THE EFFECTS OF ADOLESCENT CHRONIC MILD STRESS:

In Female Wistar-Kyoto Rats

Student Author

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Abstract

Despite years of research to understand underlying mechanisms and develop more effective treatment approaches for mood disorders, numerous challenges exist. Many chronic stress models are used to study mood disorders, however the majority have been established with adult males. This is problematic considering that affective disorders are more common in women, and generally develop during late adolescence. Studies have indicated fundamental behavioral, physiological, and neural differences between males and females in response to the same external stressors, furthering a need to develop sex-specific paradigms to accurately model the etiology of mood disorders in females. The Wistar-Kyoto (WKY) rat strain is a promising model known to demonstrate endogenous hormonal and behavioral abnormalities similar to symptom-presenting patients with depression. In this study, we test stress susceptibility of female WKY rats by using a three-week chronic mild stress (CMS) paradigm during late adolescence. We hypothesize that female WKY rats undergoing CMS will develop depressive and anxiety-like characteristics that are typically not observed in a Wistar strain. Body weight, food intake, body composition, and corticosterone levels throughout CMS are determined to evaluate physiological effects of stress. Immediately following CMS, animals undergo behavioral assessments of helplessness, anxiety, anhedonia, and locomotor activity to evaluate the development of mood disorder phenotypes. These tests are repeated during late adulthood to determine whether expected stress-induced behavioral deficits persist later in life. The validation and characterization of this sex-specific model of mood disorders allows for more studies on the underlying mechanisms driving these disorders and ultimately contribute to the development of novel therapeutic strategies.

Hallowell, A. (2020). The effects of adolescent chronic mild stress: In female Wistar-Kyoto rats. *Journal of Purdue Undergraduate Research*, 10, 27–34.

Keywords

stress, chronic mild stress, adolescence, hypothalamic pituitary adrenal axis, Kyoto, Wistar, female rats

INTRODUCTION

Mood disorders are common illnesses and are symptomatically challenging to treat. Specifically, depression has emerged as a significant mental health concern across the globe. Part of the complexity of mood disorders is due to their high comorbidity with anxiety. This stresses a need to further develop animal models to find comprehensive behavioral representations of symptoms and classify biological markers as therapeutic targets in treating affective disorders. Development of appropriate animal models has historically focused on males, as male rodents have generally been used to develop, characterize, and validate models (Kokras & Dalla, 2014).

The use of only male subjects presents a variety of issues, given that depression targets twice as many women than it does men (American Psychiatric Association, 1994). There are fundamental behavioral, physiological, and neural differences between males and females in response to external stressors (Pare & Redei, 1993). Additionally, disparities in reaction to treatment and overall symptoms allow them to be assessed separately based on sex (Giedd, Keshavan, & Paus, 2008; Kokras & Dalla, 2014). Because of these differences and the historical reliance on the use of animal models of stress-related disorders that were developed and validated in adult males, there remains a need to develop and validate paradigms to accurately model the etiology of mood disorders in females. Furthermore, studies have shown that stressors experienced during adolescence can have lasting and intense effects for the future behavioral and psychological function of an individual. Some human studies have shown that stress problems in adolescence are strongly linked with the propensity to develop depressive and/or anxiety disorders in adulthood (Turner & Lloyd, 2004). Onset, presentation, and treatment responses to stress-related dysfunction has been shown to be sex-dependent, especially when stress is experienced in adolescence. Puberty is a critical developmental period marked by an increase in the vulnerability to several psychological disorders including depression and anxiety (Conger & Petersen, 1984; Masten, 1987). Female predisposition to depression could have arisen from the effects of sex hormones throughout brain development and results in enhanced depressive-like behavior. Due to the complexity of biological, psychological, and social characteristics of depression in humans, various models are needed to assess the disorder in each sex during the adolescent period.

The chronic mild stress (CMS) paradigm exposes animals to unpredictable mild stressors that are intended to parallel the day-to-day complications reported to influence the onset of depression in some humans (Willner, 1997). In CMS, the hypothalamic pituitary adrenal (HPA) axis is responsible for the neuroendocrine adaptation element of the stress response. The release of stress hormones by the HPA axis is caused by a corticotropin-releasing hormone (CRH) and vasopressin (AVP) from the medial parvocellular division of the paraventricular hypothalamic nucleus. When CRH and AVP reach the portal system of the pituitary, this causes the anterior pituitary release of adrenocorticotrophic hormone (ACTH). ACTH stimulates the adrenal cortex to secrete glucocorticoids: cortisol in primates and corticosterone in a majority of rodent species. A neuroendocrine feedback loop indirectly regulates the stress hormones released by the HPA axis (Herman et al., 2003). Studies show that stressors have endocrine differences in stress reactivity between adolescent and adult groups. For example, some studies found that basal and stress-induced ACTH and corticosterone secretion are similar in prepubertal and adult animals; however, prepubertal animals have an elongated ACTH and corticosterone stress response compared to adults (Goldman et al., 1973; Romeo et al., 2004). Additionally, intermittent severe physical stress and variable chronic mild social and physical stress experienced during adolescence were connected with increased open-arm time in an elevated plus maze (EPM), indicating reduced anxiety-like behavior in adulthood (Pohl et al., 2007).

The Wistar-Kyoto (WKY) rat strain was generated to be used as a normotensive control for the spontaneously hypertensive rat and has been recommended as a genetic animal model for depression (Sterley, Howells, & Russell, 2011; Nam, Clinton, Jackson, & Kerman, 2014). This strain of rat is known to demonstrate endogenous hormonal and behavioral abnormalities that many symptom-presenting patients with depression exhibit, such as hyperreactivity to stress and deregulation of the HPA axis (Solberg, Olson, Turek, & Redei, 2001). Investigators have presented male-female differences in WKYs in relation to depressive profiles (Mehta, Wang, & Redei, 2013). The few studies on female WKY rats have used adults (Tizabi, Bhatti, Manaye, Das, & Akinfiresoye, 2012; Mileva & Bielajew, 2015), with even fewer studies examining female adolescent WKY rats (Sterley et al., 2011). The validation and characterization of this sex-specific model of mood disorders allows for more studies on the underlying mechanisms driving depression and

ultimately contribute to the development of novel therapeutic strategies. The aim of these experiments was to develop an adolescent model of depression in females. To do this, we age-matched and tested stress susceptibility of female Wistar (Ws) and Kyoto (Ky) rats. Ws rats are common genetic animal models used in behavioral research, while Ky rats are established models of anxiety because they naturally develop an anxiety-like phenotype. We hypothesized that chronic stress in adolescence would induce a depressive phenotype in Ws rats and exacerbate disordered behavior in Ky rats in adulthood.

METHODS

Subjects

In this study 10 female Ws and 8 female Ky rats were obtained from Charles River Laboratories. They were 36 days old upon arrival to the lab. Females were housed individually in plastic tub cages with bedding, and food and water were provided ad libitum. The females were acclimated to the laboratory for 9 days prior to exposure to CMS. The temperature of the housing room remained constant at ($20.5 \pm 1^\circ\text{C}$) with 55–65% humidity and was maintained a 12-hour light/dark cycle unless stated in the stress schedule (Figure 1).

Figure 1. Timeline of events.

Note: Adolescent stress was conducted on days 45–65. Behavioral analysis was conducted in both adolescence and adulthood. The EMP was conducted on days 67 and 91. The forced swim test was conducted on days 68 and 92. The sucrose preference test was conducted on days 69 and 93.

Experimental Subjects

Ws and Ky rats, CMS (+) or control, were tested for stress susceptibility by using a three-week CMS paradigm during late adolescence (days 45–66). Subjects experienced each stressor (Table 1) once per week and experienced each stressor a total

of three times, in a counterbalanced manner. For example, the restrain stress was conducted at a random time during the light cycle for 30 minutes each Monday of the three weeks of CMS. The cage tilt was conducted every Tuesday; for 6 hours the cage was tilted approximately 30–45 degrees on a cart in the center of the housing room. Every Wednesday, 250 ml of lukewarm water was poured into the cage during the dark cycle overnight; the cage was replaced with fresh bedding every Thursday morning. Also on Thursdays, the cages were placed in a chamber 3°C for 30 minutes at a random time during the light cycle. Each Friday there was the crowding stress in which for 6 hours at an unspecified time during the light cycle, subjects were temporarily housed with four other subjects also undergoing CMS. On Saturdays, subjects' cages were moved to a separate housing room where the lights remained on throughout the dark cycle. Each Sunday at a random time during the light cycle, a static noise (80 dB) was played in a neighboring housing room for 6 hours.

Body composition using nuclear magnetic resonance was taken in pre- and post-CMS. Body weight and food intake were measured at the same time daily. All procedures were approved by the Purdue Animal Care and Use Committee.

Food Intake and Body Weight

Food intake and body weights were collected daily to assess overall strain differences and possible stress induced changes. The graphs in Figure 2 contain a bar under an asterisk that indicates a significant difference between the groups.

Figure 2. Food intake and body weight.

Note: Food intake was calculated in kcal. Both strains developed stress-induced changes in food intake that did not continue in adulthood.

Stress Reactivity: Corticosterone Response to Last Restraint Stress and Postnatal Day 85

After restrain stress (see Table 1), blood was collected from the tail ends at time points 0, 15, 30, and 120 minutes after stress (Figure 3). Plasma corticosterone concentration was quantified in duplicates using an enzyme immunoassay (EIA) kit according to manufacturer instructions (Enzo Life Sciences, protocol ADI-900-097). An EIA kit is an in vitro quantitative assay kit that we used to quantify corticosterone levels during subjects' response to stress. Samples were diluted at a ratio of 1:40 using a steroid displacement reagent, and standards were serially diluted at a ratio of 1:50. One hundred µl of sample or standard were loaded on donkey antisheep IgG-coated plates, followed by 50 µl of conjugate, then 50 µl of sheep antibody to rat corticosterone. Plates were then incubated at

Stressor	Description	Duration
Restrain	Restrained in a clear PVC device with a perforated front at an unpredictable time during the light cycle.	30 minutes
Cage tilt	Moved to different location during different times of the day tilted 30–45 degrees in homeroom.	6 hours
Flooded cage	250 mL of room temperature tap water added to bedding in novel cage during light cycle.	overnight
Cold exposure	Moved to 4°C chamber at an unpredictable time during the light cycle.	30 minutes
Crowding	Temporarily housed with 4 unfamiliar conspecifics also undergoing CMS during the light cycle.	6 hours
Skip dark cycle	Moved to a neighboring housing room where the lights remained on during the dark cycle.	overnight
Static noise	Static noise (80dB) played in a neighboring housing room in the middle of the light cycle.	6 hours

Table 1. Table of stressors used, with animals' access to food and water only in conditions lasting 6 or more hours.

Figure 3. Ky rats had higher corticosterone than Ky+ at 60 min. Both were higher than Ws. In adulthood, Ws had higher corticosterone than Ws+ at 30min. Both Ky groups were higher than both Ws groups at 60 min.

Note: Ky rats had higher corticosterone than Ky+ at 60 minutes. Both were higher than Ws rats. In adulthood, Ws rats had higher corticosterone than Ws+ at 30 minutes. Both Ky groups were higher than both Ws groups at 60 minutes.

room temperature on a plate shaker for two hours at 500 rpm and then washed 3 times. After washing, 200 μ l of p-nitrophenyl phosphate substrate solution was added to each well and then incubated at room temperature for one hour. Fifty μ l of stop solution was then added to every well, and optical density was immediately read at 405 nm absorbance using a microplate reader (Multiskan Ascent, Thermo Scientific).

Behavioral Assessments

Anxiety-Like Behavior: Elevated Plus Maze After CMS and at Postnatal Day 90

Anxiety-like behavior was assessed using the EPM (Figure 4). The wooden EPM apparatus is 3 feet above the ground and has four arms connected at 90-degree angles, each 6 inches wide and 3 feet long. Two of the opposite side arms are enclosed, and the other two are open. All subjects habituated to the room for 15 minutes before testing. Animals were placed in the center of the EPM, and behavior was video recorded from a ceiling-mounted camera for 5 minutes. Rats were returned to home cages immediately after testing. The apparatus was sufficiently cleaned between each trial using a 10% alcohol solution. The percent of time spent in open arms and in the center was scored manually by an observer blinded to the experimental conditions, because the ANY-maze tracking software, developed by Stoelting Co., that was used to score behaviors was not sensitive enough to track the head of rats on the apparatus.

Figure 4. (a) Depicts the time spent in open arms while (b) shows the time spent in the closed arms.

Note: Time spent in open arms (a) and in closed arms (b).

Depressive-Like Behavior: Forced Swim Test

The forced swim test apparatus was a plastic cylindrical container 14 inches in height and 13 inches in diameter and contained enough water to prevent the rats from stepping on the bottom of the apparatus (Figure 5). The water temperature remained at approximately 23°C. All subjects were given 15 minutes to habituate to the testing room. Rats were monitored for the duration of the test, and the test was stopped if rats did not maintain a swim or float behavior. The test was recorded for 5 minutes using a ceiling-mounted camera. After 5 minutes, fur was immediately dried with a towel, and rats were returned to the home cage. An observer blinded to the treatment conditions assessed the time spent immobile, latency to immobility, and time spent displaying escape behaviors.

Figure 5. (a) is the % time spent escaping and (b) is % time spent immobile

Note: % time spent escaping (a) and % time spent immobile (b).

Sucrose Preference Test after CMS and at Postnatal Day 93

The sucrose preference test presented the experimental animal with two identical drinking bottles containing plain drinking water or 20% sucrose solution (Figure 6). The three days before testing were spent habituating the subjects to the two drinking bottles placed in their cage. Each day the bottles' positions were swapped until testing was complete to ensure that results were not due to a side preference.

Figure 6. Sucrose preference test of anhedonia, a loss of interest in normally rewarding stimuli, was carried out at PND 93. There was a strain difference in behavior and only Ws displayed a stress-induced difference in SPT, indicating the development of anhedonia

Note: The sucrose preference test of anhedonia, loss of interest in normally rewarding stimuli, was carried out at postnatal day 93. There was a strain difference in behavior, and only Ws rats displayed a stress-induced difference in the test, indicating the presence of anhedonia.

RESULTS

The Ws strain exhibited no significant differences in body weight changes; however, the stressed Ky strain gained less weight and had lost more fat mass than the controls. Food intake was lower for the stressed group compared to the controls for both strains. Behavioral assessments of the forced swim test and EPM exhibited behavioral helplessness and low motivation in the stressed group along with the presence of anhedonia. The Ky strain had a more profound response to stress than the Ws strain in each behavioral evaluation. Acute stress reactivity blood collection displayed that corticosterone levels in Ky rats were lower than their controls, reinforcing the notion that females have a hypoactive response to stressors.

Ws and Ky rats developed stress-induced changes in food intake; the CMS(+) rats ate significantly less than the control group. Only Ky rats had changes in body weight. These did not persist into adulthood.

There were strain differences in control and stress-induced depressive-like behavior. Ky rats overall spent less time attempting to escape from forced swim test apparatus than Ws rats. Only Ws rats exhibited stressed-induced changes in time spent immobile. The same results were seen in adulthood.

Only Ws rats demonstrated a stress-induced difference in the sucrose preference test. The Ws+ group consumed significantly less of the sucrose solution than the control group, indicating a presence of anhedonia in the stressed group.

Ky rats exhibited more anxiety-like behavior than Ws rats, with no effects of stress in either strain. These effects were consistent in adulthood.

Ky rats had higher corticosterone than Ky+ rats at 60 minutes. Both were higher than for Ws rats. In adulthood, Ws rats had higher corticosterone than Ws+ rats at 30 minutes. Both Ky groups had higher corticosterone than both Ws groups at 60 minutes.

DISCUSSION

The goal of this experiment was to elucidate the protective effects of stress experienced during adolescence to the development of stress-induced mood disorders that persist into adulthood in a preclinical model. The complex nature and varied etiology of depression have made analyzing using animal models challenging. Both strains exhibited

stressed-induced changes in food intake; the control groups ate significantly less than the stressed groups. Although these changes did not persist into adulthood, they demonstrated that eating habits were affected by CMS. The forced swim test measures depressive-like behavior by using extended time periods of swim stress to induce behavioral immobility in rodents (Porsolt, Anton, Blavet, & Jalfre, 1978). Ws rats on average spent more time trying to escape than did their Ky counterparts. Only the Ws rats displayed stressed-induced changes in time spent immobile, and these same results were seen in adulthood. The Ws rat model demonstrated exaggerated behavioral responses for depressive- and anxiety-like behaviors when compared to the Ky rat strain. This confirms previous suggestions that this model is useful for analyzing stress-related disorders (Pare, 1994).

Diminished sucrose preference, demonstrated by the sucrose preference test, may indicate desensitization of the brain reward mechanism. Since anhedonia—the inability to gain pleasure from normally pleasurable experiences—is a core symptom of depression in humans, our findings suggest that the rat CMS model may be appropriate for depressive disorders (Rygula et al., 2005). The stressed groups had a dampened corticosterone level compared to their nonstressed counterparts during the stress reactivity assessment. Increased time spent in the open arms of the EPM and decreased corticosterone in females is consistent with chronic stress experiments from other studies and suggestive of anxiety-like behavior (McCormick, Smith, & Matthews, 2008). Ky rats overall exhibited more anxiety-like behavior, with no signs of stress-induced effects in both adolescence and adulthood. They remained in the closed arms of the EPM and were hesitant to explore the open arms. This indicates that Ky rat strains naturally exhibit a depressive phenotype relative to Ws rats, and we found strain differences in stress-induced changes that were persistent in adulthood. These should be considered when choosing what aspects of mood disorders are to be modeled. Based on the data collected, the Ky strain is a preferred model for measuring depressive symptoms due to a potential glass ceiling effect regarding the measures of both depressive- and anxiety-like behaviors, whereas the Ws strain would be the better model for assessing treatment susceptibility seen through stress-reactivity tests. The inclusion of female animal models in preclinical trials is needed and will contribute to gender-based approaches for the diagnosis and treatment of depression in females.

ACKNOWLEDGMENTS

We would like to express our appreciation to Melissa McCurley, Brent Bachman, Sherrie Adjei, and Kelsey Lynch for assisting with the study.

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