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

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Abstract

Anxiety is a debilitating disorder with high prevalence and co-morbidity across multiple psychiatric diseases. Evidence from both clinical and preclinical studies suggest that the biological bases of anxiety and depression are associated with chronic exposure to stress. To identify the biological bases of anxiety-like behavior, rodent models have either studied the acute response to stress or acutely measured the effect chronic stress exposure. Little is known about the longitudinal progression of anxiety-like behavior. Most commonly used tests such as the open field test (OFT), elevated plus maze (EPM), novelty suppressed feeding (NSF) require a novelty component that undermines their ability to be used repetitively. Additionally, they measure behavior over a short duration, rely highly on housing and testing conditions and are subject to experimenter bias. Here, we have designed and validated a novel behavioral assay to measures anxiety-like behavior devoid of these limitations. Using a home cage-like apparatus (Noldus Phenotypers) we monitored baseline behavior by measuring time spent in designated zones (food and shelter zones) for a 12h period overnight. To assess anxiety-like behavior a 1h light challenge was applied over the food zone, 4h into the night. In this study, C57Bl/6 and Balb/c (n=12/group 50%♀) mice were subjected to chronic restraint stress (CRS) or unpredictable chronic mild stress (UCMS) for 5 weeks. Weekly assessments using the phenotyper plus a series of classical tests (OFT, EPM, and NSF) were performed at the end of the 5 weeks of stress exposure. Both literature and work in our lab have found that animal performances in classical behavioral tests show high heterogeneity not only across stress paradigm, strain and stress but also across test. Across experimental conditions and experiments (UCMS vs CRS, Balb/c vs C57Bl/6, male vs female) it was evident every week during the stress exposure that the phenotyper test could detect anxiety-like behavior (avoid the food zone, favor the shelter during/after challenge). We also confirm baseline and anxiogenic response. Altogether this new assay provides consistent and robust results as exhibited across different stress models, sexes and strains and can potentially be used to measure anxiety-like behavior repetitively.

This novel tool should be instrumental in the design of longitudinal studies aiming to better understand the biological bases of chronic anxiety disorders and others stress-related illnesses.

Introduction

Anxiety disorders, including phobia, post-traumatic stress disorder, social anxiety, OCD, panic disorder and generalized anxiety disorders are the most common and prevalent mental disorders worldwide(1). Anxiety can also be associated with, or accelerate the emergence of other pathologies such as depression(2), cardiovascular diseases(3), intestinal dysregulation(4) or sleep disturbance(5).

Over the years, scientists developed multiple paradigms(6) to induce and measure anxiety-like behaviors in animals(7). Anxiety-like behaviour, along with depressive-like behaviors, can be induced using diverse paradigms such as chronic stress exposure, prolonged stress, chronic social defeat or pre/post-natal stress. Behavioural tests commonly used in rodents to detect anxiety-like behavior include the elevated plus paze (EPM), the Open-Field (OF), the light/dark box, the social interaction, the novelty suppressed feeding (NSF) or the novelty induced hypophagia (NIH) tests. All these tests are based on an approach-avoidance paradigm or design (6). Indeed, most (if not all) animal models of anxiety rely on an innate conflict between their exploratory drive and their aversion to a threatening environment(8).

Despite the fact that the above-mentioned tests are commonly used in the field, determining the progression of anxiety-like deficits has proven to be difficult since these tests require the environment to be novel. Understandably, using these tests repetitively would have little success since the primary mandate of novelty will be lost when the test is repeated. Because of this caveat, classical behavioural tests are limited in providing a time-dependent trajectory of symptom development or maintenance.

In order to better understand the underlying mechanism of the emergence and progression of anxiety disorders, developing repeatable, reliable and more effective translational animal models measuring anxiety-like behaviours in rodents is necessary. To our knowledge, there is no test that can be used repetitively to assess anxiety-like behaviour in mice, and such test would be valuable to understand the dynamic emergence of anxiety in animal models of various pathologies.

Here we propose to demonstrate the limitation of the classical tests assessing anxiety-like behaviours in mice, using different mouse lines, sex, paradigms and assays performed in the same conditions. We will also demonstrate the usefulness of a new tool assessing anxiety-like behavior repetitively, reliably, and with minimal experimenter bias. Finally, following the four requirements to create a valid test of McKinney and Bunney (9) i.e. face, construct and predictive validities as well as reproducibility we developed the PhenoTyper test that can measure the influence of stress exposure by monitoring the animal's activity at baseline and in response to an aversive challenge.

Material and Methods

Animals:

Eight week-old C57bl/6 and Balb/c mice (Jackson Laboratory, Bar harbor, Maine, United States; 50% male) were housed under normal conditions, with a12hrs light/dark cycle and provided with *ad libitum* access to food and water. After 1-2 weeks habituation to the animal facility, mice were either kept in normal condition (control group) or subjected to either UCMS (unpredictable chronic mild stress) or CRS (chronic restraint stress) for 5 weeks (10). A separated cohort of C57BL/6 was used to assess the efficacy of imipramine to reverse the effect of chronic restraint stress.

Experimenters were blinded to the treatment group during behavioural testing. All procedures followed the guidelines set by the Canadian Council on Animal Care (CCAC).

Unpredictable chronic stress (UCMS): mice were subjected to randomized stressors (3-4 per day for 5 weeks). 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, exchange bedding (rotate mice into previously occupied cages), tilted cage (45º tilt), reduced space, restraint (50ml falcon tube with air hole for 15-30min), bedding change (replace soiled bedding with clean bedding), no bedding or nestlet removal. Control animals were group-housed (~3-4 per cage) to eliminate the stress of single housing, and only handled for weight assessment.

Chronic restraint stress (CRS): Animals were placed into 50mL falcon tubes with holes at either ends for breathing. Restraint stress sessions occurred twice daily for 1 hour during the light cycle (7:00am-7:00pm). A minimum interval of 2 hours was given

between each stress session. Animals were subjected to CRS for 5 weeks. Control animals were group-housed (~3-4 per cage) to eliminate the stress of single housing, and only handled for weight assessment.

Drug administration: Animals had access to imipramine at the dose of 15mg/kg, administered in the drinking water. Calculations were based on the establishment that mice drink around 8ml per day. Imipramine was prepared fresh every other day to prevent any deterioration due light exposure, room temperature.

Behavioral Tests:

Following 5 weeks of stress exposure, mice behavior was assessed in a series of classically used tests measuring anxiety-like behavior i.e. the elevated plus maze (EPM), open field (OF), and novelty induced hypophagia (NIH) and novelty suppressed feeding (NSF) tests.

EPM test: The EPM apparatus consist in four plexiglass 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 55cm above the floor and placed 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 10 minutes. A digital camera mounted on a rod recorded the animal behavior from the top. A posteriori measurement of time spent and number of entries each type of arms is performed using ANYmaze (Stoelting™).

OF test: The OF apparatus consists in a 50x50cm chamber which each mouse is allowed to explore freely for 10 minutes. A measure of time spent and total entries into the center of the arena defined (as a 20x20cm zone in the center of the apparatus) were measured. Total distance traveled in the arena was used as a measure of locomotor activity. A digital camera mounted on a rod recorded the activity of the animal from the top. A posteriori activity measurement was performed using ANYmaze (Stoelting™).

NSF test: Following a ~16 hours food deprivation period, mice are introduced to a novel arena (a dimly lit 62 x 31 cm enclosure) containing a single food pellet. Latency to approach and feed is measured during a 12 minutes period. As control of appetite drive and/or food deprivation-induced weight loss, latency to feed was measured in the animal's home cage in the same lighting conditions as in the NSF test for a maximum of 6 minutes.

NIH test: Mice are single housed in standard mouse cages for 1 week prior to the start of habituation. They remain single housed for the duration of the study. Mice are habituated to drinking a palatable liquid (Sweetened condensed milk, diluted 1:3 in water) in a little sterilised Petri dish for 1hr for 3 consecutive days. On the following 2 days, mice are given the opportunity to approach and consume the milk in 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. The latency for a mouse to approach and consume the milk from the Petri dish is recorded. Twenty four hours later, the subsequent test session occurs in a similar but new cage with bedding and the lighting is set to 500-600 lux. Latency to consume measured from the second

test (novel cage with dim lighting) is used as an index of hyponeophagia. The maximum daily duration is 10 minutes per day.

PhenoTyper™ test: The Noldus™ PhenoTyper (Leesburg VA, USA) apparatus is an observation cage designed for video-tracking mice regular behavior over an extended period using the Noldus EthoVision 10 software. The apparatus contains an integrated infrared sensitive camera that tracks activity and time spent by the animal in three designated zones (food zone and shelter) during the dark cycle (19:00-07:00). The apparatus also comprises a white LED spotlight placed above the food zone, a feature that allowed us to test whether animals would alter normal behavior in response to the application of an aversive stimulus such as a light challenge. In this case, the spotlight is turned on 4 hours into the dark cycle for a 1 hr period (23:00-24:00). This time window of the dark cycle was strategically chosen for being within the high-level plateau of time spent in the food zone by the animal. In the chronic stress studies, the PhenoTyper test was performed weekly on days 0, 7, 14, 21, 28, 36. In all experiments, the time spent in the food and shelter zones was measured hourly.

Residual avoidance: Based on the behaviour of the animal in the food zone and the shelter after the light challenge, a calculation was made to quantify how animal avoid the zone. This calculation is named the Residual Avoidance (RA) and was calculated as followed:

In the food zone:

(1-(Sum of time FZ from 12am to 4am-Time spent during the challenge))/(sum of time FZ from 12am to 4am – Time spent during the challenge)$_{\text{Control group}}$ * 100

in the shelter:

((Sum of time SZ from 12am to 4am-Time spent during the challenge)-1)/(sum of time SZ from 12am to 4am – Time spent during the challenge)$_{\text{Control group}}$ * 100

The RA calculation provides information about how animals react after the light challenge compared to controls. Control animals have a RA at 0 while positive RA means animal avoid the zone of the light by either 1) not being in the food zone or 2) being in the shelter.

Statistics:

Statistical analyses were performed using the Statview software. A student's t-test was used to determine statistical difference between 2 groups (classical tests). Repeated measure ANOVAs were used for the evaluation of the activity overnight in the PhenoTyper. If significant, the ANOVA analysis was followed by *post-hoc* Fisher's PLSD test to identify the origin of the significance.

Results

Commonly used tests assessing anxiety-like behaviours show heterogeneity between tests and between stress models or mouse strains

C57BL/6 and BalbC male and female mice were exposed to CRS (n=11/group; **Figure 1 G-L and Supplementary Figure 1**) or UCMS (n=11 C57BL6/group or N=20 BalbC/group, **Figure 1 A-F and Supplementary Figure 1**) prior to behavioral assessment using commonly used tests for anxiety-like behavior such as the OF, EPM, NIH or NSF.

Mice were subjected to CRS (**Figure 1A-F**). In the OF test, neither the percentage of time in the center (p=0.3601; **Figure 1A**), nor the number of entries in the center (p=0.0715; **Figure 1B**) revealed an effect of the CRS. In the EPM test, mice exposed to CRS displayed non-significant change in the percent time spent in the open arm (p=0.4233; **Figure 1C**) but a significant increase in the percent number of crosses ($p<0.05$) into the open arm (**Figure 1D**). Lastly, the NSF and NIH tests showed no significant difference in the latency to feed and latency to drink, respectively (p=0.1077 and p=0.5167; **Figure 1E-F**).

To parallel these assays in a different stress model, another cohort of C57BL6 mice was exposed to UCMS and tested in the OF test (**Figure 1G-L**). Statistical analysis revealed an effect of UCMS exposure, characterized by a decreased percent time in the center in the OF test ($p<0.001$) and a trend towards a decreased number of entries in the center (p=0.0746). To further analyze anxiety-like behavior, EPM test was conducted with the same mice (**Figure 1I-J**). However, time spent in the open arms and percentage of crosses were not significant (p=0.1217 and p=0.5189 respectively). Further, UCMS exposure induced a significant increase in the latency to feed in the NSF test ($p<0.05$; **Figure 1K**). However, in the NIH test, UCMS induced a decreased latency to consume the fluid ($p<0.01$; **Figure 1L**).

The same procedures and assays were performed in the same conditions with BalbC mice (**Supplementary Figure S1**). Briefly, no significant effect of either UCMS or CRS exposure was found across all used assays. All experiments were conducted on both males and

females, no significant interaction between sex and stress was found neither with strains nor models.

Baseline behavior and acute stress response changes can be monitored using the PhenoTyper apparatus

C57BL/6 and BalbC male and female mice were placed in the PhenoTyper apparatus overnight to monitor home cage-like behavior, more specifically time spent in the food and the shelter zones (n=12/group). Qualitatively, BalbC mice spend twice as much time in the food zone than C57BL/6 mice (**Figure 2A, Supplementary Figure 2A)** but a similar amount of time in the shelter zone (**Figure 2B, Supplementary Figure 2B).** Repeated measures ANOVA revealed no significant effect of sex in time spent in the food or shelter zone in BalbC mice (**Supplementary Figure 2A-B**) or in the shelter zone in the C57 mice (**Figure 2B**), suggesting a better consistency between strains regarding the results obtained in the shelter zone. However, C57BL/6 male mice spend generally more time in the food zone in the first few hours of the dark cycle (main effect of sex; $F_{1,252}$ = 4.19; p=0.05). *Post hoc* analysis however only showed a significant difference for the 4th hour within the dark cycle (p=0.01, **Figure 2A**). Repeated measure ANOVA also identified an effect of time over the night in both the food zone and the shelter zone ($F_{12,252}$>14.7; p<0.0001). Analyzed together (data not shown), the two zones (shelter and food) showed an interaction between the time of the night and the zone ($F_{12,528}$=42.3; p<0.0001). This is due to the fact that C57BL/6 mice spend equal amount of time in the food zone and the shelter during the first 6 hours of the night but more time in the shelter zone during the last 7 hours of the night (p>0.003). In the BalbC cohort, we noticed that a similar effect of time over the night in both the food zone and the shelter zone. When analyzed together, repeated measure ANOVA identified a significant interaction between the zones ($F_{12,264}$=35.7; p<0.0001). Here, BalbC mice spent more time in the food zone for the first 6 hours

of the night ($p>0.002$) while they spend more time in the shelter zone during the last 5 hours of the night ($p>0.01$).

One week after baseline monitoring, mice were placed in the PhenoTyper again and we assessed the response to an acute light challenge occurring between 11pm-12am applied on half of the animals (n=12/group) (**Figure 2C-D**). This time window of the dark cycle was strategically chosen for being within the high-level plateau of time spent in the food zone by the animal. Repeated measure ANOVA of time spent in the food zone revealed a significant interaction between time and "light condition" group ($F_{(1;252)}$ =1.971; $p=0.0273$). Post-hoc analyses identified that shining a light for 1h significantly decreased time spent in the food zone at this time point ($p=0.0241$). Parallelly, repeated measures ANOVA also revealed a significant interaction ($F_{(1;252)}$ =2.345; $p=0.0072$) between time and "light condition" group. Post-hoc analysis identified a significant increase in time spent in the shelter zone when the light was on compared to animals naïve to the light ($p<0.001$).

The Balb/c cohort was also exposed to the acute light challenge and compared to naïve controls (**Supplementary Figure 2**). Repeated measure ANOVA over the 12 hours of the dark cycle did not identify difference between groups or interaction between time and "light condition" group, probably because of a moderate reaction of BalbC mice to the light challenge in this experiment. However there was significant interaction between time and "light condition" group when the repeated measure ANOVA was performed only on the hours before, during and after the light challenge in the food ($F_{(2;38)}$=3.4;$p=0.04$) and the shelter zone ($F_{(2;38)}$=3.4;$p=0.04$).

In a separate cohort, we also tested C57BL/6 mice behavioral response to a 1 hour acute restraint stress performed immediately before placing the animal the PhenoTyper apparatus (**Figure 2E-F**). Repeated measures ANOVA of time spent in the food (**Figure 2E**) or shelter zone (**Figure 2F**) did not reveal differences between the groups or interaction between time and group (F= $p>0.29$ and F= $p>0.34$, respectively) suggesting that animals subjected to

an acute restraint stress prior testing behave in this test the same way that control mice at baseline, during or following the light challenge.

Behavioural changes induced by chronic stress exposure.

We then tested the impact of chronic stress exposure (CRS or UCMS) in C57BL6 or BalbC on home cage like behavior in the PhenoTyper test. The time spent in the shelter (**Figure 3A-E**) and food (**Supplementary Figure 3**) zones were assessed in C57BL/6 weekly from week 1 to 5 of CRS (**Figure 3A-E**) or UCMS exposure (**Supplementary Figure 4**).

Repeated measures ANOVA revealed a significant effect of the CRS exposure after 1 week of CRS (**Figure 3A**; $F_{(1;252)} = 9.49$; $p= 0.005$) and every following weeks (week 2, $F_{(1;252)} = 35.09$; $p<0.0001$, **Figure 3B**; week 3, $F_{(1;252)} = 19.9$; $p=0.0002$, **Figure 3C**; week 4, $F_{(1;252)} = 35.56$; $p<0.0001$, **Figure 3D**; week 5, $F_{(1;252)} = 20.92$; $p=0.0002$, **Figure 3E**). *Post hoc* analysis showed that CRS exposed mice spend more time in the shelter zone, in average between 12am and 4am. The same analysis was performed in the food zone where only a significant effect of the CRS was observed at week 4 ($F_{(1;252)} = 10.18$; $p= 0.004$ - **Supplementary Figure 3A-E**).

PhenoTyper tests assessing anxiety-like behaviours show consistency between stress models or mouse strains

Interestingly, the most striking differences between control and stress-exposed animal groups were not found during the light challenge but over the 5 hours following the challenge (from 12am to 4am). Stress-exposed mice continued to hide in the shelter zone after the challenge and did not return to control levels until later into the monitoring period. To establish a representative way of analyzing and presenting the differences after the challenge, we developed the “Residual Avoidance” calculation. It illustrates the difference between the behaviors of animal exposed and non-exposed to chronic stress for each week (**Figure 3F**).

Residual Avoidance is normalized to controls groups. As the non-CRS animals are used as a reference, their Residual Avoidance is null. Positive value of Residual Avoidance means that animals actively avoid the light by hiding in the shelter. At contrary, negative Residual Avoidance means that animals do not avoid the light. Repeated measures ANOVA showed a significant effect of the stress duration ($F_{(1,84)}$=49.31; p<0.0001). *Post hoc* analyses revealed an increased Residual Avoidance in CRS subjected mice on each week (p>.0.001).

As a complementary result, the same calculation was applied to the results obtained in the Food Zone (**Supplementary Figure 3F**). It illustrates how animals actively avoid being the food zone when lit. ANOVA performed on the Residual Avoidance revealed again a significant effect of the stress exposure ($F_{(1;84)}$=16.5; p=0.0006). Here, the Residual Avoidance was significantly increased after 1, 4 and 5 weeks of CRS (p>0.008).

Using the Residual Avoidance as a proxy for anxiety-like behaviour, **Supplementary Figure 4** summarises the results obtained in C57BL/6 subjected to UCMS, and BalbC subjected to CRS or UCMS. Repeated measures ANOVA on the residual avoidance calculation on the time spent in the food zone C57BL/6 mice subjected to UCMS (Supplementary Figure 4A) showed a significant effect of the stress exposure ($F_{1;88}$=6.8; p=0.015). Post hoc analysis identified a significant increase in residual avoidance only after 4 weeks of UCMS (p=0.01). Analysis of the residual avoidance calculated with the time spent in the shelter also revealed a significant effect of UCMS exposure ($F_{1;88}$=15.5; p=0.0007). Post hoc analysis identified that UCMS induced a significant increase in residual avoidance after 2 weeks of exposure (p=0.01) and beyond (p<0.05). In BalbC exposed to CRS …

When exposed to UCMS, BalbC also showed a significant residual avoidance in both the food zone and the shelter zone (ANOVAs F>17.3; p<0.001). *Post-hoc* analysis identified an significant increase in the residual after 1, 2 and 5 weeks of UCMS in the food zone, and after 1, 2, 4 and 5 weeks of UCMS in the shelter zone (p<0.05).

Chronic administration of imipramine reduces the residual avoidance observed after chronic stress exposure.

After demonstrating that chronic stress exposure in C57BL/6 or BalbC mice induces a significant increase in the residual avoidance after the light challenge, our goal was to reverse this anxiety-like behavior using known pharmaceutical agent used for their anxiolytic properties. After 2 weeks of chronic restraint stress exposure, we started a chronic administration of imipramine. The first noticeable result is that we replicated the effect of CRS exposure in C57BL/6 mice that showed significant increased residual avoidance throughout the entire stress exposure (ANOVA effect of CRS: $F_{1,220}=72.7$; $p<0.0001$; *Post-hoc* analysis: $p<0.01$). One day before the assessment of the third week, we started giving imipramine in the drinking water. Assessment at week 3 (after1day of treatment) or at week 4 (after1 week of treatment) did show any effect of imipramine neither in control mice nor in CRS mice ($p>0.05$). However, a significant difference between vehicle and imipramine administered mice, within the CRS group was identified at week 5 (after 2 weeks of treatment; $p<0.001$). Interestingly, the administration of imipramine in stress subjected mice prevented the increased residual avoidance at week 4 but did not bring the residual avoidance to the same level than control mice ($p>0.05$). At week 6, stress-exposed mice receiving imipramine showed reduced residual avoidance compared to stress-exposed animal receiving vehicle ($p=0.004$). In animals receiving vehicle, CRS-exposed mice had a significantly higher residual avoidance compared to control mice ($p<0.001$). Altogether, these results suggest that 3 weeks of imipramine treatment has an anxiolytic effect, reducing the residual avoidance in our novel assay.

Discussion

Our results demonstrated that commonly used tests assessing anxiety-like behaviours show heterogeneity between tests and between stress models or mouse strains and presented for the first time a new tool to repeatedly and reliably assess anxiety-like behaviors in mice across sex, strains and stress paradigms.

Indeed, we examined the behavioural readouts of anxiety-like behaviors from 4 different tests classically used in the field, in 2 mouse lines and with 2 chronic stress model mostly used in the literature. We showed non-consistent results, going against the idea that having multiple tests assessing the same phenotype is the best strategy(11). Over the past decades, such classical tests have been thoroughly employed and have proven their usefulness since previous work using the same models have reported behavioural “emotionality”, including previous studies performed in our lab (12-15). However, here, male and female Balb/c or C57BL/6 mice subjected either to UCMS or CRS showed highly variable “behavioural emotionality” measured across multiple behavioural tests as observed after 5 weeks of chronic stress exposure. Such findings, consistent with numerous of published and unpublished data around the world demonstrate the variability that exists. The lack of reliability is often blamed on the chronic stress model used, strain, and/or sex of animal employed and rarely attributed to the test used because they are “simple” e.g. open-field. However, this is only true when working with mice, as studies with rats have shown consistency and reproducibility across behavioural tests (16, 17). Indeed, classically-used behavioural tests such as elevated

plus maze and open field were designed to screen compounds for anxiolytic properties (18-20).

Although they are the standard tools to measure anxiety-like behavior, these classical tests present several caveats, particularly their limited ability to assess anxiety-like behaviour repetitively which can be attributed to their highly invasive properties and strong experimenter bias. Determining the progression of anxiety-like deficits has proven to be difficult since most tests such EPM, OF, NSF, and NIH require a novel environment and an anxiogenic stimulus making them ineffective in testing animal behaviour repetitively. Understandably, using either test repetitively would have little success but they can be distributed over the course of the UCMS exposure to provide a measure of the anxiety-like state of the animal; this however would offer little information on the progression of the chronic stress effect since the measurable outcomes are not comparable. It is also possible to assess the effects of chronic stress weekly in different groups of animals, although this cannot provide information on the progression of symptom development within the same group of animals, and is against the principles of the 3Rs in animal studies (Replace, Reduce and Refine)(21). Furthermore, these classical tests provide an acute behavioural output where tests can measure behaviour between 5-30 mins long which could potentially make it difficult in detecting behavioural deficits as it only provides a snapshot of the animals' behaviour. In such case, concluding about the impact of the chronic stress paradigm used is extremely difficult and can sometimes yield to misleading conclusions.

To overcome these conclusions we developed the PhenoTyper test that provides reliable results in the assessment of anxiety-like behaviour in mice. It is unique

compared to other tests as it does not require a novel environment i.e. the animals are exposed to the same environment weekly for an extended period. Also, the test is automatized allowing for the removal of potential investigator interaction or bias (22) during the test and for a longer detection period, with the ability to observe more precise changes in behaviour that might not be easily detected during an acute classical test.

In order to completely validate the PhenoTyper assay, a test for predictive validity was performed to determine antidepressant/anxiolytics efficacy in animals subjected to chronic stress. In our study, imipramine (a tricyclic antidepressant) was used to reverse the effect of the chronic stress exposure. Results showed that 3 weeks of treatment were necessary to reverse the increased anxiety-like behaviours induced by chronic stress. Imipramine was chosen as a drug to validate our assay since publications suggested that imipramine is a better option to treat generalized anxiety disorder than benzodiazepine (23). Since imipramine reduced anxiety-like behaviour in our assay, we can assume that this test can be useful in the assessment of pathological (trait-) anxiety in mice rather than state-anxiety, but further validations using other known anxiolytic are required to verify this hypothesis.

Given the necessity and usefulness of rodent behavioural models, it is evident that we need better behavioural readouts to detect the behavioural changes on the animal's regular behaviour. Here, we developed and validated an assay that is replicable, repeatable, reliable and has no bias on experimenter. We showed in both strains, both sexes and both chronic stress models that the PhenoTyper assay can be used repetitively, and we demonstrated that the residual avoidance calculation can summarize the effect in a trajectory-focused manner. Determining the progression of the

effect of chronic stress will be instrumental to pinpoint the chain of events leading to expression of the behavioural deficits and dissect, for example, the cellular changes responsible. This provided results that can chart the time-dependent trajectory from onset to emergence of the behavioural deficits, and can prove usefulness in multiple other applications, using over chronic stress models, transgenic mice, and treatment efficacy.

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Figure legends

<u>Figure 1:</u> Impact of two chronic stress models in C57BL/6 mice on anxiety-like behaviors assessed in the open field, elevated plus maze, novelty suppressed feeding and novelty induced hypophagia tests.

Male and female C57BL/6 mice were subjected either to chronic restraint stress (CRS: N=11/group) or Unpredictable chronic mild stress (UCMS; N=11/group). After completion of the chronic stress protocol, mice were assessed in a battery of tests assessing anxiety-like behavior. Animals were placed for 10 minutes in an open field chamber and a decrease in the time spent in the center as well as a decrease of the number of entry in the center was assessed as a proxy for anxiety-like behavior (A-B and G-H). Using the elevated plus maze, a decrease in the time spent in the open-arm (C and I) and the number of crosses in the open arms (D and J) was quantified. Finally, an increased latency to bite a food pellet in a novel environment after food deprivation in the novelty suppressed feeding test (E and K) and an increase in the latency to consume condensed milk in the novelty induced hypophagia (F and L) were also used to assess anxiety-like behavior. Effect of UCMS or CRS compared to Control: *$p<0.05$. Sex was put as a co-factor but was never significant.

<u>Figure 2:</u> Detection of behavioural changes in the PhenoTyper in C57BL/6.

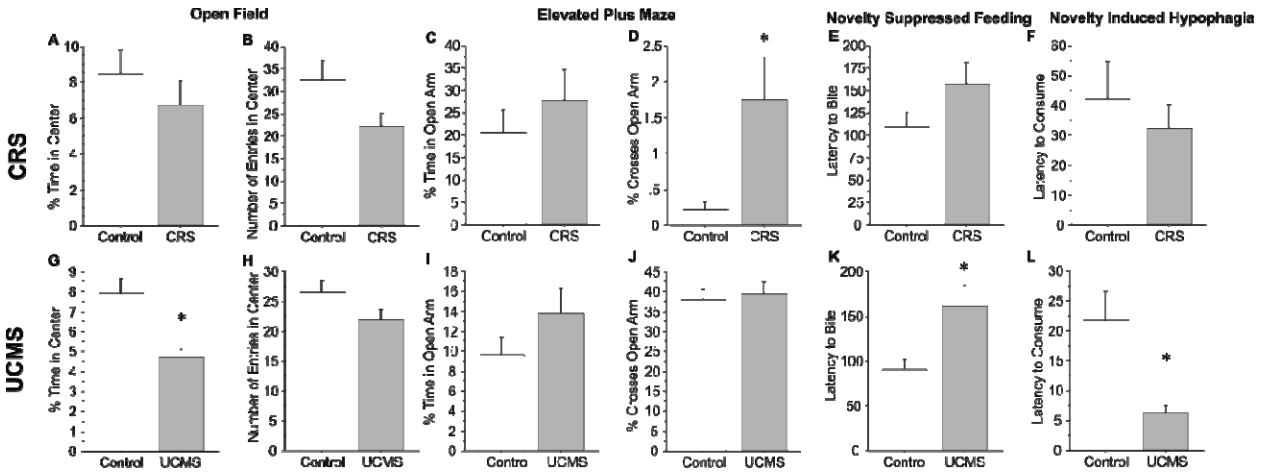
Male and female C57BL/6 mice were tested in the PhenoTyper apparatus, overnight and the time spent in the food zone (A, C and E) and the shelter zone (B, D and F) was assessed in different conditions. First, we assessed the overall “normal” behaviour of the animals in the apparatus (A-B) and assess for sex differences. The following week, animals were re-tested in the PhenoTyper apparatus with 50% of the animals receiving an acute light challenge at 11pm, for 1hr (C-D). Finally, the impact of an acute restraint stress, performed immediately before placing the animals in the apparatus (E-F). Effect of the light challenge compared to the “No Light” group: #$p<0.05$, ###$p<0.001$.

<u>Figure 3:</u> CRS exposed animals show consistent response to anxiogenic spotlight test across stress exposure.

Male and female C57BL/6 mice were subjected to chronic restraint stress (CRS) for 1 up to 5 weeks and tested in the PhenoTyper. Time spent in the shelter was quantified after 1 week (A), 2 weeks (B), 3 weeks (C), 4 weeks (D) and 5 weeks (E) of CRS exposure. The residual avoidance was calculated for each week and summarized in (F). Effect of the CRS exposure compared to the “Control” group: *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Sex was put as a co-factor but was never significant.

<u>Figure 4:</u> Imipramine reduces anxiety-like behaviors induced by chronic stress exposure after 3 weeks of treatment.

C57BL/6 male and female mice were subjected to CRS, and received imipramine or vehicle in the drinking water (N=12 per group; 50% ♀). Animals were weekly tested in the PhenoTyper test. For 2 weeks, animals received only vehicle in the drinking water. After 2 weeks, 50% of each group (Control or CRS) received imipramine (15mg/kg) in the drinking water while the other 50% kept receiving vehicle (water). The duration of the imipramine is presented by a grey rectangle from week 3 to week 6. Effect of CRS, compared to Control: *$p<0.05$; **$p<0.01$ and ***$p<0.001$. Effect of imipramine, compared to vehicle: ##$p<0.01$ and ###$p<0.001$. Sex was put as a co-factor but was never significant.



<u>Figure 1:</u> Impact of two chronic stress models in C57BL/6 mice on anxiety-like behaviors assessed in the open field, elevated plus maze, novelty suppressed feeding and novelty induced hypophagia tests.

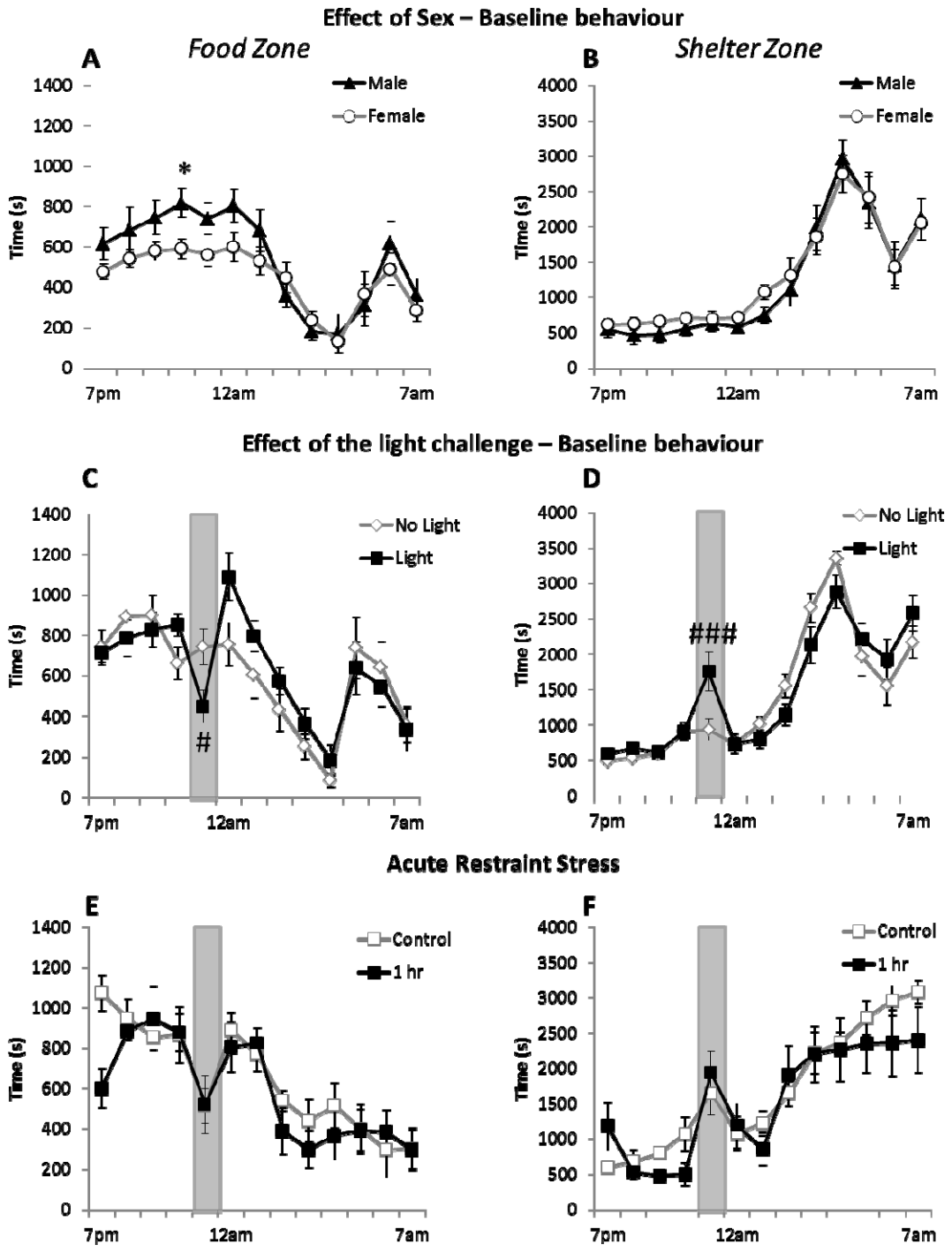


Figure 2: Detection of behavioural changes in the PhenoTyper in C57Bl/6 mice

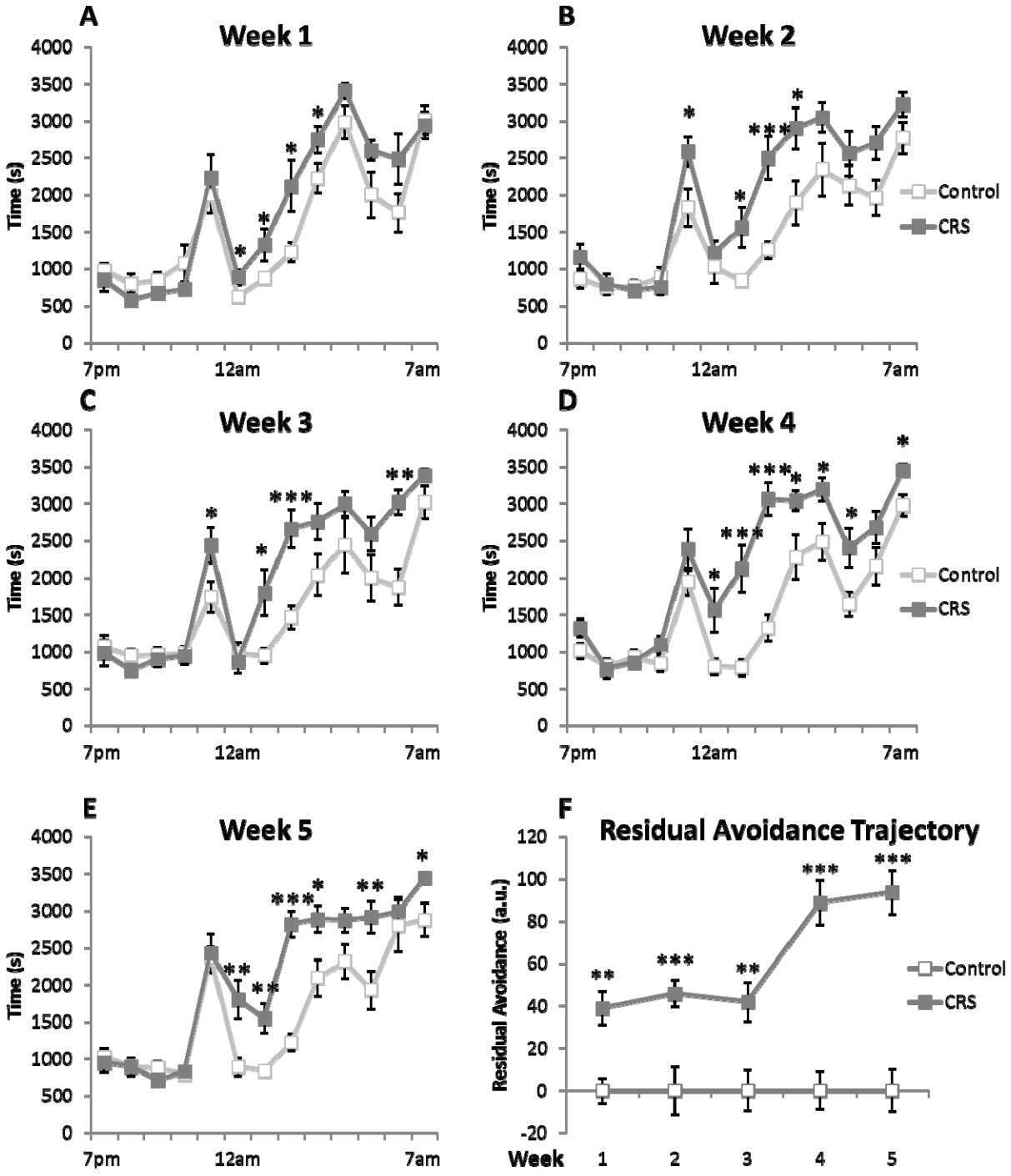


Figure 3: CRS exposed animals show consistent response to anxiogenic spotlight test across stress exposure.

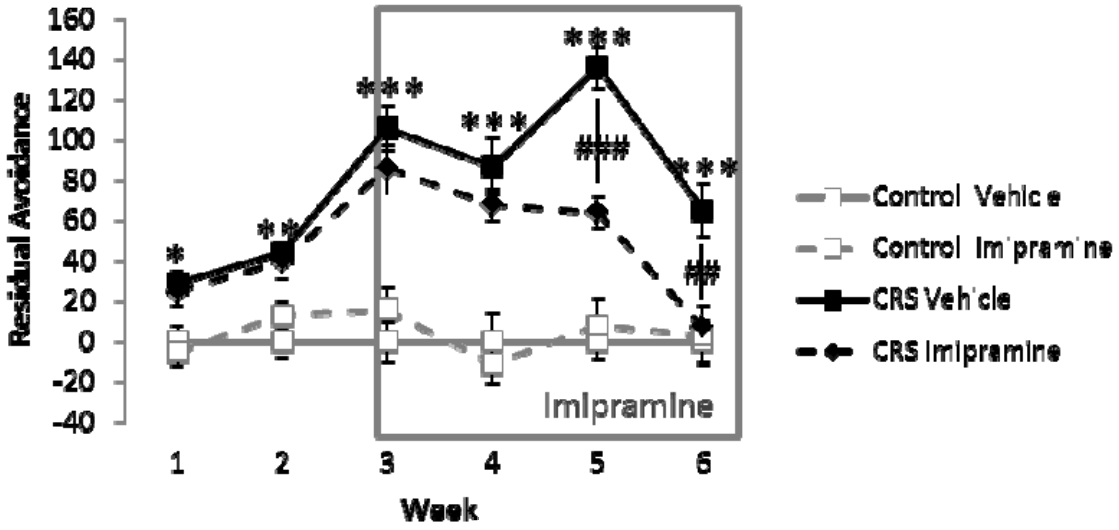


Figure 4: Imipramine reduces anxiety-like behaviors induced by chronic stress exposure, after 3 weeks of treatment.