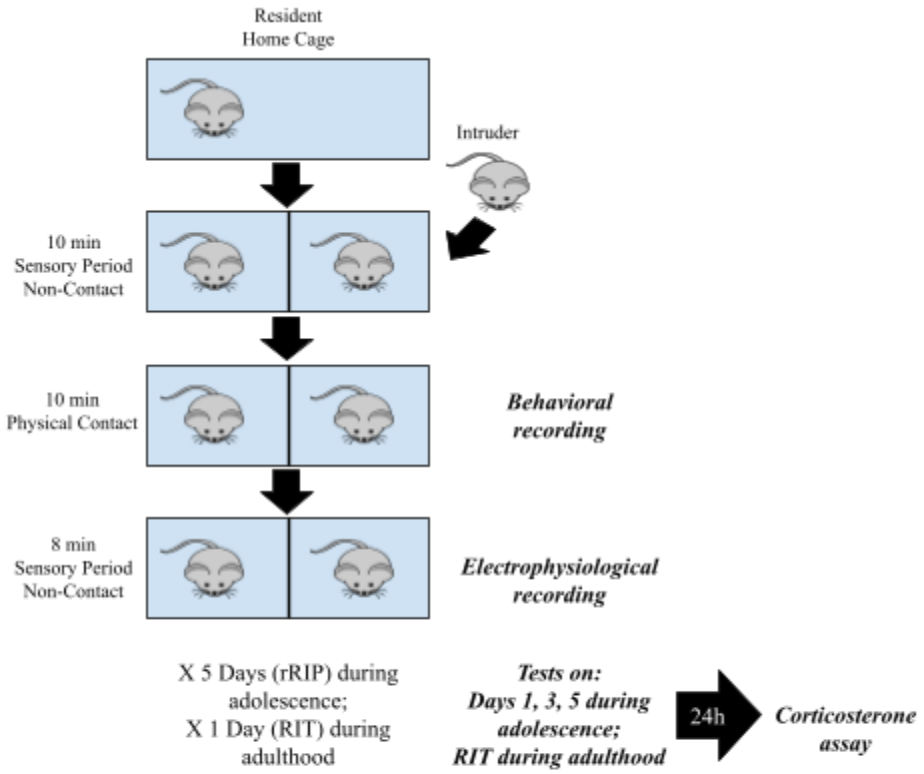

Figure 2. Timing of tests. Behavioral will be recorded during the physical contact portion of social stress. Electrophysiology will be recorded during the post-physical sensory portion, where a wire partition physically separates mice. During adolescence, five rRIP sessions will be conducted, with recording on days 1, 3, and 5. During adulthood, a single RIT session will be conducted to record long term impacts on measures of aggression. rRIP and RIT are conducted identically, but expected roles of resident and intruder are reversed. 24 hours after RIT (post-sacrifice), corticosterone assays will be conducted. Figure adapted from[11].

long term effects on aggression, and video analysis will be conducted as during rRIP (Fig. 2). Each experimental group will have n = 10 per sex (see "Rigor, Reproducibility, and Transparency").

Hypothesis

Based on previous research, PWSI groups will have higher levels of aggression in mice of both sexes during the final adulthood test[11], while chronic defeat in rRIP will decrease aggression in female mice but increase it in males[13]. CPSD is predicted to increase aggression in females[16], but may have no effect on male aggression. Furthermore, the rRIP model of social defeat in adolescents predicts that aggression will initially be low in both resident and intruder, but that over time the intruder mice will defeat the resident mice, a reversal of overwhelming tendency in adults[21]. However, during the RIT, this is expected to shift, so that the resident defeats the intruder[13]. Overall, we predict that during adolescence, aggressors exposed to PWSI will be most aggressive in both sexes, and sleep deprivation will enhance aggression in females but not males. In adults, similar effects of PWSI and CPSD are expected, but male mice defeated during adolescence and females who were aggressors during adolescence are expected to be most aggressive.

Aim 1 Alternate Outcomes and Interpretations

Results from previous studies strongly suggest some effect of all of these stressors on aggression. The effects of sleep deprivation, however, seem to be weakest[16]. This mirrors the somewhat contradictory results from human studies of sleep deprivation[5,6]. A completely absent effect or interaction of CPSD here would imply that either MMP was insufficient to produce effects or that effects seen in other studies may be influenced by other factors. Variables in

human studies are especially hard to control, so this would be a valuable first step in identifying confounding variables. The effects of PWSI[11] and CSS[13] are more robust. However, the rRIP protocol has not been tested on California mice, and may not result in social defeat. This would suggest that further adaptations are necessary to apply a chronic social defeat paradigm to adolescent female rodents. Subsequent research on Syrian hamsters instead, where females are more aggressive than males, might produce more successful results[13]. An absence of social defeat could also provide insight into other social interactions during adolescence. Similarly, the absence of effect of any form of stress may not provide insight into the original experimental question of Aim 1, but would have important implications for stress resilience.

Aim 2: Determine how activity in BF-lHb varies between groups and correlates with behavioral aggression.

Rationale

BF-lHb projections modulate aggression reward[10]. The presence or absence of reinforcement could lead to long term increases or decreases in aggression, and presents a possible mechanism for the effects of stress on aggression. Chronic social and sleep stressors alter the BF and lHb. PWSI has long term, sex-independent impacts on NAc[11]. During aggressive interactions like resident-intruder paradigms, higher BF-lHb activity leads to reward encoding, while lower activity results instead in aversion[10], which may explain differential encodings of experiences in defeated versus aggressor mice. Chronically, this might lead to tonic firing changes in one or both of these regions. Indeed, CSS can change GABAergic communication in the NAc[14] and lHb[15], although this has not yet been linked to aggression.

The BF and lHb are also closely related to sleep. Loss of sleep negatively impacts the BF cholinergic system's ability to regulate emotion and reward[17]. The lHb plays an important role in sleep homeostasis, and acute sleep deprivation elevates specific gene expression in the lHb[18]. CSS disrupts sleep homeostasis and impairs sleep recovery[20], an effect likely mediated by dysregulation of the lHb. The BF-lHb projection's ability to induce long term changes to aggressive behavior suggest it mediates stress-induced changes in aggression, which can be confirmed through in vivo electrophysiology of the BF and the lHb. The bulk of research on this connection was conducted in adult male rodents[10,18,20], so these experiments will provide novel insight into sex and age differences.

Experimental Plan

To simultaneously record activity in the BF and lHb, electrodes will be stereotaxically implanted into the NAc shell-septum transition zone BF (anteroposterior + 1.5 mm; mediolateral, + 1.6 mm; dorsoventral, −4.4 mm; angle 10°) and the lHb (anteroposterior, −1.7 mm; mediolateral, + 0.4 mm; dorsoventral, −2.5 mm; angle 0)[10]. Recordings from both mice will be taken during the first, middle, and last rRIP sessions during adolescence and during the RIT in adult mice, with the intruder still in the resident's cage, but with a wire partition to physically separate the mice while maintaining visual, olfactory, and auditory communication[21,23] (Fig. 2). Following the final experiment, post mortem histology will be used to verify electrode placements[23], and data removed from analysis if necessary.

Hypothesis

The close relationship between sleep and the lHb predicts sleep deprivation and lHb activity will show the strongest effect, but effects of all three experimental conditions are

expected to alter activity in both the BF and the lHb. These changes are expected to correlate with behavioral analysis of aggression, with higher activity in more aggressive mice. This would predict the highest activity in male mice exposed to all three stressors.

Aim 2 Alternate Outcomes and Interpretations

BF-lHb projections influence aggression, but any or all stressors may influence aggression through other circuitry, which could be the focus of future research. Alternatively, even if Aim 1 found no effect on aggressive behavior, Aim 2 may still find electrophysiological changes that may either not be behaviorally apparent or may influence behaviors unrelated to aggression. For instance, changes in the NAc and lHb after defeat in CSS have been linked to increased depressive-like behavior[14,15]. If no effects on behavior or brain activity can be found, this, like absence of effect in Aim 1 alone, suggests resistance to or different processing of stress. In this case, the locus coeruleus (LC) and the prefrontal cortex (PFC) might be regions of interest, as adolescent social stress has been shown to increase tonic LC activity and alter connectivity between these two regions[23]. Finally, it is possible that aggressive behavior measured in Aim 1 and BF-lHb activity changes in Aim 2 are both present, but do not correlate with each other. This could suggest associations between aggression and depressive-like behavior or a significant role for individual differences.

Aim 3: Compare corticosterone levels between groups and evaluate relationship between stress level and aggressive behavior.

Rationale

Corticosterone levels are indicative of stress response[24], allowing comparison of stressor impacts. Isolated rats show greater corticosterone responses to aggressive interactions later in life[11]. Chronic social stress enhanced acute corticosterone response in female California mice, and elevated baseline corticosterone levels in males[13]. The connection to aggression is unclear. In rats, nonaggressive male mice had higher levels of corticosterone than aggressive ones[10], but aggression self-administration has been shown to increase corticosterone levels[12]. Measuring corticosterone response could add insight into stressor-specific responses, as well as the relationship between corticosterone and aggression.

Experimental Plan

Blood will be collected four to twenty-four hours after the final resident-intruder and electrophysiology experiments are run[10] (Fig. 2). To compensate for high baseline corticosterone levels in California mice, we will follow assay recommendations and dilution protocols outlined by Trainor et al.[25].

Hypothesis

Despites increases in baseline corticosterone levels following social defeat in males[13], females tend to have higher baselines[25]. Within both males and females, PWSI is predicted to increase corticosterone levels[11]. Sleep deprivation is not expected to have its own effect, but may

exacerbate effects of other stressors due to impaired recovery following chronic stress[20]. Socially defeated mice are expected to have higher levels than aggressors[10].

Aim 3 Alternate Outcomes and Interpretations

It is possible that combining chronic stressors will elevate baseline corticosterone levels in males sufficiently to overtake initially higher corticosterone levels in females, which would have important implications for chronic stress in males. If, contrary to our hypothesis, corticosterone levels are higher in aggressors than in defeated mice, further research into the directionality of the relationship between aggression and corticosterone would be needed to gain a more detailed understanding of the nature of aggression.

Rigor, Reproducibility, and Transparency

1. Appropriate statistical power

   Based on similar studies[10,16,25,26], 10 animals of each sex per experimental group should be sufficient to detect statistical effects.

2. Sex as a biological variable

   We will conduct all experiments on equal numbers of female and male California mice. Previous studies[25,26] with similarly sized experimental groups support the conclusion that we have enough statistical power to detect sex differences.

3. Unbiased data collection

Experimenters conducting data collection and analysis will be blind to the experimental conditions of the animals. Behavioral analysis will be performed by a machine learning algorithm, which removes observer bias in behavioral scoring[22,27].

4. Criteria for inclusion

We will confirm the placement of electrodes implanted to satisfy Aim 2 via histological analysis. Electrophysiology data from any inaccurately implanted electrodes will be excluded from analysis.

5. Appropriate statistical analysis

For each aim, a one-way repeated measures ANOVA with experimental condition as the factor will be calculated separately for female and male mice, followed by Student-Newman-Keuls for *post hoc* comparisons between means, as described in[23]. In Aim 1, this analysis will be conducted for three dependent variables: latency to attack, number of attack bouts, and attack bout length, as determined by machine learning analysis[22]. The Pearson correlation between these three variables will be used to assess their relationship. For Aim 2, the dependent variables will be the mean spike rates for the BF and the lHb, based on the waveform analysis described in[23]. The Pearson correlation for the activity in the BF and the lHb will be used to determine the relationship between these two regions. Correlations will also be calculated between activity in each brain region and each measure of attack behavior. For Aim 3, corticosterone levels will be the dependent variable[25]. To assess correlation with aggression, the Pearson correlation between corticosterone level and each attack measure will be determined.

6. Transparency

   All publications produced in the course of this project will include detailed reports of methods and resources used to allow reproducibility of experiments.

Future Directions

This project will establish the necessary foundation for further study of aggression in adolescent females. It will also determine the individual and combined effects of PWSI and CSS and CPSD during adolescence on aggression in males and females at various points during adolescence and adulthood. Based on these results, we will optimize a model of aggression in adolescent females for use in further research. We will use this model to continue mapping neural correlates of aggression in female adolescents. To gain more insight into mechanisms of habenular control of aggression, we will look at serotonin transmission. The lHb targets nearly all midbrain neuromodulatory systems, including the serotonergic system[12,16]. 5-HT1B receptors[12] and 5-HT3 receptors[28] have been implicated in aggression. The establishment of a reliable model of female adolescent aggression is the first step to closing gaps in the study of aggression.

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