Chronic Stress and RCAN1 Expression

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Abstract

Alzheimer's Disease (AD) is a form of age-related neurodegeneration that occurs sporadically and affects 5.4 million individuals in the United States alone. Individuals with Down Syndrome (DS) develop the neuropathology of AD, suggesting the overexpression of genes on chromosome 21, like Regulator of Calcineurin1 (RCAN1), play a role in AD. RCAN1 is under the control of a stress-response promoter, but it is unknown whether chronic stress (CS) causes an elevation in RCAN1 levels. Here we show a mild CS paradigm is successful at promoting increased anxiety and elevating stress hormone levels, through behavioral tests and glucocorticoid analysis. This is a first step in looking at CS and RCAN1 expression. Based on these findings we propose to test the idea CS causes elevated glucocorticoid levels and induces RCAN1 expression in the brain to increase.

Introduction

Alzheimer's Disease (AD) is the most prevalent form of age-related neurodegeneration. Causes of AD include environmental, genetic, and lifestyle related factors, but it is thought that less than 5 percent of AD is caused by specific genetic changes [1, 2]. The greatest identified risk for AD is aging. However, most cases of AD occur sporadically, that is the underlying causal factors are unknown.

AD causes brain cell degeneration and neuronal cell death, destroying memory and mental function in individuals who express the AD phenotype [1, 2]. Individuals suffering from AD are susceptible to symptoms that often worsen as the disease progresses, including but not limited to: memory loss, difficulty thinking and concentrating, loss of communication abilities, changes in personality and behavior, reduced planning abilities, inability to do familiar tasks, decline of judgments and decision making skills, decreased spatial awareness, and loss of surrounding [1].

Two brain abnormalities, considered histopathological hallmarks of severe AD, are Amyloid-Beta protein plaques (AB Plaques) and Tau protein tangles [1,2]. AB Plaques interfere with synapses important for communication between cells,

eventually destroying synapses in the brain [1,3]. Tau protein tangles made from hyperphosphorylated tau damage the microtubule transport system necessary for carrying nutrients and other materials between nerve cells, leading to cell death [1,4]. Both AB Plaques and Tau protein tangles are thought to contribute to cognitive function loss seen in AD. Individuals with Down Syndrome (DS) display early onset of AD neuropathology and provide an opportunity to research genetic factors associated with AD.

Regulator of Calcineurin1 (RCAN1) is a gene located on chromosome 21 that codes for a protein that interacts with calcineurin to inhibit calcineurin-dependent signaling [5]. DS is caused by trisomy 21, so RCAN1 is overexpressed in DS individuals and DS model mice, as well as individuals with sporadic AD, and the elderly[6,7]. RCAN1 accumulation has been linked to oxidative stress [8, 9, 10] and mitochondrial dysfunction [11, 12, 13], features of AD, as well as being linked to aging and AD itself [6, 10, 11]. Specifically, overexpression of RCAN1.1S, a protein isoform of RCAN1, has been shown to induce hyperphosphorylation of tau, aggregate formation, synapse irregularities, and apoptosis, all features of AD pathophysiology [14, 15, 16, 17]. A mouse model has been developed ($RCAN1^{TG}$ mice) that overexpress the RCAN1 isoform RCAN1.1S. These mice have been shown to develop age-dependent cognitive and synaptic impairments, consistent with age-dependent dementia in AD [10]. RCAN1 expression is controlled by a stress response promoter induced by chronic stress (CS). Overexpression leads to RCAN1-mediated neurodegeneration.

It is hypothesized that RCAN1 overexpression due to CS contributes to AD pathophysiology, promoting cognitive deficits within the disease itself. In order to test this, mice were exposed to a CS paradigm that mimics CS in humans. Behavioral tests and glucocorticoid analysis were carried out to verify the CS paradigm was successful and mice displayed anxiogenic behavior. Further research efforts will seek evidence of elevated RCAN1 levels in the brain.

While there is no current cure for AD, identifying biomarkers such as RCAN1 may facilitate early diagnosis and treatment of the disease. It is currently unknown whether expression of RCAN1 alone is sufficient to drive brain alterations leading to

an AD phenotype or leads to an increased pre-disposition to AD, however further research may determine a correlation, if any. Research has been done at the University of Colorado, Boulder in the Molecular Signaling of Neurological Disorders Laboratory involving RCAN1 and its causative association with AD. Research for this project has been developed from peer-reviewed research articles as well as unpublished data from the Hoeffer Lab.

Methods

IACUC protocol 1311.02 (January 2014-January 3, 2017) was used throughout the entire experiment. C57 wild type (WT) mice aged 3-4 months were exposed to a CS paradigm designed to mimic chronic stress in humans [18, 19]. Following CS, the following were assessed: (1) elevated plus maze and open field activity for stress-affected behaviors. These tests were used to assess mouse correlates of anxiety and depression, behaviors impacted by exposure to CS [20, 21]. (2) Enzyme-linked immunosorbent assay (ELISA) for serum corticosterone levels were used for measurement of hypothalamic–pituitary–adrenal (HPA) axis activation. HPA axis activation measures stress response hormonally and was used to corroborate behavioral tests [22]. Mice exposed to the CS paradigm were compared against age-/sex-matched littermates not exposed to the paradigm.

Chronic Stress Paradigm

CS treatment lasted 30 days, in which the mice were exposed to behavioral stressors including intermittent inescapable shock, predator odor, and forced restraint [20, 21, 23]. These treatments were unpredictable and sporadic throughout the 30-day period.

Mouse, open field analysis (OFA) standard procedures

Animals to be tested were removed from their homeroom and brought to the behavioral testing area in home cages. The mouse was placed in the center of a clear, Plexiglas chamber (43X43X18 cm). A Plexiglas lid with 28 1-cm holes was put in place to cover the chamber. The animal remained in the chamber for 10 minutes to explore the novel environment. A computer program, Noldus, monitored the

exploration of the animal. After 10 min, the animal was removed from the chamber and returned to its home cage. The Plexiglas chamber was wiped clean with ethanol and water in-between trials. After the mice were tested they were returned to their homeroom in their home cages [24].

Mouse, elevated plus maze (EPM) standard procedures

Animals to be tested were removed from their homeroom and brought to the behavioral testing area in home cages. The elevated plus maze consists of four runways (5 cm x 30 cm) arranged perpendicularly and elevated 38 cm off of the ground. 15.5 cm white metal walls enclose two arms while the other two arms remain open. The animal was placed in a bottomless start box in the center of the elevated maze. The start box was lifted, allowing the mouse to move into the opened or closed arms. The animal remained in the maze for 10 minutes. A computer program, Noldus, monitored the exploration of the animal. After 10 minutes the animal was lifted from the maze and returned to its home cage. The maze was wiped clean with ethanol and water in-between trials. After the mice were tested they were returned to their homeroom in their home cages [24].

ELISA

Collection

Fecal samples were collected throughout the CS paradigm for analysis by Arbor Assays DetectX Corticosterone Enzyme Immunoassay Kit. Fecal samples were collected twice a week and stored in the -80°C freezer until ready for preparation.

Sample Preparation

Samples were weighed and 1mL of Ethanol was added for every 0.1gm of feces. The maximum amount of ethanol used was 1mL. Samples were shook vigorously in the cold room for 30 minutes, then centrifuged at 5,000 rpm for 15 minutes at 4°C. Supernatant was transferred to clean tubes. Supernatant was evaporated to dryness in a SpeedVac and dried extracted samples were stored in a desiccator in the -80°C freezer. When ready to run a plate, extracted samples were dissolved in 100uL Ethanol and 400uL Assay Buffer (AB), vortexed and allowed to sit for 5

minutes 3 times. The ethanol content was brought to below 5% by diluting with AB [25].

ELISA

The DetectX Corticosterone Enzyme Immunoassay Kit quantitatively measures corticosterone present in fecal samples. A standard curve was made from a provided corticosterone stock. Standards and samples were pipetted into a clear micotiter plate coated with antibodies to capture sheep antibodies. A corticosterone-peroxidase conjugate was added to wells. A polyclonal antibody to corticosterone was added to the wells to initiate binding and allowed to incubate for 1 hour. The plate was then washed and substrate added to react with bound corticosterone peroxidase conjugate. The reaction is then stopped and the intensity of color is read in a plate reader at 450nm. The concentration of coritocosterone in the samples was calculated based off the standard curve, blanks, and controls [25].

Data Analysis

Simple comparisons between experimental and control mice in weight, EPM, OFA, and ELISA were done with two-tailed independent student t-tests. Outliers were removed as necessary.

Results

Weights

Mice were weighed following conclusion of the CS paradigm and before behavior. It was observed that over the course of behavior testing, CS mice had a trend of increasing weight. No significance was found between differences in weight.

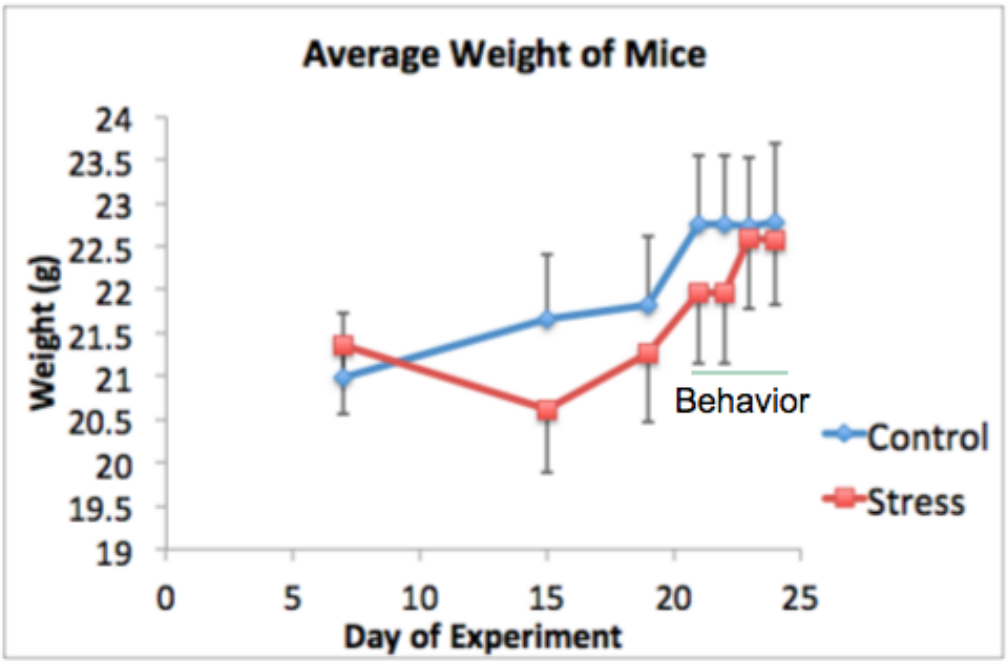

Figure 1.
Average weights of control (n=16) and CS (n=16). An independent t-test was conducted to compare average weights of control mice and mice exposed to CS. No significance was found.

OFA

To examine whether CS caused anxiety-related behaviors, OFA was carried out. Data showed CS mice spent significantly less time in the center, t(30)=2.75, p=0.0222, than controls, suggesting CS mice had more anxiety. This result was confirmed by the significantly reduced total distance moved in CS mice, t(30)=2.75, p=0.0018, compared to controls.

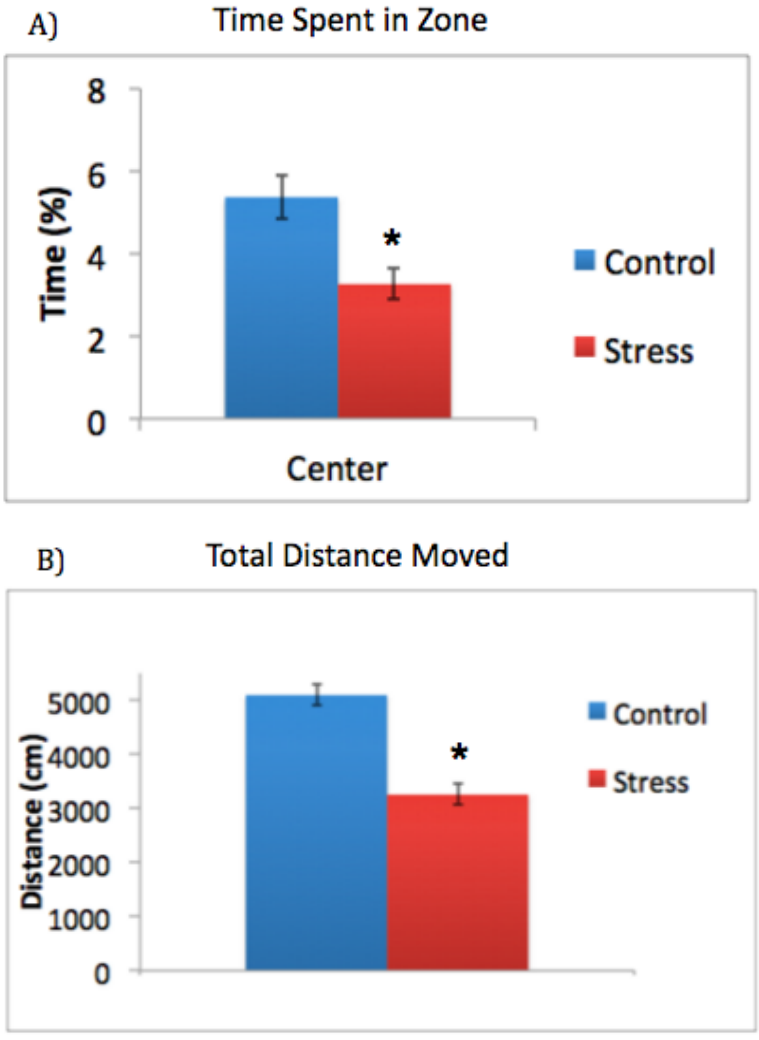

Figure 2.

A) Time spent in zones during OFA testing for control (n=16) and CS (n=16). An independent t-test was conducted to compare time spent in each zone for control mice and mice exposed to CS. There was a significant difference in the time spent in the center zone, t(30)=2.75, p=0.0222, with stressed mice spending less time in the center zone.

B) Total distance moved during OFA testing for control (n=16) and CS (n=16). An independent t-test was conducted to compare total distance moved of control mice and mice exposed to CS. There was a significant difference in the total distance moved t(30)=2.75, p=0.0018, with stressed mice moving less.

EPM

Behavior of mice was then tested in EPM. Compared to controls, CS mice spent significantly reduced time in the open arms of the EPM, t(30)=2.042, p=0.0382, instead staying in the closed arms. This is similar to OFA data. Also consistent with OFA data, CS mice moved significantly less overall, t(30)=2.75, p=0.0033, compared to controls. Time spent in closed arms of the EPM was near significant, p=0.065875, with CS mice spending more time in closed arms. Combined with OFA results, EPM behavior of CS mice support CS causing anxiogenic behavior.

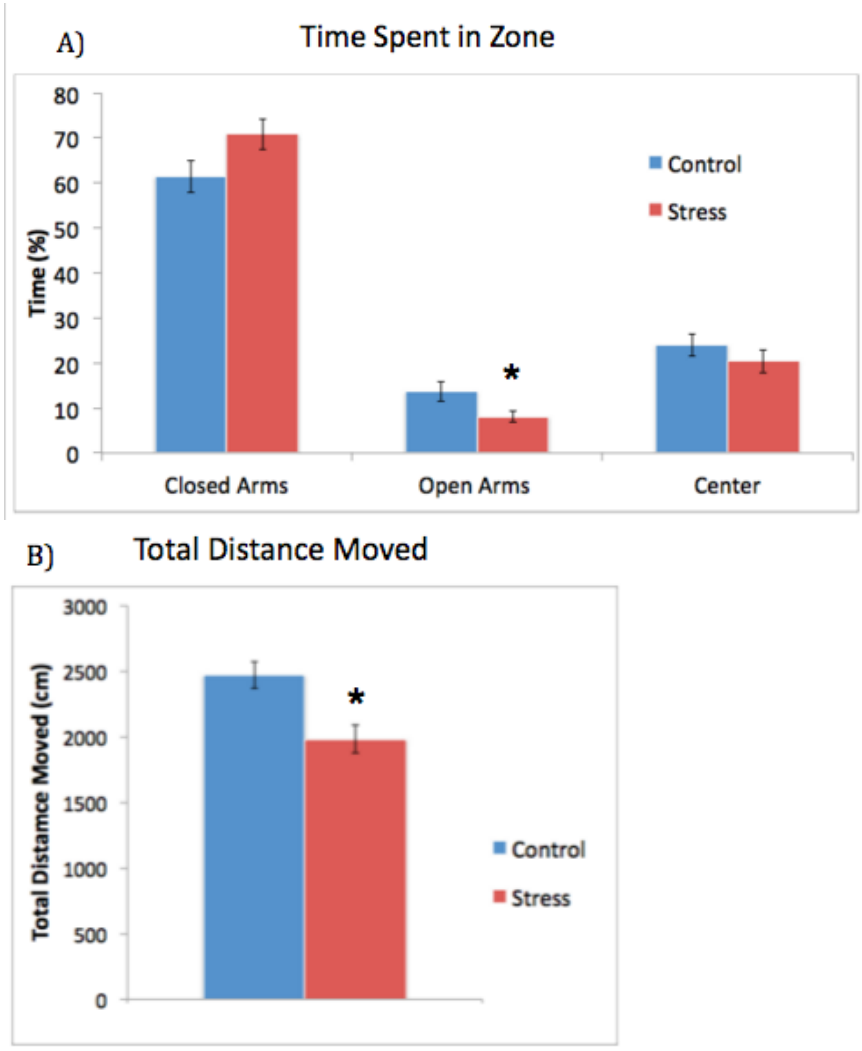

Figure 3.

A) Time spent in zones during EPM for control (n=16) and CS (n=16). An independent t-test was conducted to compare time spent in each zone for control mice and mice exposed to CS. There was a significant difference in time spent in the open arm, t(30)=2.042, p=0.0382, with stressed mice spending less time in the open arm.

B) Total distance moved during EPM testing for control (n=16) and CS (n=16)An independent t-test was conducted to compare total distance moved for control mice and mice exposed to CS. There was a significant difference in total distance moved, t(30)=2.75, p=0.0033, with stressed mice moving less.

ELISA

ELISA was run to test glucocorticoid concentration. Data show male CS mice had almost equal corticosterone concentrations 6 days into the CS paradigm compared to controls. By day 17 of the paradigm, male CS mice have significantly higher corticosterone concentrations, t(30)=, p=0.0246, compared to controls. Along with behavior data, ELISA results support the idea that the CS paradigm was successful, causing increased glucocorticoid levels and anxiogenic behavior in mice.

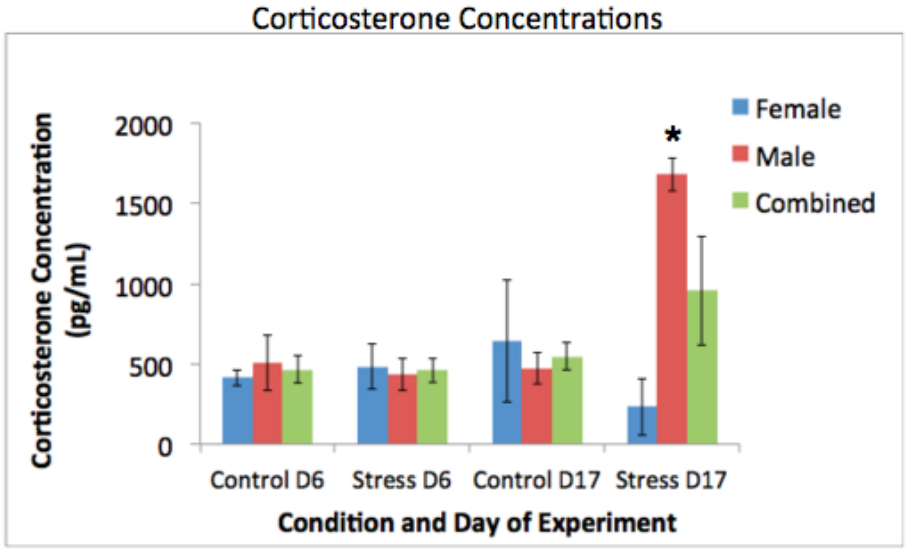

Figure 4.
Corticosterone concentrations of control (n=16) and CS (n=16) at day 6 and 17 of the CS paradigm in males and females, as well as combined sexes. An independent t-test was conducted to compare corticosterone concentrations between control mice and mice exposed to CS on the same day of the CS paradigm. There was a significant difference in corticosterone concentration in males on day 17, t(30)=, p=0.0246, with stressed males having higher corticosterone concentration.

Discussion

Following a 30-day CS paradigm, two behavioral tests measuring anxiety in rodents showed mice exposed to CS spent less time in exposed areas, indicative of increased anxiety. Using ELISA to measure glucocorticoid levels, increased anxiety due to CS was used to confirm behavior results.

CS Paradigm

While we were fairly happy with the CS paradigm, it was observed in a previous cohort that mice repeatedly exposed to predator odor appeared to physically, though not physiologically, habituated to predator odor. To combat this, mice were conditioned to the odorant using intermittent inescapable shock to heighten aversion to the odor. One drop of predator odor was added during intermittent inescapable footshock. During odorant stress, the mice will likely undergo extinction, then renewal during footshock experiments. We hypothesize that this slight change to the paradigm resulted in a significantly more stressful experience for the experimental mice.

Weights

Based on data from a previous cohort of this experiment, it was hypothesized that the absence of CS allowed CS mice to normalize weights to control counterparts. In a previous cohort CS mice were significantly smaller in weight than controls immediately after CS, and significance in weight differences declined as behavior was carried out, possibly due to the absence of CS. Here we find no significant difference in weights between experimental conditions, but observe a trend that CS mice gain weight as behavior goes on, eventually reaching weights similar to controls. Again, it is hypothesized that the absence of CS allows CS mice to normalize their weights, but a larger sample size is needed to confirm this trend in weights following the CS paradigm.

OFA

OFA data show mice exposed to the CS paradigm spent significantly less time in the center of the arena compared to controls and moved significantly less overall. This is expected behavior because mice with increased anxiety freeze more frequently

[27]. Together, these results are consistent with previous research showing CS causes increased anxiety in mice [20, 26].

EPM

EPM data show mice exposed to the CS paradigm spent significantly less time in open arms compared to controls. Similar to OFA, this is consistent with previous research showing CS causes increased anxiogenic behavior [20, 26]. CS mice also moved significant less overall, compared to controls. Again, more frequent freezing is expected in CS mice due to increased anxiety [27]. Time spent in the closed arms of the EPM by CS mice was almost significant compared to controls. This is also expected as closed arms are a safer environment than open arms, and more frequent freezing is expected in mice exposed to the CS paradigm [27]. The number of crossings between zones was similar for CS and control groups, showing a locomotor deficit does not explain increased closed arm time, but rather CS causes increased anxiety. Additionally, when closed arm time and center time are combined, CS mice spend significantly more time in the closed arm+center, compared to controls. The center area of the EPM is a kind of middle ground between the safe environment of the closed arms, and vulnerable environment of the open arms. It is hypothesized that CS mice do not find the center as anxiety-inducing as the open arms, and therefore spend more time there.

ELISA

We hypothesized that CS would increase glucocorticoid levels in both male and female CS mice, however our data does not fully support this. While male CS had a significant change in corticosterone concentration over the course of the CS paradigm, female CS did not. A possible explanation for why female CS mice did not have elevated glucocorticoid levels is they were not estrous cycling together and different hormone levels effected their glucocorticoid levels. Although female mice were housed together, they were not together since birth, causing variation in estrous cycling. In a previous study it was shown that estrogen, specifically estradiol, impairs the ability of dexamethasone (DEX) to inhibit the rise in corticosterone during the stress-induced rise in corticosterone, suggesting estradiol causes a disregulation of HPA axis negative feedback [28]. DEX is a synthetic

glucocorticoid that acts competitively with natural corticosterone in non-human mammals [29]. Because female mice were not cycling together it is possible that estrogen levels varied across subjects, and inhibition of the rise in corticosterone was not occurring simultaneously, causing variation in corticosterone levels, unlike in male CS. Research to find ways to get female mice to cycle together is underway, but implementing the task could be difficult.

Future Direction

Here it has been shown that the CS paradigm implemented was successful in causing increased anxiety in mice. While glucocorticoid levels were only significantly increased in male CS, results from OFA and EPM support evidence of anxiogenic behavior in both male and female CS. Whether this is through glucocorticoid signaling remains elusive, as only male CS mice showed significant changes in corticosterone levels throughout CS. Further research will need to be done to confirm CS causes increases in glucocorticoid signaling in both male and female mice.

Access to voluntary exercise is another condition that will be added to future cohorts to see if it will have any effect on RCAN1 levels following CS. Wheels in home cages will be attached to computer software to monitor the activity of the mice. It is hypothesized that CS mice might use exercise as an outlet to combat the CS and cause smaller increases in RCAN1 levels.

So far it is unknown whether RCAN1 levels were impacted by CS. To answer this, brain protein from three experimental test groups (immediate, aged, old) will be isolated and western blotting will determine if RCAN1 isoform expression is increased in the brain following CS [24, 30]. If found to be correct, the hypothesis that CS increases RCAN1 levels via glucocorticoid signaling will be confirmed. If CS is shown to cause increases in RCAN1 expression, and accumulation of RCAN1 has been previously linked to AD neuropathology [8, 9, 10, 11, 12, 13], this suggests individuals predisposed to AD should avoid chronic stress in order to keep RCAN1 accumulation in the brain at healthy levels.

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