Repeated assessment of anxiety-like behavior in mice: a new tool with increased reliability and consistency.

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Abstract

Stress-related illnesses such as major depressive and anxiety disorders are characterized by maladaptive physiological or behavioral responses to stressful life event. Chronic stress-based animal models have provided critical insight to a better understanding of these responses. However, currently available behavioral assays measuring chronic stress-induced phenotype in mice are limited in their design (short, not repeatable, subject to experimenter-bias) and are often inconsistent. Using the Noldus PhenoTyper apparatus, we developed a new tool to repeatedly assess behavioral changes induced by chronic stress exposure in two mouse models i.e. chronic restraint stress (CRS) and chronic unpredictable mild stress (UCMS). The PhenoTyper test consists on monitoring in home-cage setting the overnight animals' behavior before, during and after a 1hr light challenge is applied over the food zone. After, characterization of the test, we compared the reproducibility and reliability of the PhenoTyper test in assessing the effects of chronic stress exposure, with commonly-used tests such as the elevated plus maze, open-field, novelty suppressed feeding and novelty-induced hypophagia. We found that while stress mice display heterogeneous profiles in these tests, CRS- and UCMS-exposed mice showed a very consistent response in the PhenoTyper test. Indeed, CRS and UCMS mice continue avoiding the lit zone in favor of the shelter zone. This behavior, or residual avoidance after the light challenge, lasted for hours beyond termination of the challenge, was not observed after acute stress and was consistently found throughout the chronic stress exposure in both models. Chronic stress-induced residual avoidance in the shelter was alleviated by chronic imipramine treatment but not acute diazepam administration. This new tool should be instrumental in design of longitudinal studies aiming to better understanding the trajectory of chronic stress-induced deficits in animal models and potentially screen novel anxiolytic and antidepressant treatments.

1. Introduction

Depression is among the most debilitating disorders in the world (Friedrich, 2017) and commonly co-occurs with anxiety disorders (Hirschfeld, 2001). The proportion of the global population living with depression and anxiety recently reached 4.4% and 3.6%, respectively (WHO, 2017) with ~85-90% of diagnosed patients suffering from both conditions (Tiller, 2013). Human studies have been useful to identify biological deficits associated with these disorders, and since the early 1980's, great progress has been made in elucidating pathological mechanisms and exploring new avenues for therapeutic interventions through using animal models (Katz and Hersh, 1981, Katz et al., 1981Cryan and Sweeney, 2011).

The most popular rodent models employ exposure to chronic stress to study the psychopathology of mood disorders including depression (Vyas et al., 2004, Chiba et al., 2012, Eiland and McEwen, 2012, Willner, 2017a). The unpredictable chronic mild stress (**UCMS**) model has strong face and construct validities for modeling in rodents behavioral dimensions relevant to human stress-related illnesses(Willner, 1997, 2017a). Similarly, chronic restraint stress (**CRS**) or immobilisation stress paradigms are often used to study morphological, cellular, and molecular mechanisms of stress-related disorders, including changes in neuronal spine density, cell signaling, or neurotransmitter systems that parallel human cellular pathologies associated with major depressive disorders (McEwen and Magarinos, 1997, Radley et al., 2006, Qiao et al., 2016). Several lasting behavioral and physiological changes have been reported in animals exposed to UCMS and CRS, as well as other models such as social defeat stress (Nestler et al., 2002). For instance, past studies demonstrate that exposing mice or rats to chronic stress paradigms leads to persistent cognitive deficits (Sandi, 2004), helplessness- (Strekalova et al., 2006), anhedonia- (Strekalova et al., 2006), and anxiety-like (Piantadosi et al., 2016) behaviors; however, these findings have been criticized for being difficult to replicate (Cryan and Sweeney, 2011, Willner, 2017b, a, Castanheira et al., 2018, Ferreira et al., 2018). Recently, one survey of 71 experimenters employing UCMS paradigms found that 21%

experienced difficulties replicating expected results (Willner, 2017b). The lack of reliability, or the inability to induce expected phenotypes, is often attributed to individual variability (e.g., strain, sex, or rearing conditions) or non-optimal methods for assessing chronic stress-induced phenotypes. Nevertheless, some responsibility for this lack of reproducibility can be attributed to the behavioral tests used. A vast majority of behavioral tests employed to study the effects of chronic stress measure anxiety-like behaviors, and were primarily designed to detect anxiolytic and antidepressant drug efficacy in non-stress animals (Dawson and Tricklebank, 1995, Reneric and Lucki, 1998, Lucki et al., 2001). These tests are also limited by protocol variability within and across labs, experimenter bias, and the requirement for novelty precluding repetitive measurement over chronic time courses.

There are currently >15 different assays regularly employed to assess anxiety-like behavior and adapted to investigate the effects of chronic stress in rodents (Ohl, 2005), with most being relatively short (5-30 minutes) and highly susceptible to varying experimental conditions. Indeed, most commonly-used behavioral tests employ approach-avoidance paradigms (Bailey and Crawley, 2009) that rely on conflict between animals' innate exploratory drive and their aversion to a threatening environment (Rodgers, 1997). Understandably, repetitive testing of these assays is often unsuccessful due to losing the essential novelty component as animals habituate to environmental conditions across sessions. Because of this, these behavioral tests are limited in their ability to provide a time-dependent trajectory of phenotype development, maintenance, or reversal that can be of primary importance in studying chronic stress.

For a better understanding of the dynamic progression of behavioral deficits induced by chronic stress, it is necessary to develop experimenter-free, repeatable, reliable, and effective tests measuring chronic stress-induced behavior in rodents. Here, we first aimed to demonstrate the limitations of commonly-used tests using two paradigms in mice, UCMS and CRS. Then, we set out to demonstrate the usefulness of the PhenoTyper test, a new tool designed to assess chronic stress-induced behavioral emotionality repetitively, reliably, and with minimal

experimenter bias, based on the animal's activity in response to an aversive challenge in a home-cage like setting. Finally, we validated this test following the four requirements established by McKinney and Bunney (1969). Specifically, we demonstrated face, construct, and predictive validities by accurately measuring between-group differences for control/UCMS and control/CRS mice, as well as reversal with anxiolytic or antidepressant treatment. We further demonstrated reproducibility of our test both within (weekly characterization) and across experiments. In parallel, we also showed the ability of the test to assess chronic stress and antidepressant treatment behavioral trajectories.

2. Material and Methods

2.1. Animals:

Eight-week old C57B6 mice (Jackson Laboratory, Bar Harbor, Maine, USA; 50%♀) were housed under normal conditions with a 12hr light/dark cycle and provided with *ad libitum* access to food and water. All animals were kept for 1-2 weeks in the facility before the start of the experiments for habituation. Experimenters were blind to treatment group assignments during behavioral testing. All procedures followed guidelines set by the Canadian Council on Animal Care (CCAC). Six mouse cohorts were used in this study. One cohort was used to assess baseline behavior and light challenge response in the PhenoTyper apparatus (n=12/group, 50% females). The second experiment measured the effects of acute restraint stress (**ARS**) in this test (n=8/group, 50% females). Experiment 3 and 4 tested the effects of CRS and UCMS respectively and the last two mouse cohorts were used to assess the efficacy of acute diazepam (anxiolytic) or chronic imipramine (antidepressant) treatment in CRS animals.

2.1.1. Unpredictable chronic stress (UCMS):

Mice were subjected to randomized stressors (3-4/day) for 5 weeks (Nikolova et al., 2018). These stressors included: forced bath (~2cm of water in the cage for 15min), wet bedding, aversive smell (20min to 1hr exposure to fox or bobcat urine), light cycle reversal or disruption,

bedding exchange (rotate mice into previously occupied cages), tilted cage (45º angle), reduced space, restraint (50mL falcon tube with nose/tail holes for 15-30min), bedding change (replacing soiled with clean bedding), no bedding, or nestlet removal. Control animals were group-housed (~3-4/cage) to eliminate the stress of single-housing (n=12/group, 50% females).

2.1.2. Restraint stress:

Animals were placed into 50mL falcon tubes with nose/tail holes for air flow. ARS consists in one session of 1hr before testing. For CRS, restraint sessions occurred twice daily for 1hr during the light cycle (7:00am-7:00pm), separated by a minimum of 2hrs. CRS-exposed animals were single-housed throughout the stress exposure and control animals were group-housed (n=12/group, 50% females). One CRS female mouse died during the experiment and was removed from the study.

2.1.3. Drug administration:

After 3 weeks of CRS exposure, mice received diazepam (1.5 mg/kg i.p.) or vehicle (0.9% saline) (n=10-12/group, 50% females) acutely 30min before being placed in the PhenoTyper or NSF arenas. Tests were performed 48hrs apart. To assess if CRS effects were reversible by chronic antidepressant treatment, mice subjected to CRS for 3 weeks received imipramine (~15mg/kg in drinking water based on ~8mL/day fluid consumption) for the 3 following weeks while continuing CRS exposure (n=12/group, 50% females). Freshly made imipramine was provided every other day to prevent deterioration due light exposure or room temperature. Animals were tested weekly in the PhenoTyper test and once in the NSF, 48hrs after the last PhenoTyper test.

2.2. Body weight and coat state:

Body weight and fur coat deterioration of mice were assessed weekly. Weight gain was calculated using baseline weights (week 0, before chronic stress exposure), and expressed in percentage of change. Fur coat deterioration was measured using a rating scale of 0-1, with 0 equivalent to a well-groomed, smooth coat and 1 equivalent to a tousled or soiled coat with bald patches (Nollet et al., 2013). Coat quality was assessed for 7 anatomical areas (head, forepaws, hindpaws, back, neck, tail, and abdomen) and averaged for a single score of coat state deterioration per animal.

2.3. Behavioral Tests:

Following 5 weeks of stress exposure, mouse behavior was assessed in a series of commonly-used tests measuring chronic stress-induced behavioral phenotype: the elevated plus maze (EPM), open field (OF), novelty-induced hypophagia (NIH), and novelty-suppressed feeding (NSF) tests. Tests were spaced 48hrs apart to minimize between-test interaction. On non-testing days, CRS or UCMS were resumed to maintain stress-induced profiles. Tests were performed a minimum of 12hrs after the last stressor.

2.3.1. EPM test:

The EPM apparatus consist of four Plexiglas arms, where two open arms (67x7 cm) and two enclosed arms (67x7x17 cm) form a plus shape with similar arms facing opposite each other. The EPM is situated 95cm above the floor within a dimly lit room (20 lux). Mice are placed in the center of the intersecting arms, facing an open arm, and are allowed to explore the apparatus for 10min. A digital camera mounted on a rod recorded animal behavior from above. *A posteriori* measurement of time spent and the number of entries into open and closed arms is performed using ANYmaze (Stoelting Co.™, Wood Dale, IL, USA).

2.3.2. OF test:

The OF apparatus consists of a 50x50cm chamber that each mouse explores freely for 10min. Time spent and total entries into the center of the arena (digitally defined 20x20cm zone in the center of the apparatus) were measured. A digital camera mounted on a rod recorded animal behavior from above. *A posteriori* measurements were performed using ANYmaze.

2.3.3. NSF test:

Following ~16hrs food deprivation, mice are placed in a novel arena (62 x 31cm dimly lit enclosure) containing a single food pellet. Latency to feed on the pellet is measured for a 12min period. To control for appetite drive and/or food deprivation-induced weight loss, latency to feed was measured in the animal's home cage following the novel environment test for a maximum of 6min.

2.3.4. NIH test:

Mice are habituated to a palatable liquid (1mL of 1:3 sweetened condensed milk in water) in a sterilized Petri dish for 3 consecutive days. On the following 2 days, mice are timed for their milk approach and drinking latency across 2 test sessions. The first session occurs in the home cage under normal light conditions and serves as a control for potential treatment effects on appetite or activity. Home cage latency to consume the milk from the Petri dish is recorded. 24hrs later, the subsequent test session occurs in a similar but new cage and under bright lighting (500-600 lux). Latency to consume measured during the second test (novel environment) is used as an index of hyponeophagia. Tests sessions occur over a maximum duration of 10mins.

2.3.5. PhenoTyper™ test:

The Noldus™ PhenoTyper apparatus (Leesburg, VA, USA) is a commercially-available observation cage designed for video-tracking of regular mouse behavior over extended periods using the Noldus EthoVision 10 software. The apparatus contains an integrated infrared

sensitive camera that tracks activity and time spent by the animal in customizable zones. We established two designated zones, a food zone (6.5x15 cm) and a shelter zone (10x10cm), and tracked the animals' location throughout the dark cycle (19:00-07:00). The apparatus also contains a white LED spotlight placed above the food zone, a feature that we employed to test whether animals would alter normal behavior in response to the application of an aversive "light challenge". Specifically, we set the spotlight to turn on 4hrs into the dark cycle for a 1hr period (11:00pm-12:00am). This time-window was strategically chosen for being within a naturally high plateau for time spent in the food zone by the animal. For chronic stress studies, the PhenoTyper test was performed weekly on days 0, 7, 14, 21, 28, and 36. For all experiments, food and shelter zone time was measured in 1hr bins.

Based on the time spent in food and shelter zones after the light challenge, an index of avoidance was calculated as a function of the control group's response to the light challenge. This calculation was named Residual Avoidance (RA) and considers the difference between the animals' response during the light challenge and the sum of time spent avoiding the lit zone for the following 5hrs. The 5hrs cut-off was chosen to cover the time window of potential lasting response to the light challenge but exclude the last 3hrs of the dark cycle when animal's time spent in shelter or food zones plateau to the maximum and minimum, respectively. RA was calculated for each mouse as followed:

In the food zone:

$[1-(\Sigma\ Time_{(12am-5am)} - Time_{(11pm-12am)})/\ Average\ control\ group\ (\Sigma\ Time_{(12am-5am)} - Time_{(11pm-12am)})] * 100$

In the shelter zone:

$[(\Sigma\ Time_{(12am-5am)} - Time_{(11pm-12am)})/\ Average\ control\ group\ (\Sigma\ Time_{(12am-5am)} - Time_{(11pm-12am)})-1] * 100$

Residual avoidance provides information about how animals react after the light challenge, relative to the designated control group. Control group animals have a mean RA=0, while positive RA means that animal display avoidance for either food or shelter zones from the zone light challenge.

2.4. Statistics:

Statistical analyses were performed using Statview software (SAS Institute Inc., Cary, NC, USA). Student's t-test was used to determine statistical differences between groups for commonly-used behavioral tests. Repeated-measures ANCOVA (analysis of covariance) was used for within- and between-subject evaluation of behavioral changes in the PhenoTyper test, and for time course analysis of longitudinal assessments including weight gain, coat state, or weekly RA. Sex was included as a covariate for all analyses. Stress and drug effects were assessed using repeated measures ANCOVA for RA data and two-way ANCOVA for NSF data. Significant results were followed-up with *post-hoc* Fisher's test. In addition, summary scores capturing behavioral emotionality dimensionally across groups were generated using principal component analysis (PCA) on 16 major behavioral variables collected throughout the last week of testing. Variables included all key behavioral parameters from commonly-used tests, as well as before and during challenge food and shelter times and RAs for both zones. For longitudinal measures, the PCA included the last readout only. All sets of variables were submitted to PCA with a varimax rotation based on a correlation matrix with mice as one experimental unit. Two-way ANCOVA and *post-hoc* analysis was used to determine stress and sex contributions to the first 3 components. For results interpretation, individual variable loadings onto each of the newly derived components was examined and loadings >0.4 were considered significant. PCA and follow up analysis was performed using SPSS software (IBSM SPSS statistic 24).

3.Results

3.1. Commonly-used behavior tests assessing chronic stress-induced behaviors revealed heterogeneous profiles between tests and models

Mice were exposed to CRS (**Figure 1 A-F**) or UCMS (**Figure 1 G-L**) for 5 weeks before behavioral assessment using the EPM, OF, NSF, and NIH. Coat state and weight gain were assessed before and throughout stress exposure.

3.1.1. CRS:

Repeated-measures ANCOVA of coat state scores revealed a significant main effect of CRS ($F_{(1;105)}$=15.76; p<0.001), time ($F_{(5;105)}$=14.09; p<0.0001) and a stress*time interaction ($F_{(5;105)}$=10.5; p<0.0001) (**Fig. 1A**). *Post-hoc* analysis identified a significant increase in coat state deterioration induced by CRS exposure from week 1 to week 5 (all p<0.05). A significantly sex difference was found, wherein an exacerbated effect of CRS exposure was detected in males compared to females ($F_{(1;95)}$=97.64; p<0.0001) between weeks 2 and 5 *(p*<0.001; **Fig. S1**).

In the same cohort, the analysis of weight gain showed a significant effect of CRS ($F_{(1;105)}$=12.04; p<0.01), time ($F_{(5;105)}$=15.61; p<0.0001) and a stress*time interaction ($F_{(5;105)}$=7.9; p<0.0001). A significant decrease was found in weight gain induced by CRS exposure from weeks 1 to 5 *(p*<0.05, **Fig. 1B**). When sex was considered as a covariate, a significant effect was found, showing exacerbated effects of CRS exposure on weight gain in CRS-exposed males compared to females ($F_{(1;95)}$=18.78; p<0.001), and compared to control mice starting weeks 2 to 5 of CRS exposure (p<0.05; **Fig.S1**).

In the OF test **(Fig. 1C)**, neither the percentage of time in the center, nor the number of entries in the center (**Fig. 1B**) revealed effects of CRS. In the EPM test (**Fig.1D**), mice exposed to CRS displayed a non-significant change in the percent of time spent and entries in open arms. Lastly, NSF and NIH tests did not detect significant differences in the latency to feed or drink, respectively, in home cage or novel environments (**Fig. 1E-F**). Covariate analysis for sex

revealed no main effects or interaction with stress in the EPM and NSF. In the OF test, an interaction was found ($F_{(1;19)}$=5.79; p<0.05) wherein females showed greater entries in the center than males at baseline (p<0.05), and decreased entries in the center following CRS (p<0.05). In the NIH test, ANCOVA revealed a significant main effect of sex ($F_{(1;19)}$=4.95; p<0.05), wherein *post hoc* analysis identified greater latency to drink in females than males (p=0.04; **Fig. S1**).

3.1.2. UCMS:

Repeated measures ANCOVA of coat state degradation revealed a significant main effect of stress ($F_{(1;110)}$=14.134; p<0.01), time ($F_{(5;110)}$=15.808; p<0.0001) and a stress*time interaction $F_{(5;110)}$=10.7; p<0.0001). *Post hoc* analysis identified significantly increased coat state deterioration in UCMS-exposed mice from weeks 3 to 5 (p<0.01, **Fig.1G**). Considering sex as a covariate revealed a significant effect of sex ($F_{(5;100)}$=13.9; p<0.001), wherein coat state deterioration was greater in UCMS males compared to females at weeks 2, 4 and 5 (p<0.05; **Fig. S2**). for the analysisof weight gain showed a significant main effect of stress ($F_{(1;110)}$=23.8; p<0.0001), time ($F_{(5;110)}$=24.5; p<0.0001) and a stress*time interaction ($F_{(5;110)}$=20.16; p<0.0001). A significantly decreased weight gain was found among UCMS mice compared to control mice, from weeks 2 to 5 (p<0.01, **Fig.1H**). Sex was not a significant covariate on this measure.

The same cohort was then tested in the OF test (**Fig. 1I**). Statistical analysis revealed an effect of UCMS exposure, characterized by decreased percent of time spent in the center zone (p<0.001) and a trend towards decreased number of entries into this zone (p=0.07). In the EPM time spent in open arms and percentage of open arm crosses was not significantly modified by stress (**Fig. 1J**). In the NSF test UCMS exposure induced a significant increase in latency to feed in the novel arena (p<0.05; **Fig. 1K**). However, UCMS induced a decreased latency to consume milk in the NIH (p<0.01; **Fig. 1L**). Home cage latency to drink or feed was unchanged. Sex was a not a significant covariate in the OF, EPM and NIH. In the NSF, we found a

significant sex*stress interaction ($F_{(1;18)}$=4.6; p<0.05). *Post hoc* analysis revealed increased latency to feed in UCMS females compared to UCMS males (p=0.02, **Fig. S3**).

3.2.Baseline behavior and light challenge response in the PhenoTyper test

The PhenoTyper apparatus was used to monitor food and shelter zones time of male and female mice throughout the dark-cycle. One male mouse showed intermittent missing data during tracking acquisition and was excluded from the study. At baseline, repeated measures ANCOVA revealed a significant sex effect on food zone time ($F_{1,252}$ = 4.19; p<0.05). However, *post hoc* analysis revealed significant differences only for the 4th hour of the dark cycle (p<0.01, **Fig. 2A**). No significant effect of sex was found forshelter zone time (**Fig. 2B**). It is interesting to mention that qualitatively, mice spent spend equal amounts of time in the food and shelter zones during the first 6 hours of the night, but more time in the shelter zone during the last 6 hours of the night.

One week after baseline monitoring, mice were placed in the PhenoTyper again and we assessed responses to an acute light challenge applied from 11:00pm-12:00am for half of the animals (n=12/group randomly assigned) (**Fig. 2C-D**). The analysis of food zone time revealed a significant time*"light condition" interaction ($F_{(1;252)}$ =1.971; p<0.05). The 1h light challenge significantly decreased the food zone time during this time point (p<0.05, **Fig. 2C**). ANCOVA also revealed a significant time* "light condition" interaction, wherein the light challenge induced a significant increase in shelter zone time when the light was on, compared to the behavior of animals not experiencing the light challenge (p<0.001, **Fig. 2D**). No main effect of sex or sex*stress interaction was found when sex was considered as a covariate.

3.3.ARS does not alter baseline behavior and light challenge response in the PhenoTyper test

In a separate cohort, we tested the behavioral response to a 1 hour acute restraint stress performed immediately before placing the animal in the PhenoTyper apparatus (**Fig. 2E-F**). Analysis of the food (**Fig. 2E**) and shelter time (**Fig. 2F**) did not reveal differences between groups or a time*stress interaction ($F_{(1;128)}$=1.18 p>0.29 and $F_{(1;168)}$=0.94 p>0.34, respectively), suggesting that ARS animals behaved in this test in a similar manner to control mice at baseline, during or following the light challenge. No sex difference was found.

3.4. Characterization of the trajectory of behavioral changes induced by chronic stress in the PhenoTyper test

3.4.1 Effects of CRS:

Repeated measures ANCOVA revealed a significant effect of CRS exposure after 1 week of CRS (**Fig. 3A**; $F_{(1;252)}$ = 9.49; p< 0.01) that was consistent for every following week (week 2, $F_{(1;252)}$ = 35.09; p<0.0001, **Fig. 3B**; week 3, $F_{(1;252)}$ = 19.9; p<0.001, **Fig. 3C**; week 4, $F_{(1;252)}$ = 35.56; p<0.0001, **Fig. 3D**; week 5, $F_{(1;252)}$ = 20.92; p<0.001,**Fig. 3E**). CRS-exposed mice spent more time in the shelter zone at several time points between 24:00 and 4:00 (following the light challenge) for every week of testing (**Fig. 3**). A similar analysis performed on food zone time revealed stress*time interactions at week 1 ($F_{(1;252)}$=2.29; p<0.01), week 2 ($F_{(1;252}$ =1.7; p<0.05), week 3 ($F_{(1;252)}$=2.9; p<0.001), week 4 ($F_{(1;252)}$ = 2.6; p<0.01) and week 5 ($F_{(1;252)}$ = 2.8; p<0.001) (**Fig. S3**). CRS-exposed mice spent more time in the food zone at several time points between 24:00 and 4:00 (following the light challenge) for every week of testing (**Fig. S3).** No main effect of sex or sex*stress interaction were found.

3.4.2. Effects of UCMS:

Repeated measures ANCOVA revealed a significant effect of UCMS exposure after 1 week of UCMS ($F_{(1;264)}$ = 10.172; p<0.0001) that was consistent for every following week (week 2, $F_{(1;264)}$ = 10.790; p<0.0001; week 3, $F_{(1;264)}$ = 16.34; p<0.001; week 4, $F_{(1;264)}$ = 6.569; p<0.05; week 5,

$F_{(1;264)}$ = 12.608; p<0.01) in the shelter zone. UMCS-exposed mice spent more time in the shelter zone at several time points following the light challenge (between 24:00 and 4:00) for every week of testing. Mirror results were found in the food zone, where a significant effect of UCMS was observed in week 1($F_{(1;264)}$ = 4.624; p<0.05), week 2 ($F_{(1;264)}$ = 11.546; p<0.01), week 3 ($F_{(1;264)}$ = 6.771; p<0.05), week 4 ($F_{(1;264)}$ = 5.833; p<0.05) and week 5 ($F_{(1;264)}$ = 8.413; p<0.01). No main effect of sex or sex*stress interaction was found.

3.4.3. Residual avoidance (RA) provides a summary readout of CRS- and UCMS-induced behavioral deficits in the PhenoTyper test :

Interestingly, the most striking differences between control and chronic stress-exposed groups were not found at baseline or during the light challenge, but in the hours following the light challenge. Chronic stress-exposed mice continued to hide in the shelter zone after the challenge and did not return to control group levels until later in the monitoring period. To establish a method for analyzing and presenting these post-challenge differences, we developed the “Residual Avoidance” (RA) calculation. RA illustrates the difference in shelter time post-challenge between chronic stress-exposed and control mice for each week (**Fig. 3F**). Repeated measures ANCOVA revealed a significant effect of stress exposure ($F_{(1,84)}$=49.31; *p*<0.0001). *Post hoc* analysis revealed increased RA among CRS mice for each week (all *p*>.0.001). Sex was not a significant covariate.Mirror results were obtained when RA calculations were applied to food zone time. Analysis of food zone RA revealed a significant effect of stress exposure ($F_{(1;84)}$=16.5; *p*<0.001). Here, RA was significantly increased after 1, 4, and 5 weeks of CRS (*p*<0.01, **Fig. S3**) with no sex differences on this measure.

Analysis of shelter zone RA in the UCMS model provided similar results, as summarized in **Fig.3G.** A significant effect of UCMS exposure ($F_{1;88}$=15.5; *p*<0.001) was found without sex or stress*sex interaction. UCMS induced a significant increase in RA at 2 weeks (*p*<0.01) and for

every subsequent week of testing (p<0.05). Similar analysis of food zone RA revealed a significant main effect of stress-exposure ($F_{1;88}$=6.8; p<0.05) with no main effect of sex or stress*sex interaction. Analysis identified a significant increase in RA for UCMS-exposed mice at the 4 week time point (p<0.05, **Fig. S3**).

3.4.4. Chronic stress exposure, and its interaction with sex, account for the majority of variance across 16 behavioral test parameters

We employed dimension reduction via principal component analysis (PCA) to identify the main factors contributing to the variance among behavioral tests employed in each of the abovementioned experiments, and to determine if PhenoTyper parameters such as RA belong to similar categorical dimensions as anxiety parameters measured with commonly-used tests. PCA of 16 behavioral parameters indicated 2 components capturing 25.5% (PC1) and 21.2% (PC2) of behavioral variance across CRS and control groups (**Fig. S4**). PC1 had the strongest loadings (>0.4) from anxiety/weight gain-related variables in individual tests and weaker (<0.4) notsignificant loadings from variables such as fur coat deterioration, home cage latency to feed or drink, or PhenoTyper pre-challenge behavior (**Table 1**). ANCOVA revealed that CRS-exposed mice had significantly higher PC1 scores compared to controls ($F_{(1;19)}$=22.5; p<0.0001; **Fig. 4A**) with no main effect of, or interaction with, sex (**Fig. S4**). PC2 captured variance from the majority of the commonly-used tests employed as well as that from home cage feeding or drinking, and fur coat deterioration. ANCOVA of PC2 scores revealed significant main effects of stress ($F_{(1;19)}$=12.4; p=0.002), sex ($F_{(1;19)}$=10.0; p=0.005) and a stress*sex interaction ($F_{(1;19)}$=10.0; p<0.01, **Fig. 4A and S4**), wherein CRS males had significantly lower PC2 scores than CRS females. Our results demonstrated that animals with higher PC1 scores had greater elevations in behavioral emotionality across dimensions and groups, whereas PC2 scores differentiated the influence of sex for CRS groups (**Fig. 4A**).

PCA was also performed on data collected from the UCMS cohort. In this case, PCA of 16 variables indicated 2 components capturing 27.3% (PC1) and 17.4% (PC2) of behavioral variance across UCMS and control groups (**Fig. S4**). ANCOVA of PC1 scores revealed significant main effects of stress ($F_{(1;20)}$=53.1; p<0.0001), sex ($F_{(1;20)}$=16.1; p<0.001) and a stress*sex interaction $F_{(1;19)}$=16.1; p<0.001, **Fig. 4B**). No effects of stress, sex, or and stress*sex interaction were found for PC2 scores (**Fig. S4)**. Our results indicate that animals with lower PC1 scores had greater behavioral emotionality across dimensions and groups, wherein UCMS female mice displayed lower PC1 scores than UCMS males and control animals (**Fig. 4B**).

In both experiments there were other components with eigenvalue >1, which were, however, independent of sex and/or stress (**Fig. S4**) and probably capture variance from other sources. Finally, in both studies, shelter zone RA loaded strongly on PC1, that captured the behavioral variance attributed to chronic stress, along with the variables that measured approach/avoidance conflict or aversion in the commonly-used tests, such as the entries and times in the center in the OF or the latency to feed in the NSF (**Table1**).

3.5. CRS-induced increases in residual avoidance are reversed by treatment with chronic imipramine, but not acute diazepam.

A separate cohort of mice was added to identify the effects of DZP in control and CRS conditions (Figure 4A-B). Analysis of shelter zone time revealed a significant main effect of stress ($F_{(1;504)}$=12.76; p<0.001), no main effect of drug treatment ($F_{(1;504)}$=0.15; p=0.69), and an interaction between stress*drug conditions ($F_{(1;504)}$=7.5; p=0.008). However, *post hoc* analysis revealed that CRS mice spent significantly more time in the shelter zone regardless of drug treatment (p<0.05). Despite a significant stress*drug interaction, no significant differences were identified at particular time points between control or CRS mouse group receiving vehicle or DZP. However, RA analysis revealed a significant main effect of stress ($F_{(1;42)}$=26.29; p<0.0001) and its interaction with drug treatment ($F_{(1;42)}$=4.3; p=0.04), wherein *post hoc* analysis showed

decreased RA in control animals receiving DZP (p=0.02, **Fig. 5A**). We further confirmed increased RA among CRS mice (p=0.0035) and found no significant differences between CRS animals treated with vehicle or DZP (p=0.5). No significant effect of sex was found.

To determine if DZP could reverse CRS effects in another test, mice then were tested in the NSF (**Fig. 5C**). Two-way ANCOVA revealed significant main effects of stress ($F_{(1;48)}$=24.7; p<0.001) and drug treatment ($F_{(1;48)}$=7,1; p<0.05), but no stress*drug interaction. DZP had no effects on control animals, but significantly reduced latency to feed in CRS animals (p<0.05). Consideration of sex as covariate revealed a main effect of stress ($F_{(1;44)}$=13.6; p<0.001) and a stress*sex interaction ($F_{(1;44)}$=3.3; p<0.001), but no effect of drug*stress*sex interaction for latency to feed, reflecting a general increase in latency to feed being greater among CRS females compared to males.

Finally, after 1-week washout and continued stress exposure, CRS animals were administered with a higher dose of DZP and re-tested in the PhenoTyper test. At a higher dose (3 mg/kg, i.p.), DZP again failed to reverse CRS-induced behavioral alterations (data not shown). We also assessed the effects of DZP (1.5 mg/kg, i.p) 30mins before the light challenge and found no effects of this regimen in CRS animals (data not shown).

In an additional cohort, we replicated the effects of CRS on RA throughout 6 weeks of stress exposure ($F_{(1;220)}$=72.7; p<0.0001), and assessed the effects of chronic imipramine treatment starting on day 21. We found a significant stress*drug ($F_{(1;220)}$=5.4; p=0.023) and stress*drug*time interactions ($F_{(5;220)}$=3.25; p=0.007) on RA. There was no significant effects of imipramine at week 3 (after 1 day of treatment) or at week 4 (after 1 week of treatment) in control or CRS mice (p>0.05, **Fig. 5D**). However, a significant difference between vehicle- and imipramine-treated CRS mice was identified on weeks 5 and 6 (after 2 and 3 weeks of treatment, respectively; p<0.01, **Fig. 5D and E**). No significant effect of sex was found.

48 hours later mice were tested in the NSF test. ANCOVA revealed a significant main effect of drug ($F_{(1;44)}$=4.5; p<0.05), and a stress*drug interaction ($F_{(1;44)}$=4.4; p<0.05). Chronic imipramine

had no effect in non-stress animals, but significantly reduced latency to feed in CRS animals (p<0.05, **Fig.5F**). Analysis of sex as a covariate revealed a significant main effect ($F_{(1;44)}$=14.7; p<0.001), a stress*drug interaction ($F_{(1;44)}$=7.8; p<0.01), and a drug*stress*sex interaction ($F_{(1;44)}$=8.8; p<0.01) on latency to feed. These changes were driven by an overall greater increase in latency to feed following CRS and decreases following imipramine in females compared to males.

Discussion

Our findings confirmed that mice subjected to UCMS or CRS displayed clear responses to chronic stress exposure on longitudinal physical readouts such as weight gain and coat state deterioration. However, when tested under similar experimental conditions, commonly-used tests assessing chronic stress-induced behavioral deficits such as the EPM, OF, NSF, and NIH show highly heterogeneous results between tests and stress models. Here, we designed a novel method for assessing normal exploratory behavior, response to a light challenge in a home cage-like setting, and residual avoidance following the light challenge, called the PhenoTyper test, based on many other tests named after their cognate apparatus. Mice subjected to UCMS or CRS showed a common response after the light challenge i.e. continued avoidance of the lit zone in favor of a shelter zone, lasting for hours beyond termination of the challenge. This “residual avoidance” (RA) after the light challenge was replicated across the 4 experiments performed in this study using 2 stress models and including both males and females. In addition, chronic stress-induced elevations in RA were detected rapidly, following the first/second week of UCMS or CRS exposure, and lasted throughout the experiments. RA was not altered by acute restraint stress, but was reduced by acute DZP in control conditions. Chronic treatment with imipramine for 2-3 weeks, but not acute treatment with DZP or imipramine, was sufficient to reverse CRS-induced RA. Altogether, our findings demonstrate that shelter RA is a highly consistent readout for chronic stress-induced “emotional reactivity” that responds to chronic antidepressant treatment.

The main objectives of the current study were: 1) to develop an experimenter-free, repeatable, behavioral test that would consistently detect the effects of chronic stress, 2) to compare chronic stress-induced behavioral changes among the most widely-used tests with those measured by the PhenoTyper test, 3) to determine whether the PhenoTyper test could identify similar profiles of elevated behavioral emotionality between two well-documented chronic stress models and, 4) to investigate the effects of an anxiolytic and an antidepressant in this test.

In designing experiments, generally researchers choose a chronic stress model and one (or a few) test(s) that produce consistent results under lab-specific conditions. This approach has proven its usefulness, since previous work reports increased behavioral emotionality following chronic stress, including studies from our lab (Edgar et al., 2011, Soumier and Sibille, 2014, Lin and Sibille, 2015, Nikolova et al., 2018). However, here we demonstrate that UCMS or CRS procedures, while clearly effective (i.e. robust effects on coat state and weight gain assessments) induced highly variable effects across multiple behavioral tests, even after 5 weeks of stress exposure. In accordance with published and unpublished data around the world, we demonstrated inconsistent results arguing against (or in favor of, depending on the reasoning) the idea that having multiple tests assessing the same phenotype is the best strategy (Ramos, 2008). This lack of reliability is often blamed on issues with chronic stress models, mouse strain, and/or sex differences, and rarely attributed to the tests used because of their inherent “simplicity” (e.g. OF). In addition, this lack of consistency is common when working with mice, whereas rats show greater reliability across experiments and tests (Willner, 2005, Nollet et al., 2013).

Standard tools are also relatively short (Ohl, 2005), providing a snapshot of the animal’s behavior and are highly sensitive to experimenter-bias with regards to how animals are handled before and during testing. Indeed, given that each previous time a mouse from the stress group was handled a stressor was applied, animals may naturally develop a rapid and transitory experimenter-induced hyperlocomotor activity. Since most commonly-used tests are short and highly dependent on exploratory drive, more often than not animals subjected to CRS and UCMS may display a greater number of entries in open arms of the EPM or in center zone of the OF, results usually interpreted as decreased anxiety-like behavior. In addition, it is not uncommon to find (as illustrated in this study) that the same UCMS animals display opposite phenotypes in very similar tests such as the NSF and NIH. In these cases, drawing conclusions

about the impact of chronic stress from these tests can be extremely difficult. Since chronic stress-induced behavioral effects are expressed heterogeneously, recent studies have employed z-emotionality scores (averaging z-normalized scores across multiple tests) as an attempt to improve consistency and reliability between tests and to establish a type of quantitative scale for rodent emotionality (Guilloux et al., 2012); loosely comparable to the Hamilton anxiety or depression scales used in humans (DSM-V).

Here, we designed the PhenoTyper test based on the rationale that a longer automatized test in a home-cage-like setting would address the aforementioned caveats and provide a more reliable readout of anxiety-like behavior throughout and following chronic stress exposure. This home cage-like setting also offers the unique advantage of being non-reliant on novelty, therefore allowing repetitive testing (Aarts et al., 2015). This test shares several features with light/dark box tests (e.g., using light as stressor to measure avoidance behavior), with the added benefit of monitoring animal behavior for a relatively extended period of time (overnight), and before, during and after the stressor experience (the light challenge). We previously used the PhenoTyper test to assess the UCMS effects longitudinally in BalbC male mice (Nikolova et al., 2018) and as a single readout in male and female C57B6 mice (Maluach et al., 2017). In BalbC mice, we found increased shelter zone time before, during, and after the light challenge (Nikolova et al., 2018) and in C57B6 mice, a reduction in shelter zone time after the light (Maluach et al., 2017). Here, the weekly monitoring of C57B6 mice revealed no major effects of either chronic stress model at baseline (i.e. before the light challenge), and a variable increased response during the light challenge exhibited some weeks and not others. We believe that this non-systematic effect of CRS or UCMS during the challenge is akin to findings one may see in commonly-used tests, and is likely due to the inherent difficulty of reliably capturing differences during a short testing period. Further testing would be required to determine if a longer light challenge may improve consistency of readouts for both UCMS and CRS models during the challenge.

Importantly, we demonstrated that UCMS and CRS induced consistent effects on RA in C57B6 mice. This finding was replicated using the UCMS model in parallel experiments with BalbC mice (data not shown), and by analysing the RA of animals from our previous two studies (Maluach et al., 2017, Nikolova et al., 2018). The fact that this deficit was found using both models and both strains and lasted throughout stress exposure, but was not observed after an acute stressor, suggests that it is highly specific to chronic stress. The underlying cause of this maladaptive inability to resume normal behavior, induced by chronic stress, is open to speculation. It is clear that a rapid response to an aversive stimulus helps organisms avoid potential threat or harm, but the ability to flexibly adapt to changing environments after the stress response is crucial for the capacity to recover from a challenge or stressor (Feder et al., 2009, McEwen et al., 2015). In this context, RA could be a readout for excessive stress generalisation, which when maladaptive can lead to fear responses that are too strong or occur in inappropriate situations. One could also interpret increased RA as enhanced freezing, decreased exploration, and disengagement of the animal towards its environment which would seem related to human neuropsychiatric symptoms such as psychomotor retardation or loss of interest in usual activities, such as food consumption or exploration (i.e., anhedonia). This decrease in the pursuit of rewarding activity due to inappropriate fear is a cardinal feature of anxiety disorders (Luyten et al., 2011, Lissek, 2012, Dunsmoor and Paz, 2015), and found in other stress-related illness, such as post-traumatic stress and major depressive disorders (DSMV, REF).

DZP and imipramine were used to test the predictive validity of RA as an anxiety measure. Indeed, DZP reduced RA in control conditions, suggesting that RA reflects a component of anxiety-like behavior. The principal component analysis suggests an anxiety-related dimension shared with other stress sensitive-parameters that measured approach/avoidance conflict or at the very least, a different but stress-related construct. Nevertheless, DZP had no effect on CRS mice for RA, but was efficacious in the NSF. Although this may appear to contradict the

interpretation of RA as readout of anxiety-like behavior, it is important to mention that differential effects of acute DZP can be similarly observed in other tests. The closest reported examples would be that DZP has lower efficacy in light/dark-related tests while showing clear anxiolytic-like effects in the EPM when tested in the same animals at the same dose (Kshama et al., 1990, Rodgers and Shepherd, 1993, Santucci et al., 1994, Rodgers and Johnson, 1995). One could also suggest that differential DZP effects in the PhenoTyper test and NSF may reflect different anxiety-like states/endophenotypes measured by the two tests; one that responds acutely to DZP and the other which may require longer treatment with DZP or that responds to a different class of drugs. Reversal of chronic stress-induced RA by long-term imipramine treatment supports the later hypothesis. Indeed, imipramine is often described as a better option than benzodiazepines for the treatment of generalized anxiety in clinical settings (Huh et al., 2011). It is also possible that RA encompasses an additional dimension independent of anxiety such as loss of interest, helplessness, or anhedonia, which are classically more sensitive to antidepressant treatment (Willner, 2005, Valentine et al., 2008, Frisbee et al., 2015). This would explain why treatment with imipramine, but not DZP, reversed chronic stress-induced RA. However, further experiments using other classes and regiments of antidepressants or anxiolytics would be needed to validate either hypothesis.

Finally, while we included sex as a biological variable in all experiments, our studies were not designed to examine sex differences and may be underpowered to identify meaningful changes. We confirmed that weight gain and coat state are primary readouts differentially affected by sex in response to stress (Nollet et al., 2013, Stanley et al., 2014, Piantadosi et al., 2016, Moench and Wellman, 2017). Yet, overall, our results confirm similar effects in males and females on behavioral emotionality following CRS or UCMS. The stress and sex interaction found in the PCA mainly illustrates a greater variability in the response to chronic stress in females vs. males. Such variability is frequently documented and can be attributed to factors such as oestrus cycle, level of stress susceptibility, or the dimension being measured by each test (e.g.,

consumption, exploratory, conflict, or social) (Seney and Sibille, 2014, Diaz et al., 2016, Lam et al., 2018).

Given the necessity and usefulness of rodent behavioral models, it is evident that better behavioral readouts are needed to detect changes to animals' regular behavior. Here, we developed and validated an assay that detects replicable and repeatable residual avoidance deficits after a light challenge following chronic stress exposure. This new tool will be instrumental to pinpoint the chain of events leading to expression of behavioral deficits associated with chronic stress and chronic anxiety, and to potentially screen novel anxiolytic and antidepressant treatments.

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Figure legends

Figure 1: **Physical and behavioral changes induced by two chronic stress paradigms.**
Mice were subjected to chronic restraint stress (CRS; A-F) or unpredictable chronic mild stress (UCMS; G-L). Fur quality (A,G) and wait gain (B,H) were assessed on a weekly basis, throughout the stress exposure. After the 5 weeks of chronic stress exposure, mice were tested in the open-field test (C, I) in which the time and the number of entries in the center were quantified. Mice were also tested in the elevated plus maze (D, J) in which the time and the number of crosses in the open arms were assessed. Mice were also tested in the novelty suppressed feeding test (E, K) and the novelty-induced hypophagia (F, L) in which the latency to bite a food pellet or consume the solution was measured. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls.

Figure 2: **Hourly detection of behavioral changes in the PhenoTyper test.**
Male and female mice were placed overnight (from 7pm to 7am) in the PhenoTyper boxes. Food zone (A) and shelter zone (B) times were assessed every hour. The following week, the same mice were placed in the PhenoTyper boxes, this time with 50% of each sex exposed to a light challenge between 11pm and 12am over the food zone (C, D). The same parameters (time in the food and shelter zones) were assessed. Finally, another cohort of mice was subjected to acute restraint stress (ARS) for 1hr, 1hr before being placed in the PhenoTyper boxes (E, F). A light challenge was applied from 11pm to 12am, over the food zone. Time spent in the food and shelter zone was quantified. *$p < 0.05$ compared to the corresponding sex, # $p < 0.05$, ### $p < 0.001$ compared to group receiving no light.

Figure 3: **Chronic restraint stress induces behavioral alterations of the shelter zone time inthe PhenoTyper test**.
Mice were subjected to chronic restraint stress (CRS) for 5 weeks, and tested in the PhenoTyper test every week with a light challenge occurring between 11pm and 12am. The shelter zone time was assessed every week (A-E). Using the residual avoidance (RA) calculation, each week's shelter zone time was transformed into a single value resuming the trajectory of the effect of chronic stress exposure (F). The same calculation was applied to the shelter zone time of mice subjected to unpredictable chronic mild stress (UCMS; G) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with controls.

Figure 4: **Principal component (PC) analysis of behavioral data obtained from mice exposed or not to chronic stress**
Data obtained from animals subjected to chronic restraint stress (CRS) and respective control group tested in the elevated plus maze, open-field, novelty suppressed feeding, novelty-induced hypophagia and the PhenoTyper test were used for factor analysis (A). PC1 and PC2 captured behavioral variance of stress and sex across groups. Similar factor analysis was performed on data collected from mice subjected to unpredictable chronic mild stress (UCMS) and respective control group. PC1 captured the behavioral variance of stress across groups. (B).

Figure 5: **Reversal of behavioral deficits after anxiolytic or antidepressant treatments**
Mice were tested in the PhenoTyper test (A-B) and in the novelty-suppressed feeding (C) after being subjected to chronic restraint stress (CRS). Animals also received acute diazepam treatment 30min prior being placed in the apparatus. The shelter zone time was quantified in the PhenoTyper test (A), and the residual avoidance was calculated based on these results obtained in the shelter zone (B). Mice were also tested in the novelty-suppressed feeding, in which the latency to bite was monitored. A separate cohort of animals was subjected to CRS and received imipramine chronically in the drinking water after the second week on CRS exposure, and for the next 4 weeks. Residual avoidance in the shelter zone was calculated for every week (D). Effect of imipramine on shelter RA obtained after 4 weeks of treatment was quantified (E). Finally, after 6 weeks of CRS and 4 weeks of imipramine treatment, mice were tested in the novelty-suppressed feeding test in which the latency to bite the food pellet was measured (F). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to CRS vehicle treated animals #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to control vehicle treated animals.

Figure 1

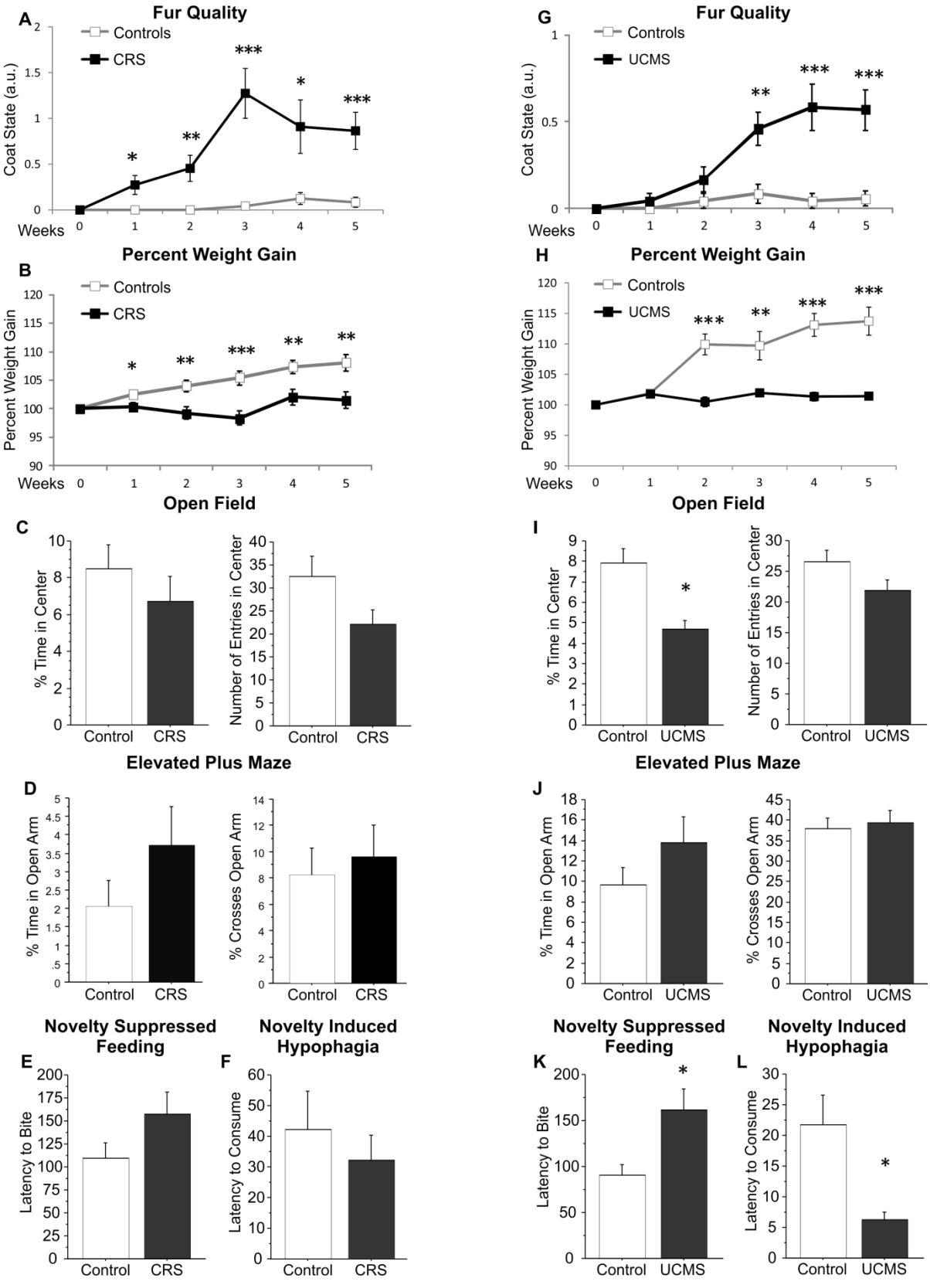
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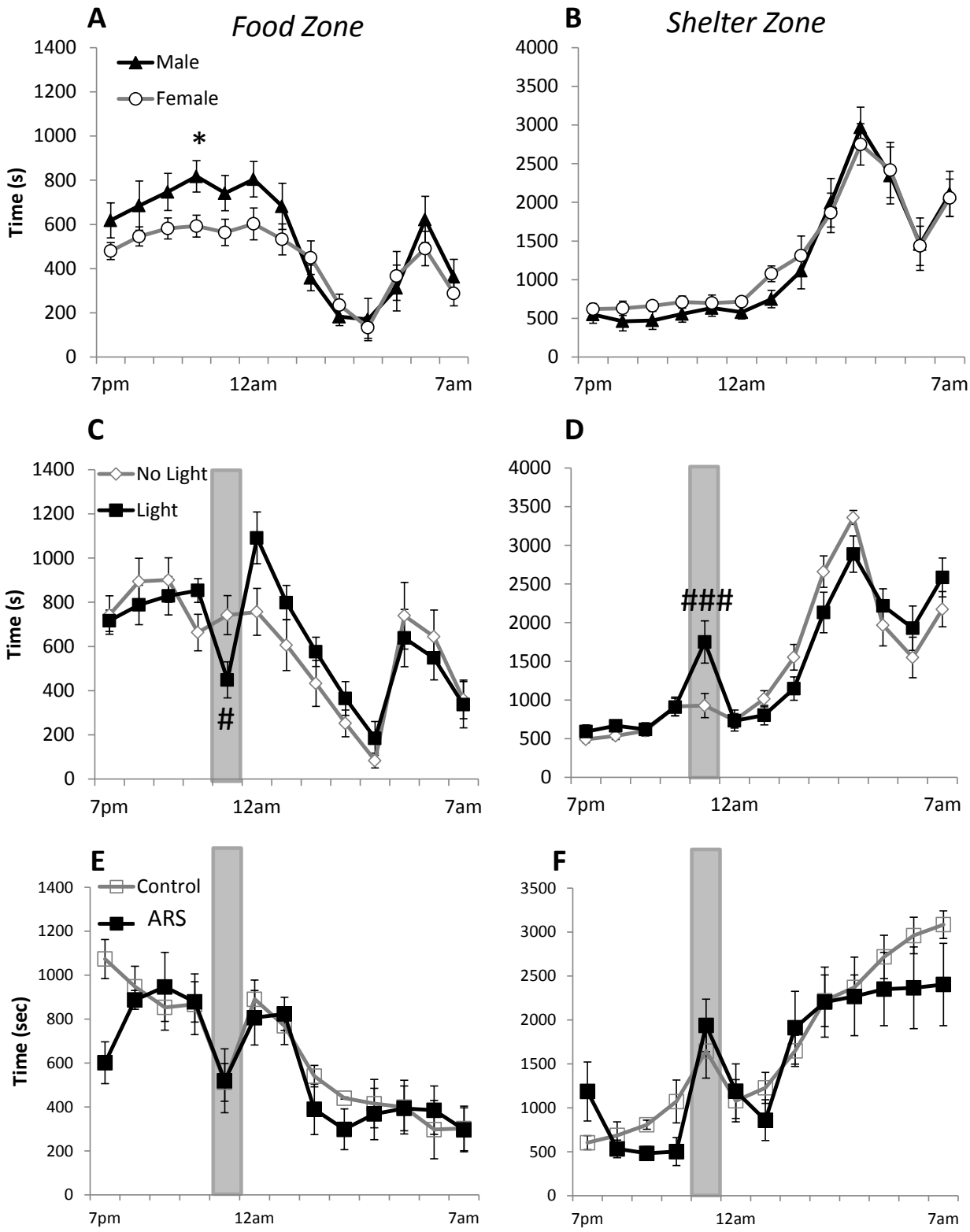
Figure 2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12am
7am
C
No Light
Light
#
Time (s)
1400
1200
1000
800
600
400
200
0
7pm
12am
7am
D
###
4000
3500
3000
2500
2000
1500
1000
500
0
7pm
12am
7am
E
Control
ARS
Time (sec)
1400
1200
1000
800
600
400
200
0
7pm
12am
7am
F
3500
3000
2500
2000
1500
1000
500
0
7pm
12am
7am

Figure 3

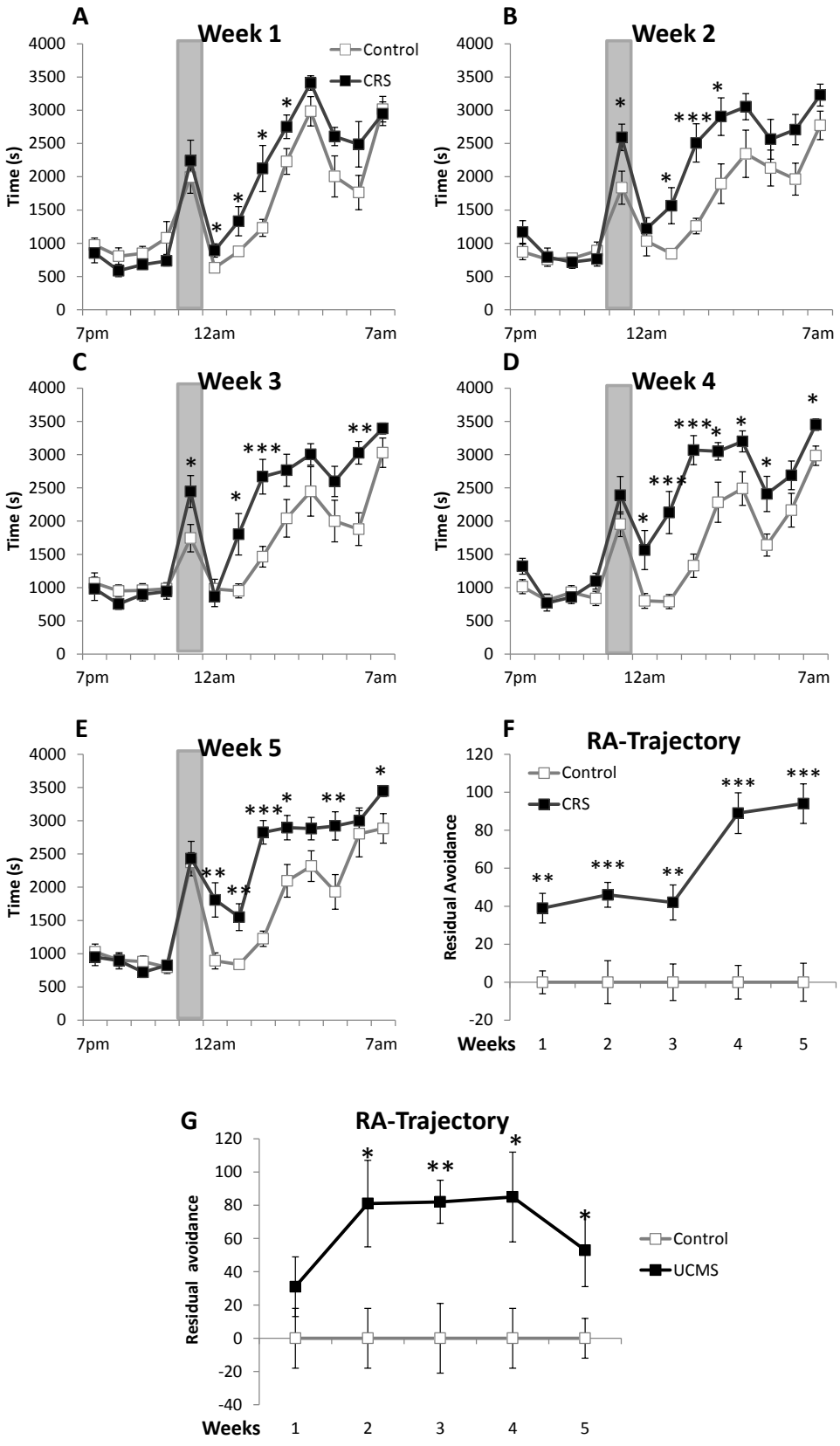

A
Week 1
Control
CRS
Time (s)
7pm
12am
7am
B
Week 2
C
Week 3
D
Week 4
E
Week 5
F
RA-Trajectory
Residual Avoidance
Weeks
G
RA-Trajectory
Residual avoidance
UCMS

Figure 4

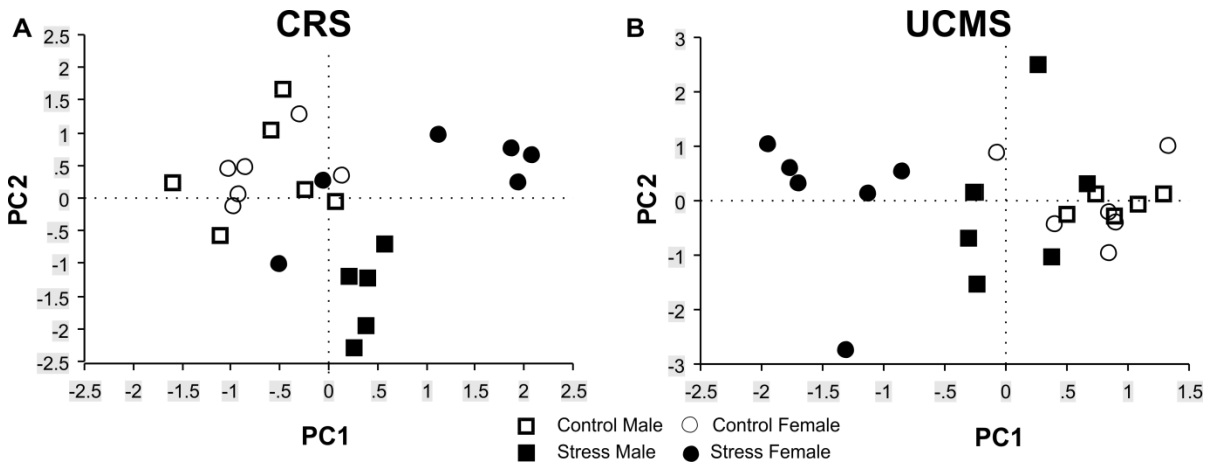
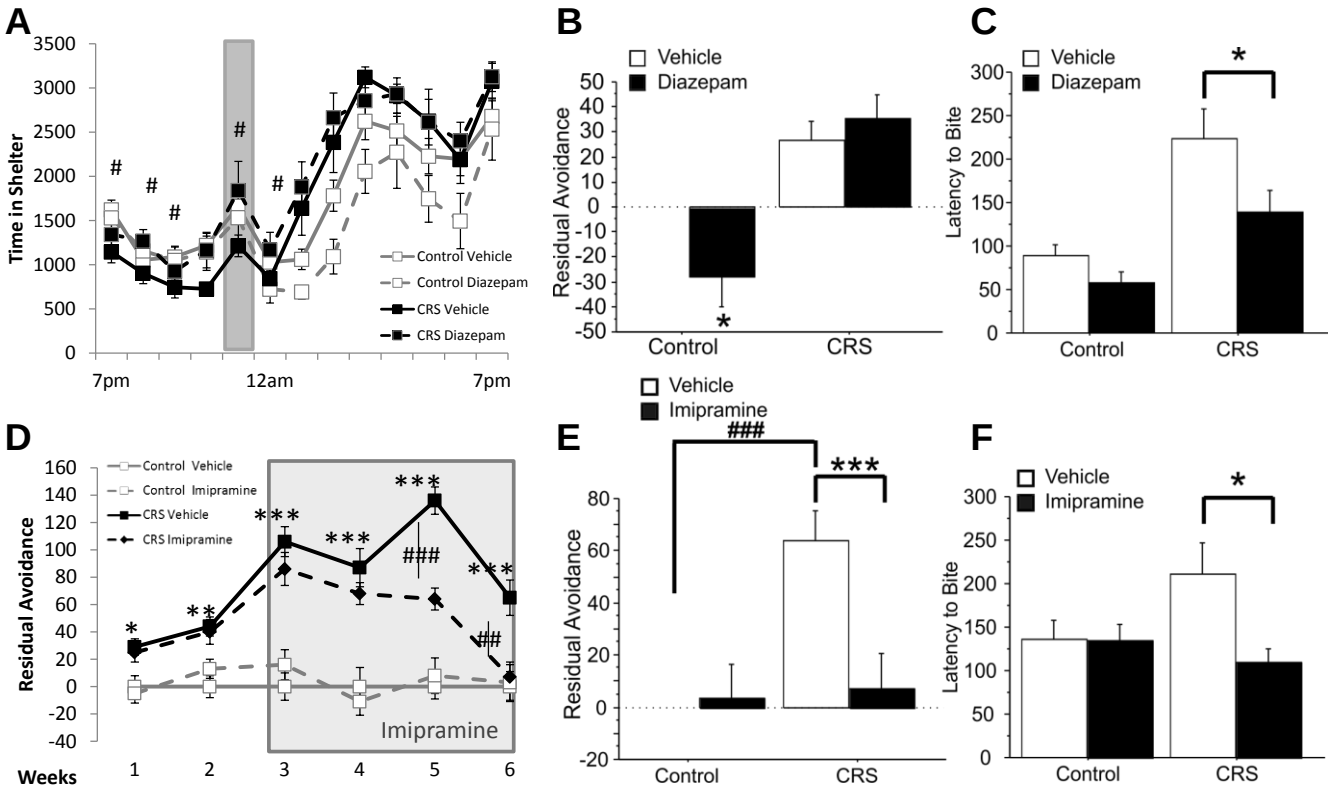


Table 1: Principal component analysis of the behavioral variables measured after 5 weeks of chronic stress.

Behavioral variables	CRS		UCMS	
	PC1	PC2	PC1	PC2
Coat state	_	-0.749	-0.702	_
% Weight gain	-0.579	_	0.714	_
OF Entries in center	-0.701	-0.410	0.490	0.608
OF % Time in center	-0.660	-0.426	0.740	_
EPM- % Open arm entries	0.435	0.620	_	0.791
EPM % Time in open arms	0.600	0.589	_	0.717
NSF Latency to bite	0.556	_	-0.750	_
NSF Home cage latency to eat	_	0.600	_	_
NIH Latency to drink	_	0.570	0.581	_
NIH Home cage latency to drink	_	0.543	0.580	_
Shelter zone time before challenge	_	_	0.781	_
Shelter zone time during challenge	0.855	_	_	_
Shelter zone residual avoidance	0.596	-0.444	-0.605	_
Food zone time before challenge	_	-0.403	_	0.607
Food zone time during challenge	-0.682	_	_	0.549
Food zone residual avoidance	0.424	_	_	_

For clarity only loading s on the first 2 components and > 0.4 are shown.

Figure 5


A
Time in Shelter
3500
3000
2500
2000
1500
1000
500
0
7pm
12am
7pm
Control Vehicle
Control Diazepam
CRS Vehicle
CRS Diazepam
B
Vehicle
Diazepam
Residual Avoidance
Control
CRS
C
Vehicle
Diazepam
Latency to Bite
Control
CRS
D
Control Vehicle
Control Imipramine
CRS Vehicle
CRS Imipramine
Residual Avoidance
Imipramine
Weeks
E
Vehicle
Imipramine
Residual Avoidance
Control
CRS
F
Vehicle
Imipramine
Latency to Bite
Control
CRS