M.Sc. Thesis

Investigating Cross-Resilience to Chronic Social Defeat and Learned Helplessness Stress

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Abstract

Title: Investigating cross-resilience to chronic social defeat and learned helplessness stress

Background: The chronic social defeat (CSDS) and learned helplessness (LH) rodent models of stress have facilitated investigation of the molecular mechanisms underlying resilience and susceptibility to major depressive disorder (MDD) and post-traumatic stress disorder (PTSD). In this study, we wanted to determine whether there is a shared resilience to these two distinct paradigms of chronic stress.

Methods: 2-3 months old C57BL/6J male and female mice were subjected to 10 days of CSDS, resulting in depression-like phenotypes in susceptible individuals. Mice were tested in the social interaction (SI) test, elevated plus maze (EPM), open field (OF) test and sucrose preference test (SPT), to identify anxiety and depressive-like phenotypes. This was followed by a 30-day LH protocol using inescapable foot shocks, and a second SI test 24 hours after the final LH test day.

Results: Our research suggests that there is a cross-resilience to these two types of stress in males. We found that a higher proportion of males that were resilient following social defeat were also resilient following LH, compared to individuals that were susceptible and non-defeated controls. We also identified different patterns of resilience for males and females, with defeated females acquiring resilience to LH stress earlier than defeated males.

Conclusion: Our results point to a cross-resilience to chronic social and trauma-type stress in males, and we identified sex differences in resilience. This is relevant to the understanding of the underlying mechanisms of resilience to MDD and PTSD, especially in the context of comorbid MDD and PTSD.

Résumé

Titre: Étude de la résilience croisée entre la défaite sociale chronique et le stress d'impuissance acquise.

Contexte: Les modèles de stress chez les rongeurs de la défaite sociale chronique (CSDS) et de l'impuissance acquise (ou Learned Helplessness : LH) ont permis l'étude des mécanismes moléculaires sous-jacents à la résilience et à la susceptibilité au trouble dépressif majeur (TDM) ainsi qu'au trouble de stress post-traumatique (SSPT). Pour notre étude, nous avons voulu déterminer s'il existe une résilience partagée entre ces deux paradigmes distincts du stress chronique.

Méthodes: Des souris mâles et femelles C57BL / 6J âgées de 2-3 mois ont été soumises à 10 jours de CSDS (Défaite Sociale Chronique), ce qui a entraîné des phénotypes de type dépression chez les individus sensibles. Les souris ont été testées dans le test d'interaction sociale (SI), le test du labyrinthe en croix surélevé (EPM), le test en champ ouvert (OF) et le test de préférence au saccharose (SPT), pour identifier l'anxiété et les phénotypes de type dépressif. Cela a été suivi par un protocole de résignation acquise (Learned Helplessness - LH) de 30 jours utilisant des chocs électriques inévitables, et d'un second test d'Interaction Sociale 24 heures après le dernier jour du test LH.

Résultats: Nos résultats suggèrent qu'il existe une résistance croisée à ces deux types de stress chez les mâles. Nous avons constaté qu'une proportion plus élevée de souris mâles résilientes pour la défaite sociale chronique étaient également résilientes après le protocole de l'impuissance acquise, par rapport aux souris vulnérables. Nous avons également identifié différents profils de résilience pour les mâles et les femelles, les femelles vulnérables au stress ayant une extinction plus rapide lors des stress de chocs électriques par rapport aux mâles vaincus.

Conclusion: Nos résultats permettent d'identifier pour la première fois une résistance croisée entre le stress chronique social et le stress traumatique chez les mâles. Nous avons également identifié des différences de résilience entre les sexes. Ces résultats permettent de mieux comprendre des mécanismes sous-jacents partagés de la résilience à ces deux types de stress ayant des origines différentes.

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PREFACE AND CONTRIBUTION OF AUTHORS

The project described in this thesis will be used in manuscript(s) submitted to peer-reviewed journals for publication. Dr. Giros and Dr. Bairachnaya proposed the idea for this project and established the experimental design. Dr Bairachnaya and I performed the behavioural experiments together for the first cohort of males. I performed the rest of the behavioural experiments, with assistance from Dr. Bairachnaya when needed. I performed the female social defeat experiments and established the changes to the protocol. Dr. Bairachnaya provided guidance on data analysis.

INTRODUCTION AND STATEMENT OF PROBLEM

Major depressive disorder (MDD) is a highly prevalent psychiatric disorder (Kessler et al., 2007) that causes significant impairments (Otte et al., 2016), and affects twice as many women as men (Seedat et al., 2009). The lifetime prevalence of MDD in Canada is 11.3% (Lam et al., 2016). Symptoms of this disease include depressed mood, loss of interest, fatigue, impaired cognition, along with sleep and appetite problems (Otte et al., 2016), and those with MDD are at a greater risk of suicidality than healthy individuals (Cai et al., 2021). While there are several treatments for MDD, these do not work for everyone, and around 30% of patients do not recover even after receiving multiple treatments of antidepressant medication (Cipriani et al., 2018). There are many factors involved in the development of MDD, including genetic heritability, environmental factors such as child abuse, and changes in regional brain volumes and brain circuits (Otte et al., 2016). While there have been advances in understanding different aspects of the etiology of MDD, the physiological mechanisms are still being investigated and knowledge of the pathophysiology remains limited (Fava and Kendler, 2000).

Post-traumatic stress disorder (PTSD) is another debilitating psychiatric disorder which can occur following exposure to a traumatic event. It is characterized by four groups of symptoms, which are re-experiencing symptoms, negative changes in mood and thinking, avoidance symptoms, and abnormal arousal or reactivity (Lancaster et al., 2016). PTSD, like MDD, affects twice as many women as men, and the symptoms tend to be more debilitating for women (Yehuda et al., 2015). Altered brain circuitry and genetic heritability are among the factors involved in the etiology of PTSD (Fenster et al., 2018). There are several treatments for this disorder such as psychotherapy and pharmacological treatments (Lancaster et al., 2016). However, like MDD, PTSD

also has variable treatment outcomes, with only 20-30% of patients achieving complete remission following pharmacological treatment (Berger et al., 2009). The molecular changes involved in vulnerability to PTSD are not well understood (Fenster et al., 2018) making this an important area of research in order to identify targets for pharmacological treatment.

Stress does not affect each individual in the same way, and following life events or repeated stresses some individuals will develop MDD or PTSD whereas others will not. The capacity of an individual to rebound from traumatic events or stresses is known as resilience (Franklin et al., 2012). Understanding the underlying vulnerabilities that cause some individuals to be more susceptible to these disorders could promote the development of new treatment approaches, as well as potentially guiding prevention of these disorders. This is important because along with causing suffering to the individual and contributing to the significant economic burden of mental disorders (Trautmann et al., 2016), both MDD and PTSD are associated with an increased risk of suicidality (Cai et al., 2021: Sareen et al., 2007). This study will contribute to the understanding of behavioural mechanisms of resilience to MDD and PTSD, through the investigation of cross-resilience to both of these disorders.

LITERATURE REVIEW

Stress Resilience:

Stress can be defined as a circumstance in which our homeostasis is threatened, or what we perceive as being threatened. Throughout life we encounter a multitude of stressors, and these trigger important adaptive responses of the central and peripheral nervous systems, including immunological, behavioural and endocrine responses, in what is known as the stress response (Charmandari et al., 2005), or allostasis (McEwan, 2005). One of the major components of the stress response is the Hypothalamic Pituitary-Adrenal (HPA) Axis. This begins in the hypothalamus, which upon being activated by stress releases corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from its paraventricular nucleus (PVN). This triggers the activation of the anterior pituitary gland, which releases adrenocorticotropic hormone (ACTH). ACTH travels to the adrenal glands where it triggers the release of cortisol from the adrenal cortex (Jacobson and Sapolsky, 1991). Cortisol affects the functioning of a range of processes and results in adaptive stress responses – including increased heart and respiratory rate, along with increased blood pressure and glucose levels (Chrousos and Gold, 1992). This response is ‘switched off’ when cortisol regulates its own secretion via a negative feedback mechanism (Gjerstad et al., 2018). The second major aspect of the stress response is the autonomic nervous system (ANS), and involves the activation of the sympathetic nervous system (SNS). The adrenal medulla is activated and releases epinephrine into the blood, and this results in several adaptive responses such as increased heart rate, smooth muscle contraction and a rise in blood pressure (Seyle, 1950). The locus coeruleus-norepinephrine (LC-NE) system is also involved in the stress response, as it has been shown that levels of norepinephrine (NE) increase during stress (Koob, 1999).

Although the stress response is an adaptive process in the short term, chronic or repeated activation of these systems can result in negative health consequences (Kemeny, 2003). Consequences include the development of psychiatric disorders such as major depressive disorder (MDD) and posttraumatic stress disorder (PTSD), and there is a growing body of research that shows how these disorders may be associated with maladaptive stress responses. Firstly, researchers investigating cortisol responses to physiological stressors in individuals with MDD found that they had significantly higher levels of cortisol than healthy individuals during the recovery period. This suggests that MDD is associated with the inability to turn off the production of cortisol via negative feedback of the HPA-axis (Burke et al., 2005). In contrast to this, PTSD appears to be associated with low levels of circulating cortisol (Yehuda and Seckl, 2011). Researchers investigating the role of cortisol in PTSD in an animal model found that injecting rats with corticosterone an hour before exposing them to stress significantly reduced the prevalence of extreme behavioural changes associated with PTSD (Cohen et al., 2006). The role of the cortisol in PTSD has also been implicated in human studies. For example, one study of car crash victims found that mean cortisol levels of individuals who had PTSD six months after the accident were significantly lower than those who did not develop PTSD (McFarlane et al., 1997).

However, while some individuals develop these disorders following stressful events, others do not, and the ability to rebound from stressful events is known as resilience (Franklin et al., 2012). Many factors have been implicated in stress resilience, including physiological, environmental, and psychological factors, and this has been elucidated largely through the use of animal models. Firstly, direct epigenetic regulation of CRF may determine the long-term behavioural responses to stress, as chronic stress resulted in long-term demethylation of the CRF gene in mice (Elliot et

al., 2010). Secondly, hippocampal neurogenesis likely has a role in the behavioural and neuroendocrine responses to stress, as neurogenesis-ablated animals presented increased susceptibility when exposed to stress (Levone et al., 2015). Also, individual differences in the peripheral immune system may determine whether an individual is resilient or susceptible to stress, as researchers found that replacing a stress-naive animal's peripheral immune system with that of a stressed animal increased susceptibility to social stress (Hodes, 2014). Early life adversity also appears to be a risk factor for the development of MDD and PTSD. One study using mice found that early adversity such as maternal separation, limited nesting and mild chronic stress was associated with increased susceptibility to chronic stress in adulthood, which suggests that some early adversity programmes an individual's susceptibility and resilience to stress later in life (Peña et al., 2019).

Several neural circuits have been implicated in stress resilience and vulnerability. The two major circuits that have been identified as having important roles in resilience are the mesolimbic dopamine (DA) reward circuit and the fear circuit. The mesolimbic DA pathway involves the ventral tegmental area (VTA) and nucleus accumbens (NAc), and regulates an individual's response to rewarding stimuli. DA neurons in the VTA project to the NAc along with other areas of the brain, and are activated in response to a reward (Hyman et al., 2006). The firing pattern of DA neurons in this circuit appears to be important in determining resilience, with increased firing of DA neurons in the VTA being associated with susceptibility to chronic stress in mice (Krishnan et al., 2007). Further to this, functional magnetic resonance imaging (fMRI) studies have provided evidence of dysfunction in the reward system in patients with MDD as well as those with PTSD (Sailer et al., 2008; Pizzagalli et al., 2009). The second major circuit, the fear circuit, involves several brain areas including the hippocampus,

amygdala, medial prefrontal cortex (mPFC), and the locus coeruleus (LC) (Feder et al., 2009). Abnormalities of the fear circuit appear to be involved in PTSD, as a study of individuals with PTSD found that the amygdala is hyperresponsive in this disorder (Rauch et al., 2000). While progress has been made in understanding the neural circuits and molecular mechanisms underlying resilience, there is much left to be understood, making this an important area of research.

The LC-NE System in Stress Resilience:

Norepinephrine (NE) is another neurotransmitter that has been demonstrated to be involved in resilience to MDD and PTSD. When investigating cerebrospinal fluid (CSF) NE concentrations in men with PTSD, researchers found that CSF NE concentrations were significantly higher in those with PTSD than in the healthy individuals (Geracioti Jr et al., 2001). Klimek et al. (1997) examined brain tissue collected post-mortem from subjects diagnosed with MDD, and found decreased NE transporter (NET) binding in the LC in samples from the depressive subjects compared to healthy subjects. This low NET density in the LC points to reduced NE in MDD, and the abnormal levels of NE and NET in these disorders suggests that NE has an important role in mediating resilience to stress.

Animal studies have demonstrated that the LC-NE system is involved in resilience via upstream modulation of different brain regions and circuits. Firstly, the LC-NE system influences resilience to chronic stress via downstream inhibition of the mesolimbic circuit. It was found that NE from the LC is necessary for resilience to chronic stress through the inhibition of downstream DA neurons in the VTA. In this study, brain-specific NE-depleted knockout (KO) mice were less likely to be resilient following chronic stress than wild-type (WT) mice. In vivo extracellular single-unit recordings on

VTA DA neurons showed that there was an increase in both the spontaneous firing rate and bursting activity of these neurons in the KO mice, suggesting that the lack of NE results in greater activity of the VTA DA neurons (Isingrini et al., 2016). In another experiment using the same strain of KO mice, the KO mice were resilient and had decreased activity of LC-NE neurons after being subjected to inescapable foot-shock stress (Isingrini et al., 2020), suggesting that NE depletion promotes resilience to trauma-type stress. Since NE depletion increases susceptibility in chronic stress, but promotes resilience in trauma-type stress, there may be a modality-dependent role of the LC-NE system in resilience (Isingrini et al., 2016; Isingrini et al., 2020; Zhang et al., 2019).

Animal Models of Stress Resilience:

Animal models are very useful for studying the biological mechanisms of MDD and PTSD, and several experimental approaches have been developed that allow us to investigate the mechanisms of resilience in rodents. One widely used behavioural model is the chronic social defeat stress paradigm (CSDS), which induces a depression-like phenotype by repeatedly exposing naïve mice to aggressor mice (Kudryavtseva et al., 1991). It results in susceptible mice displaying depression-like phenotypes that are characteristic of human symptoms, such as social avoidance and anhedonia (Golden et al., 2011), giving this model face validity. It also has predictive validity, since the effects of CSDS can be reversed by chronic antidepressant administration (Krishnan et al., 2007), making it an effective model of depression.

Other behavioural tests can be used to assess whether the resilient and susceptible mice display the expected anxiety and depression-like phenotypes following social defeat. These include the open field (OF) test and elevated plus maze (EPM), which

are used to assess exploratory behaviour and anxiety in rodents (Walsh & Cummins, 1976; Pellow et al. 1985). Since mice are adverse to unknown, large, bright and open environments (Choleris et al., 2001), the most anxious mice should spend the least time in the center zone of the OF arena and in the open arms of the EPM. Regardless of whether they are resilient or susceptible, all mice exposed to 10 days of CSDS are characterized by this anxious behaviour (Krishnan et al., 2007). Another test that is commonly used following CSDS is the sucrose preference test (SPT) as this can determine whether mice display anhedonia, which is an indicator of a depression-like phenotype. Anhedonia is characterized by decreased preference for sucrose water compared to plain water, and is associated with susceptibility to chronic stress (Krishnan et al., 2007).

Another major paradigm used to model stress in animals is learned helplessness (LH), which can be used as a model of PTSD. In this the animals are exposed to unpredictable and uncontrollable stress, in the form of electric shocks. This is followed by several days of controllable stress during which the animal is able to escape from the stressful stimulus. Mice that undergo foot-shock stress exhibit behavioural and physiological effects that persist for several weeks (Schöner et al., 2017). Similarly to CSDS, the LH paradigm produces two distinct groups of helpless and non-helpless mice, with helpless mice being susceptible and non-helpless being resilient. The phenotypes of susceptible individuals are similar to human PTSD symptoms, such as passive behaviour (not attempting to escape foot-shocks), increased emotional stress, and failure to learn that responding can cause relief (Seligman, 1972). This gives the LH model face validity, and it also has predictive validity because drugs that are used to treat PTSD in humans can reverse the effects of foot-shock stress in mice (Siegmund and Wotjak, 2007).

An important difference between the CSDS and LH paradigms is the type of stressor that is used. CSDS involves a social stress through direct antagonism with another rodent (Golden et al., 2011), while LH involves a physical stress, for example through electric foot-shocks (Schöner et al., 2017). Due to this difference in stressors, these two paradigms may call on different brain circuits and adaptive processes.

MDD and PTSD Comorbidity:

MDD and PTSD are highly comorbid disorders, and it is estimated that around half of PTSD patients also suffer from MDD (Kessler et al., 1995). It is important to understand this comorbidity because individuals that have both of these disorders are at a higher risk of suicidal behaviour, compared to individuals that have either MDD or PTSD alone (Oquendo et al., 2003). Several ideas have been proposed to explain the comorbidity. Flory & Yehuda (2015) suggested that comorbidity of these disorders reflects a separate trauma-related phenotype, with this phenotype representing an overall risk for both MDD and PTSD after exposure to trauma. Another explanation is that there are shared mechanisms of vulnerability to MDD and PTSD. When looking at the prevalence of psychiatric disorders and exposure to traumatic events in a sample of women, Breslau et al. (1997) found that pre-existing MDD increased vulnerability to developing PTSD following traumatic events. In another study, researchers examined MDD risk after exposure to traumatic events and found that people with PTSD are more likely to develop MDD after traumatic events than those without PTSD (Breslau et al., 2000). This gives support to the idea that there are shared mechanisms of vulnerability to MDD and PTSD, and suggests that there could also be shared mechanisms of resilience. It is important to understand the basis of the comorbidity because there are currently no specific treatments for patients that have both MDD and PTSD (Flory & Yehuda, 2015).

However, while there have been many preclinical studies using CSDS to study resilience to chronic stress in the context of depression, and LH to study resilience to trauma-type stress in the context of PTSD, there is limited research looking at resilience to both chronic and trauma-type stress in the same individual animals. Therefore, very little is known about the relationship between resilience to chronic and trauma-type stress. Amat et al. (2010) found that experiencing controllable stress may 'block' the negative effects of later exposure to CSDS and LH in rats. In this study rats that underwent CSDS were more likely to have escape deficits in a subsequent LH tail-shock experiment. However, if the rats were exposed to escapable shocks 7 days before CSDS, then this negative effect on escape deficits was blocked, suggesting that escapable stress can have stress-blunting effects. While this shows that exposure to controllable stress can influence responses to uncontrollable stress, it does not consider individual resilience to CSDS and how this may be associated with resilience to LH stress, highlighting the need to study CSDS and LH together in the context of individual resilience.

Sex Differences:

There is a difference in the prevalence of MDD and PTSD between men and women, with women being twice as likely to develop these disorders (Seedat et al., 2009; Yehuda et al., 2015). One idea proposed to explain this is that females are more likely to seek professional help, which leads to more diagnoses (Shi et al., 2021), but it could also point to females having greater susceptibility. The neurobiological basis of this difference in prevalence is not understood, but studies have begun to identify the underlying mechanisms. Human studies have shown that there are differences in the HPA axis between males and females in response to stress, and this has been proposed as a possible explanation for the sex difference in the prevalence of PTSD

(Olff et al., 2007). Kirschbaum et al. (1999) found that a psychosocial stress test induced an increase in ACTH levels in both men and women, but that this increase was higher in men. Sex differences in the HPA axis may also explain differences in the prevalence of MDD, as Chopra et al. (2009) found that females with MDD secrete significantly higher amounts of cortisol in response to a stressor than males.

However, despite the fact that females are disproportionately affected, male models have predominantly been used in preclinical research of the neurobiological mechanisms of these disorders (Blanchard et al., 1995). Studying resilience to chronic stress in females has been limited due to the traditional CSDS paradigm only being appropriate for male rodents (Golden et al., 2011). However, female protocols for CSDS have recently been developed, which will allow the advancement of knowledge of female resilience (Harris et al., 2018; Takahashi et al., 2017; Newman et al., 2019). In the protocol developed by Harris et al. (2018) male urine is applied to the females in order to induce the male aggressors to attack them. Using this protocol, Ortiz et al. (2022) found that CSDS causes hyperactivity of VTA DA neurons in susceptible but not resilient females, which is consistent with what has been observed in males (Krishnan et al. 2007). This study demonstrates the importance of including females in preclinical research, as it reveals that some common mechanisms underlie resilience to MDD in both sexes. They also found that injecting the susceptible females with ketamine reversed the depressive-like phenotype, showing that the CSDS protocol by Harris et al. (2018) has predictive validity.

Other preclinical research that has included females highlights the importance of a shift to using female models because of the sex differences that have been observed. Researchers used the subchronic variable stress (SCVS) model, which involves exposing mice to multiple different stressors over six days, and found that following

stress exposure female mice spend less time grooming, have reduced sucrose preference, and have increased corticosterone levels, none of which was observed in male mice (Hodes et al., 2015). Brancato et al. (2017) looked at the effects of SCVS on glutamatergic synapses in the NAc in both male and female mice, and found that vesicular glutamate transporter 1 (VGLUT1) levels were decreased only in stressed females. They also found that following SCVS increases in NAc vesicular glutamate transporter 2 (VGLUT2) levels were three times higher in stressed females compared to males. The LC-NE system may also have a role in the increased stress vulnerability of females, as Curtis et al. (2006) found that when exposed to hypotensive stress, the LC of female rats was more highly activated than that of males. Further investigation of the circuitry underlying stress resilience in female rodents is important in order to understand the sex differences in resilience to MDD and PTSD.

RATIONALE, AIMS AND HYPOTHESES

While there has been a considerable amount of research investigating the pathophysiology of MDD and PTSD, the distinct molecular mechanisms underlying resilience to these disorders remain unclear. The majority of animal studies investigate MDD or PTSD individually, and there are very few studies looking at both chronic social and trauma-type stress together. Moreover, there is less research that includes females as well as males, and so more needs to be elucidated about sex differences in resilience. It is important to continue to investigate mechanisms of resilience in order to determine novel therapeutic targets.

The objectives of this study are to determine whether resilience to chronic social stress and trauma-type stress are the same resilience at the behavioural level, and investigate sex differences in this resilience. This will be done using CSDS, an animal model of MDD, and LH, an animal model of PTSD. The overall aim of this study is to contribute to the understanding of resilience to both MDD and PTSD, which is important in order to facilitate the development of new therapeutics.

Aim 1 of this study is to determine whether mice that are resilient to chronic social stress are also resilient to trauma-type stress, in order to assess whether this is the same resilience. We will identify whether there is a cross-resilience by subjecting mice to CSDS, and then subjecting the same individuals to LH, to determine whether mice that are resilient to CSDS are also resilient following LH. We will perform the open-field (OF) test, elevated plus-maze (EPM) and sucrose preference test (SPT) to determine whether susceptible mice display the anxious and anhedonia phenotypes that are expected following chronic social stress, before beginning the LH protocol. The CSDS protocol will be carried out first because the resilient phenotype typically

persists for 30 days following defeats (Meduri et al., 2013), and this gives sufficient time to complete the other tests. Following this, the LH paradigm will consist of four test days over a period of 30 days, to determine the rate of extinction of the susceptible phenotype. Our hypothesis is that there is a cross- resilience to chronic social and trauma-type stress, and that individuals that are resilient to CSDS are more likely to be resilient to LH from the first test day.

Aim 2 of this study is to determine whether there are differences between males and females in the pattern of resilience to chronic social and trauma-type stress. We want to investigate whether the same proportion of females and males share resilience to both CSDS and LH, and also whether there are differences in the rate of extinction of the susceptible phenotype in LH between CSDS resilient males and females. Firstly, we hypothesize that females that are resilient to CSDS are more likely to be resilient to LH by test day 30 than those that are susceptible. Secondly, because there is a higher prevalence of PTSD in women (Yehuda et al., 2015), we hypothesize that there will be a different pattern of resilience in females than males, in that the susceptible phenotype during LH will persist longer in females than in males.

METHODS

Animals and Housing

C57BL/6J mice were obtained from the Giros colony and CD-1 mice were obtained from Charles River Lab, and both were housed in the Douglas Institute Research Center. The mice were kept under standard conditions at 22±1°C, 60% relative humidity, and a 12 hour light-dark cycle with food and water available *ad libitum*. We used C57BL/6J mice that were between 8-11 weeks old at the beginning of testing, and they were individually housed once the experiment began. CD-1 mice were obtained as 3-month old retired breeders, and individually housed.

AIM 1:

We used 38 test and 29 control males altogether, over three different cohorts.

Chronic Social Defeat Stress

Prior to beginning the defeats CD-1 aggressor mice were screened over 3 consecutive days, and selected according to their response to a novel juvenile male C57BL/6J mouse that was placed in their home cage. They were selected if they had an attack latency under 60 seconds, consistent attacks over 180 seconds and if they attacked on 2 consecutive days (Golden et al. 2011).

Male C57BL/6 mice were subjected to a daily 5 minute defeat with a different CD-1 mouse on each of 10 consecutive days. A defeat lasted for either 5 minutes or 10 attacks, whichever occurred first, and defeats were carried out in large rat cages with two halves separated by a plexiglass divider. After each defeat the mouse remained in the aggressor's home cage but separated from the aggressor by a perforated translucent Plexiglas divider. This allowed the mouse to have visual, olfactory and

auditory interaction with the aggressor until the next defeat. Control mice were housed in pairs separated by a Plexiglas divider and were moved to a different cage each day, but never had physical contact with each other and they were not exposed to CD-1 aggressors. The controls were housed in a different room to the defeated mice, where there were no CD-1 mice present.

Social Interaction Test

24 hours after the last defeat, the defeated mice were tested in the social interaction (SI) test. This was carried out in an open field box (45 cm × 45 cm × 45 cm) with a Plexiglas wire mesh enclosure (10 cm wide × 6.5 cm deep × 42 cm high) placed in a section of the box designated as the 'social interaction zone' (SIZ). In the first phase of this test, the mouse was placed in the box for 150 seconds with an empty wire mesh enclosure. After this, the mouse was placed back in his home cage for 30 seconds. For the second phase, the mouse was placed in the box for another 150 seconds, but this time with a novel CD-1 aggressor present in a different wire mesh enclosure. The time the mouse spent in the SIZ during each phase was measured, and this was used to calculate the individual's SI ratio and determine whether they were resilient or susceptible.

SI ratios were calculated using the formula from Henriques-Alves and Queiroz (2016), by dividing the time spent in the SIZ with an aggressive CD-1 mouse present by the sum of time spent in the SIZ with and without the aggressor present. This is a slightly adjusted version of the original equation used by Golden et al. (2011). We chose to use this as it allows the inclusion of mice that spent 0 seconds in the SIZ without the aggressor present, something that does not work in the original equation. Resilience is defined by a ratio greater than 0.5 – meaning they spent more time in the SIZ when

the aggressor was present. The SI tests were recorded using Anymaze software, and time spent in the SIZ was determined using TopScan tracking software.

A second SI test was carried out 24 hours after the last day of the LH protocol, to determine whether there had been extinction of the CSDS resilient phenotype.

Open Field Test

24 hours after the SI test, all mice were tested in OF chambers. Locomotor activity was measured with an Omnitech digiscan activity monitor. The movements of the mice were measured for 10 minutes in open field chambers (40 cm × 40 cm × 40 cm) with photocells and plexiglass walls and floors. The data were recorded using VersaMax software.

Elevated Plus Maze

The elevated plus maze (EPM) is made up of 2 opposing open arms (30 × 5cm) and 2 opposing closed arms (30 × 5 × 11cm) branching out from a center zone (5 cm^2), and is 50cm high. The mice were placed in the center zone of the EPM, facing an open arm, and were allowed to explore the maze for 10 minutes. The mouse was considered to be inside an open or closed arm when the center of its body was inside it. We recorded the amount of time spent in the open and closed arms. The EPM tests were recorded using Anymaze software, and time spent in each arm of the maze was determined using TopScan tracking software.

Sucrose Preference Test

The sucrose preference test (SPT) was carried out in the animal's home cage over 7 days using the protocol described by Liu et al. (2018), but without the apparatus adaptation step, since we did not use the same apparatus. *Habituation:* During the first

48 hours the mice were presented with two bottles along with regular food – one with water, and one with 1% sucrose solution. The bottles were swapped each day to avoid place preference. *Baseline 1 measurement:* At 5pm on day 3 the bottles were removed from the cage, filled with fresh water and sucrose solution, and weighed. At 8pm on day 3, food was removed and the water and sucrose bottles were placed in the cage. At 8am on day 4, the bottles were removed as quickly as possible and weighed. The baseline measurement was calculated as a percentage of the weight of the sucrose bottle over the total weight of the sucrose and water bottles. *Baseline 2 measurement:* The mice were given normal food and water until 8pm on day 4. All the steps were then repeated for the second baseline measurement, which took place from 8pm on day 4 to 8am on day 5. *Sucrose preference:* Mice had access to regular food and water until 8pm on day 5, when these were removed from the cage and they underwent food and water deprivation for 24 hours. At 8pm on day 6, the mice were given fresh water and sucrose solution, after these bottles had been weighed. At 8am on day 7, the bottles were removed and weighed again. Sucrose preference was calculated as a percentage of the weight of the sucrose bottle over the total weight of the sucrose and water bottles.

Learned Helplessness

A shuttle box apparatus was used to subject the mice to foot-shocks, consisting of a Plexiglas shock chamber with a gate separating two compartments, along with a stainless-steel grid floor. The mice were first subjected to two training sessions 24 hours apart, which each consisted of 120 shocks over 30 minutes. Shock intensity was set as 0.25mA, the shocks lasted for 3 seconds each, and interval lengths between shocks were randomized. During these training sessions the gate between the compartments was closed. *Test day 1:* 24 hours later they were subjected to an

assessment session, which consisted of 20 shocks. Before the initial shock, there was a 3 minute habituation period during which the mouse could explore the two compartments. The gate between the compartments was closed before the first shock, but it opened each time a shock occurred, giving the mouse the choice to escape to the other compartment. Each shock was terminated either as soon as the animal shuttled to the other compartment (defined as an escape), or at the end of the 24 second shock period (defined as a failure). This session lasted approximately 15-20 minutes, depending on how many escapes the mouse made and how long it took to make each escape. Latency to escape each shock and the number of escapes and failures were recorded. Foot-shock escape behaviour is an indicator of resilience, so susceptible (helpless) mice had a high escape latency and number of failures, while resilient (non-helpless) mice had a low escape latency and number of failures. Following the completion of test day 1, they were also subjected to assessment sessions on test days 10, 20 and 30, in order to investigate changes in helpless behaviour over time. Controls underwent the same procedure as the defeated mice.

Using MATLAB software, a k-means (k=2) cluster analysis using the escape latency and number of escape failures for each individual was applied in order to categorize the mice into resilient (non-helpless), and susceptible (helpless) groups for each test day.

Z-Score Analysis

Z-scores are standardized scores that allow for the normalization of results when several different tests are used within a study. For each observation, they reveal how many standard deviations this is away from the mean of a control group. Z-score analysis was used to give each mouse its own individual score of emotionality by

integrating the data from each behavioural test, along with change in weight during CSDS. To do this, the mean and standard deviation of the control group were calculated for each test. These values were then used to calculate the Z-score of each individual. For each test, the mean value was subtracted from the observation for each individual, and then this was divided by the standard deviation. The z-scores were used to identify how many standard deviations an individual observation was from the mean of the control group - 0, 0.25, 0.5, 0.75 or 1 - giving a susceptibility index for each measurement. These indexes were averaged to form an overall susceptibility index for each individual.

AIM 2:

We used 18 test and 13 control females altogether, over two different cohorts. The same tests and analyses were carried out for females, but with a different CSDS protocol.

Chronic Social Defeat

The female social defeat protocol described by Harris et al. (2018) was used, with some adjustments. Screening and defeats were carried out in the same way as male CSDS, but urine from male mice was applied to the females directly before each defeat to ensure that the CD-1 mice displayed the necessary level of aggression towards them. *Urine collection:* Urine was gathered from male C57 mice, with a mixture from 2-3 mice being used for each day of defeats. Urine was collected into 1.5ml Eppendorf tubes by scruffing the mice. This was collected up to 1 week prior to use, and stored at +4°C. Each female was given the same mixture, but the mixture was different each day to ensure that the aggressors did not become familiar with the scent. *Urine application:* As described by van Doeselaar et al. (2021), urine was applied to the

females using a paintbrush on the base of the tail, vaginal orifice, back and face, using approximately 0.4ul per mouse. As soon as urine was applied, the female was placed with the aggressor. *CD1 behaviour:* Defeats lasted for either 5 minutes or 10 attacks, but were ended immediately if mounting occurred. If an aggressor underperformed on more than one day, it was replaced with a new aggressor. *Controls:* Control females were housed in pairs and moved between cages every day. They had no physical contact with each other, and did not have urine applied to them.

Social Interaction Test

This was performed in the same way as it was for males.

Male vs Female Comparisons

Learned Helplessness: To investigate whether there are sex differences in latency to escape LH foot-shocks in both resilient and susceptible groups, mean latency to escape on test day 1 and test day 30 were compared between males and females.

Chronic Social Defeat and Learned Helplessness Resilience: The proportion of individuals that were resilient to learned helplessness were calculated for each LH test day, for CSDS resilient, CSDS susceptible, and control groups. This data was used to determine whether the proportion of mice that were resilient on each LH test day was different between males and females in CSDS resilient and susceptible groups.

Susceptibility Indexes: Susceptibility indexes were compared between CSDS resilient males and females, and between CSDS susceptible males and females. The same male control group was used to calculate female susceptibility indexes because this allows the comparison between the two groups, as was described by Guilloux et al. (2011).

Statistical Analyses:

Social Interaction Test: Normality of distribution of the data was assessed using the Shapiro-Wilk test. Data from the first SI test were non-normally distributed, so the Kruskal-Wallis one-way analysis of variance test was used to investigate whether there was a significant difference in the SI ratios between control, resilient and susceptible groups. Data from the second SI test were normally distributed, so a one-way analysis of variance (ANOVA) test was used to investigate differences in SI ratios between the groups.

Elevated Plus Maze, Open Field and Sucrose Preference Tests: For all of these tests, normality of distribution of the data was assessed using the Shapiro-Wilk test. The data were non-normally distributed, so the Kruskal-Wallis one-way analysis of variance test was used to investigate whether there was a significant difference in time spent in the center zone and margins of the OF chamber, time spent in the open arms of the EPM, and sucrose preference between control, resilient and susceptible groups.

Learned Helplessness: Normality of distribution of the data for latency to escape was assessed using the Shapiro-Wilk test. The data were non-normally distributed, so the Kruskal-Wallis one-way analysis of variance test was used to investigate whether there was a significant difference in the latency to escape between control, resilient and susceptible groups.

The proportion of mice that were resilient on each LH test day were determined for CSDS resilient, susceptible and control groups. Fisher's exact test was used determine whether the proportion of mice that were resilient on each LH test day was significantly different between the groups.

Mean latency to escape on test day 1 and test day 30 were compared between males and females. Normality of distribution of the data was assessed using the Shapiro-Wilk test. The unpaired t-test was used for normally distributed data and the Mann-Whitney U test was used for non-normally distributed data.

Susceptibility Indexes: Unpaired t-tests were used to compare susceptibility indexes between CSDS resilient and susceptible mice, CSDS resilient mice and controls, CSDS susceptible mice and controls, and between males and females.

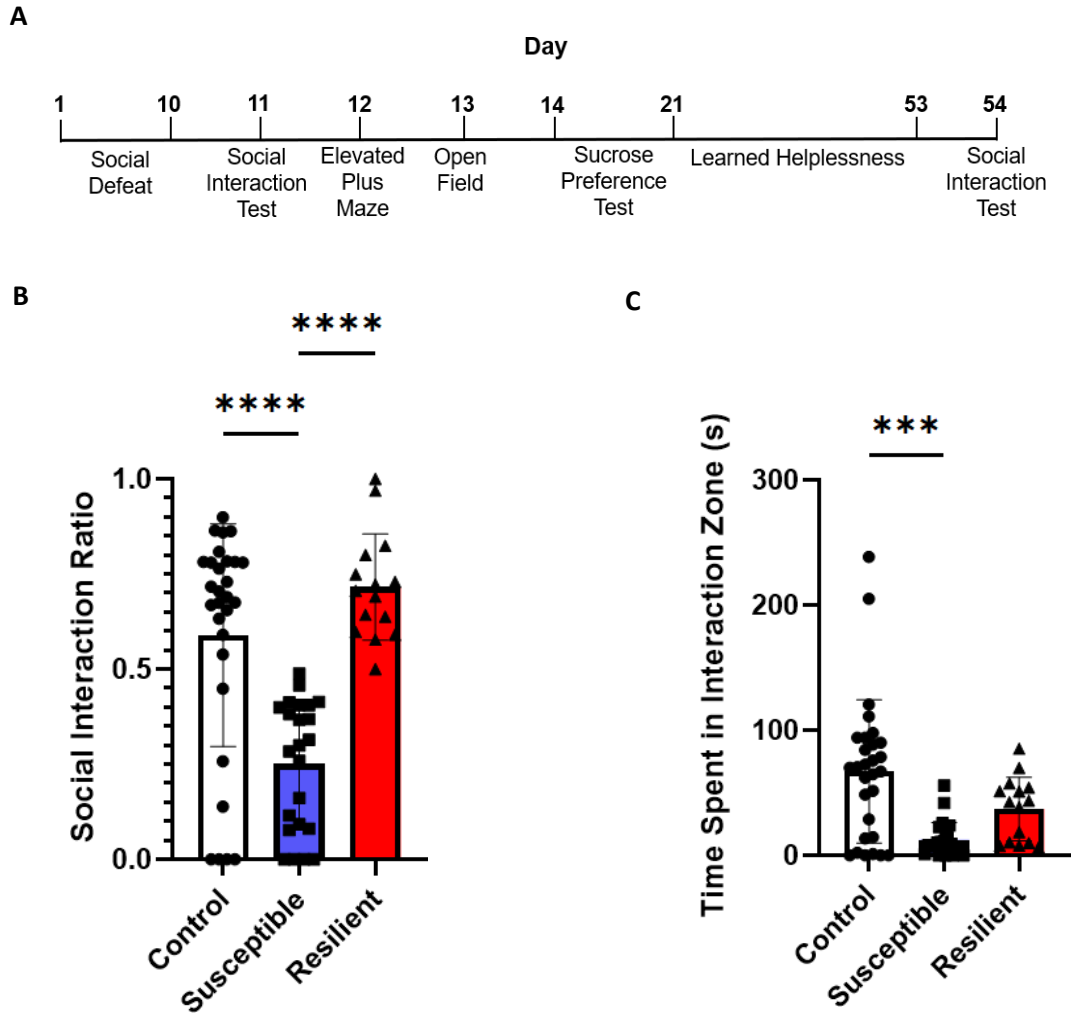
Chronic Social Defeat and Learned Helplessness Resilience: Chi-square analyses or Fisher's exact test were used determine whether the proportion of mice that were resilient on each LH test day was significantly different between males and females in CSDS resilient and susceptible groups.

RESULTS

<u>Aim 1:</u> Resilience to chronic social stress is associated with resilience to trauma-type stress in male mice:

To investigate cross-resilience to chronic social and trauma-type stress we carried out several behavioural tests (Fig. 1A), beginning with the CSDS model. Male mice in the defeated group (n=38) were subjected to 10 days of CSDS, which was followed by a social interaction (SI) test for both defeated males and controls (n=29) to screen for depressive-like social avoidance behaviour (Fig. 1B). Defeated males were categorized into resilient (n=15) and susceptible (n=23) groups depending on their SI ratio, with a ratio of <0.5 being an index of susceptibility and a ratio of >0.5 being an index of resilience. 39.5% of the defeated males were categorized as resilient and 60.5% as susceptible, which validates the social defeat protocol as 30-40% of males are expected to show a resilient phenotype following defeats (Golden et al. 2011). There was a significant difference between the SI ratios of the control, susceptible, and resilient groups (Kruskal–Wallis, $\chi 2=27.8$, $P<0.0001$ for main group effect; Control vs Susceptible $P<0.0001$ (n=29, 23), Susceptible vs Resilient $P<0.0001$ (n=23, 15), Control vs Resilient $P=0.89$ (n=29, 15)) and susceptible males had the lowest SI ratios (Fig. 1B). Susceptible males exhibited increased social avoidance, spending significantly less time in the SIZ when the CD-1 aggressor was present compared to the controls, which indicates a depressive-like phenotype (Kruskal–Wallis, $\chi 2=17.0$, $P=0.0002$ for main group effect; Control vs Susceptible $P=0.0001$ (n=29, 23), Susceptible vs Resilient $P=0.07$ (n=23, 15), Control vs Resilient $P=0.62$ (n=29, 15)) (Fig. 1C).

Figure 1: Susceptible males display depressive-like behavior following chronic social defeat stress.

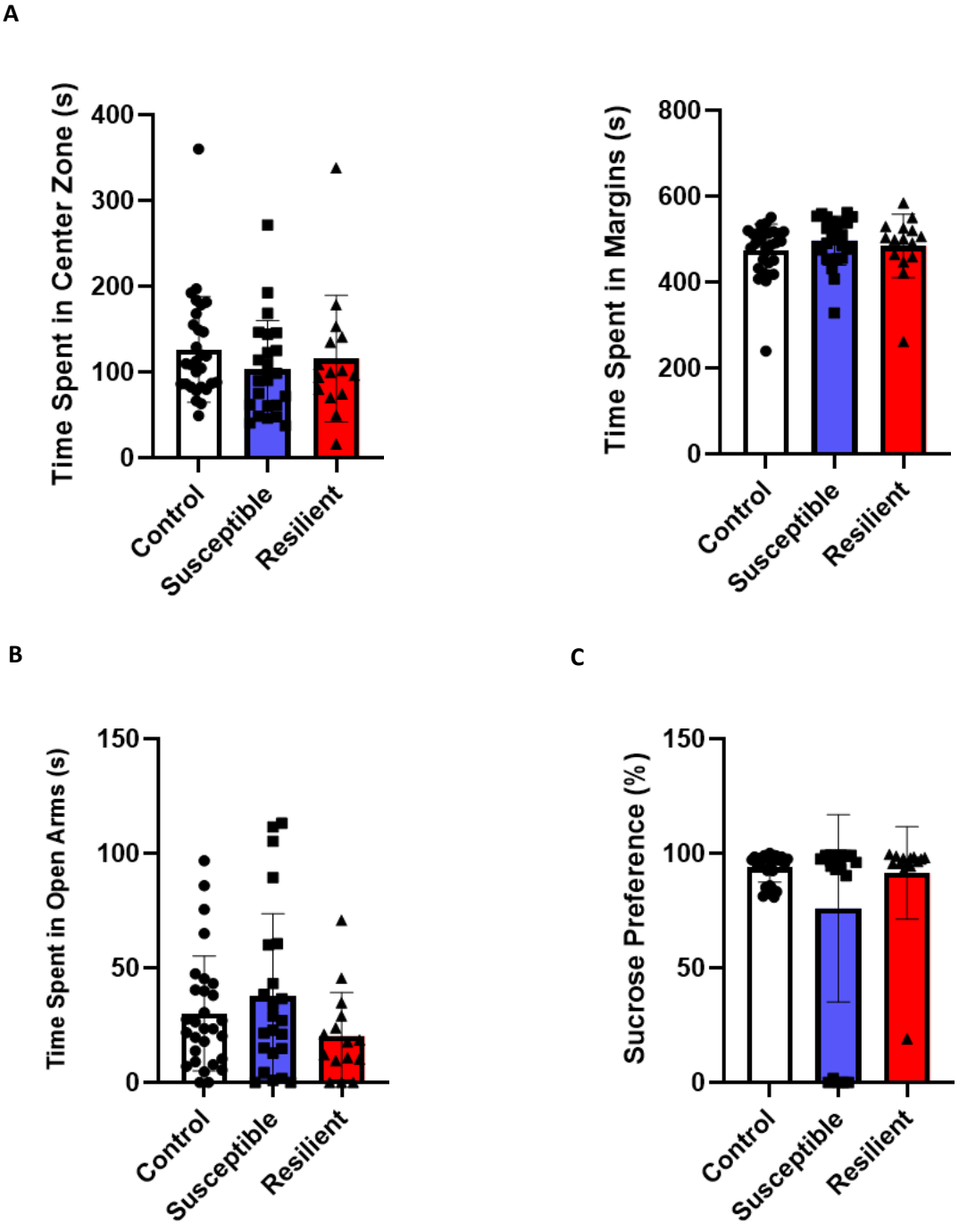


A: Experimental timeline. **B:** Social interaction ratio following the social interaction test, with mice separated into control, susceptible and resilient categories (Kruskal–Wallis, $\chi2=27.8$, $P<0.0001$; n=29, 23, 15). **C:** Time spent in the social interaction zone with an aggressive mice present during the social interaction test, by control, susceptible and resilient mice (Kruskal–Wallis, $\chi2=17.0$, $P=0.0002$; n=29, 23, 15). Error bars represent SD.

24 hours after the SI test, the males were screened for the appearance of anxiety in an OF arena followed by the EPM. During the OF test, susceptible males spent less time in the center zone and more time in the margins which is an indicator of an anxious state, however, this was not significantly different from control and resilient males (Kruskal–Wallis, $\chi2=3.0$, $P=0.23$; for main group effect; Control vs Susceptible

P=0.27 (n=29, 23), Susceptible vs Resilient P>0.99 (n=23, 15), Control vs Resilient P>0.99 (n=29, 15)) (Fig. 2A). Time spent in the open arms of the EPM was not significantly different between groups (Kruskal–Wallis, $\chi 2=2.7$; P=0.26 for main group effect; Control vs Susceptible P>0.99 (n=30, 23), Susceptible vs Resilient P=0.40 (n=23, 15), Control vs Resilient P=0.46 (n=30, 15)) (Fig. 2B). One mouse from the control group was identified as an outlier in the EPM because the data point was extremely high, so was removed from all analyses at the end of the experiment. 24 hours after the EPM the males were tested in the SPT. Low preference for sucrose is associated with anhedonia, which is another depressive-like phenotype (Liu et al. 2018). While CSDS susceptible mice had the lowest preference for sucrose, the difference was not significant between the groups (Kruskal–Wallis, $\chi 2=0.02$; P=0.99 for main group effect; Control vs Susceptible P>0.99 (n=30, 23), Susceptible vs Resilient P>0.99 (n=23, 15), Control vs Resilient P>0.99 (n=30, 1)) (Fig. 2C) and it was observed that some susceptible males demonstrated very high sucrose preference.

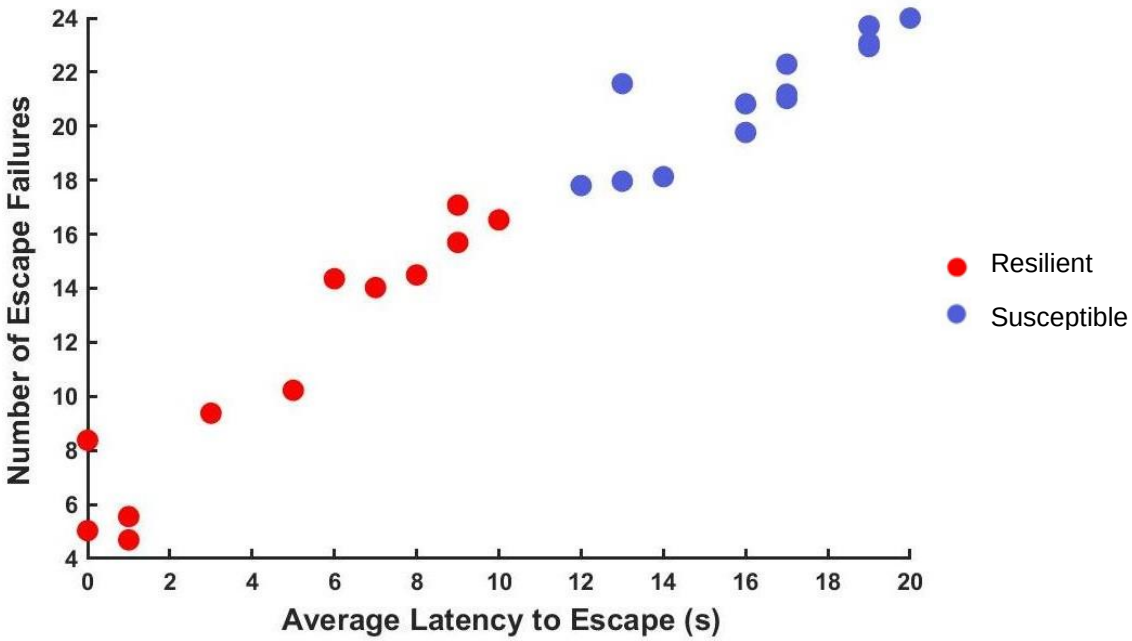
Figure 2: Tests to measure anxiety and depressive-like phenotypes in male mice following chronic social defeat stress.



A: Time spent in the center zone (left) and the margins (right) during the open field test for CSDS control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=3.0$, P=0.23; n=29, 23, 15). **B:** Time spent in the open arms of the elevated plus maze by CSDS control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=2.5$, P=0.29; n=29, 23, 15). **C:** Sucrose preference of CSDS control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=0.14$, P=0.93; n=29, 23, 15). Error bars represent SD.

To investigate whether mice that that have been categorised as resilient to chronic social stress are also resilient to trauma-type stress, we subjected the same individuals to a LH uncontrollable foot-shock stress protocol. Control mice were also subjected to the same foot-shock stress, to investigate differences in resilience between individuals that underwent chronic social stress and those that didn't. The foot-shock escape behavior (number of failures to escape and latency to escape) was assessed and mice were classified into stress-resilient (non-helpless) or stress-susceptible (helpless) using k-means clustering algorithm. Figure 3 depicts an example of a K-means cluster analysis, calculated from test day 1 data from males.

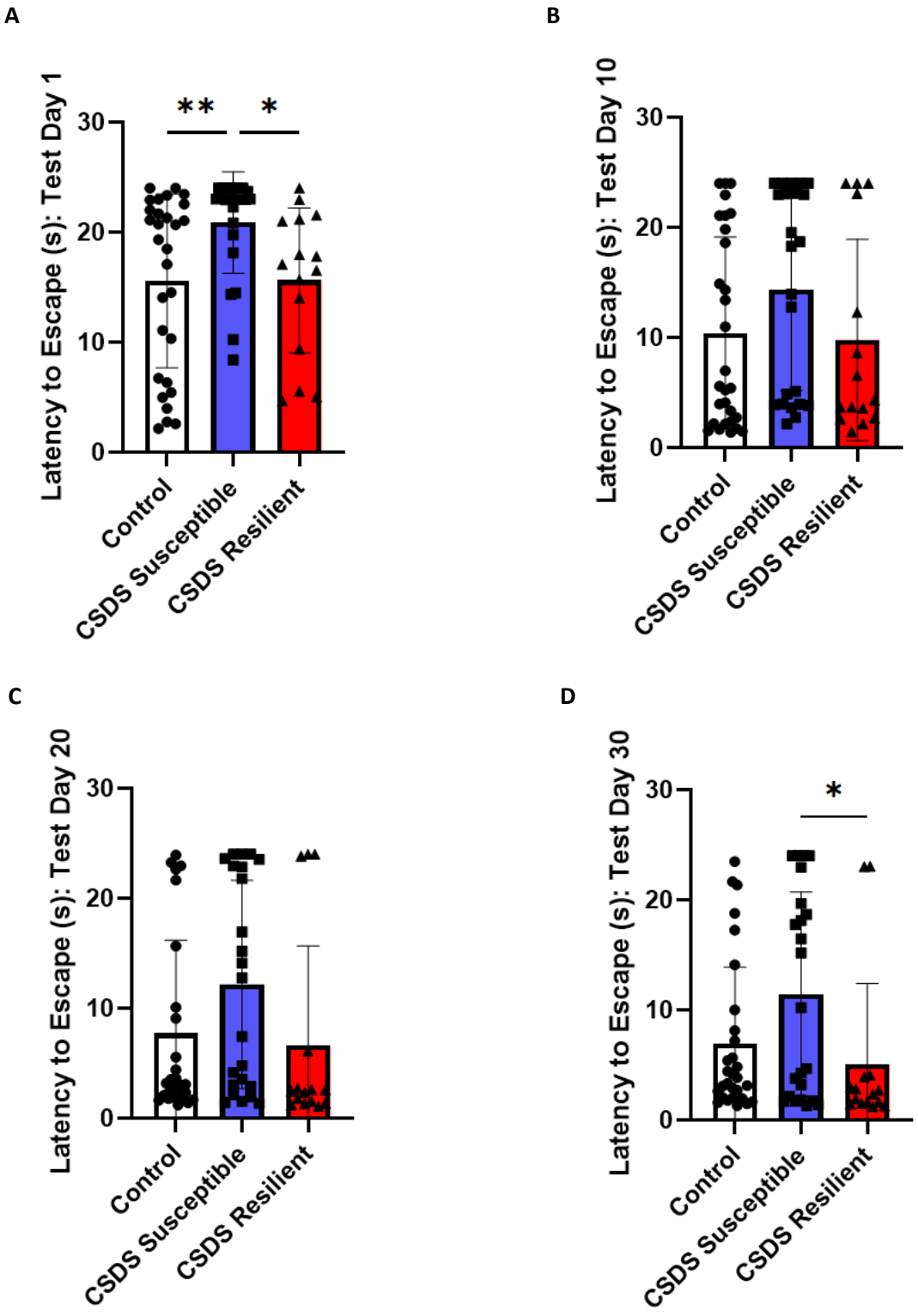
Figure 3: Example of a K-means cluster analysis.



K-means cluster analysis for learned helplessness test day 1. Male mice were categorized into two clusters: resilient (non-helpless) and susceptible (helpless) based on average latency to escape foot-shocks and number of escape failures. Each mouse belongs to the cluster with the nearest mean.

Figure 4 shows the average latency of mice to escape foot-shocks following the 10 days of CSDS, over a 30 day period of LH test days. On test day 1, CSDS resilient males took significantly less time to escape the foot-shocks than those that were susceptible, and CSDS susceptible mice had a significantly higher latency to escape than controls (Kruskal–Wallis, $\chi2=11.2$; $P=0.0038$ for main group effect; Control vs Susceptible $P=0.014$ ($n=30, 23$), Susceptible vs Resilient $P=0.011$ ($n=23, 15$), Control vs Resilient $P>0.99$ ($n=30, 15$)) (Fig. 4A). There were no significant differences between groups for latency to escape on test day 10 (Kruskal–Wallis, $\chi2=3.2$; $P=0.20$ for main group effect; Control vs Susceptible $P=0.31$ ($n=30, 23$), Susceptible vs Resilient $P=0.46$ ($n=23, 15$), Control vs Resilient $P>0.9999$ ($n=30, 15$)) (Fig. 4B) and test day 20 (Kruskal–Wallis, $\chi2=5.3$; $P=0.071$ for main group effect; Control vs Susceptible $P=0.41$ ($n=30, 23$), Susceptible vs Resilient $P=0.074$ ($n=23, 15$), Control vs Resilient $P=0.87$ ($n=30, 15$)) (Fig. 4C). However, by test day 30, CSDS resilient mice had a significantly lower latency to escape the foot-shocks than CSDS susceptible mice (Kruskal–Wallis, $\chi2=6.5$; $P=0.04$ for main group effect; Control vs Susceptible $P=0.99$ ($n=30, 23$), Susceptible vs Resilient $P=0.034$ ($n=23, 15$), Control vs Resilient $P=0.22$ ($n=30, 15$)) (Fig. 4D). This data suggests that resilience to chronic social stress is associated with resilience to trauma-type stress in male mice.

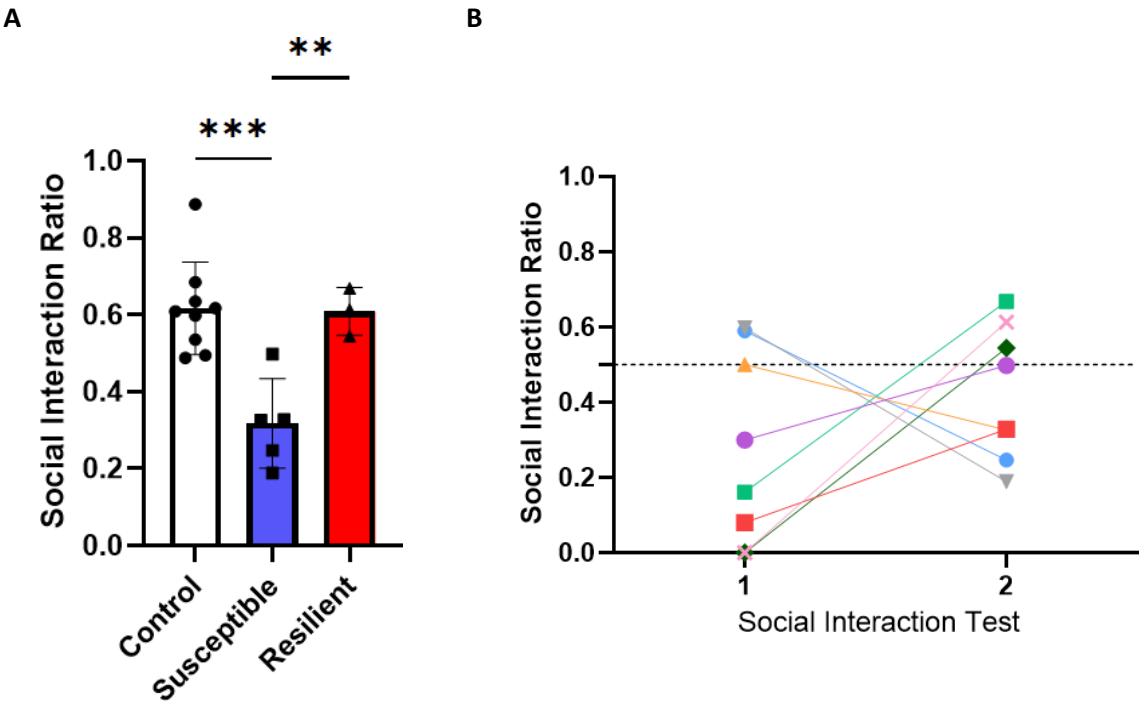
Figure 4: Males susceptible to chronic social defeat stress take longer to escape foot-shock stress in the learned helplessness test.



Average latency to escape 20 0.25mA foot-shocks in the learned helplessness shuttle-box for CSDS control, susceptible and resilient mice on **A:** Test day 1 (Kruskal–Wallis, $\chi2=12.2$, $P=0.0023$; n=29, 23,15) **B:** Test day 10 (Kruskal–Wallis, $\chi2=3.6$, $P=0.16$; n=29, 23,15) **C:** Test day 20 (Kruskal–Wallis, $\chi2=5.59$, $P=0.061$; n=29, 23, 15) **D:** Test day 30 (Kruskal–Wallis, $\chi2=6.5$, $P=0.04$; n=29, 23,15). Error bars represent SD.

To investigate extinction of CSDS susceptibility and resilience over the 30 days of LH, we carried out a second SI test 24 hours after test day 30. We decided to do this following completion of some cohorts, so this was only carried out for the final cohort of defeated males (n=8) and controls (n=9). Defeated males were categorized into resilient (n=3) and susceptible (n=5) groups depending on their SI ratio: 37.5% of the defeated males were resilient and 62.5% were susceptible. There was a significant difference between the SI ratios of the control, susceptible, and resilient groups, and susceptible males had the lowest SI ratios (One-way ANOVA, $F(2,14) = 12.23$; $P=0.0008$ for main group effect; Control vs Susceptible $P=0.0008$ (n=9, 5), Susceptible vs Resilient $P=0.009$ (n=9, 3), Control vs Resilient $P=0.99$ (n=9, 3)) (Fig. 5A). 100% of mice that were resilient following the first SI test were categorized as susceptible in the second SI test, and 80% of the mice that were susceptible following the first SI test were categorized as resilient in the second SI test (Fig. 5B). As the resilient and susceptible states are expected to last around 30 days after CSDS (Meduri et al., 2013), and the second SI test was carried out 43 days after the first SI test, we would expect some extinction of resilience and susceptibility. Also, the loss of novelty between the first and second SI test might be a confounding variable. However, it is surprising that there was a complete reversal of both phenotypes in most individuals. This data suggests that being exposed to trauma-type stress such as foot-shocks changes an individual's response and vulnerability to chronic stress.

Figure 5: Reversal of susceptibility and resilience of male mice in a second social interaction test following learned helplessness.



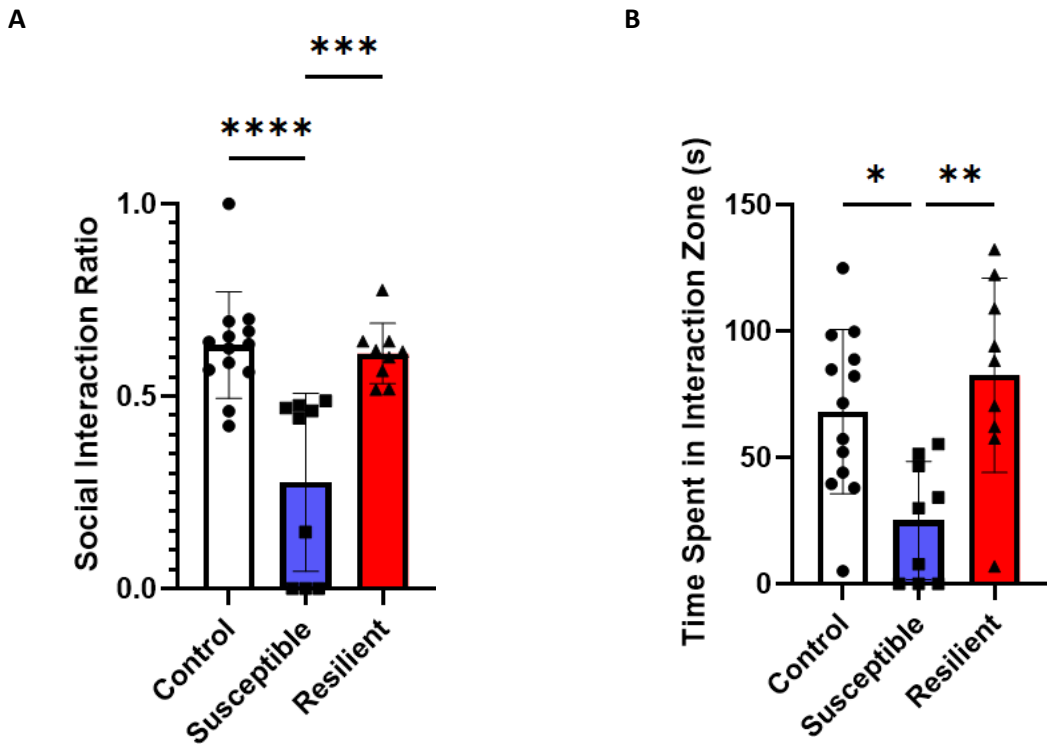
A: Social interaction ratio following the second social interaction test, with mice separated into control, susceptible and resilient categories (One-way ANOVA, F(2,14)=12.23; P=0.0008; n=9, 5, 3). Error bars represent SD. **B:** The change in SI ratio of each mouse between the first and second SI test. The dotted line is the threshold for resilience (>0.5 = resilience).

<u>Aim 2:</u> Females that are susceptible following chronic social defeat stress may be less vulnerable to learned helplessness stress than susceptible males:

To investigate sex differences in resilience to chronic social and trauma-type stress we subjected females to the same set of behavioural tests as males, beginning with CSDS. Mice in the defeat group (n=18) were subjected to 10 days of social defeat, which was followed by the SI test for both defeated mice and controls (n=13). Defeated mice were categorized into resilient (n=9) and susceptible (n=9) groups depending on their SI ratios. 50% of the defeated mice were categorized as resilient and 50% as susceptible, percentages that have been obtained by other researchers using the same female social defeat protocol (Ortiz et al., 2022). There was a significant

difference between the SI ratios of the control, susceptible, and resilient groups, and susceptible females had the lowest SI ratios (Kruskal–Wallis, $\chi 2 = 15.4$; $P=0.0005$ for main group effect; Control vs Susceptible $P=0.0007$ ($n=13, 9$), Susceptible vs Resilient $P=0.0047$ ($n=9, 9$), Control vs Resilient $P>0.99$ ($n=13, 9$)) (Fig. 6A). Susceptible females exhibited increased social avoidance, spending significantly less time in the SIZ when the CD-1 aggressor was present compared to the controls, indicating a depressive-like phenotype (Kruskal–Wallis, $\chi 2=12.1$; $P=0.0024$ for main group effect; Control vs Susceptible $P=0.022$ ($n=13, 9$), Susceptible vs Resilient $P=0.0027$ ($n=9, 9$), Control vs Resilient $P>0.99$ ($n=13, 9$)) (Fig. 6B).

Figure 6: Susceptible female mice display depressive-like behavior following chronic social defeat stress.

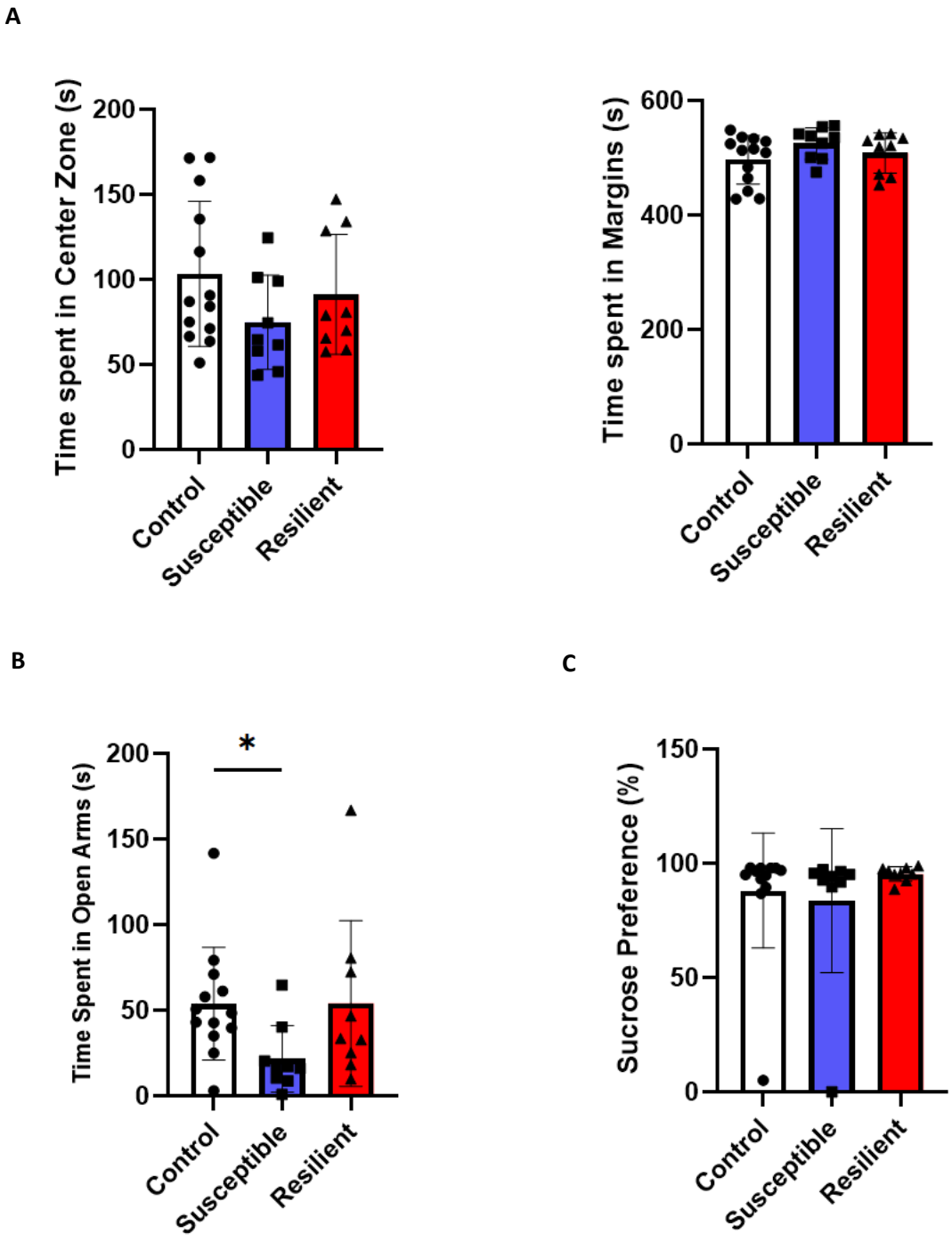


A: Social interaction ratio following the social interaction test, with mice separated into control, susceptible and resilient categories (Kruskal–Wallis, $\chi2=15.4$, P=0.0005; n=13, 9, 9). **B:** Time spent in the social interaction zone with an aggressive mice present during the social interaction test, by control, susceptible and resilient mice (Kruskal–Wallis, $\chi2=12.1$, P=0.0024; n=13, 9, 9). Error bars represent SD.

24 hours after the SI test, the females were screened for the appearance of anxiety in the OF arena followed by the EPM. Susceptible females spent less time in the center zone and more time in the margins during the OF test than control and resilient females, but this difference was not significant between groups (Kruskal– Wallis, $\chi2=3.5$; P= 0.18 for main group effect; Control vs Susceptible P=0.19 (n=13, 9), Susceptible vs Resilient P=0.94 (n=9, 9), Control vs Resilient P>0.99 (n=13, 9)) (Fig. 7A). However, susceptible females spent significantly less time in the open arms of the EPM compared to the controls, indicating that they were more anxious (Kruskal–Wallis, $\chi2=7.5$; P=0.023 for main group effect; Control vs Susceptible P=0.023 (n=13, 9), Susceptible vs Resilient P=0.138 (n=9, 9), Control vs Resilient

P>0.99 (n=13, 9)) (Fig. 7B). While CSDS susceptible females had the lowest preference for sucrose, the difference was not significant between groups and similarly to the males, most CSDS susceptible females had very high sucrose preference (Kruskal–Wallis, $\chi 2=2.2$; P=0.33 for main group effect; Control vs Susceptible P=0.92 (n=13, 9), Susceptible vs Resilient P=0.44 (n=9, 9), Control vs Resilient P>0.99 (n=13, 9)) (Fig. 7C).

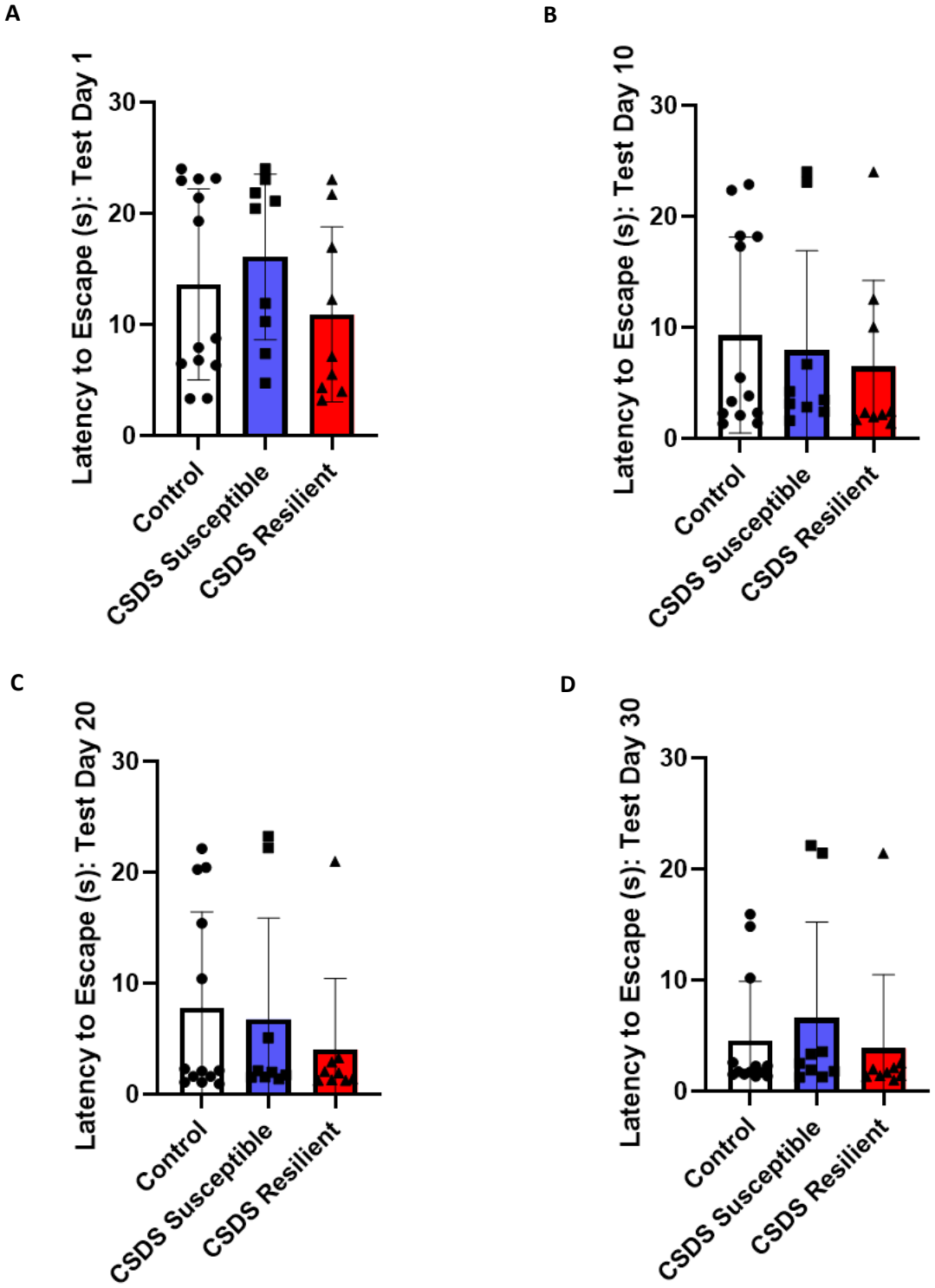
Figure 7: Tests to measure anxiety and depressive-like phenotypes in female mice following chronic social defeat stress.



A: Time spent in the center zone (left) and the margins (right) during the open field test for control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=3.5$, $P=0.18$; n=13, 9, 9). **B:** Time spent in the open arms of the elevated plus maze by control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=7.5$, $P=0.023$; n=13, 9, 9). **C:** Sucrose preference of control, susceptible and resilient mice (Kruskal–Wallis, $\chi 2=2.2$, $P=0.33$; n=13, 9, 9). Error bars represent SD.

To investigate whether females that are resilient to chronic social stress are also resilient to trauma-type stress, we subjected the same individuals to the LH foot- shock stress protocol. Figure 8 shows the average latency of mice to escape foot- shocks following 10 days of CSDS, over 30 days of LH. Low latency to escape is one of the indicators of resilience. Females that were resilient to CSDS took less time to escape the foot-shocks than CSDS susceptible females, but there wasn't a significant difference between groups on test day 1 (Kruskal–Wallis, $\chi 2=2.4$; $P=0.30$ for main group effect; Control vs Susceptible $P>0.99$ ($n=13, 9$), Susceptible vs Resilient $P=0.37$ ($n=9, 9$), Control vs Resilient $P=0.97$ ($n=13, 9$)) (Fig. 8A), test day 10 (Kruskal–Wallis, $\chi 2=1.2$, $P=0.55$ for main group effect; Control vs Susceptible $P>0.99$ ($n=13, 9$), Susceptible vs Resilient $P=0.86$ ($n=9, 9$), Control vs Resilient $P>0.99$ ($n=13, 9$)) (Fig. 8B), test day 20 (Kruskal–Wallis, $\chi 2=0.88$; $P=0.64$ for main group effect; Control vs Susceptible $P>0.99$ ($n=13, 9$), Susceptible vs Resilient $P>0.99$ ($n=9, 9$), Control vs Resilient $P>0.99$ ($n=13, 9$)) (Fig. 8C), or test day 30 (Kruskal–Wallis, $\chi 2=1.5$; $P=0.47$ for main group effect; Control vs Susceptible $P>0.99$ ($n=13, 9$), Susceptible vs Resilient $P=0.67$ ($n=9, 9$), Control vs Resilient $P>0.99$ ($n=13, 9$)) (Fig. 8D). This data suggests that, unlike males, resilience to chronic social stress is not strongly associated with resilience to trauma-type stress in female mice.

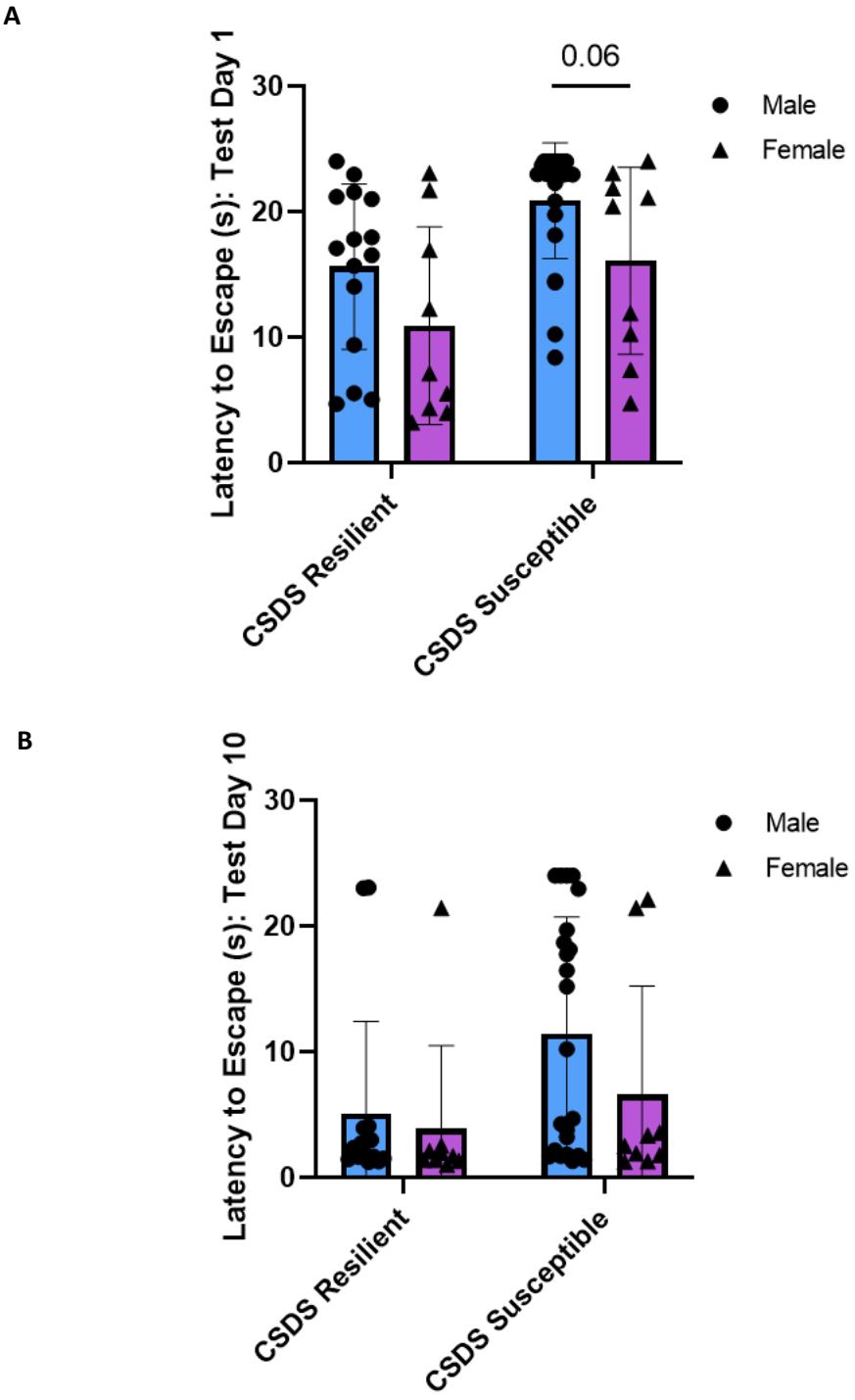
Figure 8: Latency to escape foot-shocks on each learned helplessness test day for female mice that underwent chronic social defeat stress.



Average latency to escape 20 0.25mA foot-shocks in the learned helplessness shuttle-box for CSDS control, susceptible and resilient mice on **A:** Test day 1 (Kruskal–Wallis, $\chi 2=2.4$, P=0.30; n=13, 9, 9) **B:** Test day 10 (Kruskal–Wallis, $\chi 2=1.2$, P=0.55; n=13, 9, 9) **C:** Test day 20 (Kruskal–Wallis, $\chi 2=0.88$, P=0.64; n=13, 9, 9) **D:** Test day 30 (Kruskal–Wallis, $\chi 2=1.5$, P=0.47; n=13, 9, 9). Error bars represent SD.

We compared average latencies to escape foot-shocks of males and females, looking at CSDS resilient and susceptible mice separately, to investigate whether there are sex differences in resilience to chronic social and trauma-type stress. There was a trend towards significance in the difference between CSDS susceptible males and females on test day 1, as CSDS susceptible females had a lower average latency to escape than the males (Mann-Whitney test, U=59, P=0.06; n=9, 23) (Fig. 9A). By test day 30 there was no difference in latency between the CSDS susceptible males and females (Mann-Whitney test, U=70, P=0.17; n=23, 9) (Fig. 9B). There was no significant difference between CSDS resilient males and females on test day 1 (Unpaired t-test, P=0.13; n=15, 9) or test day 30 (Mann-Whitney test, U=47, P=0.24; n=15, 9). This difference on the first day of the LH test, although not significant, could be indirectly related to the different sensitivity to the chronic social stress between males and females.

Figure 9: Sex differences in latency to escape foot-shocks on learned helplessness test days 1 and 30.



Average latency to escape 20 0.25mA foot-shocks in the learned helplessness shuttle-box, comparing males and females on **A:** Test day 1 1) CSDS resilient (Unpaired t-test, P=0.13; n=15, 9) 2) CSDS susceptible (Mann-Whitney, U=59, P=0.06; n=9, 23) **B:** Test day 10 - CSDS resilient (Mann-Whitney, U=47, P=0.24; n=15, 9), CSDS susceptible (Mann Whitney, U=70, P=0.17; n=9, 23).

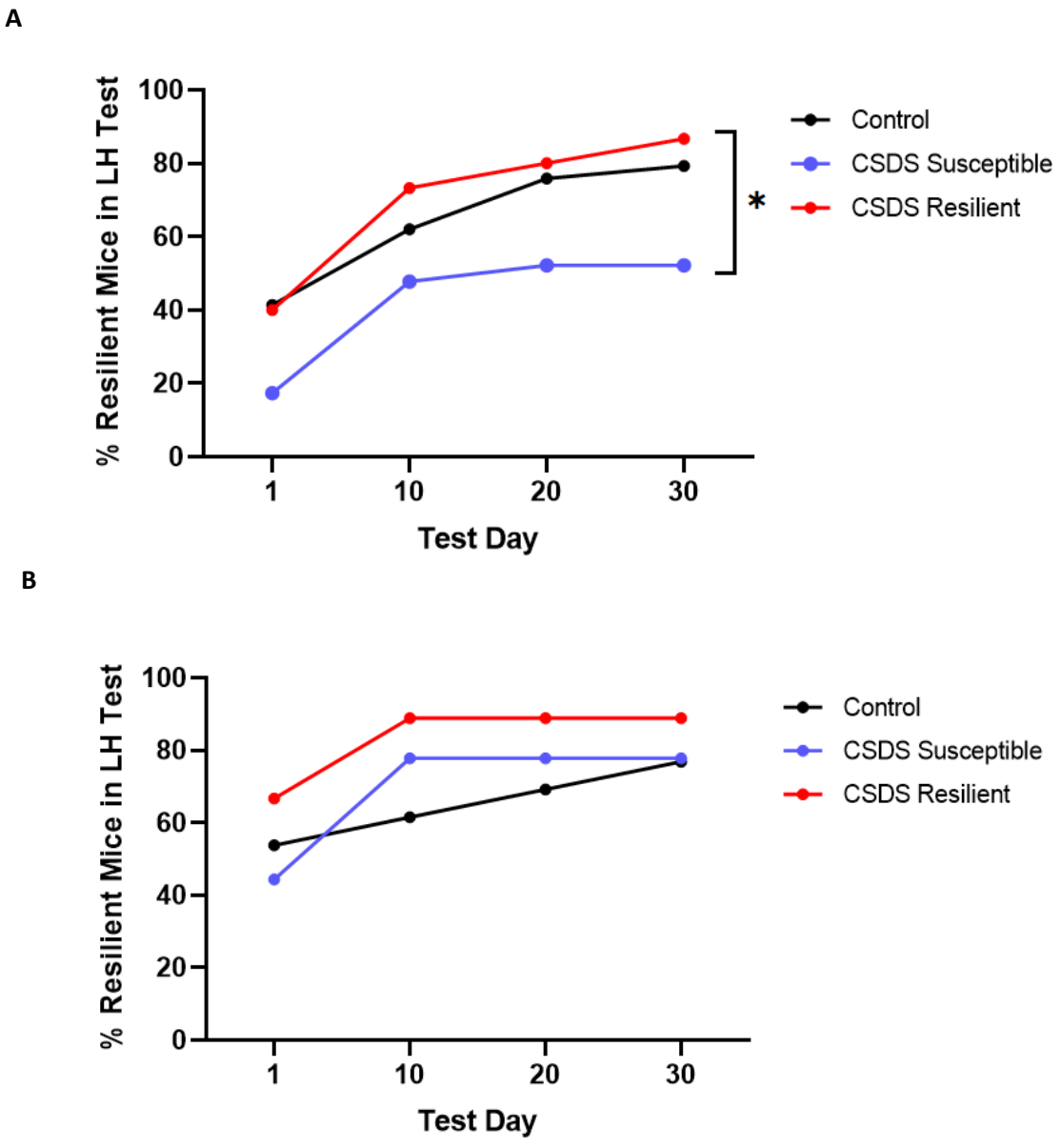
To further investigate cross-resilience to chronic social and trauma-type stress, we calculated the proportions of individuals that were resilient on each LH test day for CSDS resilient, susceptible and control groups. Fisher's exact tests revealed that there were no significant differences between CSDS resilient (n=15) and susceptible (n=23) males on days 1 (P=0.66), 10 (P=0.21) and 20 (P=0.10). However, the proportion of CSDS resilient males that were resilient to LH was significantly higher than CSDS susceptible mice by test day 30 (P=0.04) (Fig. 10A). This data suggests that there is a relationship between resilience to CSDS and extinction of the susceptible phenotype following LH stress in males.

There appears to be a different relationship between resilience to social defeat and LH stress in females. A higher proportion of CSDS resilient females (n=9) were resilient to LH from the first test day than CSDS susceptible females (n=9) (Fig. 10B), which suggests there could be a cross-resilience to both types of stress. However, Fisher's exact tests revealed that there were no significant differences between these groups on test day 1 (P=0.64). There were also no significant differences on test days 10 (P >0.99), 20 (P >0.99) and 30 (P>0.99). Interestingly, by test day 10 a higher proportion of CSDS susceptible females were resilient to LH than controls. While this difference was not significant (P=0.65), it could imply that experiencing chronic social stress might promote resilience to trauma-type stress in females.

In addition, 44.4% of females that were CSDS susceptible were resilient on test day 1, while in males it was only 17.4%. This suggests that females vulnerable to chronic social stress are less likely to also be vulnerable to trauma-type stress compared to males. However, using Fisher's exact test we found that the difference in proportion was not significant (P=0.18). By the final test day 77.8% of the CSDS susceptible females were resilient, compared to 52.2% of the CSDS susceptible males, but this

was also not significant (P=0.25). We used a lower number of females than males, and this may explain why the data was not significant.

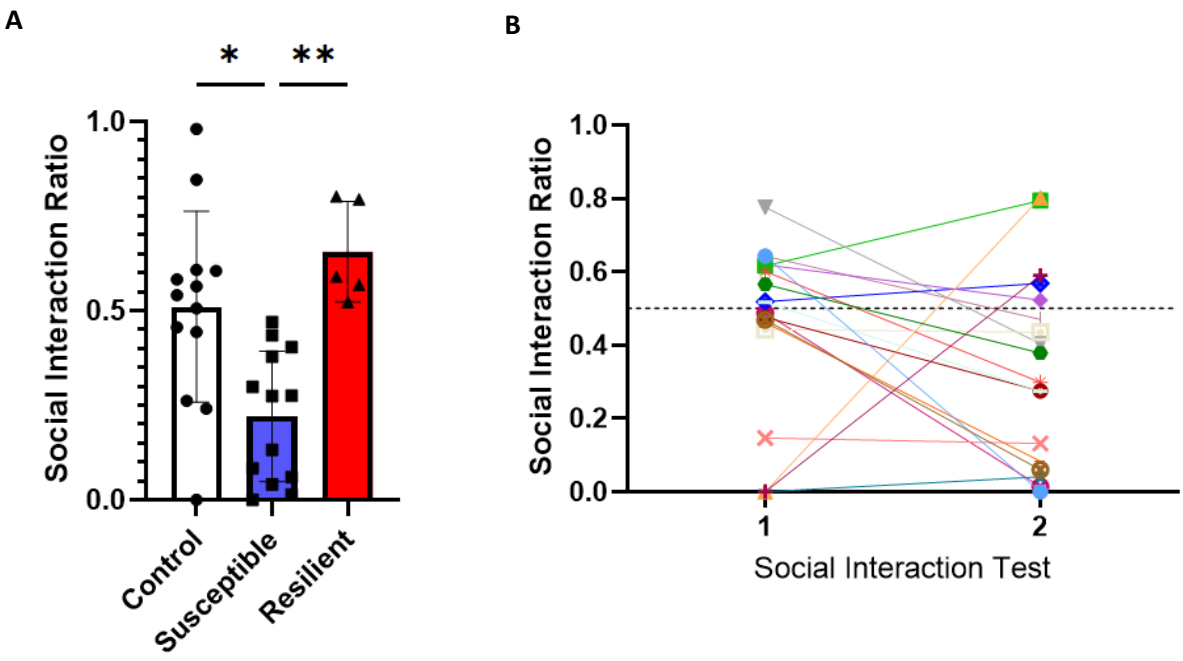
Figure 10: Resilience to learned helplessness stress is associated with resilience to chronic social defeat stress in male mice.



Percentage of mice resilient to learned helplessness foot-shock stress, for control, CSDS susceptible and CSDS resilient mice for **A:** Males. **B:** Females.

To investigate extinction of CSDS susceptibility and resilience over the 30 days of LH in females, we conducted a second SI test 24 hours after LH test day 30. The females were categorized into resilient (n=5) and susceptible (n=13) groups depending on their SI ratio: 27.8% of the defeated females were resilient and 72.2% were susceptible. There was a significant difference between the SI ratios of the control, susceptible, and resilient groups, and susceptible females had the lowest SI ratios (One-way ANOVA, $F(2, 28)=10.6$; $P=0.0004$; n=13, 9, 9 for main group effect; Control vs Susceptible $P=0.004$ (n=13, 13), Susceptible vs Resilient $P=0.001$ (n=13, 5), Control vs Resilient $P=0.39$ (n=13, 5)) (Fig. 11A). 66.7% of mice that were resilient following the first SI test were categorized as susceptible in the second SI test, and 22.2% of the mice that were susceptible following the first SI test were categorized as resilient in the second SI test (Fig. 11B). This data is different to the males, denoting that there are sex differences in extinction of CSDS susceptibility or resilience.

Figure 11: Social Interaction Test 2

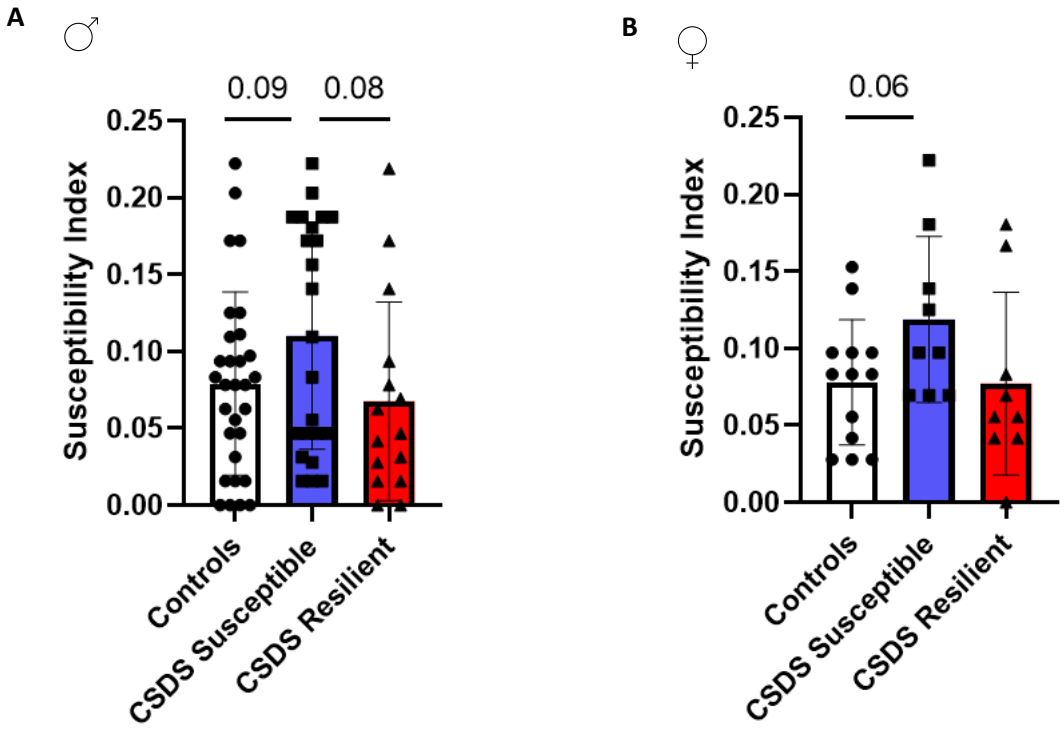


A: Social interaction (SI) ratio following the second SI test, with mice separated into control, susceptible and resilient categories (Kruskal–Wallis, $\chi 2=14.0$, P=0.0009; n=13, 13, 5). **B:** The change in SI ratio of each mouse between the first and second SI test. The dotted line is the threshold for resilience, with an SI ratio of 0.5 or representing resilience.

To investigate differences in individual stress vulnerability between CSDS resilient, susceptible and control groups, we gave each mouse its own individual score of emotionality. To do this, we used z-score analysis to integrate the data from each behavioural test, along with change in weight during CSDS. Z-scores were used to identify how many standard deviations an individual observation was from the mean of a control group, giving each mouse a susceptibility index for each test. These indexes were averaged to form an overall susceptibility index for every individual, which were analysed using unpaired t-tests. Susceptibility indexes of control (n=30) and CSDS resilient males (n=15) were not significantly different (P=0.56). However, there was a trend towards significance for differences in the susceptibility indexes of CSDS resilient and susceptible males (n=23) (P=0.08), and CSDS susceptible and

control males (P=0.09) (Fig. 12A). There were no significant differences in susceptibility indexes between female controls (n=13) and CSDS resilient females (n=9) (P=0.97), and CSDS susceptible (n=9) and resilient females (P=0.12). However, differences in the susceptibility indexes of female CSDS susceptible mice and controls had a trend towards significance (P=0.06) (Fig. 12B). This data suggests that CSDS susceptible mice have higher overall vulnerability to stress, for both males and females. This was validated when comparing the susceptibility indexes between males and females, as there were no significant differences between CSDS resilient males and females (P=0.72), and between CSDS susceptible males and females (P=0.76).

Figure 12: Susceptibility indexes indicate that individuals susceptible to chronic social defeat stress may have greater overall stress vulnerability.



A: Susceptibility indexes of CSDS control, susceptible, and resilient males. Unpaired t-tests revealed a trend towards significance for differences between CSDS resilient and susceptible males (P=0.08; n=15, 23), and CSDS susceptible and control males (P=0.09; n=23, 30). **B:** Susceptibility indexes of CSDS control, susceptible, and resilient females. There was a trend towards significance in the difference between CSDS susceptible and control females (Unpaired t-test, P=0.06; n=9, 13). Error bars represent SD.

DISCUSSION

The current study demonstrated that there is an association between resilience to CSDS and LH stress. Our findings suggest that males have a cross-resilience to chronic social and trauma-type stress, and that being resilient to one of these types of stress may predict resilience to the other. In addition, we identified sex differences in the pattern of resilience, as CSDS susceptible females had higher resilience to LH stress than males. Overall this study suggests that there are shared mechanisms underlying resilience and vulnerability to both chronic social and trauma-type stress, and this has potential implications for the treatment of individuals with comorbid MDD and PTSD.

Cross-Resilience to Chronic Social Defeat and Learned Helplessness Stress in Males

When exposed to stress some individuals will develop MDD or PTSD whereas others will not, and individuals that rebound from stressful events are considered to be resilient (Franklin et al., 2012). These disorders are highly comorbid, as it is estimated that half of PTSD patients also suffer from MDD (Kessler et al., 1995). Human studies point to shared mechanisms of vulnerability to MDD and PTSD, as it was found that the risk of developing PTSD after exposure to a traumatic event is higher in individuals with MDD (Koenen et al., 2002). Preclinical studies that have investigated vulnerability and resilience to MDD and PTSD indicate that there are some shared mechanisms underlying these disorders, such as the role of the LC-NE system. For example, Olsen et al. (2011) found that mice that were susceptible following exposure to traumatic stress had increased c-Fos expression in LC-NE neurons, and NE from the LC was found to be necessary for resilience to chronic stress through the inhibition of

downstream VTA DA neurons (Isingrini et al., 2016). However, there is limited research that considers resilience to both chronic and trauma-type stress in the same individual mice. As a result, very little is known about the association of resilience to both types of stress.

Our results indicate that there is a cross-resilience to CSDS and LH stress in male mice. We found that a higher proportion of males that were resilient following 10 days of social defeat were also resilient when exposed to 30 days of LH foot-shock stress, compared to males that were susceptible following social defeat. By the final test day, this difference in proportion was significant, indicating that CSDS resilient individuals exhibit faster extinction of the LH susceptible phenotype. These results suggest that males that are resilient to chronic social stress are more likely to be resilient to trauma-type stress, which supports our hypothesis that there is a cross-resilience. In addition, we found that CSDS susceptible males had a higher susceptibility index than CSDS resilient males and controls. While there was only a trend towards significance for these differences, there may have been significance if we had used a larger number of mice. The difference in susceptibility indexes suggests that mice that are susceptible to chronic social stress have greater overall stress vulnerability and those that are resilient have the lowest, which provides further support for the cross-resilience hypothesis. Overall, this gives support to the idea that there are shared underlying mechanisms of vulnerability and resilience to MDD and PTSD.

Data from the EPM and OF tests did not show that defeated mice were significantly more anxious, and the susceptible males actually displayed the least anxious behaviour in the EPM compared to control and resilient males. Due to aggressive CD-1 mice causing severe injuries to some individuals during social defeat, we reduced maximum attack length from 5 seconds to 3 seconds, and this may explain why the

defeated mice did not display higher levels of anxiety. It could also be due to the number of mice used or that moving control mice from their housing room increased their anxiety. Mice from all three groups had similar high preferences for sucrose in the SPT. While this may indicate that the mice did not display the anhedonia phenotype, since the males previously exhibited social avoidance in the SI test it could also indicate that the SPT protocol needs to be adjusted. There are several factors that can affect the results of the SPT, such as lack of a standardized apparatus (Liu et al., 2018), test duration (Tordoff & Bachmanov, 2002), and dietary influences (Bertino & Wehmer, 1981). While we used the protocol by Liu et al. (2018) which predicts a decrease in sucrose preference to 60% following social defeat, we did not use their standardized apparatus and this may have affected the results. In future experiments we will use the splash test instead of the SPT, as this is an alternative way of measuring anhedonia in rodents. Sugar water is splashed onto the back fur of each individual and the time they spend grooming following this is recorded. Reduced grooming time is thought to be an indicator of anhedonia (Isingrini et al., 2010), and so mice that are susceptible following social defeat would be expected to spend less time grooming in the splash test compared to control and resilient mice.

Since the behavioural tests in our experiment took 54 days altogether, it is important to consider the change in age of the mice throughout the experiment. The mice were almost eight weeks older by the second SI test and so it is possible that the results from this test could have been impacted by the difference in age. However, the effects of age on behaviour following social defeat was examined by Oizumi et al. (2019), who found that 24 month old males had similar behaviour to 8-12 week old males following social defeat, with defeated mice of both ages exhibiting similar levels of social avoidance in the SI test. This suggests that the difference in age between the first and

second SI test in our experiment did not impact their behaviour. Therefore, the phenotype reversal that we observed between the first and second SI test is likely not due to age. Instead, being exposed to trauma-type stress such as foot-shocks may change an individual's response to chronic stress.

Sex Differences in Resilience

Women are twice as likely to develop MDD and PTSD (Seedat et al., 2009; Yehuda et al., 2015), which may point to females having greater susceptibility following exposure to stress. Our study aimed to fill gaps in the preclinical research of female stress vulnerability, because research of the neurobiological mechanisms underlying resilience and vulnerability has predominantly been carried out on males (Blanchard et al., 1995). Some studies have shown similar mechanisms underlying these disorders in rodents, such as Ortiz et al. (2022), who found that susceptibility to stress can be induced in females by the positive modulation of α7 nicotinic acetylcholine receptors (nAChRs), an effect previously observed in male mice (Morel et al., 2017). However, other research points to sex differences in mechanisms underlying resilience. Curtis et al. (2006) found that in females, the amount of CRF required to activate the LC-NE arousal system following stress exposure is lower. By determining whether behavioural patterns of resilience are the same in both sexes, it can guide further research of the neurobiological mechanisms underlying resilience in females. Therefore, we wanted to determine whether females have the same cross-resilience to CSDS and LH stress that we identified in males.

Our results suggest that there are sex differences in the patterns of resilience to chronic social and trauma-type stress. While there were a higher proportion of CSDS resilient females that were resilient to LH on each test day compared to CSDS

susceptible females, there were no significant differences on any test day. This suggests that vulnerability to chronic social stress does not predict vulnerability to trauma-type stress in females, so they do not have the same pattern of cross-resilience as males. A higher proportion of CSDS susceptible females were resilient to LH on each test day compared to CSDS susceptible males, and by the final test day 77.8% of the CSDS susceptible females were resilient compared to 52.2% of the susceptible males. This could indicate that females are more likely to be resilient to trauma-type stress than males. In addition, females did not show the same phenotype reversal in the second SI test that the males did, suggesting there are sex differences in extinction of CSDS susceptibility or resilience. On test days 10 and 20 of LH, a higher proportion of CSDS susceptible females were resilient compared to controls, which is something we did not observe in males and could indicate that females that undergo chronic social stress have greater resilience to trauma-type stress than those that have not previously been exposed to stress. However, in contrary to this, the susceptibility indexes of CSDS susceptible females were almost significantly higher than those of the controls, which suggests that CSDS susceptible females are more vulnerable to stress overall. Also, because the results were not significant there are limited assumptions that can be made from our data. By adding more females to the experiment we may gain a clearer understanding of the sex differences in resilience.

The only difference in experimental design between males and females cohorts was the adapted social defeat procedure. Whilst this female adaptation of CSDS resulted in the same proportion of resilient to susceptible females as previous studies (Ortiz et al., 2022), the CD-1 mice displayed less aggression towards the females than they did towards males. If a CD-1 was underperforming for more than one day, or displayed mounting behaviour, we swapped it for another. However, even though attack

frequency was similar to what we observed in male defeats on attack days, there were less days of attacks: Females had an average of 7 days of attacks, compared with 9 days for males. We cannot exclude the possibility that the increased proportion of females that were resilient following both CSDS and LH was due to them experiencing less days of attacks during social defeat. However, other studies have also found that female rodents display less helpless behaviour than males after exposure to uncontrollable foot-shock stress (Dalla et al., 2007; Padilla et al., 2009), which is in support of our data.

Another important variable to consider is the effect of the estrous cycle on female response to stress. The rodent estrous cycle consists of four stages - proestrus, oestrus, metestrus and diestrus – and lasts approximately 5 days (Goldman et al., 2007). We did not monitor the estrous cycles of the females in our experiment, so we cannot exclude the possibility that this had an effect on their behaviour. Calipari et al. (2017) found that the activity of VTA DA neurons fluctuates throughout the estrous cycle, and VTA DA activity influences resilience to CSDS (Krishnan et al., 2007). However, when designing their female CSDS protocol, Harris et al. (2018) determined which stage of the estrous cycle each female was in during every defeat day and on the day of the SI test. They found that estrous cycle stage did not impact the behaviour of the defeated or the control mice during the SI test, which suggests it may not be necessary to consider estrous cycle stages when performing CSDS. In addition, Padilla et al. (2009) found that estrous cycle phase did not affect the escape behaviour of female rats in a learned helplessness experiment, which also suggests that the estrous cycle did not influence the behaviour of the females in our experiment.

Future Experiments

Our next aim is to clarify the role of NE in resilience to chronic social and trauma-type stress, and to accomplish this we will carry out the same behavioural experiments on brain-specific NE-depleted KO mice developed by the Giros lab. Previous work from the Giros lab using these KO mice demonstrated that NE depletion increases susceptibility to CSDS (Isingrini et al., 2016) but promotes resilience to LH (Isingrini et al., 2020). These tests were carried out on different individuals, so in our next experiment we will subject the same individuals to both CSDS and LH. Our hypothesis is that KO mice will not have a cross-resilience to both types of stress, but instead will be susceptible following CSDS and resilient to LH stress. We will carry out experiment with KO females initially because CSDS has not previously been performed using these mice, and it is important to determine whether there are sex differences in how NE mediates resilience to chronic social stress.

Another future aim is to identify biomarkers of MDD and PTSD, because this is important in order understand the molecular background of these disorders and identify targets for therapeutic intervention. Several potential biomarkers of resilience in the brain are being investigated. One of these is brain-derived neurotrophic factor (BDNF). Preclinical research using the CSDS and LH models have shown that stress affects levels of BDNF in several brain regions. It was found that CSDS increases BDNF levels in the NAc, and this increase in the NAc is associated with susceptibility to stress (Krishnan et al., 2007). Yang et al. (2015) found that rats that were susceptible to LH foot-shock stress had lower BDNF levels in the mPFC compared to resilient rats, but had higher BDNF levels in the NAc. Other potential biomarkers of psychiatric disorders are the extracellular signal-regulated kinases (ERK) 1/2. Lio et al. (2011) subjected rats to 5 weeks of CSDS and found that ERK1/2 expression was

decreased in the hippocampus of the defeated rats. ERK1/2 have also been implicated in vulnerability to LH stress. Dwivedi & Zhang (2016) subjected rats to inescapable tail or foot-shock stress and then measured their latency to escape when subjected to escapable shocks. Helpless rats had reduced expression of ERK1/2 as well as phosphorylated ERK1/2 (p-ERK1/2) in the frontal cortex and hippocampus. Although this research points to the role of BDNF and ERK1/2 signalling in stress and depression, it is not understood exactly how they mediate resilience and susceptibility.

Therefore, we will investigate the role of these potential biomarkers of resilience and also investigate c-Fos expression as a marker of neuronal activation. We will use the western blot technique to determine levels of expression of these in the NAc, LC, VTA, amygdala, hippocampus and mPFC, and compare levels of expression between resilient and susceptible mice, along with stress-naïve mice. Our hypothesis is that BDNF, ERK1/2 and c-Fos expression in different brain regions is associated with resilience to CSDS and LH, and therefore resilient and susceptible mice will have differential expression in different brain areas. Specifically, we hypothesize that males susceptible to both tests will have greater expression of BDNF in the NAc, as this has been observed previously in male mice following CSDS (Krishnan et al., 2007) and LH (Yang et al., 2015). In addition, we aim to clarify the role of NE in mediating the expression of these biomarkers during chronic social and trauma-type stress. To do this, we will determine expression in the KO mice that have been subjected to CSDS and LH, and compare this to WT mice that were subjected to both types of stress as well as naïve KO mice. The LC is the main source of NE in the brain (Bennaroch, 2009), so our hypothesis is that KO mice will have lower expression of c-Fos in the LC. We also hypothesize that KO mice will have higher c-Fos expression in the NAc,

because NE inhibits the VTA-DA pathway that projects to the NAc (Isingrini et al., 2016).

CONCLUSION

Overall, our results indicate that there is a cross-resilience to both chronic social and trauma-type stress in males, since males that were resilient following CSDS were more likely to be resilient to LH stress compared to those that were susceptible to CSDS. This supports the idea that there are shared mechanisms of resilience to MDD and PTSD. Our results also suggest that there are sex differences in resilience. Females may have greater resilience to trauma-type stress because defeated females acquired resilience to LH stress earlier than the defeated males. Whilst this does not explain why females are twice as likely develop PTSD and MDD (Kessler et al., 1995), it highlights important differences in patterns of resilience that may indicate different underlying neurobiological mechanisms.

Although we observed these differences in females, our results are not confirmative. Therefore, it is important to further establish the association between resilience to chronic social and trauma-type stress in females. In addition, in order to understand how these behavioural patterns of resilience and susceptibility are associated with differences in neurobiological mechanisms, we should now determine the role of NE in this resilience. We will do this by investigating the patterns of cross-resilience in brain-specific NE-depleted KO mice.

This research has implications for the guidance of research of the neurobiological mechanisms of resilience and vulnerability to stress. This is relevant to the understanding of the underlying mechanisms of MDD and PTSD, and could guide the development of therapeutics. Our finding that there is a cross-resilience to chronic social and trauma-type stress in males is especially relevant for patients with comorbid PTSD and MDD.

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List of Abbreviations

ACHT - Adrenocorticotropic hormone

ANOVA - Analysis of variance

ANS - Autonomic nervous system

AVP - Arginine vasopressin

BDNF - Brain-derived neurotrophic factor

CRF - Corticotropin-releasing factor

CSDS - Chronic social defeat stress

CSF - Cerebrospinal fluid

DA – Dopamine

EPM - Elevated plus maze

ERK1/2 - Extracellular signal-regulated kinases 1/2

fMRI - Functional magnetic resonance imaging

HPA Axis - Hypothalamic pituitary-adrenal axis

KO – Knockout

LC – Locus coeruleus

LH - Learned helplessness

MDD - Major depressive disorder

mPFC - Medial prefrontal cortex

NAc - Nucleus accumbens

nAChRs - Nicotinic acetylcholine receptors

NE – Norepinephrine

NET – Norepinephrine transporter

OF - Open field

p-ERK1/2 - Phosphorylated ERK1/2

PTSD - Post-traumatic stress disorder

PVN - Paraventricular nucleus

SCVS - Subchronic variable stress

SI - Social interaction

SIZ - Social interaction zone

SNS - Sympathetic nervous system SPT - Sucrose preference test

VGLUT1 - Vesicular glutamate transporter 1

VGLUT2 - Vesicular glutamate transporter 2

VTA – Ventral tegmental area

WT – Wild-type

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