Modulation of behavioral adaptation to aversive experience by accumbal circuits

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ABSRTACT

Depression remains the leading cause of disability worldwide. With limited treatment efficacy for a significant portion of the affected population and high risk for recurrence, there is a serious need for more targeted treatments and interventions for disease prevention. Stress is a major factor in the development of depression, but there is considerable variability in how individuals respond, and only a minority will develop depression. Rodents also show variability in their behavioral response to stress with some animals developing severe behavioral deficits and others maintaining a control-like phenotype. Pre-clinical animal models can exploit this variability to interrogate the neural circuit mechanisms underlying stress induced behavioral adaptation. This has allowed for the probing of functional, morphological and molecular changes that define resilience versus susceptibility following stress, but little is known about how pre-existing differences in neural circuits may predispose animals to susceptibility to future stress.

The nucleus accumbens (NAc) integrates information from upstream structures to modulate behavior and plays an important role in motivation and reward as well as in behavioral adaptation to stress. It is predominantly composed of medium spiny neurons (MSNs) that can be divided based on primary expression of dopamine receptor subtypes into D1 and D2 MSNs. Using fiber photometry calcium imaging to interrogate cell type specific activity in the NAc medial shell before animals are exposed to stress, I define activity profiles that associate with future resilience, providing evidence that circuit mechanisms associated with susceptibility may be present before and influence an animal's adaptation to stress, and are not merely a consequence of stress exposure.

NAc MSNs are known to require coincident input from glutamatergic inputs to fire action potentials. The ventral hippocampus (vHIP) is one major source of glutamatergic projections to the NAc, providing information about emotional context and regulating behavioral adaptation to chronic stress. By interrogating this pathway before and after a chronic variable stress paradigm in both male and female animals, I can examine how pre-existing differences in ventral hippocampal activity and behavior may predict future stress susceptibility and how

stress modulates this signal. I find that individual differences in vHIP activity associate with variability in social interaction and open field exploration before stress, and this is predictive of future susceptibility. I also find that stress increases neural activity and, using ex vivo electrophysiology, I determine that this occurs via a presynaptic mechanism.

Finding that the vHIP-NAc pathway is modulated by stress in both sexes, I then investigate how aversive information is encoded in this projection as well as in projections from the infralimbic prefrontal cortex (PFC-IL), which are known to interact with the vHIP to drive NAc firing. Using a discriminative fear conditioning paradigm, I find that, while both pathways encode aversive and neutral stimuli, discrimination is encoded in a sex specific manner with PFC inputs discriminating in females and vHIP inputs, in males. Probing the behavioral significance of these neural signals, I find only weak associations with classical fear behavior of freezing and identify a sex-specific role for these pathways in suppression of reward seeking by aversive cues.

Taken together, these results establish that pre-existing differences in neural activity in NAc circuitry influence how an animal engages with its environment and may also predispose individuals to susceptibility to future stress. Glutamatergic projections to the NAc integrate aversive information in a sex-specific manner to bias reward motivated behavior. These findings provide important evidence for neural signatures of vulnerability, identifying a potential target for preventative interventions.

RESUMÉ

La dépression est la principale cause d'invalidité au monde. Les traitements n'ayant que des effets limités, et le risque de récurrence de la dépression étant très élevé, il est urgent de développer des interventions pour prévenir la maladie. Le stress est un facteur de risque majeur pour la dépression, mais seule une minorité de personnes exposées au stress souffriront de dépression. Cette variabilité est également présente chez les rongeurs, seulement certains d'entre eux développant des déficits importants dans leur comportement. Les modèles animaux pré-cliniques peuvent utiliser cette variabilité comportementale afin d'explorer les mécanismes neuronaux qui sous-tendent l'adaptation comportementale au stress. Néanmoins, on ignore comment des différences neuronales préexistantes pourraient prédisposer des animaux à la susceptibilité au stress.

Le noyau accumbens (NAc) joue un rôle important dans l'adaptation comportementale au stress. Le NAc est composé principalement de neurones épineux moyens (MSNs) qui peuvent être divisés en deux sous-types, dépendamment du niveau d'expression des récepteurs dopaminergiques D1 et D2. En utilisant la photométrie par fibers optiques, pour interroger l'activité de cellules de types spécifiques, j'ai défini des profils d'activité neuronale associés à une future résilience au stress. Ceux-ci démontrent que des mécanismes de circuits neuronaux associés à la susceptibilité ne seraient pas une conséquence de l'exposition au stress, mais seraient plutôt présents au préalable et influenceraient l'adaptation d'un animal au stress.

Les MSNs du NAc ont besoin d'entrées glutamatergiques pour déclencher des potentiels d'action. L'hippocampe ventral (vHIP) est l'une des sources principales de projections glutamatergiques vers le NAc et il régule l'adaptation comportementale au stress chronique. En explorant cette voie anatomique avant et après exposition à un modèle de stress chronique variable chez des souris mâles et femelles, j'observe que des différences individuelles dans l'activité du vHIP sont associées avec la variabilité observée dans des tests de comportements pertinent à la dépression, et que ces différences peuvent prédire la future susceptibilité au stress. Je trouve également que le stress augmente l'activité neuronale, et je détermine par de l'électrophysiologie ex vivo que ceci est dû à un mécanisme présynaptique.

Ayant démontré que la voie vHIP-NAc est modulée par le stress dans les deux sexes, j'explore ensuite comment une information aversive est encodée dans cette projection, ainsi que dans des projections du cortex préfrontal (PFC) infralimbique qui interagissent avec le vHIP afin d'activer le NAc. En utilisant un paradigme de conditionnement de peur discriminative, j'observe que les deux voies anatomiques encodent les stimuli aversifs, mais que la discrimination d'un indice neutre est encodée de façon sexo-spécifique, étant régulée par les entrées du PFC chez les femelles, et par les entrées du vHIP chez les mâles. Etonnamment, je ne trouve que des associations faibles avec le comportement de peur classique d'immobilité totale. En revanche, en utilisant une technique d'inhibition chimiogénétique, j'identifie un rôle-clef pour ces voies anatomiques dans la suppression de la quête de récompense après exposition à des indices aversifs.

Ces résultats établissent des différences d'activité neuronale préexistantes dans le circuit du NAc qui influencent la façon dont un animal réagit à son environnement. Ces différences pourraient également prédisposer certains individus à être susceptibles à des stress futurs. Des projections glutamatergiques vers le NAc intègrent les informations aversives de façon sexo-spécifique afin d'affecter le comportement motivé par la récompense. Ces découvertes apportent une preuve décisive de l'existence de signaux neuronaux de vulnérabilité au stress, et identifient une cible potentielle pour des interventions de prévention de la maladie.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Although stress is a major risk factor for the development of depression, not everyone who encounters stress will develop the disorder. Current antidepressant treatments remain ineffective in a large portion of individuals with a high risk for recurrence, pointing to a serious need to prevent the disorder before its emergence. This work is driven by the hypothesis that pre-existing differences in circuitry impact an individual's response to stress and may leave certain people more vulnerable to stress induced depression.

In **chapter 1**, I introduce background on the disorder and present evidence for a circuit-based model of depression. I review pre-clinical animal models for depression and look at the current literature linking pathway-specific dysregulations with stress induced depressive-like behavior. Finally, I focus in on nucleus accumbens circuitry, detailing its role in behavior and adaptation to chronic stress.

Chapter 2 presents a paper published in Neuropsychopharmacology in which I identified cell type specific differences in neural activity in the nucleus accumbens of animals that are resilient vs susceptible to stress in home cage recordings and during social interaction. This is one of the first pieces of evidence linking circuit activity to stress vulnerability demonstrating that pre-existing differences in neural activity impact susceptibility to stress in males. In completing this work, I also generated a novel analysis method for calcium imaging data to quantify changes in activity in tasks without discrete behaviors that has been used by other research groups and integrated into an analysis package for fiber photometry (1).

Chapter 3 presents a paper published in Biological Psychiatry in which I looked at the glutamatergic afferents from the ventral hippocampus and their role in stress vulnerability. I define a role for the ventral hippocampal projections to nucleus accumbens in depressive and anxiety-like behavior in females and identify behavioral and neural signatures of stress vulnerability in both sexes. I also define stress induced changes in nucleus accumbens projecting ventral hippocampal cell activity and identify a mechanism for this change. This paper extends the work from chapter 2 to implicate alterations in an upstream pathway. This work builds upon and extends earlier work that had identified a role for ventral hippocampal projections to the

nucleus accumbens in susceptibility in male mice in chronic social defeat stress, by demonstrating that a different stress, chronic variable stress, induces alterations in this pathway in both males and females. Moreover, it defines a mechanism of pre-existing vulnerability in both sexes as well as providing a mechanism for stress induced circuit dysregulations.

Chapter 4 presents a manuscript submitted for review in which I probe both the role of the prefrontal cortical and ventral hippocampal afferents to the nucleus accumbens in encoding aversive cues, finding a sex specific role for these pathways in discriminating aversive from neutral cues. I discover that although these pathways contribute very little to classical fear behavior (freezing), they play an important role in cue-induced suppression of reward seeking behavior in a sex specific manner. This paper interrogates two pathways implicated in behavioral adaptation to stress and their role in encoding threat, identifying a surprising double dissociation between the sexes and providing a potential mechanism by which stress leads to disruptions in behavior.

CONTRIBUTION OF AUTHORS

Chapter 1

Jessie Muir wrote the general introduction with Joelle Lopez and input from Rosemary Bagot. Parts of the introduction were adapted from Muir et al (2018)(2).

Chapter 2

Rosemary Bagot, Erin Calipari and Zachary Lorsch performed the experiments. The virus was obtained from Karl Deisseroth and Charu Ramakrishnan. Eric Nestler and Rosemary Bagot funded the projects. Jessie Muir developed a novel method for analyzing population calcium imaging data and performed all data analysis. Jessie Muir and Rosemary Bagot wrote the manuscript with input from other authors.

Chapter 3

Jessie Muir and Rosemary Bagot conceived the experiments. Jessie Muir and Yiu Tse Chung performed the experiments. Julia Biris and Eshaan Iyer performed behavioral scoring. Vedrana Cvetkovska took images for histology. Jessie Muir performed the data analysis. Jessie Muir and Rosemary Bagot wrote the paper.

Chapter 4

Jessie Muir and Rosemary Bagot conceived the experiments. Jessie Muir and Yiu Chung Tse performed the experiments with assistance from Eshaan Iyer, Rand Eid, Karen Wassef and Sarah Gostlin. Karen Wassef and Jessie Muir performed behavioral scoring. Vedrana Cvetkovska took images for histology. Jessie Muir performed the data analysis with assistance from Eshaan Iyer, Julian Sorensen and Nick Spencer. Jessie Muir and Rosemary Bagot wrote the paper.

Chapter 5

Jessie Muir wrote the discussion with input from Rosemary Bagot. Parts of the discussion were adapted from Muir &Bagot (2021)(3).

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LIST OF ABBREVIATIONS

ACTH adrenocorticotropin hormone

AMY amygdala

CaM calmodulin

ChR2 chanel rhodopsin

CRH corticotrophin releasing hormone

CMS chronic mild stress

CPP Conditioned Place Preference

CSDS Chronic Social Defeat stress

CVS Chronic Variable Stress

DA Dopiminergic/Dopamine

DMS Diagnostic and Statistical Manual of Mental Disorders

DREADD Designer Receptors Exclusively Activated by Designer Drugs

ELS Early Life Stress

EPSC Excitatory Post Synaptic Potential

- **m**EPSC mini
- **s**EPSC spontaneous

GC glucocorticoids

GFP Green Fluorescent Protein

GP Globus Pallidus

HPA Hypothalamic-Pituitary-Adrenocortic

IEG immediate early gene

IPSC Inhibitory Post Synaptic Potential

LH learned helplessness

MDD Major Depressive Disorder

MSN Medium Spiny Neuron

NAc Nucleus Accumbens

NAcc Nucleus Accumbens Core

NAcs Nucleus Accumbens Shell

NpHR Halorhodopsin

NTG Nose to grid

OFT Open Field Test

PFC Prefrontal Cortex

PrL PreLimbic Area

IL Infralimbic Area

PIT Pavlovian to instrumental transfer

PR Progressive ratio

PVN Paraventricular Nucleus of the hypothalamus

SCVS Sub Chronic Variable Stress

SP Social Preference

TST Tail Suspension Test

vHIP Ventral Hippocampus

VP Ventral Pallidum

VTA Ventral Tegmental Area

CHAPTER 1

Introduction

1.1 Depression

1.1.1 Major depressive disorder

Major depressive disorder (MDD) (also known as depression) is a psychiatric condition characterized by a number of cognitive and emotional symptoms. Diagnosis relies on the Diagnostic and Statistical Manual of Mental Disorders (DMS)-5, which is the identification of at least 5 symptoms associated with depression that persist for at least 2 weeks and that cannot be associated with other conditions, medications, etc. These symptoms include depressed mood, anhedonia, significant changes in weight, appetite or sleep, fatigue, thoughts of suicide and a number of others (4).

Depression is the leading cause of disability worldwide; 280 million people currently suffer from the disorder (WHO), with the prevalence 50% higher in women compared to men (5) and is expected to be the leading cause of burden of disease by 2030 (6). The lifetime risk of MDD sits between 15 and 18% (4, 7), and the COVID-19 pandemic is expected to further increase this risk (8). Depression carries a substantial economic burden as well, with an estimated cost of 51 billion dollars per year in Canada (9).

Unfortunately, treatment outcomes are poor for a large portion of the population, with only half of patients achieving remission following treatment. In line with this, the disorder carries a high risk of recurrence, with 50% of patients exhibiting at least one more episode of depression after initial recovery; this jumps to an 80% chance after two episodes (10). Due to this high risk or recurrence, which increases with every subsequent episode, as well as the risk for treatment resistant depression (11, 12), which can occur in previously successfully treated patients, there is a serious need to prevent rather than treat the disorder before the emergence of symptoms.

1.1.2 Stress and depression

Epidemiological studies identify stress as a major risk factor for depression (13, 14), with depressive episodes being highly correlated with a preceding major negative life event (15). Although most literature has focused on acute stressors, chronic stress has been found to be a much stronger predictor of future depression (16, 17), a fact that is particularly relevant given

the recent pandemic. Depression is associated with dysregulated stress responses. Typically, an acute stressor activates the hypothalamic-pituitary-adrenocortical (HPA) axis, triggering the release of stress hormones, which allow animals to appropriately respond (18). Briefly, corticotrophin releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus (PVN) which stimulates release of adrenocorticotropin hormone (ACTH) from the pituitary; this triggers the release of glucocorticoids (GC) from the adrenal glands, triggering physiological responses that allow individuals to meet the challenges of an acute stressor (18, 19). The HPA axis is under a GC-modulated negative feedback loop whereby increased GC release in response to a stressor signals to the PVN to inhibit the release of CRH, effectively terminating the stress response (19). However, chronic activation of the HPA axis and elevated circulating levels of GCs can lead to desensitization of this negative feedback loop, increasing risk for various psychopathologies and other diseases (20, 21).Indeed, depressed patients exhibit dysregulated HPA responses to stress as well as baseline alterations(22) .

Nevertheless, only a minority of people who experience stress will develop depression. Most are resilient, and adapt appropriately to stress and other adversity, allowing them to avoid behavioral dysregulation and maintain homeostasis (23). Although it is important to note that resilience is not the absence of a stress response, but it is an active process allowing an individual to adapt to stress (24). The question is: why do some individuals react poorly to stress while others are resilient? A wide body of literature has identified interactions between genetics, stress and environment that modulate risk for the disorder (4, 25, 26). Furthermore, variability in an individual's stress-induced cortisol response (27) and cortisol awakening response (28) is predictive of recurrence of the disorder, indicating that how an individual responds to acute stress may be predictive of vulnerability. Being able to predict which individuals will react poorly to stress is essential to identifying who is at risk for depression and to prevent the disorder before exposure to stress and, more importantly, before the emergence of the disorder. Again, this disorder carries a high risk for recurrence; stress induced neural and behavioral dysregulation that occur in an initial episode of depression increase risk for subsequent episodes. Being able to identify at risk individuals and target treatments to mechanisms of vulnerability may allow these individuals to adapt to stress and prevent the disorder all together.

1.1.3 Rodent models for depression

Human studies provide important insight into potential mechanisms of depression. fMRI studies and post-mortem tissue analyses have identified regions that are functionally and structurally changed in depressed patients (29-31) as well as transcriptional/ epigenetic markers associated with the disorders (32, 33) while blood samples have identified inflammatory markers (34). However, human studies are limited in that it is not possible to target and manipulate specific pathways, genes and receptors in humans, making it impossible to determine causality and difficult to find targetable mechanisms for therapeutic intervention. Thus, in pre-clinical research, we turn to animal models to elucidate causal mechanisms underlying depression. Most animal models use stress, chronic or acute, to induce depression-like behavior, based on the well-established connection between stress and depression (13, 14). Depression-relevant behavior is assessed in a range of simple tests thought to measure behavioral phenotypes that parallel symptoms of depression, and often also anxiety (see Table 1 & Figure 1). Commonly used depression models include social defeat, learned helplessness and chronic mild/variable stress, summarized in Figure 1 and Table 2.

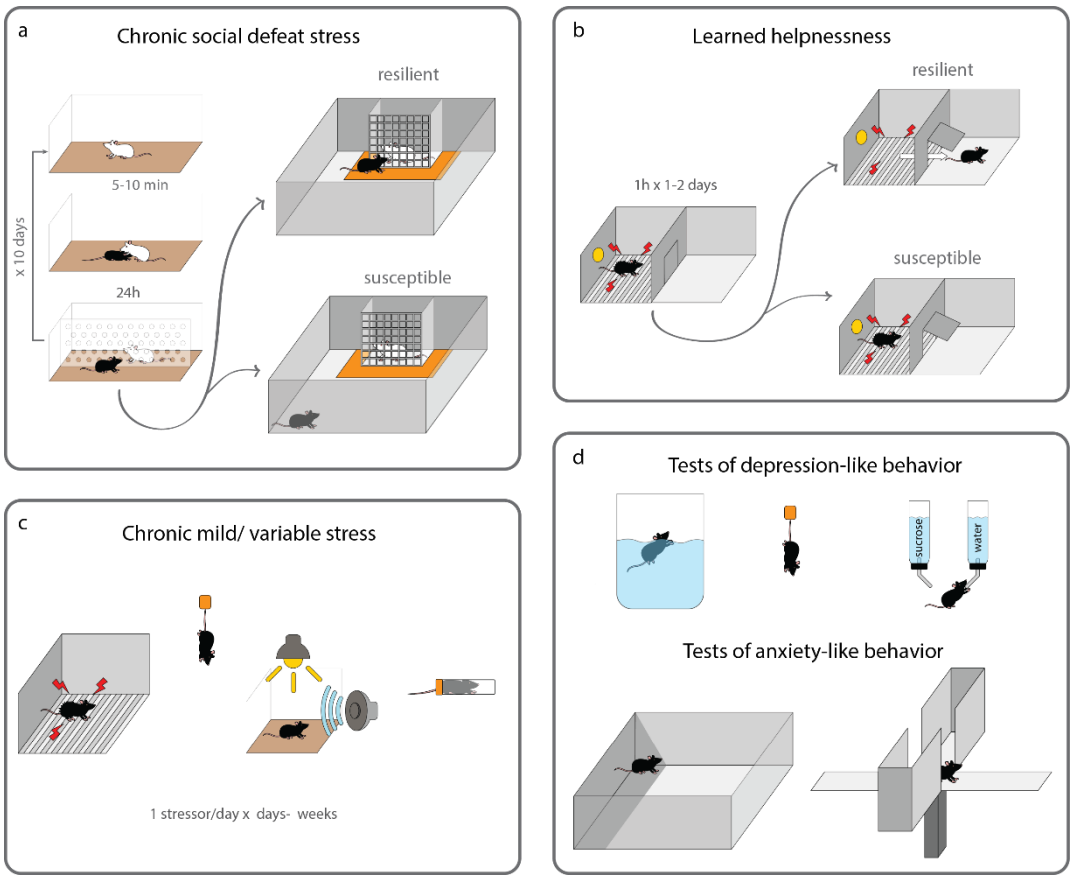

Figure 1. *Schematic overview of commonly used animal models of depression and tests of depression- and anxiety-like behavior. (a) Chronic social defeat stress (CSDS). (b) Learned helplessness. (c) Chronic mild stress or chronic variable stress (d) Susceptibility is assessed by increases in depression- and anxiety-like behavior which is assessed by tests such forced swim, tail suspension, or sucrose preference. Anxiety-like behavior is also assessed in an open field or elevated plus test assess. See Table 3 for a complete description of paradigms and tests.*

Test	Procedure	Common Interpretation	Indicator of Susceptibility
Forced Swim	Mouse/rat is placed in an inescapable cylinder of water. After initial struggling (swimming, climbing), animals will eventually stop struggling and float immobile. Latency to first become immobile and total time immobile are measured.	'Behavioral despair' or passive vs active coping	Increased immobility
Tail Suspension	Mouse is suspended by tail. After initial struggling, mice stop struggling and hang immobile. Latency to first become immobile and total time immobile are measured.	'Behavioral despair' or passive vs active coping	Increased immobility
Sucrose Preference	Mouse/rat has free access to bottles of water and sucrose solution in home cage or in a discrete test. The volume of each solution consumed is measured and compared to calculate a preference score.	Anhedonia	Decreased preference for sucrose
Social Interaction	Mouse is placed in an arena with a mesh enclosure that is empty on an initial 'no target' trial and contains a novel mouse on a 'target' trial. Time spent in vicinity of the enclosure (interaction zone) while the social target is present vs absent is measured and used to calculate a social interaction ratio.	Social withdrawal	Reduced time interacting with social target
Open Field	Mouse/rat is placed in an empty arena and time spent exploring the center vs periphery is measured. Manipulations associated with increased anxiety reduce center exploration.	Anxiety	Reduced time in center
Elevated Plus Maze	Mouse is placed in the center of a plus (+) shaped maze with two walled 'closed' arms and two exposed 'open' arms. Time spent in open and closed arms is measured. Manipulations associated with increased anxiety reduce time spent in open arms.	Anxiety	Reduced time in open arms

Table 1. *Overview of behavioral assays of depression- and anxiety-like behavior, outlining the method as well as the common interpretation of results and behavioral profile associated with susceptibility.*

Chronic social defeat stress (CSDS) (Figure 1a) (35, 36), is particularly useful in modeling differential susceptibility. Many people remain resilient to depression in the face of stress. After CSDS, susceptible mice exhibit a depression-like phenotype characterized by reduced social interaction (also reduced sucrose preference, increased immobility in a forced swim test (FST), circadian and metabolic changes) whereas resilient mice continue to seek out social interaction and, unlike susceptible mice, are similar to non-stressed controls on a range of depression-relevant metrics, although, like susceptible mice, they show increased anxiety indicated by reduced center exploration in an open field (35). Importantly, both resilience and susceptibility are associated with mechanisms that differ from non-stressed controls, indicating that resilience to CSDS is an active mechanism (37); this allows us to compare how circuits successfully adapt to

stress compared to those that do not. Due to this phenotypic divergence, CSDS is considered a powerful and clinically relevant model to look at mechanisms of stress-induced behavioral adaptations. In line with this, CSDS has recapitulated many of the changes seen in depressed humans such as reduced deltaFosB in the nucleus accumbens (NAc) of susceptible mice and depressed humans (33), decreased immediate early gene (IEG) expression in the prefrontal cortex (PFC) (38), and others (32, 39). However, it also presents with a key limitation: it is not easily adaptable to females as CD1 aggressors will not readily attack a female intruder. Manipulations of the experimental female (40) or the aggressor (41, 42) can induce attacks, but this brings into question ethological relevance of the model, given females are not generally subjected to attacks in naturalistic environments. Witness social defeat stress is an adaptation to this model where females observe the CD1 attacking an experimental male mouse and are then returned to the home cage of another CD1, providing a purely psychosocial stressor (43, 44). However, this protocol leads to less severe deficits in social interaction behavior (43, 45), making it difficult to define resilient and susceptible populations and thus taking away from the strength of the model, although some groups have looked to define these populations using other tests to define social interaction behavior (46). Furthermore, in studies where it is useful to compare males and females, it may be preferable to expose both sexes to the same stressor. Nevertheless, CSDS is an incredibly powerful model to look into mechanisms of stress induced susceptibility and resilience and is widely used in the field. Furthermore, it provides the potential to investigate mechanisms of vulnerability and to look at how pre-existing differences may be differentially associated with susceptibility versus resilience, a fact that had not yet been taken full advantage of in the literature.

Learned helplessness (Figure 1b) is another commonly used model for depression. After experiencing repeated inescapable footshock, susceptible animals fail to escape from signaled shocks when given the opportunity, but some animals will escape, demonstrating resilience. Like the Tail Suspension Test (TST) and FST, it has good predictive validity in drug screening (47, 48). Unfortunately, helplessness seems to be short lived, lasting only about 2-3 days, bringing into question its validity as a model for depression which is characterized by enduring alterations in brain and behavior.

Chronic mild stress (CMS) or chronic variable stress (CVS) (Figure 1c) refers to a number of protocols in which mice or rats are repeatedly exposed to a single stressor or a variety of stressors over days or weeks. Stressors may include a wide range of manipulations such as tail suspension, restraint, unpredictable footshock, noise and bright light. Susceptibility is assessed by increases in depression- and anxiety-like behavior. Depression-like behavior is typically assessed by tests such FST, TST or sucrose preference (Figure 1d). Anxiety-like behavior is also assessed in an open field or elevated plus maze (Table 1). CMS/CVS protocols are easily adaptable to females, and have revealed sex differences in susceptibility and associated mechanisms (49-51) A potential limitation is that CVS uses purely physical stressors as opposed to CSDS which integrates physical and psychological stressors encountered within social behavior (48). Furthermore, there are questions about reproducibility given methodological details are limited in most protocols and this model seems to be sensitive to small changes (48, 52). Despite these limitations, the power of these models is the ability to examine mechanisms of susceptibility in both male and female mice as well as sex differences. Specifically CVS has been used to compare both sexes, revealing increased vulnerability in females compared to males which mirrors the increased incidence of depression seen in women (49, 51, 53). Again, this is a powerful way to extend the question of vulnerability to females by not only asking why females are more vulnerable but to also identify mechanisms of vulnerability in both sexes with similar protocols.

Paradigm	Duration	Description	Outcomes	Advantage	Disadvantage
Chronic Social Defeat Stress	Chronic (10 day)	Experimental mice experience brief aggressive encounters (5-10 min) in the home cage of an aggressive mouse and are then housed in the same cage separated by a divider to maintain sensory exposure with the aggressor. This is repeated with a new aggressor daily.	Social avoidance, reduced center time in open field, immobility in FST/TST, reduced sucrose preference, circadian and metabolic changes	Standardized protocol produces both susceptible and resilient mice modeling phenotypic divergence in stress adaptation using an ethologically relevant social stress	Not readily adaptable to female mice
Chronic mild stress/chronic unpredictable mild stress/ chronic variable stress	Chronic (6 day -12 week)	Mice are exposed to daily mild stressors for a variable period. Stressors vary considerably between protocols but can include: Cage tilt, foot shock, tail suspension, restraint, wet cage, social isolation, perturbation of light/ dark cycle, cage changing, temperature perturbation, cage shaking, intruder, noise	Reduced sucrose preference, immobility in FST/TST, increased latency to eat in a novel environment, decreased grooming.	Identical manipulations can be employed in males and females	Lack of standardization & detailed reporting of some protocols obstructs efforts to reproduce across labs
Learned Helplessness (LH)	Sub-chronic (2-3 days)	Mice are exposed to one or more sessions of repeated inescapable electric shock and later are given the opportunity to escape further signaled shocks	Failures to escape from escapable, signaled shock	Reproducible protocol across labs can produce a phenotypic split between learned helpless and non-learned helpless animals	Relevance to females has been questioned
Forced Swim/ Tail Suspension	Acute (5 min - 1 hour)	Mice are suspended from their tail (TST) or placed in an inescapable body of water (FST)	Immobility in FST/TST	Quick & easy to conduct	Questions of relevance of single stressor

Table 2. *Overview of stress paradigms used to model depression in rodents.*

Despite the utility of models for stress susceptibility, depression remains a fundamentally human condition. Tests for depressive and anxiety-like behavior provide metrics for behaviors that parallel those seen in depression, but symptoms such as sadness, suicidal thoughts and guilt do not readily translate to mice and we should be cautious not to anthropomorphise based on these simple metrics. A further caveat of animal depression studies is the almost exclusive use of males. This is particularly concerning given the increased prevalence of depression in women (54). Enough evidence of sex differences exists to caution against assuming that findings from males will translate directly to females (55). Even the assumption that males and females will behave similarly on these behavioral tests is pre-mature. For example, females exhibit more fear generalization and active escape responses in classical fear conditioning paradigms (56). Another important consideration is the need to expand the repertoire of depression-relevant behavior.

Existing studies have largely employed simple behaviors that can be difficult to directly compare to humans, and often rely on loosely defined constructs with validity based on superficial similarity or efficacy of existing antidepressant drugs. Expanding to include an alternative approach, exemplified by the work of Soares-Cunha et al. (2016), that uses more structured behaviors to probe endophenotypes of depression will help to increase the translational potential of this field. Finally, one major caveat of the current use of different animal models is that most work looks to identify differences in mice after stress, effectively studying mechanisms underlying depressive and anxiety-like behavior. Although these are interesting and important questions, in such experiments it is impossible to separate the mechanisms of stress induced behavioral adaptation (susceptibility) from pre-existing differences contributing to vulnerability (factors that contribute to increased risk to susceptibility). Looking into mechanisms of vulnerability, as opposed to susceptibility, could allow for the prediction of high-risk individuals before the onset of the disorder and yield targetable mechanisms for prevention as opposed to treatment of the disorder. Given high risks of recurrence which increase with every episode, addressing dysregulations that precede stress may yield better treatment outcomes.

1.1.4 Circuit based model of depression

There are many theories of the mechanisms by which depression leads to behavioral dysregulations. Some focus on the role of dysregulation of the HPA axis (57) whereby prolonged stress and subsequent overactivation of the HPA system leads to an imbalance in the system in vulnerable individuals. In line with this, abnormalities such as increased release of stress hormones to an acute stressor have been identified in depressed patients and have been shown to predict subsequent episodes (58, 59). Mouse models also link abnormalities in glucocorticoid receptors, corticotropin releasing hormone and its receptor to depressive-like behavior (60, 61). (62, 63). The monoamine hypothesis which was formulated in the 1950s following the observation that patients treated for hypertension developed depression (64, 65) suggests that depression is due to depletion of monoamines in the central nervous system (65, 66). This hypothesis was strengthened by observing the effects of monoamine oxidase inhibitors in treating depression and led to the use of selective serotonin uptake inhibitors (67), which are still used as antidepressants today. Other models link the immune system and gut to the disorder.

Depression is associated with increased levels of pro-inflammatory cytokines in the peripheral and central nervous system (34, 68) likely caused by increased microglia and astrocyte activation (69). More recently, the gut microbiome has been shown to influence behavior and stress response in rodents (70, 71), with differences in the gut microbiota of depressed versus healthy patients that relate to depression metrics.

Depression is a heterogeneous disorder and likely all of these systems may play a role with different processes implicated in different individuals (72). This thesis applies a neural circuit-based model of depression (73) focusing on how neural populations and pathways which are known to regulate motivation, reward and other functions are disrupted in depression, leading to specific sets of symptoms. (74). Monoamines, stress hormones, inflammation and microbiome-mediated effects may all converge in the pathophysiology of depression by ultimately influencing neural circuit function. Evidence of disrupted neural circuit function in depression comes from neuroimaging data in depressed patients as well as basic findings in animal models (72, 73) which has been significantly aided by the advent of optogenetics and chemogenetics, tools for manipulating precise circuits (section 1.2,1.3).

1.2 Circuit Interrogation of depression-relevant states

In the previous section, I introduced depression as a circuit-based disorder driven by dysregulations in neural populations that underly affective behavior. In order to properly study this, there is a need for tools that allow for the manipulation and measurement of activity *in vivo* to look at causal relationships and associations between specific circuits and behavior. Importantly, given the heterogeneity of the disorder, it is likely that specific pathways and populations underly specific behavioral dysregulations thus requiring tools to target genetically, functionally and spatially defined populations.

1.2.1 The optogenetic and chemogenetic toolbox

The optogenetic and chemogenetic toolbox includes a diversity of tools and techniques to achieve cell-type and pathway-specific control of neuronal activity. Various opsins, light-sensitive channels, have been engineered to inhibit or excite cells. Chemogenetics is synonymous with designer receptors exclusively activated by designer drugs (DREADDs), modified human

muscarinic receptors with no affinity for endogenous ligands, little constitutive activity, and activated by an experimenter-administered ligand. Opsins and DREADDs enable greatly enhanced spatial, cell-type and temporal specificity compared to traditional pharmacological techniques, allowing for precise dissection of the neural circuits underlying depression-like behavior.

Opsins

The first demonstration of optical control of neuronal firing came from channelrhodopsin (ChR2), a class of non-specific channels that open when illuminated with blue light (460nm), depolarizing the cell (75) and triggering action potentials with high temporal precision (76). The optogenetic toolbox then grew to include hyperpolarizing channels: initially, halorhodopsin (NpHR), light-gated chloride channels activated by yellow light, allow for temporally-specific hyperpolarization with single spike precision (77, 78) and later, archaerhodopsin- 3 (Arch), a yellow-light (590 nm) activated outward proton pump that spontaneously recovers from inactivation, with more rapid recovery than NpHR and fewer concerns of rebound excitation (79) and increased light sensitivity. Modifications to these tools have been made to offer greater light sensitivity, more efficient targeting and distinct optical properties and kinetics (80-83).

DREADDs

The predominant excitatory and inhibitory DREADDs, hM3Dq and hM4Di, were derived from the human muscarinic M3 and M4 receptors, respectively. When activated by CNO, hM3Dq couples to the Gq signaling pathway, leading to increased intracellular calcium, enhancing excitability and increasing firing (84-86). hM4Di couples to the Gi signaling pathway, leading to hyperpolarization via decreased cAMP, and decreased firing (84, 87). hM4Di can also silence neuronal activity by inhibiting neurotransmitter release (88, 89). DREADDs coupled to the Gs signaling pathway (rM3Ds) phosphorylate DARPP-32 (Dopamine-and cyclic AMP-regulated phosphoprotein) to increase cAMP levels (88, 90). A DREADD for non-canonical G-protein signaling pathways, the Rq (R165L), specifically activates β-arrestin signaling (89). DREADD activation with systemic CNO injection induces behavioral effects lasting up to 6 hours (87). CNO administered in drinking water can mediate prolonged regulation of neural activity in the time

scale of days or even weeks. The kinetics of chemogenetic tools render them ideal for studies requiring relatively long manipulations of neural activity (minutes, hours or even days), and for studying prolonged behavioral manipulations (e.g. long period of stress, feeding). In contrast, the optogenetic toolbox is ideally suited for short, temporally-precise manipulations on the scale of milliseconds to minutes, and so for investigating fast-paced behaviors (e.g. decision making, social interaction behavior).

1.2.2 Strategies for targeting specific cell populations

Opsins and DREADDs allow precise *in vivo* control of neural activity via targeted expression in specified cellular populations with viral vectors and/or transgenic mice. Stereotaxic viral injection allows spatial specificity, with specific promoters (e.g. CAMKII) conferring cell type-specificity (87, 91, 92). Cre-recombinase transgenic mice along with Cre-dependent viral vectors allow further cell-type specificity and double-viral strategies using Cre-dependent opsin or DREADD viruses with retrograding or anterograding Cre-expressing virus can target expression to projection-defined populations (93, 94). Inducible expression using IEG-based transgenic lines can restrict expression to cells activated by temporally defined stimuli (95-97). Strategies for ever-greater specificity continue to emerge. FLARE and Cal-Light confer targeted expression upon coincident occurrence of increased calcium and blue-light, to restrict expression to neurons active during an experimenter-defined time window (98, 99). vCAPTURE offers activity dependent, pathway-specific opsin expression (100), while FLiCRE allows for temporally specific activity dependent opsin expression in genetically inaccessible populations of cells (101).

1.2.3 Optogenetics & chemogenetics: advantages, limitations and caveats

Both optogenetics and chemogenetics are powerful tools for dissecting the neural circuits underlying behavior. The great promise of optogenetics is the ability to manipulate neuronal populations with greater spatial, temporal and cell-type specificity than traditional techniques. Electrical stimulation excites and inhibits cells but lacks spatial and cell-type specificity. Gene expression manipulations (e.g. potassium channel to alter excitability) and pharmacological techniques offer precise control but poor temporal resolution. Optogenetics offers control of cell populations precisely defined by spatial location, genetic identity, circuit connectivity or even

temporally-defined activity, with fast temporal precision in awake behaving animals to probe the causality between brain and behavior (91).. However certain limitations necessitate caution in interpreting results. Limited penetrance of light in brain tissue is an inherent challenge to *in vivo* optogenetic manipulations: generally only ~1mm^3 is effectively stimulated posing a particular challenge in larger animals (e.g. non-human primates) (102). Chemogenetics, in contrast, has high spatial reach; systemic administration allows diffusion across the entire nervous system, while targeted DREADD expression allows manipulation of specific cell populations. As chemogenetics uses a chemical actuator, systemic administration will affect all DREADD-expressing cells. Although intra-cranial infusions of CNO can limit effects to a specific region of interest (85), diffusion of the actuator can be hard to control, rendering this technique less spatially precise.

Concerns with these techniques center around the fact that they have unexpected effects on neuronal firing (103) and cell morphology as well as potential unknown downstream effects (104). Both require the use of appropriate controls as laser stimulation (105, 106) and CNO alone can have effects (107).

Another important consideration is the difference between demonstrating that modulating a circuit produces a behavior and that this occurs under physiological conditions. To optogenetically elicit behavior, experimenters have at times induced cells to fire at frequencies outside physiological range. Findings based on non-physiological stimulation protocols should be interpreted with caution. While efforts to achieve more physiological stimulation protocols are important, as generally employed, optogenetic stimulation results in synchronous activation (or inhibition) of an entire cell population (108-110). Inhibition of circuit activity can theoretically be more informative concerning the necessity of a circuit to behavior, although there are a number of technical concerns. For example, optogenetic inhibition can induce rebound excitation in cells once released from non-physiological inhibition and inhibition of terminal activity has been problematic (111).

Reviewing multiple studies in animal models of depression points to regulation of similar behaviors by divergent brain regions. It is common to assert *sufficiency* of a brain circuit when optogenetic or chemogenetic excitation elicits a particular behavior and *necessity* when

inhibition suppresses this behavior. However, concluding that a brain circuit is *sufficient* to drive a behavior because activating it increases the behavior can erroneously imply that this is the unique and complete mechanism (112). Establishing true sufficiency would require demonstrating not only that circuit activation *can* elicit behavior, but that it is the *only* requirement, something rarely true. A more appropriate conclusion is that circuit activity *induces* the behavior, which does not exclude the possibility that other circuits may also exert control over the same behavior. In fact, with complex, motivated behaviors, as examined in animal models of depression, it would be surprising for a single brain region or circuit to exert unique and complete mechanistic control. In this light, it is to be expected that optogenetic and chemogenetic studies will identify multiple brain regions mediating the same depression-relevant behaviors. While chemogenetics and optogenetics are powerful tools to probe the circuitry underlying depression-related circuits. These limitations point to a need to measure endogenous activity in vivo to look at how circuits respond to various stimuli as opposed to what they can do when stimulated.

1.2.4 *In vivo* imaging advances

Many of these limitations highlight a potential disconnect between control of cellular activity and behavior by optogenetics and chemogenetics and the *in vivo* physiological realities of circuit function. A solution that is currently gaining popularity is to complement perturbation experiments with population or single-cell *in vivo* imaging such as fiber photometry, microendoscopes (113) and head fixed two photon calcium imaging. Fiber photometry (114), in particular, has emerged as a powerful tool to interrogate neural pathways during ongoing behavior. This technique makes use of a calcium indicator, GCaMP which was constructed with a green fluorescent protein (GFP) tethered to calmodulin (CaM), a calcium binding protein, and CaM-interacting M13 domain. This protein goes through a conformational change when binding calcium, which increases the brightness of GCaMP (115). Initial designs suffered from slow kinetics and low sensitivity but since then it has been optimized to have vastly improved signal to noise ratio (116). A fiber optic canula gives access to brain tissue and attaches to a patchcord to allow for the delivery of a 470nm excitation light. The fiber also collects fluorescence emitted by GCaMP and projects it onto a photodetector, giving a proxy measure for levels of neural

activity in a population of cells. Advances have been made in this relatively new technology to allow for dual color, multisite imaging, giving access to multiple populations of cells simultaneously (117) through the use of alternative colored calcium indicators and CMOS cameras instead of photodetectors. Much of the strength of fiber photometry is similar to optogenetics in interrogating populations of cells precisely defined by spatial location (118), genetic identity, (119, 120) or circuit connectivity (121). Use of a fiberoptic canula and patchcord allows for animals to engage in naturalistic behaviors without being head fixed (122). Furthermore, it gives the ability to describe naturally occurring neural activity during these behaviors as well as offers the potential for longitudinal studies that examine changes in activity across different events such as stress. Initial work introducing the technique recorded fluorescence in dopaminergic (DA)- ventral tegmental area (VTA) cells as well as NAc projecting DA-VTA cell, showing elevations in activity in these pathways coincident with social interaction (114). Fiber photometry has been rapidly and widely adopted and used to probe many circuits, neurotransmitters and neuromodulators across a wide range of contexts (100, 119, 121).

However, despite its utility, fiber photometry has certain limitations. First, GCaMP dynamics do not offer the temporal precision that is seen with electrically based techniques as it exhibits a slow decay in fluorescence (115, 123). As this is a population recording (imaging an entire population of cells), fluorescence from each neuron sums together at each instance, meaning it is impossible to tease apart individual firing dynamics of these cells and there is quite a high signal to noise ratio. Furthermore, the signal produced suffers from many artifacts due to motion, bleaching and other sources. A 410nm isosbestic wavelength is projected simultaneously through the patchcord to record non calcium related fluorescence and control for these artifacts, but it is certainly not perfect. As with many techniques to measure neural activity, analyses are complex and need to be carefully thought out in order to make appropriate conclusions. Despite these, it remains a powerful technique to examine the relationship between neural activity and behavior.

1.2.5 Probing Circuitry underlying depression and stress susceptibility

Although depression involves widespread dysregulations across a number of bodily systems, human imaging studies have provided evidence for a neural circuit model of the disorder,

identifying changes in multiple regions such as the PFC, amygdala (AMY), hippocampus and basal ganglia associated with depression (124). In clinical and pre-clinical research, a large focus has been put on how dysregulations in reward circuitry drive maladaptive behaviors following stress, given the profound deficits seen in reward related behaviors in depression (125). fMRI studies have identified decreases in activity in the NAc during reward related tasks (29, 30) and decreases in PFC during reversal learning tasks (31). Post-mortem analyses show decreased cortical volume in depressed patients which is associated with neuronal atrophy and loss of glia cells (126), fueling the hypothesis that depression involves a reduction in excitatory control of reward circuits (125). The hippocampus is a structure important for encoding emotionally salient memories and thus is thought to play an important role in depression. However, human imaging studies are less clear, with inconsistent reports of hippocampal atrophy in depressed patients (124). Studies in rodents reconfirm a role for reward circuitry in driving susceptibility to stress (127-130). Advances in tools for probing specific neural populations have allowed for the functional interrogation of circuits that regulate susceptibility, implicating various regions such as the NAc, PFC, AMY, VTA and others in behavioral adaptation to stress (Figure 2). In particular, a lot of work has focused on accumbal circuitry and its role in stress response (36, 92, 129, 131, 132). The NAc receives dopaminergic input from the VTA, which is heavily implicated in motivation and reward (133). Thus, this projection/region has been heavily studied in the context of stress. (37, 127, 129). However, DA is simply a neuromodulator, regulating glutamatergic input from other projections to the NAc (134). In future sections, we investigate the role of the NAc and its afferent glutamatergic projections in goal directed behavior as well as how stress-induced dysregulations in this circuitry drives stress susceptibility.

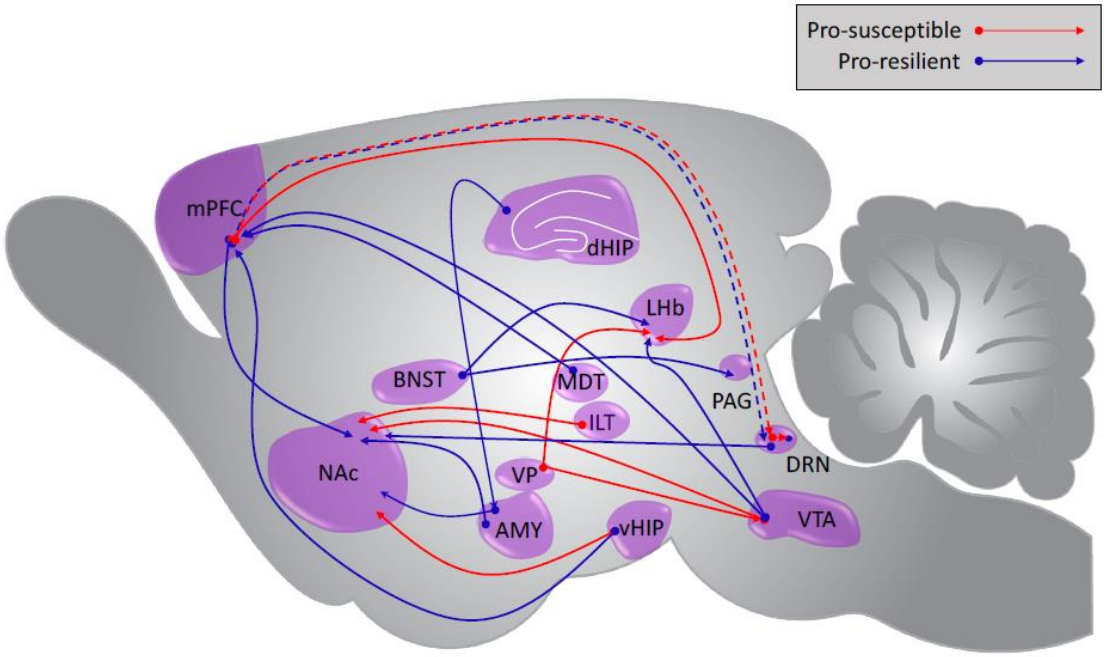

Figure 2. *Schematic illustrating neuronal pathways implicated in depression-like behavior by optogenetic or chemogenetics. Red arrows indicate pro-susceptible projections where activation leads to increased depression-like behavior (social avoidance, immobility, etc.) and blue arrows indicate pro-resilient projections. Dashed lines indicate pathways in which conflicting findings exist.*

1.3 Accumbal Circuitry

The NAc and its related circuitry play an important role in depression and stress susceptibility (35, 132). This circuitry regulates motivated behavior and reward (133), making it a good candidate for driving deficits in valence processing and motivated behavior following stress. Here, I review studies highlighting the structure and function of the NAc, and two of its major glutamatergic afferents, PFC and ventral hippocampus (vHIP), as well as their contribution to behavioral adaptation to stress.

1.3.1 Nucleus accumbens anatomy and connectivity

The NAc is a major structure in the ventral striatum that can be divided into two anatomically and functionally distinct regions: core (NAcc) and shell (NAcs). These regions are defined based on histochemical markers, connectivity and distribution of certain neuropeptides. These subregions also have different efferent projections, with the core projecting to the lateral globus pallidus (GP), dorsolateral ventral pallidum (VP), dopaminergic cells in the VTA and Substantia

Nigra pars reticulata (135) and to the NAcs, while the NAcs projects to the ventromedial VP, VTA and hypothalamus (135, 136). These differing outputs allow them to contribute uniquely to behavior and the pathophysiology of disease (see section below).

This region contains mostly (>95%) medium spiny neurons (MSN) (137), which are the main output projection for the NAc. These cells release GABA, an inhibitory neurotransmitter, have a negative resting membrane potential and low excitability (138, 139); thus, they do not have the properties to be able to generate recurrent spontaneous activity as is the case with cortical projection cells (140). Therefore, they require significant excitatory drive from upstream structures to be able to fire action potentials. The vHIP, AMY, PFC and thalamus (TH) send glutamatergic inputs to the NAc (121, 141-143) and interact to drive action potential firing. Some of the most important interactions occur between the vHIP and mPFC projections (144), which monosynaptically converge in the NAc (143). Activation of vHIP projections induces a bistable state in NAc MSNs, whereby their membrane potential is increased, but they are not firing action potentials. Without this switch to a bistable state, PFC inputs are incapable of driving action potentials, indicating that the vHIP gates PFC input (139). Conversely, vHIP projections are incapable of driving action potential firing without the PFC (145), unless LTP is induced, while PFC burst firing leads to desynchronization of vHIP and NAc, suggesting that this closes the gate for vHIP to influence the NAc (142). Evidence suggests that dopaminergic innervation from the ventral tegmental area (VTA) may modulate input from these other structures via dopamine receptor expression (134, 146). A model of ensemble encoding has been proposed in the NAc, whereby specific groups of MSNs are recruited by synchronous upstream input in order to influence behavior (140, 147, 148).

NAc MSNs can be subdivided based on their primary expression of either D1 or D2-type dopamine receptors (137). These receptors exert opposing influences over the cell via their regulation of cAMP signaling, with D1Rs increasing excitability in MSNs and D2Rs decreasing excitability (149); their dopaminergic signaling to the NAc is thought to influence the responsiveness of these cells to glutamate from upstream structures (134, 149-151). Although previously there has been controversy over the amount of overlap between these populations, more recent studies using transgenic mice expressing reporter genes encoding fluorescent proteins under the control of D1

and D2 receptor promoters have reported less than 2% co-localization of these receptor sub-types (152-154). Aside from expression of dopamine receptors, these cells also differ on expression of other genes such as enkephalin (enriched in D1-MSNs) and substance P (enriched in D2-MSNs) (155, 156) as well as their output projections, with both D1 and D2-MSNs projecting to the VP and D1-MSNs also projecting to the VTA (153, 157, 158).

Surprisingly, tracing studies have found that these two cell subtypes receive similar inputs from upstream structures including the PFC and hippocampus (159), indicating that they have access to similar information. Although, in the NAcc, hippocampal inputs contact more distal dendritic spines, making them less likely to trigger action potentials compared to PFC inputs (160), indicating that interactions between afferent projections to drive NAc firing may be region specific.

1.3.2 Nucleus accumbens function

The NAc is thought to integrate information about emotional salience from various glutamatergic and dopaminergic projections in order to influence motor output (134, 161). It is important to note that, despite the popular view that the NAc acts as a reward center, this region actually serves to bias the direction of behavior to achieve certain goals (162). In fact, studies also implicate this region in responding to aversive stimuli (163-165).

Although structurally, the NAc core and shell are well defined, there is controversy over the functional role of these two subregions. Floresco et al (162) suggested that the NAcc drives approach behavior while the NAcs inhibits inappropriate actions. In support of this, multiple studies have confirmed that inactivation of NAcc suppresses reward seeking/ approach behavior (166, 167). On the other hand, inactivation of the NAcs inhibits extinction, increases unrewarded actions (167) and suppresses actions leading to larger rewards (168). However, West et al (169) have suggested that the NAcc plays a bigger role in action selection while the shell tracks hedonic value. In line with this, although both subregions respond to reward, the NAcc plays an important role in reward learning, being necessary for reward conditioned behavior (170). NAcs but not the NAcc encodes both the motivational value of a reward based on internal state as well as the relative value of food rewards (171, 172). Furthermore, following extinction, NAcc encodes

flexible behavior, while NAcs firing tracked the value of the reward (173). Despite this complexity, it is clear that both subregions play a role in biasing motivated behavior.

Dissecting cell-type specific functionality in motivation and reward

Classically, D1- and D2-MSNs are thought to play opposing roles in motivation and reward (174-177) , with the vast majority of studies supporting this theory. Stimulation of D1-MSNs can drive instrumental behavior to a stimulation paired manipulandum, while D2-MSN stimulation biases behavior to a non-stimulation paired lever (178). Inhibition of D2-MSNs has been shown to increase motivated behavior at the cost of goal directed efficiency despite not impacting sensitivity to reward value (179, 180). These two cell types seem to be necessary for learning about reward and aversion, with D1R antagonism impairing food driven conditioned place preference (CPP) while D2R antagonism blocked passive avoidance learning (181). Studies looking at drugs of abuse have solidified a role for these cell subtypes in regulating reward. Cocaine and associated cues increase activity in D1-MSNs and decrease activity in D2-MSNs (119). Similarly, stimulation of D1-MSNs and D2-MSNs respectively increase and decrease cocaine preference (174) as well as preference for morphine (176).

Despite the majority of studies supporting classical roles for D1- and D2-MSNs in reward and aversion, there is literature to suggest that motivated behavior, in fact, requires both cell types. Pavlovian to instrumental transfer (PIT) measures the ability of a reward-predicting cue to enhance a rewarded operant behavior, and progressive ratio (PR) assesses willingness to work for reward. Optogenetic excitation of either D1- or D2-MSNs increases motivation in both these tasks and surprisingly, inhibition of D2-MSNs decreases motivation (182). Moreover, in rats exposed to *in utero* glucocorticoids, a manipulation that leads to deficits in motivation and hypodopaminergia, targeted optogenetic stimulation of NAc D2-MSNs increases motivation in both tests, suggesting a more complex role for D2-MSNs. Furthermore, stimulation of both D1- and D2-MSNs can drive conditioned place aversion and preference depending on the length of stimulation (183), while both subtypes contributed positively to goal directed, food motivated behavior (184). . Recent data shows that D2-MSN stimulation during reward predicting cues but

inhibition during reward consumption increases motivation (185). These studies suggest that the function of these neurons may be context, task and condition dependent

1.3.3 Nucleus accumbens and stress adaptation

Human studies show decreased activity in the NAc in depressed patients during reward related tasks as well as decreased volume (29, 30). Furthermore, downregulation of RAC1 (32), a regulator of synaptic structure, is seen in post-mortem studies, implicating the NAc in depression. D1 and D2 MSNs play important opposing roles in reward and motivation depression (133, 174, 178) and experiments have also identified opposing roles for these cell types in stress susceptibility. Chronic restraint stress leads to depression of excitatory transmission onto D1-MSNs (186) while, following CSDS, susceptible mice display a decreased mini excitatory post synaptic current (mEPSC) frequency in D1-MSNs and increased mEPSC frequency in D2 MSNs, as well as increase spine density in D2-MSNs (187), indicating respective decreases and increases in excitatory inputs onto these cells. Optogenetically stimulating D1-MSNs in susceptible mice following CSDS promotes resilience while stimulating D2-MSNs in stress-naïve mice induces susceptibility to subthreshold defeat (132). Inhibiting D1-MSNs by hM4Di induced susceptibility in resilient mice. Cell type specific manipulations, ultimately, confirm earlier findings using electrophysiology and genetic manipulations and directly identify a causal role for D1 and D2-MSN activity in modulating resilience and susceptibility respectively. (132). Single cell calcium imaging was also recently applied to interrogate D1-MSN firing following social defeat. At the single cell level, defeat reduced the frequency of events in D1-MSNs, although somewhat paradoxically, the average neural response during social interaction was increased (188).

Recent work from Pignatelli et al (189) shows that a single cue pavlovian fear conditioning paradigm, in which animals are exposed to a shock predictive cue, leads to anhedonia and passive coping. D1- but not D2-MSNs exhibit increased excitability due to reduced inwardly rectifying calcium channel activity. These results seem at odds with previous literature. However, decreasing excitability in these cells via a dominant negative channel rescues depressive-like behavior but also impairs fear conditioning, indicating that this manipulation may impact how animals learn about aversive stimuli, rather than simply driving a negative affective state.

Molecular players in the NAc are also implicated in adaptation to stress. ΔFosB, an IEG which acts to increase GluR2 subunits in AMPA receptors, leading to decreased inward rectification at a synapse, has been implicated in depression with reduced levels seen in the NAc of depressed patient and increased levels in resilient animals compared to susceptible following defeat (33). SSRI treatment, which rescues social avoidance behavior, also led to increases in ΔFosB. In line with this, overexpression of ΔFosB in the NAc, using a transgenic mouse line, promotes resilience following defeat (33). Interestingly, this mouse line seems to overexpress the IEG in D1-MSNs specifically (190), indicating that enrichment in these cells is what drives resilience. Another study by Lobo et al (177) confirms an increase of ΔFosb in D1-MSNs of resilient mice and in D2-MSNs of susceptible mice following defeat. Furthermore, Egr3 knockdown increased social interaction behavior and normalized stress induced changes in D1-MSN firing (188)

1.3.4 Contribution of afferent projections to behavior: focus on prefrontal cortical and ventral hippocampal input

The vHIP and PFC are key nodes in brain circuits mediating emotional behavior (191). As mentioned above, both regions send glutamatergic projections to the NAc and interact to drive MSN firing. Both the vHIP and PFC target the entirety of the NAc, although the vHIP afferents are concentrated in the medial NAc shell. The vHIP is capable of eliciting the largest EPSCs in MSNs in this region although these projections do not differ in other synaptic properties such as PPR or NMDA/AMPA receptor ratios (141). Furthermore, although optically evoked AMPAR currents do not differ between pathways, vHIP cells pass proportionally higher peak inward current at negative holding potentials, indicating that even at negative resting potentials, they may contribute to excitatory transmission (141).

Behaviorally, the vHIP projection has been shown to be important for reward motivated behavior, with stimulation supporting instrumental behavior and CPP (141). LTP induction in this pathways also drove CPP while vHIP- NAc silencing blocked social interaction conditioning (192), consistent with work showing this pathway to be important for social memory (193). On the other hand, work by Yoshida et al defines a role for the vHIP as a whole in behavioral inhibition as a suppression in activity is necessary for goal directed behavior (194) while consummatory

behavior is associated with suppression in vHIP projections to the rostral NAc shell (121). Other work has found that this pathway integrates aversive information as well, showing increases in activity following shock and a shock predictive cue (189).

Both the infralimbic (IL) and the prelimbic (PrL) subregions of the mPFC project to the NAc, with the IL targeting the shell and PrL, the core. Evidence also points to a role for this pathway in reward, with the mPFC-NAc also supporting instrumental behavior and CPP. PrL projections exhibit increased activity in response to reward predictive cues compared to neutral cues (195). However, increases in activity are also seen in response to shock, but show inactivation before a lever press in a shock associated context, indicating that this pathway must be inhibited to initiate reward seeking behavior. A population of functionally defined projection cells in this pathway respond to aversive stimuli and, while stimulating the entire pathway had no effect, stimulating this subpopulation of cells inhibited lever pressing and drove real time place aversion (100). Similarly, inactivation of both the PrL and IL inhibit conditioned suppression of reward seeking (196). IL-NAc connectivity is also required for PIT (197) and is essential for discriminating between and responding to aversive and appetitive cues in adaptive environments (198).

Although it is clear that these projections play a role in regulating motivated behavior, there are a number of conflicting findings. This lack of consistency could be attributed to a number of factors such as targeting, stimulation parameters and context/ behavioral paradigms. For example, many papers do not specify the specific mPFC subregion that is targeted, despite known differences in anatomy and function, making it difficult to compare results across studies.

1.3.5 Hippocampal projections and stress adaptation

A large body of work has implicated the ventral hippocampus and many of its downstream projections, such as to the PFC , AMY and NAc, in stress adaptation. Its projections to the NAc have been found to be activated following acute restraint stress (199), indicating that this projection is sensitive to aversive stimuli. The vHIP projection to the NAc has been implicated in stress susceptibility. CSDS induces differences in pre-synaptic glutamate release at vHIP-NAc synapses between susceptible and resilient mice (Bagot et al, 2015). *In vivo*, low frequency stimulation of this pathway, a manipulation that induces lasting synaptic plasticity to reduce post-

synaptic activity (long-term depression), induces a pro-resilient effect in defeated mice, increasing social interaction in a later test. An opposing manipulation to acutely increase vHIP-NAc signaling during testing, induces susceptibility in defeated mice, suppressing social interaction. Thus, through its interaction with NAc, the vHIP bi-directionally modulates susceptibility to depression-like behavior (92). This pathway has also been shown to drive sex differences in stress susceptibility. Males show a testosterone dependent decrease in excitability in their vHIP-NAc compared to females which is associated with resilience to a 6d-subchronic CVS (SCVS) which leads to susceptibility in females. Androgen receptor antagonism in males drives susceptibility to this subchronic stress by increasing excitability in this pathway while testosterone injections in females decrease excitability and drives resilience. Chemogenetic excitation of this pathway in males recapitulates this effect, driving reduced sucrose preference in males after SCVS while inhibition in females rescues stress induces deficits in sucrose preference (200).

Other work has looked into firing properties of ventral subiculum pyramidal neurons, identifying regular spiking, weak bursting and strong bursting neurons (201). The majority of NAc projecting cells were identified as regular spiking neurons in naïve mice. CSDS decreased the number of regular spiking neurons NAc projecting neurons, with susceptible mice showing decreased numbers compared to resilient. Susceptible mice show increased strong bursting cells compared to control and resilient as well as increased firing frequency in their regular spiking cells (202). These studies provide evidence that stress may fundamentally change the properties of these cells. Using a 3-day Pavlovian fear conditioning paradigm, Pignatelli et al used fiber photometry and found that the vHIP-NAc exhibited an increase in activity at shock onset throughout all three days of training as well as increased activity during the cue starting on day 2. They find that vHIP projections onto D1- but not D2-MSNs are potentiated, exhibiting increased AMPA/NMDAR ratio and EPSC amplitude. Inducing LTD at the synapse via low frequency optogenetic stimulation does not affect fear conditioning but rescues stress induces anhedonia and passive coping, indicating that these projections reflect a negative affective state.

Seemingly at odds with the above body of literatures, LeGates et al (192) found that stress induced reward deficits were driven by weakening of the vHIP-NAc. Chronic multimodal stress

was found to induce anhedonia which was associated with decreased synaptic strength at vHIP-D1-MSN synapses specifically as well as an impairment in LTP, all of which was rescued by antidepressant treatment. However, these inconsistencies may relate to differential targeting of projections; LeGates targeted intermediate hippocampus and probed the lateral NAc shell while the majority of studies target ventral hippocampus inputs to NAc medial shell (189, 192).

1.3.6 Prefrontal cortical projections and stress adaptation

Reduced PFC activation is observed in depressed humans as well as in rodent depression models, making it a key area of interest (38, 203). Optogenetically stimulating the mPFC reverses depression-like behaviors such as social avoidance and anhedonia in defeated mice (Covington et al., 2010). This may be specifically mediated by NAc-projecting mPFC neurons as targeted stimulation of this pathway increases social interaction in defeated male mice (38, 92). However, despite evidence implicating increased mPFC-NAc activity in resilience, suppression of this pathway does not induce social avoidance, suggesting a potential dissociation between circuit mechanisms of resilience and susceptibility (92). Indeed, other molecular players act in concert with glutamatergic mPFC projections to regulate susceptibility. Upregulation of ΔFosB induces susceptibility following CSDS and increases expression of CCKB, the CCK receptor, in the prelimbic cortex (PrL) which has the potential to decrease PrL activity, leading to social avoidance, reduced sucrose preference and increased immobility in FST (130). Infusing CCK into the PrL reduces social interaction in mice after defeat and optogenetic stimulation of PrL-NAc projections counteracts the effects of increased CCK signaling to reverse social avoidance. By integrating optogenetics with pharmacology, this experiment sheds light on the molecular mechanisms underlying susceptibility and resilience.

However, literature on the PFC-NAc seems slightly at odds. More recent work by Bittar et al (53) implicates functional and morphological changes in this pathway with susceptibility. Following a 21d-CVS, stressed females show increased spontaneous (s)EPSC and decreased spontaneous inhibitory post synaptic current (IPSC) frequency and amplitude compared to control as well as E/IPSC frequency while males show decreases in sIPSC frequency and increases in E/IPSC frequency. Both males and females also show increased spine density. Pathways specific

chemogenetic manipulations show that excitation of these pathways drives susceptibility to a SCVS across a range of behavioral tests in both males and females, while inhibition rescues stress induced depressive-like behavior in females alone. Although, again, targeting may at least partially account for such inconsistencies.

RATIONALE AND AIMS

Depression is one of the leading causes of disability worldwide, yet current antidepressant treatment remains ineffective in approximatively 50% of the affected population. This points to a serious need for more targeted treatment but also prevention of the disorder. Stress is a major risk factor leading to the development of depression, but there is significant variability in how individuals respond to stress, with only a small percentage developing depression as a result. Previous sections highlight a large body of work looking into how accumbal circuitry drives behavioral adaptation to stress. Most of this work looks at differences after stress, comparing resilient and susceptible animals to control in order to investigate how stress changes the brain in animals with a depressive-like versus a control-like phenotype. An important question brought up by this literature is why genetically inbred animals, reared in similar environments and exposed to identical stresses are behaviorally diverging in such a profound way. Of course, in humans, risk factors such as early life adversity can increase one's chance of developing depression (204), but in the absence of major divergences in environment such as laboratory reared rodents, what contributes to these differences in stress susceptibility, and how can we detect these changes? Until very recently, the literature addressing this phenomenon was sparse with work identifying differences in mGlu2 expression in the hippocampus (205) as well as levels of leukocytes and IL-6 release (206) associated with susceptibility to stress. However, possible functional differences in neural activity associated with behavioral adaptation to stress had not been explored.

The goal of this thesis is to examine the mechanisms underlying this differential adaptation to stress and identify pre-existing differences that might pre-dispose individuals to stress induced susceptibility, allowing us to identify vulnerable individuals before the development of the disorder. My goal was to interrogate how neural activity in accumbens circuitry relates to behavior as mice interact with their environment and determine how this activity predicts stress susceptibility and how stress itself modulates activity. Finding a link between an animal's neural activity and stress vulnerability, I then sought to determine how aversive information, as is encountered in chronic stress paradigms, is encoded in this circuitry and how this threat encoding

modulates behavior in order to gain a better understanding of how chronic exposure to aversive stimuli and subsequent neural dysregulations may contribute to susceptibility.

In **Chapter 1**, I identify individual differences in cell type specific activity in the NAc that associate with future stress susceptibility following chronic social defeat stress. I hypothesize that, given the role of this structure in modulating behavior as well as stress adaptation, it is a prime candidate for pre-existing differences that predispose individuals to stress. Using in vivo fiber photometry calcium imaging, I interrogate cell type specific neural activity in the NAc in the home cage and while interacting with a social target in order to define signatures of neural activity that associate with future susceptibility. **Chapter 2** expands upon this work to look at glutamatergic projections from the vHIP to the NAc. Knowing that these pathways are involved in behavioral adaptation to stress in males, I look to show how these pathways relate to ongoing depressive and anxiety-like behavior in both sexes and understand how individual differences in neural activity and behavior may predict/ predispose individuals to stress induced susceptibility. Using a longitudinal design and in vivo calcium imaging, I interrogate neural activity before and after a chronic stress, I examine how stress modulates activity in this pathway as mice interact with their environment. Furthermore, I look to use pre-stress behavioral and neural metrics to predict future susceptibility. In **Chapter 3,** I look at both vHIP and PFC projections to the NAc and examine how they encode aversive experiences in both sexes as well as how this neural activity relates to ongoing behavior. Appropriately responding to threatening stimuli is a behavior dysregulated in many psychopathologies including depression and anxiety. Learning about how these pathways integrate aversive information in order to control behavior is essential to our understanding the mechanisms underlying stress induced behavioral dysregulations. Using in vivo calcium imaging in combination with pathway specific chemogenetic manipulations, I interrogate how these pathways respond to unconditioned aversive stimuli and the cues that predict them and probe the functionality of this signal in the control of behavior.

Framing the questions: Chapter 2

The nucleus accumbens plays an important role in regulating stress susceptibility, with D1-MSN activity driving resilience and D2-MSN activity driving susceptibility in male mice after stress. However, prior to conducting this study, the majority of the literature had looked to identify mechanisms of susceptibility, interrogating differences in neural activity in resilient and susceptible animals following stress that associated with behavior. Our goal in this first chapter was to determine if differences in cell-type specific nucleus accumbens activity present before stress associated with post-stress phenotype and to examine how these animals differentially encode rewarding stimuli. This was one of the first pre-clinical studies looking into mechanisms of vulnerability and pointing to fundamental differences in how animals that do not yet exhibit a stress phenotype process environmental stimuli.

CHAPTER 2

In vivo fiber photometry reveals signature of future stress susceptibility in nucleus accumbens

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Abstract

Recognizing why chronic stress causes only a subset of individuals to become depressed is critical to understanding depression on a basic level and, also to developing treatments that increase resilience. Stress-induced alterations in the activity of reward-related brain regions, such as the nucleus accumbens (NAc), are linked to the pathophysiology of depression. However, it has been difficult to determine if differences in stress susceptibility are pre-existing or merely an effect of chronic stress. The NAc consists largely of medium spiny neurons (MSNs), distinguished by their predominant expression of either D1 or D2 dopamine receptors. Mice that develop depressive-like symptoms after chronic social defeat stress (CSDS) show distinct changes in the activity of these two cell subtypes. Until now it has not been possible to determine if such effects are merely a consequence of stress or in fact precede stress and, thus, have utility in pre-identifying stress-susceptible individuals. The goal of this study was to define a cell-type specific signature of stress susceptibility and resilience. Using fiber photometry calcium imaging, we recorded calcium transients in NAc D1- and D2-MSNs in awake behaving mice and found that D1-MSN activity is a predictive marker of depression susceptibility: prior to stress, mice that will later become resilient had increased baseline D1- MSN activity, and increased calcium transients specific to social interaction. Differences in D2- MSN activity were not specific to social interaction. Our findings identify a pre-existing mechanism of stress-induced susceptibility, creating the potential to target preventative interventions to the most relevant populations.

Introduction

Epidemiological studies highlight the importance of stress in the etiology of depression (Kendler *et al*, 1999; Kessler, 1997). However, in reality only a minority of people who experience stress become depressed. Identifying the neural mechanisms that increase risk for depression is of fundamental importance to both treatment and prevention. The chronic social defeat stress (CSDS) paradigm captures variation in stress susceptibility; genetically-inbred mice exposed to the same experience phenotypically diverge: susceptible mice exhibit depressive-like symptoms such as anhedonia and decreased social interaction, whereas resilient mice fail to show these changes (Berton *et al*, 2006; Krishnan *et al*, 2007). Animal models of depression, such as CSDS, have provided important insights into key neuronal changes associated with depression-like states (Bagot *et al*, 2015; Berton *et al*, 2006; Chaudhury *et al*, 2013; Covington *et al*, 2010; Dias *et al*, 2014; Francis *et al*, 2015; Krishnan *et al*, 2007; Lim *et al*, 2012; Lobo *et al*, 2013; Sun *et al*, 2015; Tye *et al*, 2013; Vialou *et al*, 2010), however, understanding the neuronal mechanisms that guide the initial development of susceptibility or resilience remains to be understood. One major challenge in identifying risk factors is the lack of prospective mechanistic studies: assessments are made post-stress, making it impossible to distinguish stress-induced neuronal plasticity from the intrinsic factors that render an individual susceptible to stress. Understanding the neuronal mechanisms that support susceptibility vs. resilience *after* stress can inform treatment but identifying pre-existing features that differentiate individuals that will become susceptible and those that will remain resilient when challenged by stress opens the door to prevention.

The nucleus accumbens (NAc) is an important structure in motivation and reward (Schultz, 2006; Wise, 2004). It is comprised predominantly of medium spiny neurons (MSNs) distinguished by their relative expression of D1 or D2 dopamine receptors (Gerfen and Surmeier, 2011). These two cell types have different downstream targets and play opposing roles in motivation, with D1-MSNs implicated in reward and D2-MSNs primarily implicated in aversion (Baik, 2013; Kravitz *et al*, 2012; Lobo *et al*, 2010). Balanced activity between these two cell populations supports normal behavior, with selective dysregulation in the cell types implicated in the pathophysiology of depression (Dias *et al*, 2014; Francis *et al*, 2015; Lim *et al*, 2012). Differential activity of D1- and D2-MSNs has been observed after stress in susceptible and resilient mice (Francis *et al*, 2015).

Optogenetic activation of D1-MSNs after chronic stress promotes resilience, whereas pharmacological inhibition promotes susceptibility. Furthermore, activation of D2-MSNs promotes susceptibility after acute stress and, following chronic social defeat, excitatory inputs onto D2-MSNs are increased, but decreased onto D1-MSNs, in susceptible mice (Francis *et al*, 2015). It is not known if differences in D1 and D2 activity emerge only *after* stress or if pre-existing differences exist that might identify those individuals that will go on to develop depressive-like symptoms in the face of stress.

We hypothesized that pre-existing differences in D1- and D2-MSN activity may play a role in regulating stress susceptibility, ultimately making some mice more susceptible to stress. I aimed to identify the pre-existing endogenous patterns of in vivo neuronal activity from which susceptibility vs. resilience emerge. Using fiber photometry (Gunaydin *et al*, 2014) , a novel technique that allows for the measurement of calcium transients from selected cell types in vivo, we measured real-time neuronal activity in NAc D1 and D2-MSNs in awake behaving mice prior to stress and identified pre-stress differences in D1 signaling as a key risk factor for stress susceptibility. We report that resting D1-MSN activity and temporally-specific calcium transients during social interaction associate with emergent phenotypes after social defeat, while D2-MSN activity appears to play a more complex role in regulating stress susceptibility.

Materials and Methods

Experimental animals.

Male 8-16 week-old D1-Cre and D2-Cre BAC transgenic mice on C57BL/6J background mice initially obtained from NINDS/GENSAT (www.gensat.org/index.html) were bred at Icahn School of Medicine at Mount Sinai and 6-month old CD1 retired breeders were obtained from Jackson labs. All mice were maintained on a 12-h light-dark cycle at 22-25^{o}C with ad libitum access to food and water. D1-Cre and D2-Cre mice were group housed 5 per cage until the start of defeat. A total of 21 mice were included in this study: 9 D1-cre transgenic mice and 12 D2-cre transgenic mice. All experimental manipulations occurred during the light cycle. All experiments were conducted in accordance with guidelines of Mount Sinai's Animal Care and Use Committee.

Stereotaxic fiber implantation and virus injection.

To achieve cell-type specific GCaMP6f expression, D1-Cre and D2-Cre were injected with AAVdj-EF1α-DIO-GCaMP6f-WPRE virus (5 x $10^{-12/}$mL). Stereotaxic surgery was performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia to target the NAc (A/P +1.3, L/M +0.5, D/V, -4.4 mm) and 0.5 μL virus was infused at a rate of 0.1 μL per min and allowed to diffuse for 10 min before the needle was withdrawn. Chronically implantable optic fibers (Doric Lenses) with 400 μm core, 0.48 N.A. optic fiber threaded through metal ferrules were then implanted above the viral injection site (A/P +1.3, L/M +0.5, D/V, -4.3). Recordings began a minimum 4 weeks after surgery to allow sufficient time for stable and robust virus expression.

CSDS and social interaction.

An established CSDS protocol was used (Berton *et al*, 2006; Golden *et al*, 2011). Briefly, D1-Cre and D2-Cre mice were subjected to 10 daily 5 min aggressive encounters with a novel CD1 mouse after which they were separated from the CD1 mouse by a plexiglas divide to allow for sensory but not physical contact for the remainder of the 24 h period. This protocol yields two phenotypes (susceptible and resilient) identified by an animal's social interaction with a novel CD1 mouse in a social interaction test 24 h after CSDS. Mice were tested in a social interaction test prior to the start of CSDS and again 24h after CSDS. Mice were not pre-exposed to the interaction arena

before the social interaction tests. In the first 2.5 min test (no target), mice explore an arena (44 x 44 cm) containing a plexiglas mesh enclosure on one wall (10 x 6 cm). In the second 2.5 min test (target), a novel CD1 mouse (social target) is placed in the plexiglas mesh enclosure and mice again explore freely. Time spent in the interaction zone around the plexiglas mesh enclosure (14 x 26 cm), and the corner zones (10 x 10 cm), was recorded by video tracking software (Ethovision XT, Noldus). To generate precise behavioral time-stamps to align with the neuronal calcium signal, the timing of corner entries and proximal interaction events were annotated by an experimenter blind to condition (Observer XT, Noldus); proximal interaction events were scored when the mouse's nose contacted the mesh grid of the enclosure and corner entries were scored when the mouse's nose crossed into the corner zones.

Fiber photometry.

This technique allows for the measurement of neuronal calcium transients in real time (Calipari *et al*, 2016; Gunaydin *et al*, 2014) . Two light emitting diodes at 490 nm (GCaMP stimulation wavelength) and 405 nm (control for artifactual fluorescence) (Thorlabs) were reflected off dichroic mirrors (FF495; Semrock) and coupled to a 400 µm 0.4 N.A. optical fiber (BFH48-600, Thorlabs) using a 400 x 0.4 N.A. microscope objective (Olympus) and fiber launch (Thorlabs). The emission light was collected by the same optical fiber, passed through a GFP filter and focused onto a photodetector (model 2151 femtowatt photoreceiver; Newport) where the two output signals were separated based on modulation frequency. Samples were collected at a frequency of 381 Hz.

Recordings were made from NAc D1- or D2-MSNs in mice during both target (2.5 min) and no target (2.5 min) social interaction tests 24 h prior to the start of the defeat protocol and in the defeat cage (separated from aggressor by plexiglas divider) immediately before (2 min) and after (5 min) the first aggressive encounter.

Data were extracted and analyzed using custom-written scripts in Matlab R2016b (The MathWorks). To normalize the data, the control channel was fitted to and then subtracted from the raw trace, giving the ΔF/F. For peak detection, data were high-pass filtered and transformed to a Z-score. Peaks were detected as follows: High amplitude events (local maxima 2 MAD above

the median) were filtered out and the median of the resultant trace calculated. The peak detection threshold was set at 3 times this median and the resultant average peak amplitude and peak frequency were compared across groups. Fiber photometry recordings with no detectable peaks (i.e. no significant increases in D1- or D2- MSN activity above baseline) were included in analysis of peak frequency but were not considered in the analysis of average peak amplitude. All animals included in analyses also showed peaks time-locked to behavioral events in at least one of the conditions affirming that recordings without detectable peaks during pre-defeat baseline recordings were not attributable to technical limitations.

For behavior time-locked activity, the maxima within a 5 second window centered on the behavioral event (corner entry or proximal interaction) were identified in the Z-score transformed ΔF/F and the amplitude of these peaks were compared across groups. As the number of proximal interaction and corner entries is different between susceptible and resilient mice, to ensure comparability, only the first incidence of each event was examined. Animals that did not display a particular behavior (i.e. proximal interaction or corner entry) were not included in the analysis for that stage of the test (target or no target) but were included in the data for the subsequent stage.

Statistics.

GraphPad Prism 7 was used for statistical analysis. Grubb's test was used to identify and exclude statistical outliers. Two-way repeated measures ANOVA with Sidak's correction for post-hoc testing was used.

Results

Pre-existing baseline differences in D1- but not D2-MSNs associate with post-defeat stress phenotype.

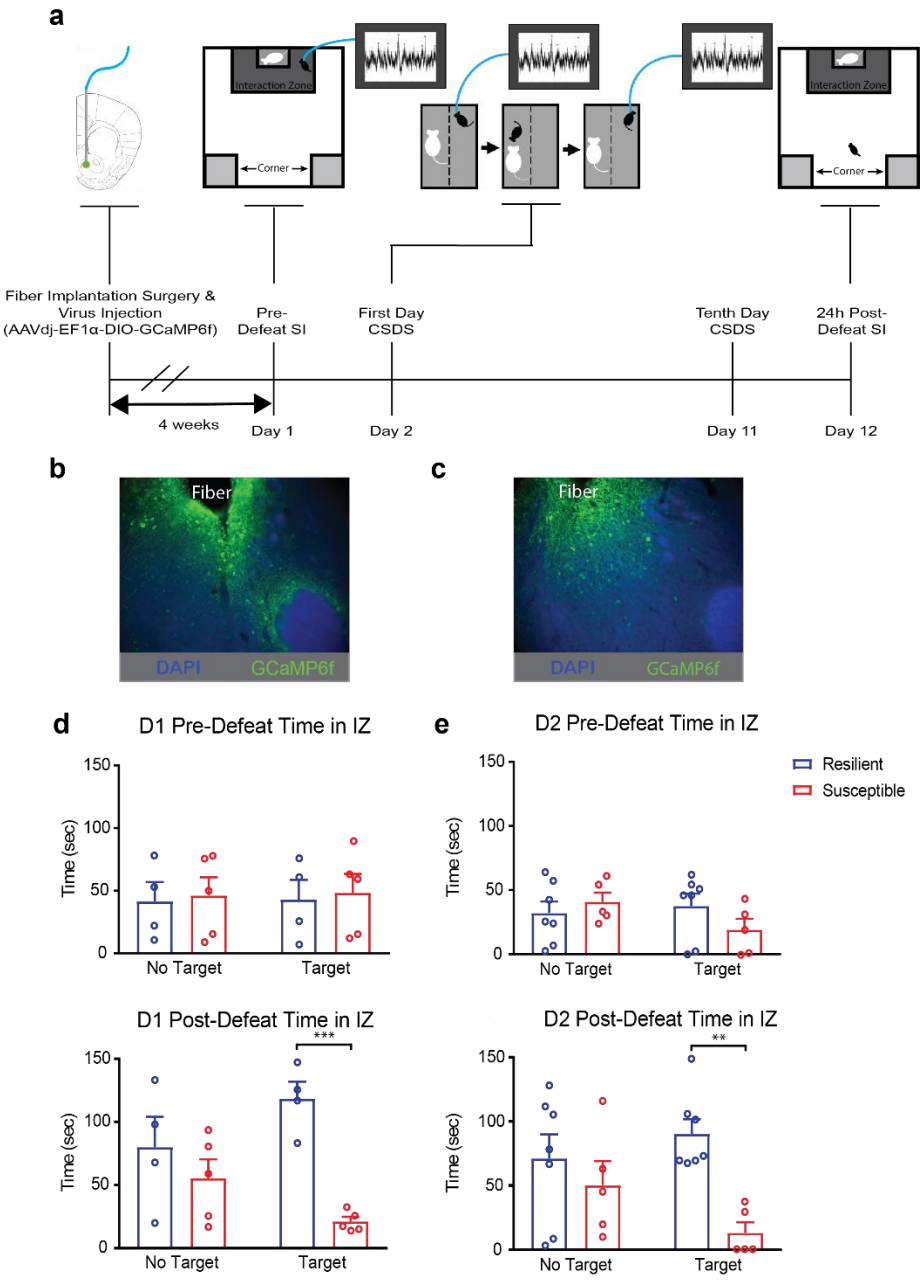

Figure 1. In vivo fiber photometry imaging to identify latent mechanisms of susceptibility and resilience. *(a) Experimental timeline. Immunohistochemistry images show viral GCaMP6f expression and fiber placement in (b) D1-MSNs and (c) D2-MSNs. (d,e) Time spent in the interaction zone during pre-defeat social interaction (SI) test did not differ between groups during either target or no target trials for either D1-cre (n=4,5) (d) or D2-cre (n=7,5) (e) transgenic mice. After CSDS, resilient mice spent more time in the interaction zone during the target trials than susceptible mice for both D1- cre (d) (group effect, p< 0.05, interaction effect p<0.05, n=4, 5, post-hoc ***p=0.0008) and D2-cre (group effect, n=7, 5, post-hoc **p=0.0046) (e) transgenic mice. Data are presented as mean ± S.E.M.*

To record cell-specific activity in awake behaving mice, we injected AAVdj-EF1α-DIO-GCaMP6f-WPRE into the NAc of D1- and D2-Cre transgenic mice and recorded Ca^{+2} transients in a

population of infected cells through an implanted optic fiber prior to and after the first aggressive encounter (Fig. 1A-C). Naïve mice were tested in a social interaction test prior to any stress manipulations and were later defined as resilient or susceptible based on time spent interacting with a social target in a SI test 24 h after ten days of social defeat (resilient mice >60 sec in interaction zone with target, susceptible, <60 sec). Pre-defeat, time in the interaction zone did not differ (Fig. 1D,E) between mice that would become susceptible or resilient after defeat. Post-defeat, resilient mice spent significantly more time in the interaction zone than susceptible mice when the target was present, an effect seen in both D1-cre ($F_{(1,14)}$=16.84, p=0.0011, $F_{(1,14)}$= 5.96, p=0.0285, n=4, 5, post-hoc ***p=0.0008) and D2-cre transgenic mouse lines ($F_{(1,20)}$=9.86, p=0.0052, n=7, 5, post-hoc **p=0.0046). Although mice that would become susceptible or resilient did not differ behaviorally prior to CSDS, we nevertheless observed differences in neuronal activity prior to defeat. In neuronal activity recordings immediately before the first aggressive encounter, D1-MSN baseline peak amplitude was larger in mice that later became resilient than in mice that later became susceptible ($F_{(1,5)}$= 7.56, p=0.0404, n=3, 4, pre-defeat post-hoc, p =0.0123; Fig. 2A,C); this difference was not significant in recordings immediately after the first aggressive encounter (post-defeat post-hoc p=0.3412). The frequency of peaks was not different between groups (Fig. 2B,C). In contrast to D1-MSNs, pre-defeat baseline peak amplitude in D2-MSNs did not differ between groups (Fig. 3A,C). Although the frequency of peaks was increased in mice that later became susceptible when averaging across pre- and post-defeat recordings, this effect did not reach statistical significance when assessed by individual post-hoc tests at either pre- or post- defeat time-points ($F_{(1,9)}$=8.69, p=0.0163, n= 6, 5) (Fig. 3B,C). These results suggest that lower D1-MSN activity precedes the behavioral state of susceptibility that is revealed after chronic stress.

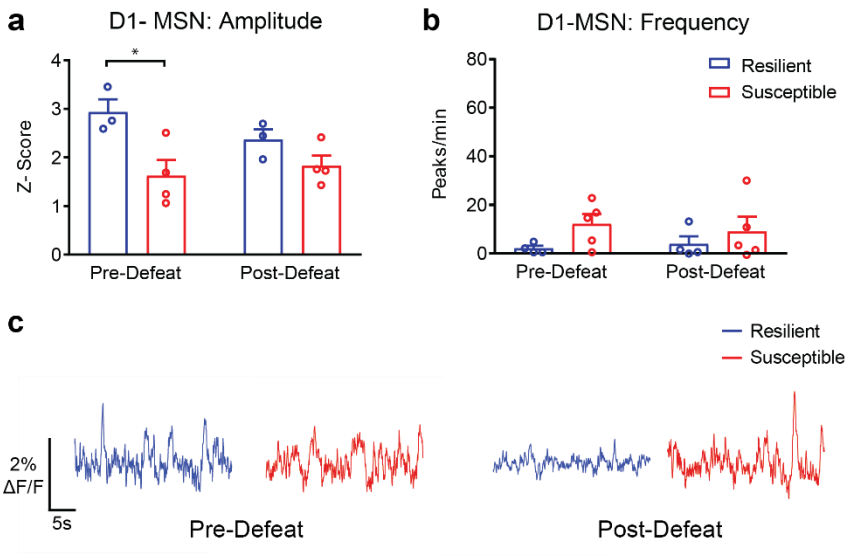

Figure 2. Pre-existing differences in baseline D1-MSN activity associate with later stress phenotype. *(a) Amplitude (Group effect, $p < 0.05$, post-hoc $*p < 0.05$, n=3, 4) but not (b) frequency (n=4, 5) of calcium transients in D1-MSNs differed between mice that later exhibited resilience vs. susceptibility prior to CSDS. (c) Representative 30-sec segments of fiber photometry traces. Data are presented as mean ± S.E.M.*

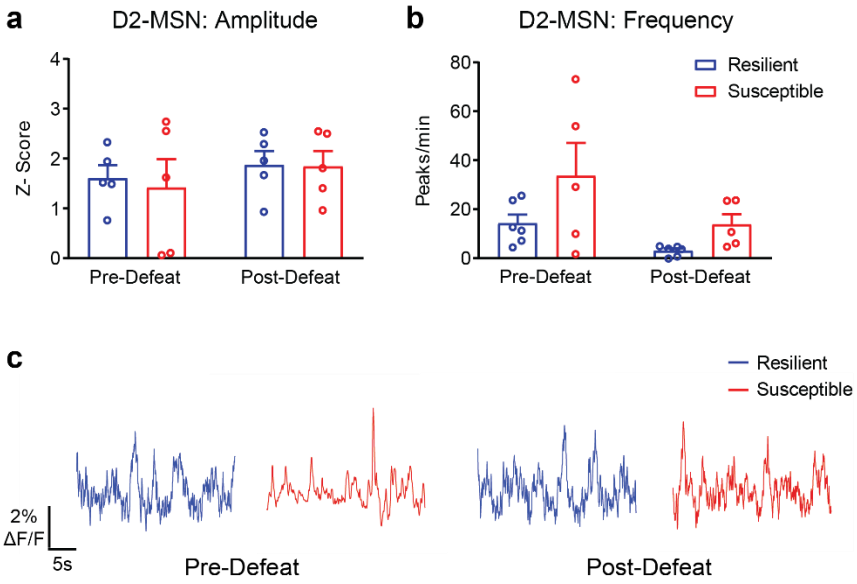

Figure 3. Baseline D2-MSN activity does not associate with later stress phenotype. *No differences in (a) amplitude (n=5, 5) or (b) frequency (group effect, $p<0.05$, post-hoc tests non-significant, n= 6, 5) of calcium transients were detected in D2-MSNs during baseline home-cage recordings prior to CSDS. (c) Representative 30-sec segments of fiber photometry traces. Data are presented as mean ± S.E.M.*

Pre-defeat D1-MSN activity in resilient but not susceptible mice is specific to social interaction.

Having identified baseline differences in D1-MSN activity, we then asked if temporally-specific signaling of NAc MSNs associated with specific behaviors during social interaction prior to CSDS

(Fig. 1A). Previous work identified increased activity in the ventral tegmental area (VTA) during

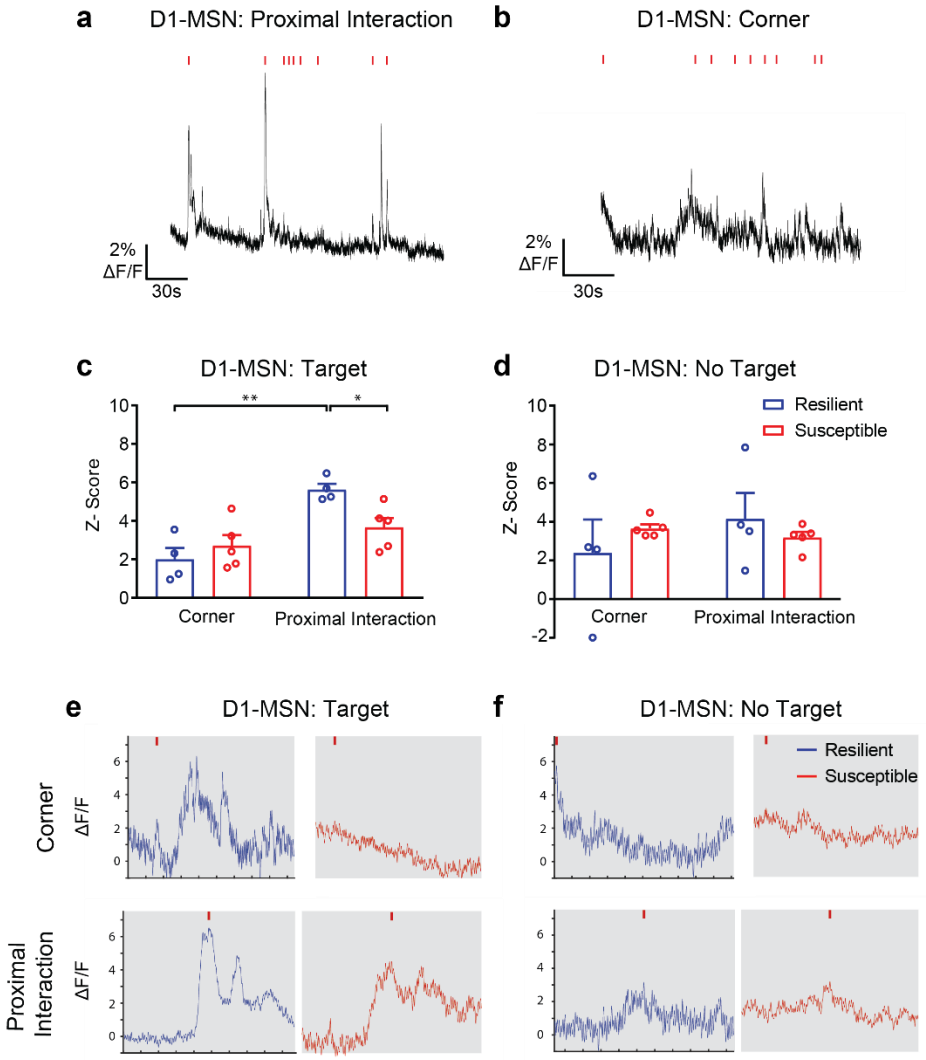

Figure 4. Pre-defeat D1-MSN activity temporally correlated with social interaction is increased in future resilient mice. *Photometry traces from mice during a pre-defeat social interaction (SI) target test show large increases in calcium transients in D1-MSNs during SI (proximal interaction with target) (a) and smaller increases during corner entries (b) (red dashes at top of panel indicate occurrence of behavioral events). (c) When a target was present, peak amplitude of calcium transients differed between future resilient and susceptible mice during the first proximal interaction event, and between the first corner entry and first proximal interaction in resilient mice (effect of event p<0.05, post hoc *p<0.05, **p<0.05, n=4,5). (d) In the absence of the target, peak amplitude of calcium transients during first proximal interaction and corner entry event did not differ between groups (n= 4,5). Representative 10-sec segments of fiber photometry traces aligned with first corner entry (upper panel) or first proximal interaction (lower panel) during target (e) or no target (f) tests. Data are presented as mean ± S.E.M.*

bouts of interaction with a novel mouse that predicted the onset of social behavior, with activation of VTA-NAc projections favoring social behavior (Gunaydin *et al*, 2014). To determine if pre-defeat D1-MSN activity associates with later CSDS-induced behavioral states, we examined peaks in activity that were time-locked with behavioral events including direct social (proximal) interaction and corner entries and then compared the amplitude of these time-locked peaks across groups (Fig. 4). Similar to previous findings in VTA, we found that the largest time-locked

peaks in D1-MSN activity occurred during social interaction (Fig. 4A), although we also observed smaller peaks associated with corner entry (Fig. 4B). Between group comparisons revealed that proximal interaction peaks were larger than corner peaks in mice that later exhibited resilience, but not susceptibility, when a social target was present and that proximal interaction peaks in the presence of the social target were larger in future resilient than susceptible mice (Fig. 4C, E; $F_{(1,7)}$ = 15.87, p=0.0053, n=4,5, post-hoc **p<0.0077, *p<0.0346). However, peaks did not differ in either susceptible or resilient mice in the absence of the social target, nor was the amplitude of peaks different between susceptible and resilient mice (Fig. 4D, F). Thus, we observed that increases in D1-MSN signaling are specifically associated with social interaction in mice that will become resilient, again pointing to a role for pre-stress D1-MSN signaling in determining the outcome of stress-induced adaptations.

Temporally-associated D2-MSN activity is not modulated by social interaction.

We then compared peaks in D2-MSN activity time-locked with behavioral events (Fig. 5A,B). As with D1-MSNs, we observed peaks during social interaction and corner entries (Fig. 5A). In contrast to D1-MSN signaling, the amplitude of D2-MSN events did not differentiate proximal interaction and corner entry events in the presence of the social target (Fig. 5C). However, in the absence of the target, D2-MSN peaks in resilient mice were smaller during proximal interaction events compared to corner events ($F_{(1,9)}$=13.53, p=0.0051, n= 6,5, post-hoc, **p=0.0049; Fig. 5F). We observed no differences in susceptible mice. These data suggest that D2-MSNs may convey differential information in resilient compared to susceptible mice but this signal is not specific to social interaction.

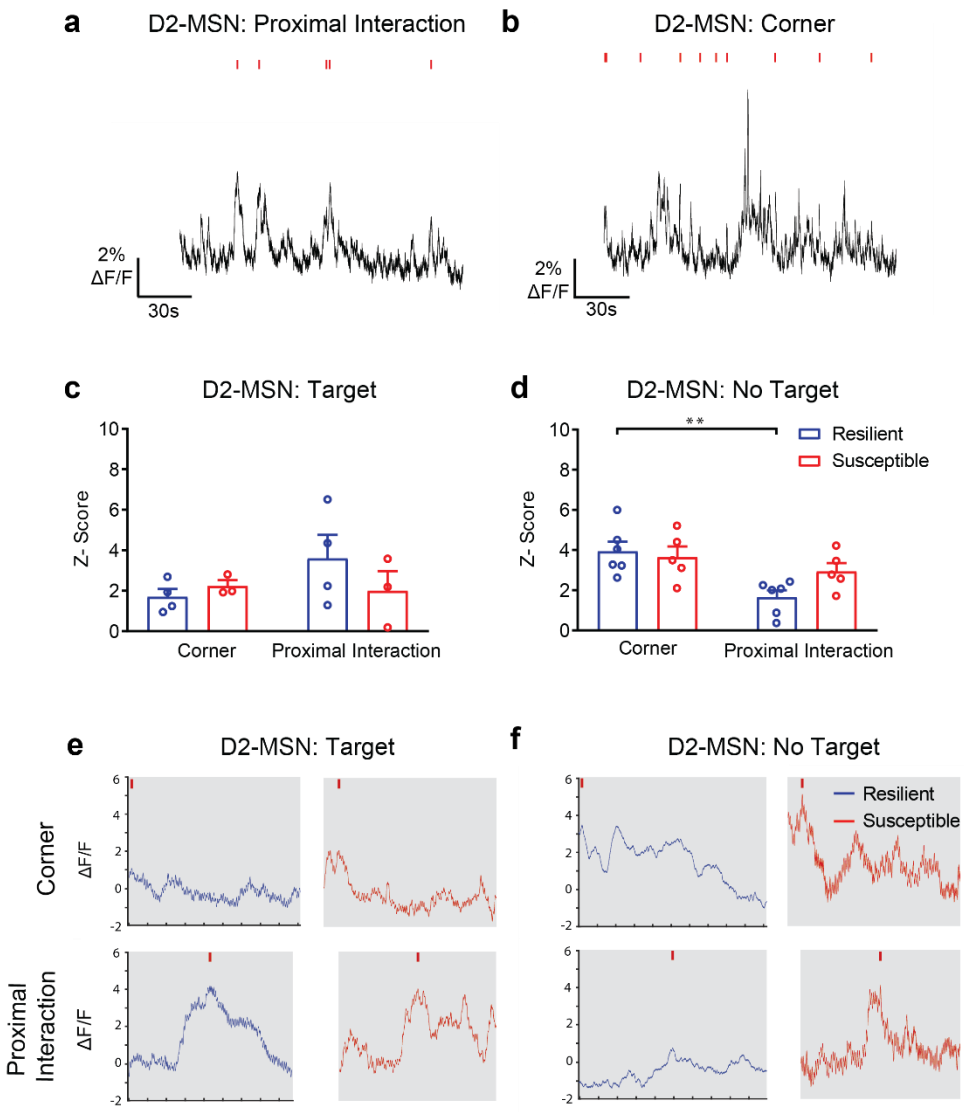

Figure 5. Pre- defeat D2-MSN activity varies with behavioral event. *Photometry traces from mice during a pre-defeat social interaction (SI) test show increased calcium transients in D2-MSNs during SI (proximal interaction with target) (a) and corner entries (b), (red dashes at top of panel indicate occurrence of behavioral events). When a target was present, peak amplitude of calcium transients during the first proximal interaction and first corner entry did not differ between groups (n=4, 3). (d) In the absence of the target, peak amplitude of calcium transients was lower for resilient mice during proximal interaction compared to corner entry (effect of event, $p<0.05$, post hoc, $^{**}p<0.05$, n= 6,5). Representative 10-sec segments of fiber photometry traces aligned with first corner entry (upper panel) or first proximal interaction (lower panel) during target (e) or no target (f) tests. Data are presented as mean ± S.E.M.*

Discussion

Identifying the endogenous mechanisms that lead some individuals to become susceptible to stress and others resilient is essential for developing effective treatments and, ultimately, preventing the emergence of depression. Here, we identified pre-existing differences in baseline D1-MSN activity that associate with the subsequent development of stress susceptibility. While much previous work has examined how activity in brain reward areas is altered by stress (Bagot *et al*, 2015; Chaudhury *et al*, 2013; Christoffel *et al*, 2015; Francis *et al*, 2015; Gunaydin *et al*, 2014; Lim *et al*, 2012; Tye *et al*, 2013), very little is known about the root cause of stress susceptibility. We report that, prior to encountering stress, mice that will go on to become resilient already have higher baseline D1-MSN activity as compared to mice that go on to become susceptible. In contrast, we observed no baseline differences in D2-MSN activity. Further, we find that D1-MSN, but not D2-MSN, signaling is specific to social interaction in resilient, but not susceptible, mice. Together, these data highlight that pre-existing reward circuit dysfunction is a critical mediator of depression vulnerability.

Fiber photometry provides a population recording of calcium transients and as such the precise interpretation of differences in peak amplitude and frequency are somewhat complex. An increase in peak amplitude could indicate either an increase in the number of cells firing or in increase in the synchronicity of firing from a constant population of cells, whereas an increase in peak frequency could reflect an increase in the number of cells firing, an increase in the frequency of firing of the same number of cells or less synchronous firing of the same number of cells firing at the same frequency. In the present study we found an increase in peak amplitude with no significant change in peak frequency for D1-MSNs. One possible, but not the only, interpretation of our data is that the number of D1-MSNs firing at baseline is increased in mice that go on to become resilient. While we cannot definitively conclude the precise nature of the activity change in D1 MSNs from population recordings, such data satisfies our primary goal to identify a cell-specific signature of stress susceptibility, despite a certain degree of ambiguity inherent in population recordings. It would be interesting to pursue this finding in a follow-up study focusing on single cell activity in-vivo with alternative calcium imaging techniques.

Previous studies of stress-induced changes in NAc activity also point to a specific role for D1-MSNs in mediating resilience to stress (Francis *et al*, 2015; Lim *et al*, 2012; Lobo *et al*, 2013). Artificially increasing D1-MSN activity through optogenetic manipulations after defeat was pro-resilient and pharmacological inhibition of these cells increased susceptibility (Francis *et al*, 2015). In contrast, manipulating D2-MSN activity after defeat did not influence susceptibility. Moreover, after CSDS, excitatory input to D1-MSNs in susceptible mice was reduced relative to stress-naïve mice, an effect not observed in resilient mice (Francis *et al*, 2015). Decreased synaptic strength was also observed in D1-MSN in mice exhibiting anhedonia after chronic restraint stress (Lim *et al*, 2012). Our findings suggest that these changes observed after stress may in part reflect the pre-existing increases in D1-MSN activity that we identify in vivo in stress-naïve mice during social interaction. We suggest that, similar to the pro-resilient effect of optogenetic activation of D1-MSNs (Francis *et al*, 2015), enhanced intrinsic activity prior to defeat may buffer against the deleterious effects of stress, rendering mice resilient. Taken together with previous evidence of post-stress alterations in neuronal activity, we conclude that pre-existing individual differences in D1-MSNs play an important role in determining later stress susceptibility, with increased activity promoting resilience to chronic stress.

Intriguingly, the magnitude of temporally-specific D1-MSN activity differentiated social interaction from corner events only in resilient mice. This finding suggests that, even in the absence of pre-defeat behavioral differences, social interaction may be processed differently at the neuronal level prior to stress in mice predisposed to be resilient vs. susceptible after chronic stress. Previous work showed that activity of VTA dopamine (DA) neurons projecting to NAc is increased during social interaction and that activation of D1-MSNs, but not D2-MSNs, is necessary and sufficient to initiate social interaction (Gunaydin *et al*, 2014). Profiling the endogenous signaling of D1- and D2-MSNs suggests that this basic mechanism may be compromised in mice that become susceptible. D1-MSN activity in susceptible mice was not different between interacting with the target mouse and corner entry. Potentially, this lack of differential D1-MSN activation could suggest that social interaction is processed differently in susceptible mice even before they encounter stress. While the mechanism underlying this difference in susceptible mice remains unknown, it is not genetic given that we are utilizing genetically inbred mice, despite the

fact that genetic factors are also known to contribute to differences in stress responses among outbred populations.

Dopaminergic projections from the VTA provide important modulatory input to NAc MSNs (Baik, 2013) and previous work has identified a role for VTA-NAc projections in regulating stress susceptibility. Following chronic mild stress (CMS), optogenetic VTA DA activation reverses depressive-like symptoms, an effect dependent upon NAc DA receptors. Conversely, inhibition of VTA DA neurons causes depressive-like symptoms and CMS reduces VTA activity (Tye *et al*, 2013). In contrast, after CSDS, increased firing of VTA DA neurons is observed specifically in susceptible mice. Moreover, bulk activation of VTA DA neurons or targeted activation of NAc-projecting VTA DA neurons is pro-susceptible (Chaudhury *et al*, 2013). While these findings appear contradictory, mild and more severe forms of stress have been shown to exert different effects on VTA DA firing (Valenti *et al*, 2012). It is possible that the balance between VTA projections to D1- and D2-MSNs may be differentially affected by different stressors and may also play a role in regulating future stress susceptibility. While the clearest effects we observed were in D1-MSN signaling, we also found modest differences in D2-MSN signaling, although these did not associate with social interaction behavior. Our findings suggest that, pre-stress, mice that will become resilient may already have increased DA input to D1-MSNs as evidenced by increased neuronal activity during social interaction. Integrating our findings with published data, we speculate that CSDS may decrease VTA input to D1-MSNs to drive depression-like states and, that in mice which become resilient, pre-existing increases in VTA input to D1-MSNs mitigate against this later pro-susceptible stress-induced plasticity. However, it is important to note that the pro-susceptible effect of enhanced VTA-NAc activity during CSDS may be mediated by brain-derived neurotrophic factor (BDNF) rather than DA, with BDNF effects predominating on D2-MSNs (Wook Koo *et al*, 2016). This underscores the complexity of factors that control NAc MSN activity under normal and stressful conditions, and emphasizes the need for future work to identify the specific inputs onto D1-MSNs that are responsible for their enhanced baseline activity in a social setting in mice that are inherently resilient.

Overall, we conclude that pre-existing differences in D1-MSN activity are an important determinant of resilience. Our findings offer insight into the mechanistic basis of differential

adaptation to stress and hold promise for identifying those individuals most at risk of becoming susceptible in the future, prior to encountering significant life stress. The incidence of depression, and the associated costs to affected individuals and society, continue to increase. Identifying neuronal signatures of risk prior to the emergence of observable behavioral change will open the door to targeted treatments for at-risk groups and, thus, the potential to address the underlying pathology even before the disorder emerges.

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Framing the questions: Chapter 3

The previous chapter identifies differences in cell type specific activity as well as the encoding of social stimuli in the NAc that associate with resilience to stress. NAc MSNs firing is dependent upon drive from upstream structures. Glutamatergic projections from the vHIP synapse onto NAc MSNs and play a role in regulating behavioral adaptation to stress, having been found to be pro-susceptible after stress. However, questions remain about the role of these projections such as how they regulate susceptibility in females and how neural activity in these pathways may encode vulnerability.

In this chapter, I look to investigate (1) how vHIP-NAc activity relates to anxiety and depressive-like behavior in males and females (2) if these pre-stress neural and behavioral metrics are predictive of future susceptibility and (3) how stress itself modulates vHIP-NAc activity.

CHAPTER 3

Ventral-hippocampal afferents to nucleus accumbens encode both latent vulnerability and stress-induced susceptibility

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Abstract

BACKGROUND: Stress is a major risk factor for depression, but not everyone responds to stress the same way. Identifying why certain individuals are more susceptible is essential for targeted treatment and prevention. In rodents, nucleus accumbens (NAc) afferents from the ventral hippocampus (vHIP) are implicated in stress-induced susceptibility but little is known about how this pathway might encode future vulnerability or specific behavioral phenotypes.

METHODS: We use fiber photometry to record *in vivo* activity in vHIP-NAc afferents during tests of depressive and anxiety-like behavior in male and female mice, both before and after a sex-specific chronic variable stress (CVS) protocol to probe relationships between pre-stress neural activity and behavior and potential predictors of post-stress behavioral adaptation. Furthermore, we examine CVS-induced alterations in vHIP-NAc activity *in vivo* and use *ex-vivo* slice electrophysiology to identify the mechanism of this change.

RESULTS: We identify behavioral specificity of the vHIP-NAc pathway to anxiety-like and social interaction behavior. We also show that this activity is broadly predictive of stress-induced susceptibility in both sexes while pre-stress behavior is only predictive of anxiety-like behavior. We observe a stress-induced increase in *in vivo* vHIP-NAc activity coincident with an increase in sEPSC frequency.

CONCLUSIONS: We implicate vHIP-NAc in social interaction and anxiety-like behavior and identify markers of vulnerability in this neural signal with elevated pre-stress vHIP-NAc activity predicting increased susceptibility across behavioral domains. Our findings indicate that individual differences in neural activity and behavior play a role in pre-determining susceptibility to later stress, providing insight into mechanisms of vulnerability.

Introduction

Depression is the leading cause of disability worldwide (1). Current therapies treat observable symptoms, rather than known mechanisms, and remain ineffective for many. Depression is a chronic, recurrent disorder with 50% of individuals experiencing repeated episodes, and each episode further increasing risk of recurrence (2). Strategies to prevent emergence of the disorder before symptoms occur would have enormous clinical and societal impact (3). Stress is a major risk factor, yet only a minority of people who encounter stress will develop depression. Identifying at-risk individuals and the neural mechanisms underlying differential vulnerability is essential to developing targeted interventions for treatment and prevention.

Chronically stressed rodents exhibit individual differences in stress susceptibility, such that some develop depressive- and anxiety-like behavior while others remain resilient (4). Differential adaptation can reveal mechanisms of vulnerability to stress-induced disorders such as depression (5-8). However, most rodent research has exclusively considered males despite the increased depression prevalence in women (9). Chronic variable stress (CVS) (10, 11), a model for depression in which mice experience repeated inescapable stressors, is easily applied to males and females and can reveal interesting sex-differences in stress susceptibility, with females developing depressive-like behavior following shorter stress durations. With sufficient stress, both sexes exhibit behavioral susceptibility (11-13), rendering this is a useful model for studying stress-induced neural adaptations accompanying comparable behavioral susceptibility in both sexes.

The ventral hippocampus (vHIP) is sensitive to early life experience (14) and regulates anxiety-like behavior in male rodents (15-17), suggesting a potential role in encoding latent vulnerability to stress. Through projections to nucleus accumbens (NAc), vHIP regulates susceptibility to chronic social defeat stress (CSDS) in male mice (18). Immediate early gene expression in vHIP-NAc projecting neurons and altered glutamate release probability suggest increased vHIP-NAc activity in susceptible mice. Optogenetically increasing vHIP-NAc activity increased stress-induced susceptibility, decreasing social interaction and increasing passive coping, whereas attenuating neural activity increased social interaction, fostering resilience. Stress may also

increase neuronal excitability in vHIP-NAc projecting neurons in females inducing reduced sucrose preference, commonly interpreted as an indicator of anhedonia (19). While *ex vivo* electrophysiology and optogenetic experiments broadly implicate this pathway in stress-induced susceptibility, how *in vivo* physiological vHIP-NAc activity regulates specific behavioral phenotypes is unknown. Moreover, the prior focus on *consequences* of chronic stress cannot differentiate whether the vHIP-NAc is simply a locus of stress-induced alterations or if pre-existing differences in vHIP-NAc activity drive differential vulnerability. This is an important distinction because, if the latter is true, vHIP-NAc may shape the initial development of susceptibility to chronic stress, providing a potential therapeutic target for early intervention.

Despite sex differences in prevalence, depression afflicts both men and women, and it is essential to understand mechanisms of depression-like behavior in both sexes. To this end, we employed sex-specific stress protocols to probe the role of vHIP-NAc under conditions in which behavioral susceptibility is observed in each sex. We interrogated vHIP-NAc neural activity in both male and female mice during depressive and anxiety-like behavior tests both before and after chronic stress to investigate the behavioral significance of this neural signal, and if variation in this signal predicts future susceptibility and/or is then consequently modified by stress. We hypothesized that, not only would CVS alter vHIP-NAc neural activity, but that pre-stress differences in basal activity may pre-identify susceptible individuals.

Materials and Methods

See Supplemental Methods for extended details.

Chronic Variable Stress (CVS)

CVS was performed as previously described (10) with one of three stressors administered for 1h daily (100 foot-shocks, tail suspension, restraint). Males and females were subjected to sex-specific differing length CVS protocols: 21d CVS for males and 4d for females.

Behavioral Assessments

Open field, social preference and tail suspension tests assessed depressive and anxiety-like behavior before and again after CVS.

In Vivo Fiber Photometry

To measure calcium-associated fluorescence changes, we recorded vHIP NAc-projecting cells during behavior before and after stress (5). Data were extracted and normalized to $\Delta F/F$ using custom written Matlab scripts.

Statistics. Statistical analyses were performed using GraphPad Prism 7.

Results

vHIP-NAc activity associates with baseline anxiety-like behavior and social interaction, but not passive coping

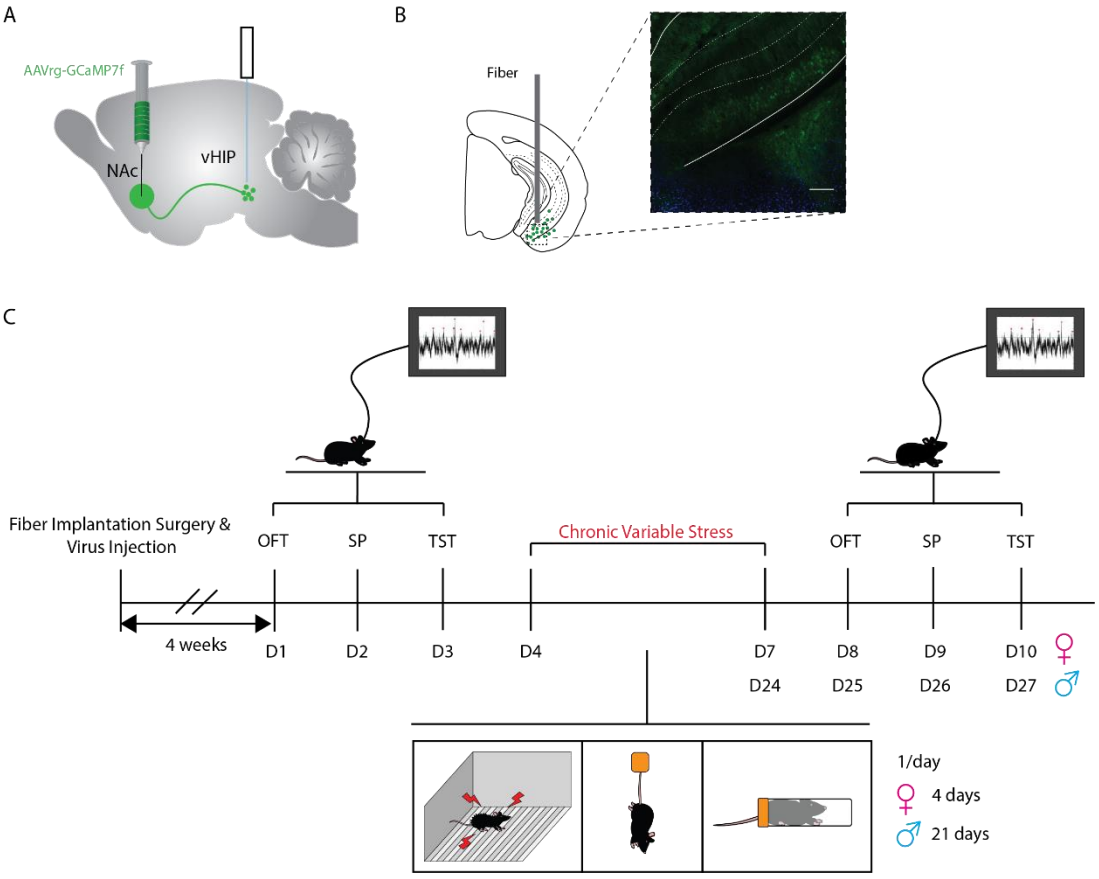

***Figure 1**. (a) Schematic of virus injection and fiber implantation sites (b) depicting site of retrogradely-infected cells and fiber placement. Immunohistochemistry images show retrograde GCaMP7f expression (Scale bars represent 300 µm (leftmost image) and 75um (rightmost image). (c) Experimental timeline.*

To examine *in vivo* behaviorally-relevant modulation of vHIP-NAc neural activity, we injected retrograding AAV-GCaMP7f into NAc and recorded Ca^{2+}-associated fluorescence via an implanted optic fiber above vHIP during tests of anxiety- and depressive-like behaviors in stress-naïve male and female mice and interrogated how the photometry signal varied with behavior (Figure 1). The open field test (OFT) is a standard test for anxiety-like behavior wherein reduced center time is commonly interpreted as indicating increased anxiety-like behavior. Using linear regression,

we found that peak frequency, but not amplitude, of vHIP-NAc events negatively correlated with center time in females (Figure 2A), and males (Figure 2B), implicating increased vHIP-NAc activity in increased anxiety-like behavior. Passive coping is one behavioral strategy employed during prolonged challenge that may help manage energy expenditure (20, 21). This behavior can become maladaptive and has been associated with depression and other mood disorders (22). Passive coping, assessed from time immobile in a tail suspension test (TST), did not correlate with peak frequency in either sex (Figure 2C,D), suggesting vHIP-NAc is not a primary mediator of this behavior.

Mice generally seek out social interaction, (23) however, chronically stressed mice display social avoidance, reflecting dysregulation in reward circuitry (4). vHIP-NAc mediates reward (24) but, paradoxically, its increased activity is also implicated in stress-induced social avoidance in defeated mice (18). The interpretation of this test as indicating reduced social reward has been challenged as the social target is commonly an aggressive mouse conspecific to social aggressors (25). To examine if vHIP-NAc regulates social interaction in stress-naïve animals, we probed activity during proximal social interaction, defined as nose-to-grid (NTG) bouts, in a social preference (three chamber sociability) test wherein a mouse freely explores a 3-chamber arena, containing both an empty grid enclosure and another containing a same-sex conspecific. In females, increased peak frequency of vHIP-NAc events within individual NTG bouts associated with increased NTG interaction bout number (Figure 2E). In males, a related metric, higher mean Z-score during NTG bouts, associated with increased bout number (Figure 2F). There was no relationship between social interaction behavior and mean Z-score in females (Figure S1A) or peak frequency in males (Figure S1B). Our data implicate increased vHIP-NAc activity in preference for same-sex social interaction in stress-naïve animals of both sexes.

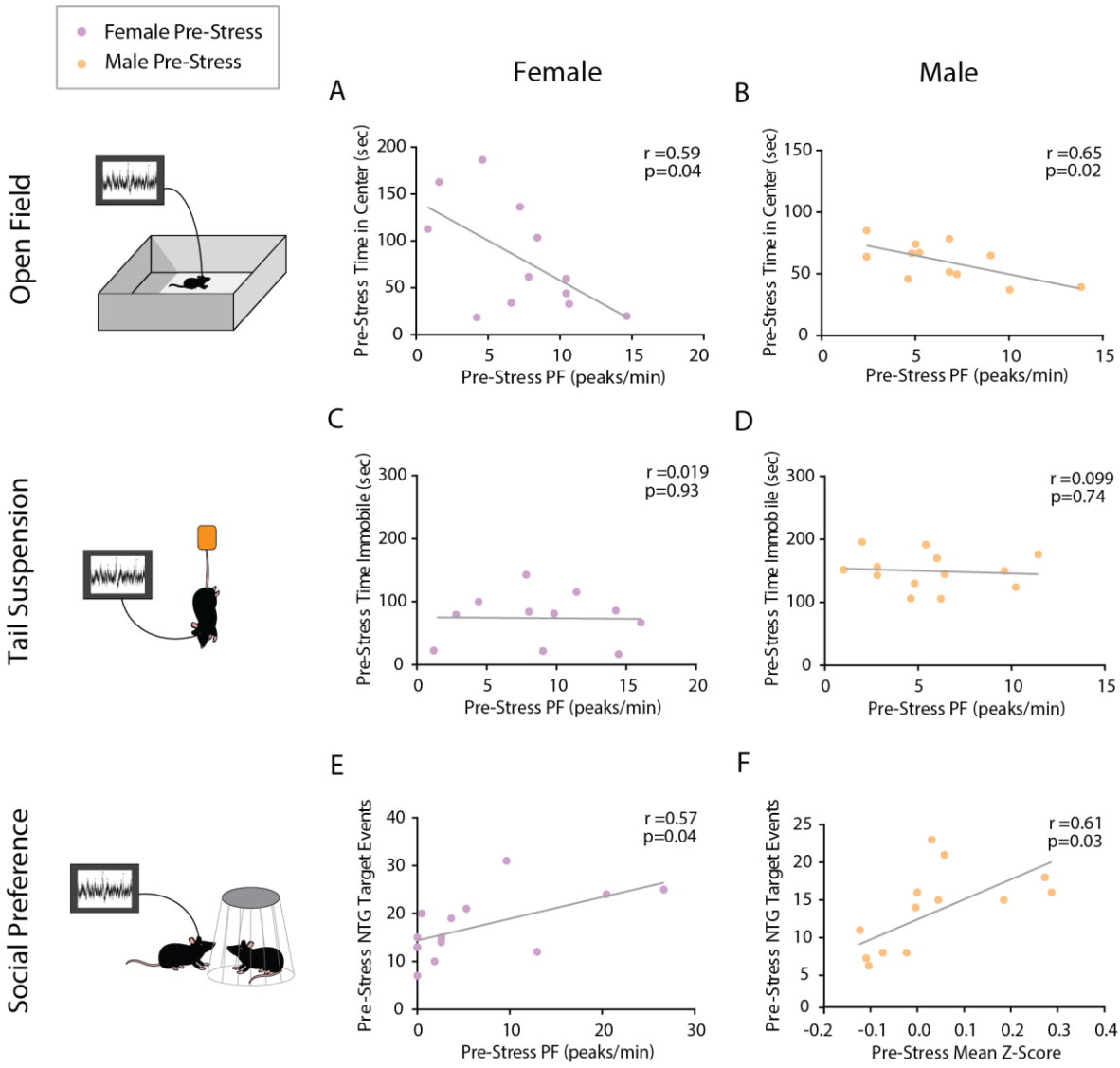

Figure 2. *Baseline relationships between vHIP-NAc activity and behavior. Increased peak frequency (PF) is predictive of decreased time in center in the open field test before stress in females (a; $F_{1,10}$=5.36, n=12, r=0.59, p=0.04) and males (b; $F_{1,10}$=7.11, n=12, r=0.65, p=0.02). Peak frequency is not related to time immobile in the tail suspension test in either sex (c; $F_{1,9}$=0.003, n=11, r=0.019, p=0.93, d; $F_{1,11}$=0.1095, n=13, r=0.099, p=0.74). Increased peak frequency is predictive of increased number of nose-to-grid (NTG) events during a social interaction test in females (e; $F_{1,11}$=5.45, n=13, r=0.58, p=0.04), while increased mean Z-score is predictive of increased number of NTG events in males (f; $F_{1,11}$=6.33, n=13, r=0.61, p=0.03). *p<0.05*

Predictors of stress-induced increases in depressive- and anxiety-like behavior

Stress-induced changes in vHIP-NAc synaptic activity (18) and excitability (19) regulate differential susceptibility. A key clinically relevant challenge is identifying individuals that will be vulnerable to stress-induced pathology, *before* stress. We hypothesized that individual differences in vHIP -NAc may precede stress, representing a latent vulnerability that is revealed by future stress. To test this, following baseline behavioral testing we exposed the same mice to sex-specified intermediate CVS to induce individual variability in stress-induced anxiety- and depressive-like behavior in both sexes. While previous work has identified sex differences in stress susceptibility using identical stress protocols in both sexes (11, 19), depression afflicts both men and women, and thus we asked if vHIP-NAc activity modulates stress-induced behavioral adaptation in both sexes under the relevant conditions whereby behavioral susceptibility is observed in each sex. In female mice, 6d CVS induces robust susceptibility and 3d results in a sub-threshold effect (11) whereas, consistent with reported sex differences in stress susceptibility and lower incidence of mood and anxiety disorders in men, 28d CVS is necessary to elicit robust depressive- and anxiety-like behavior in male mice (26). Thus, we exposed females and males to 4d, and 21d CVS, respectively, and then recorded vHIP-NAc activity during behavior tests.

Intermediate CVS robustly increased depressive and anxiety-like behavior in both males and females with considerable within group variability. On average, CVS decreased open field center time, indicating increased anxiety-like behavior (*females*: Figure 3A; *males*: Figure 3B). Both male and female mice increased immobility during tail suspension, suggesting increased passive coping (*females*: Figure 3C; *males*: Figure 3D). Having previously identified a role for vHIP-NAc in defeat-induced social avoidance (18), we examined if CVS, which does not involve social aggression, also impacts social behavior. Although total social preference (time in social /time in non-social area) was unaffected (Figure S2), proximal interaction (nose to grid; NTG) with the social target after CVS was reduced in both sexes (*females*: Figure 3E; *males*: Figure 3F), indicating that even non-social stress of CVS can induce social avoidance.

Having established stress-induced susceptibility across anxiety- and depressive-like behaviors in both sexes, with considerable individual variability, we then examined if the degree of susceptibility could be predicted from either pre-stress behavior or neural activity in NAc-

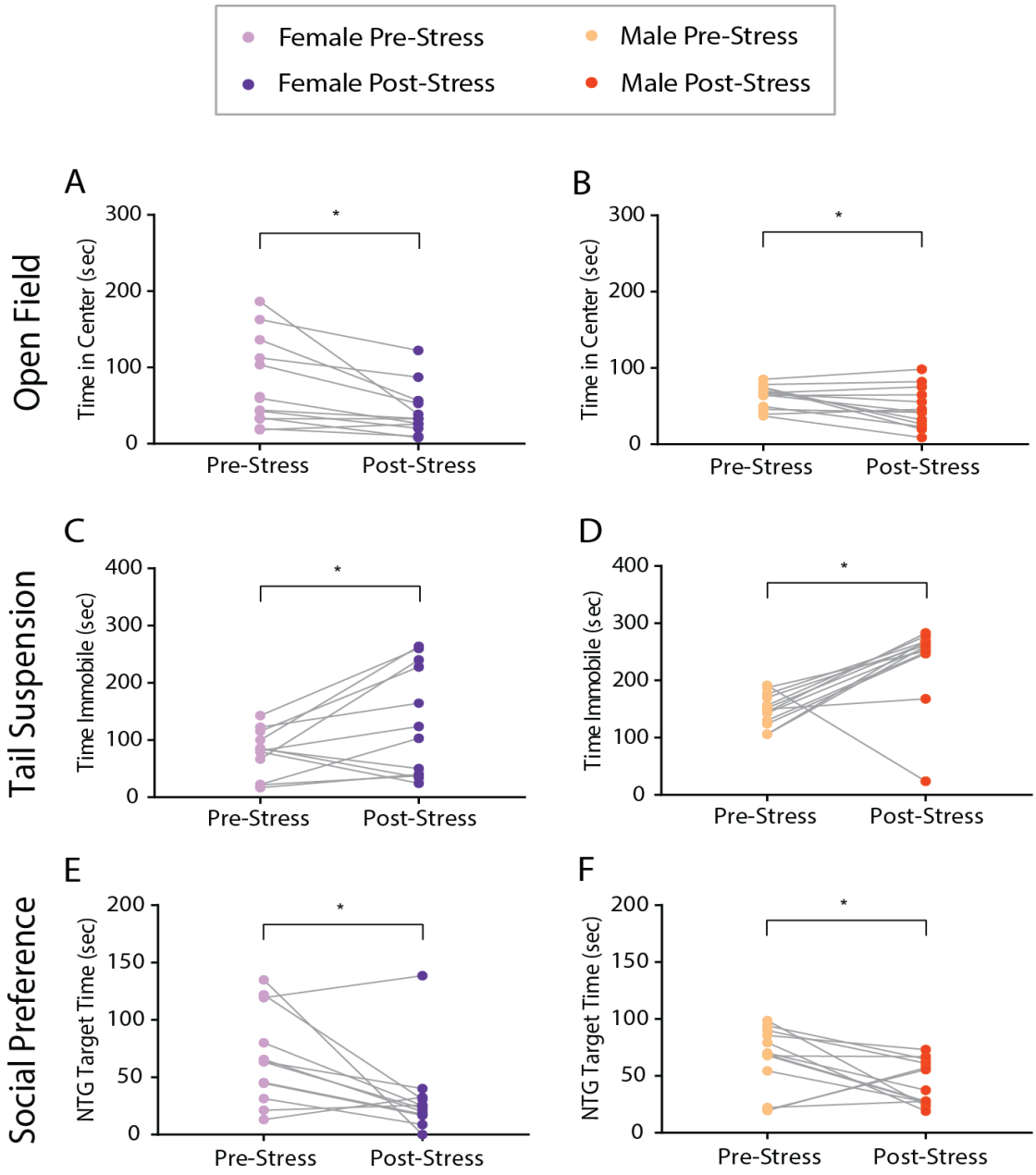

Figure 3. *Chronic variable stress modulates depressive and anxiety-like behavior. Following 4- and 21d of stress, females (a; n=12, p=0.01) and males (b; n=13, p=0.03), respectively, show decreased time in center on the open field test as well as increased time immobile in the tail suspension test (c; n=12, p=0.04, d; n=13, p<0.01) and decreased time interacting with a social target in a social preference test (e; n=12, p=0.02, f; n=13, p=0.04) following stress. *p<0.05*

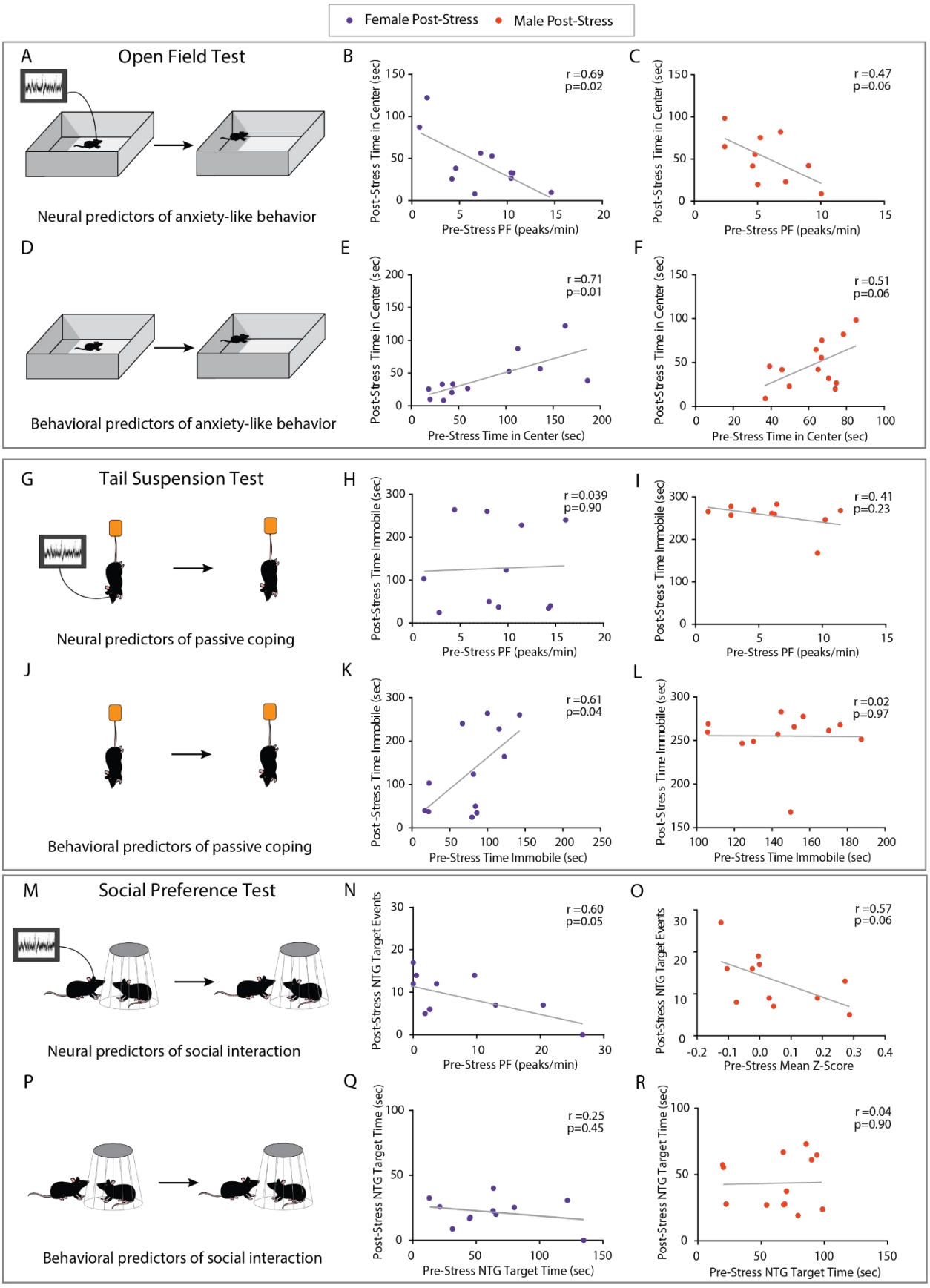
Female Post-Stress
Male Post-Stress
A
Open Field Test
Neural predictors of anxiety-like behavior
B
r =0.69
p=0.02
Post-Stress Time in Center (sec)
Pre-Stress PF (peaks/min)
C
r =0.47
p=0.06
Post-Stress Time in Center (sec)
Pre-Stress PF (peaks/min)
D
Behavioral predictors of anxiety-like behavior
E
r =0.71
p=0.01
Post-Stress Time in Center (sec)
Pre-Stress Time in Center (sec)
F
r =0.51
p=0.06
Post-Stress Time in Center (sec)
Pre-Stress Time in Center (sec)
G
Tail Suspension Test
Neural predictors of passive coping
H
r =0.039
p=0.90
Post-Stress Time Immobile (sec)
Pre-Stress PF (peaks/min)
I
r =0. 41
p=0.23
Post-Stress Time Immobile (sec)
Pre-Stress PF (peaks/min)
J
Behavioral predictors of passive coping
K
r =0.61
p=0.04
Post -Stress Time Immobile (sec)
Pre-Stress Time Immobile (sec)
L
r =0.02
p=0.97
Post-Stress Time Immobile (sec)
Pre-Stress Time Immobile (sec)
M
Social Preference Test
Neural predictors of social interaction
N
r =0.60
p=0.05
Post-Stress NTG Target Events
Pre-Stress PF (peaks/min)
O
r =0.57
p=0.06
Post-Stress NTG Target Events
Pre-Stress Mean Z-Score
P
Behavioral predictors of social interaction
Q
r =0.25
p=0.45
Post-Stress NTG Target Time (sec)
Pre-Stress NTG Target Time (sec)
R
r =0.04
p=0.90
Post-Stress NTG Target Time (sec)
Pre-Stress NTG Target Time (sec)

Figure 4. *Predictors of anxiety and depressive-like behavior. In the open field test, pre-stress peak frequency (PF) predicts post-stress time in center (a) in both females (b; $F_{1,10}$=8.44, n=12, r=0.69, p=0.02) and males (c; $F_{1,8}$=4.70, n=10, r=0.61, p=0.06). Pre-stress time in center also predicts post-stress time in center (d) in females (e; $F_{1,10}$=10.08, n=12, r=0.71, p<0.01) and weakly in males (f; $F_{1,11}$=4.42, n=13, r=0.54, p=0.06). In the tail suspension test, pre-stress peak frequency did not predict time immobile (g) in either females (h; $F_{1,9}$=0.01, n=11, r=0.04, p=0.90) or males (i; $F_{1,8}$=1.64, n=10, r=0.41 p=0.23). Pre-stress time immobile predicts post-stress time immobile (j) in females (k; $F_{1,10}$=5.77, n=12, r=0.60, p=0.04) but not males (l; $F_{1,10}$=0.0032, n=12, r<0.02, p=0.97). In the social preference test, number of nose-to-grid (NTG) interaction events following stress is predicted by peak frequency during these bouts (m) in females (n; $F_{1,9}$=5.09, n=11, r=0.60, p=0.05) and by mean Z-score during these events in males (o; $F_{1,10}$=4.41, n=12, r=0.57, p=0.06). Pre-stress time interacting does not predict time interacting after stress (p) in females (q; $F_{1,9}$=0.63, n=11, r=0.25, p=0.448) or males (r; $F_{1,11}$=0.02, n=13, r=0.04, p=0.90). *p<0.05*

projecting vHIP neurons. In OFT, both pre-stress behavior and neural activity predicted post-stress anxiety-like behavior. In females, center time post-stress was robustly predicted by pre-stress peak frequency (Figure 4B) and by pre-stress center time (Figure 4E). Similarly, in males, stress-induced anxiety-like behavior was marginally, although not statistically significantly, predicted by pre-stress peak frequency (Figure 4C) and pre-stress center time (Figure 4F). Thus, stress-induced anxiety-like behavior can be predicted by either pre-stress individual differences in vHIP-NAc activity or anxiety-like behavior. In contrast, pre-stress vHIP-NAc activity did not predict post-stress passive coping in either sex (Figure 4H,I), further suggesting that vHIP-NAc is not a critical mediator of this behavior. Post-stress passive coping could be predicted from pre-stress behavior in females (Figure 4K) but not males (Figure 4 L). Consistent with previous findings (5), post-stress social interaction (cumulative duration of social target NTG bouts) was not predicted by pre-stress social interaction in either sex (Figure 4R,Q). Neural activity had greater predictive value. Post-stress proximal interaction frequency (number of NTG events) was marginally predicted by pre-stress vHIP-NAc peak frequency during social NTG bouts in females (Figure 4N) and by pre-stress social NTG bout mean Z-score in males (Figure 4O). Number of SI bouts were not predicted by mean Z-score in females (Figure S1C) or peak frequency in males (Figure S1D). This suggests that vHIP-NAc may be one, although likely not the primary, mediator of stress-induced social interaction deficits.

Stress modulates in vivo vHIP-NAc neural activity across behavioral domains

After identifying that pre-stress vHIP-NAc activity predicts specific domains of stress-induced behavioral adaptation, we next asked if *in vivo* vHIP-NAc activity is also altered by chronic stress. Previous *ex vivo* analyses found stress-induced alterations in glutamate release probability in male mice after CSDS (18) and increased excitability in females after CVS (19) but how the integrated impact of synaptic and intrinsic changes regulate behaviorally-relevant *in vivo* neural signaling is not known. In females, CVS increased vHIP-NAc peak amplitude (Figure 5E) and marginally increased peak frequency (Figure 5C) while mice explored an open field (Figure 5A). In males, CVS increased peak frequency (Figure 5D), but not (Figure 5F) amplitude. To probe the evolution of increased neural activity with emerging behavioral susceptibility, we examined an intermediate time-point in males. 15d CVS did not impact anxiety-like behavior (Figure S3A) however, peak frequency was already significantly increased (Figure S3B), and did not increase further after 21d CVS, indicating that changes in vHIP-NAc activity precede emergence of stress-induced behavioral adaptations.

CVS also increased peak amplitude during tail suspension (Figure 5G) in both sexes (Figure 5K,L), but peak frequency was only significantly increased in males (Figure 5J). In contrast to broadly consistent CVS-induced increases in vHIP-NAc activity during OFT and TST in both sexes, neural activity during social interaction revealed sex differences. Following CVS, both mean Z-score (Figure 5Q) and peak frequency (Figure 5O) of fluorescence increased during proximal social interaction (NTG bouts) (Figure 5M) in female mice, consistent with a general stress-induced increase in vHIP-NAc activity across behavioral contexts. However, in males, CVS did not impact either measure (Figure 5P,R), suggesting greater task-specificity in modulation of vHIP-NAc neural activity in male mice.

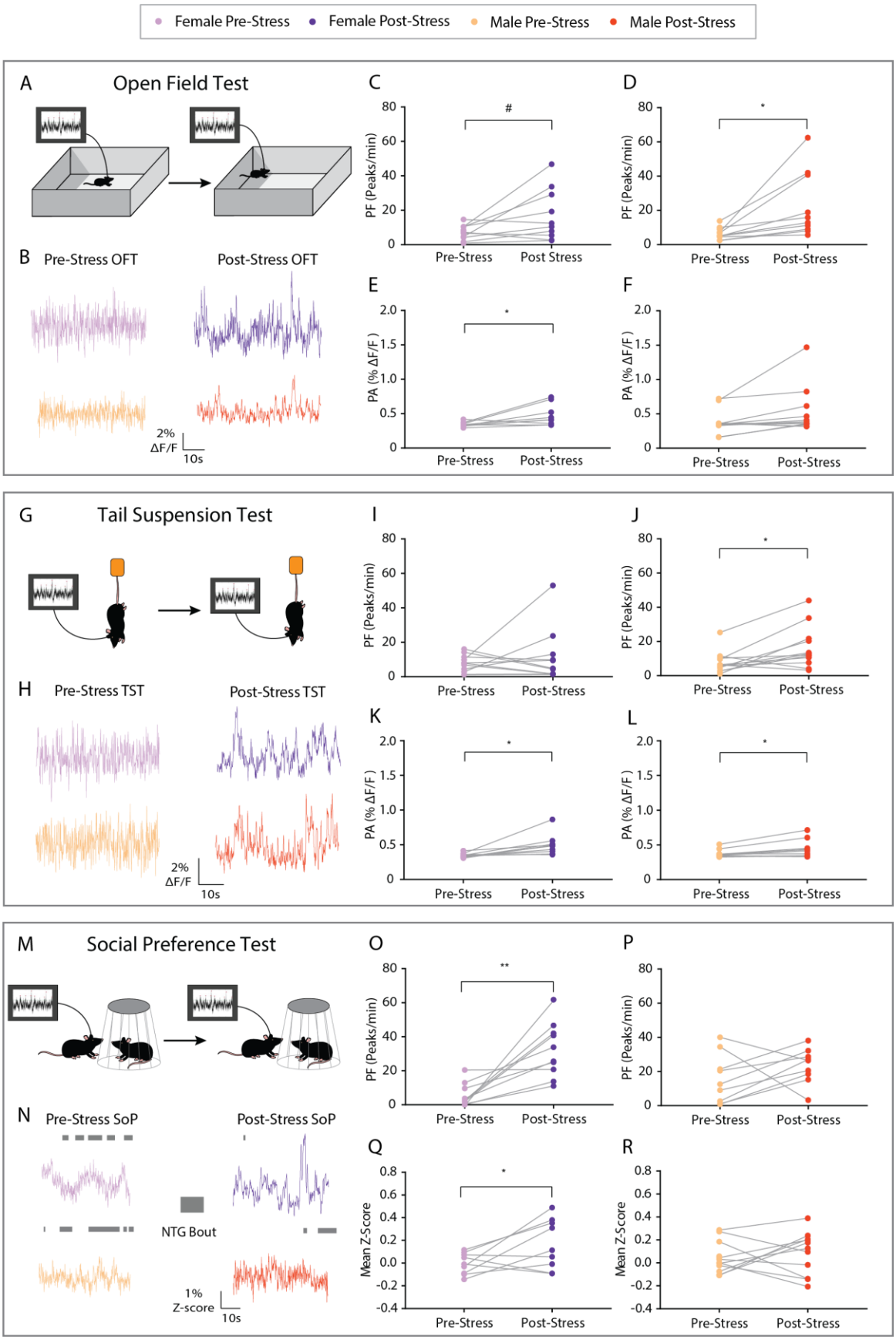
Female Pre-Stress
Female Post-Stress
Male Pre-Stress
Male Post-Stress
A
Open Field Test
B
Pre-Stress OFT
Post-Stress OFT
2%
ΔF/F
10s
C
D
E
F
PF (Peaks/min)
PA (% ΔF/F)
Pre-Stress
Post Stress
Post-Stress
#
*
G
Tail Suspension Test
H
Pre-Stress TST
Post-Stress TST
I
J
K
L
M
Social Preference Test
N
Pre-Stress SoP
Post-Stress SoP
NTG Bout
1%
Z-score
O
P
Q
R
**
Mean Z-Score

Figure 5. *Chronic variable stress (CVS) increases vHIP-NAc activity. During the open field test (a), in females, CVS increases peak frequency (PF) (c; n=11, p=0.05) and amplitude (PA) (e; n=10, p=0.03) while peak frequency (d; n=11, p=0.02) but not amplitude (f;n=10, p=0.12) is increased in males. During the tail suspension test (g), CVS increases peak frequency in males (j; n=12, p=0.01) but not females (I; n=10, p=0.30) and peak amplitude in both females (k; n=10, p<0.01) and males (l; n=11, p=0.02). During the social preference test (m), CVS increases peak frequency and mean Z-score during nose-to-grid (NTG) interaction bouts in females (o; n=10, p<0.01, q; n=9, p=0.05). but not in males (p; n=11, p=0.58, r; n=9, p=0.25). (b, h, n) Representative traces for all conditions. **p< 0.005*p<0.05, # p<0.1.*

CVS increases pre-synaptic input to vHIP-NAc projection neurons

Fiber photometry identified key relationships between *in vivo* vHIP-NAc neural activity and specific anxiety- and depressive-like behaviors and further revealed stress-induced increases in neural activity in multiple behaviorally relevant contexts. While a powerful technique for probing *in vivo* neural activity during behavior, capturing gross changes in neural activity, as the photometry signal represents integrated activity of a population of neurons, resolving precise mechanisms of altered neural activity necessitates complementary approaches. To investigate mechanisms of stress-induced increase in vHIP-NAc activity we used *ex vivo* patch-clamp electrophysiology. We injected mice with retrograding AAVrg-hSyn1-GCaMP6s-P2A-nis-dTomato (Figure 6A,B) to visualize and target recordings to the same population of neurons studied *in vivo*, i.e. vHIP neurons projecting to NAc, and exposed mice to 21d (male) or 4d (female) CVS, and then prepared acute brain slices (Figure 6C). We examined spontaneous EPSCs to assay pre- and post-synaptic alterations. CVS increased sEPSC frequency relative to stress-naïve controls in both sexes (Figure 6D,H,E,I), but amplitude was unaffected (Figure 6F,G), suggesting a pre-synaptic effect of CVS. We probed AMPA: NMDA receptor ratio and confirmed that CVS also did not alter this metric of post-synaptic function in either sex (Figure 6J-M). Thus, CVS increases neural activity in the vHIP-NAc pathway via a pre-synaptic alteration that increases synaptic input to NAc-projecting vHIP neurons similarly in both males and females.

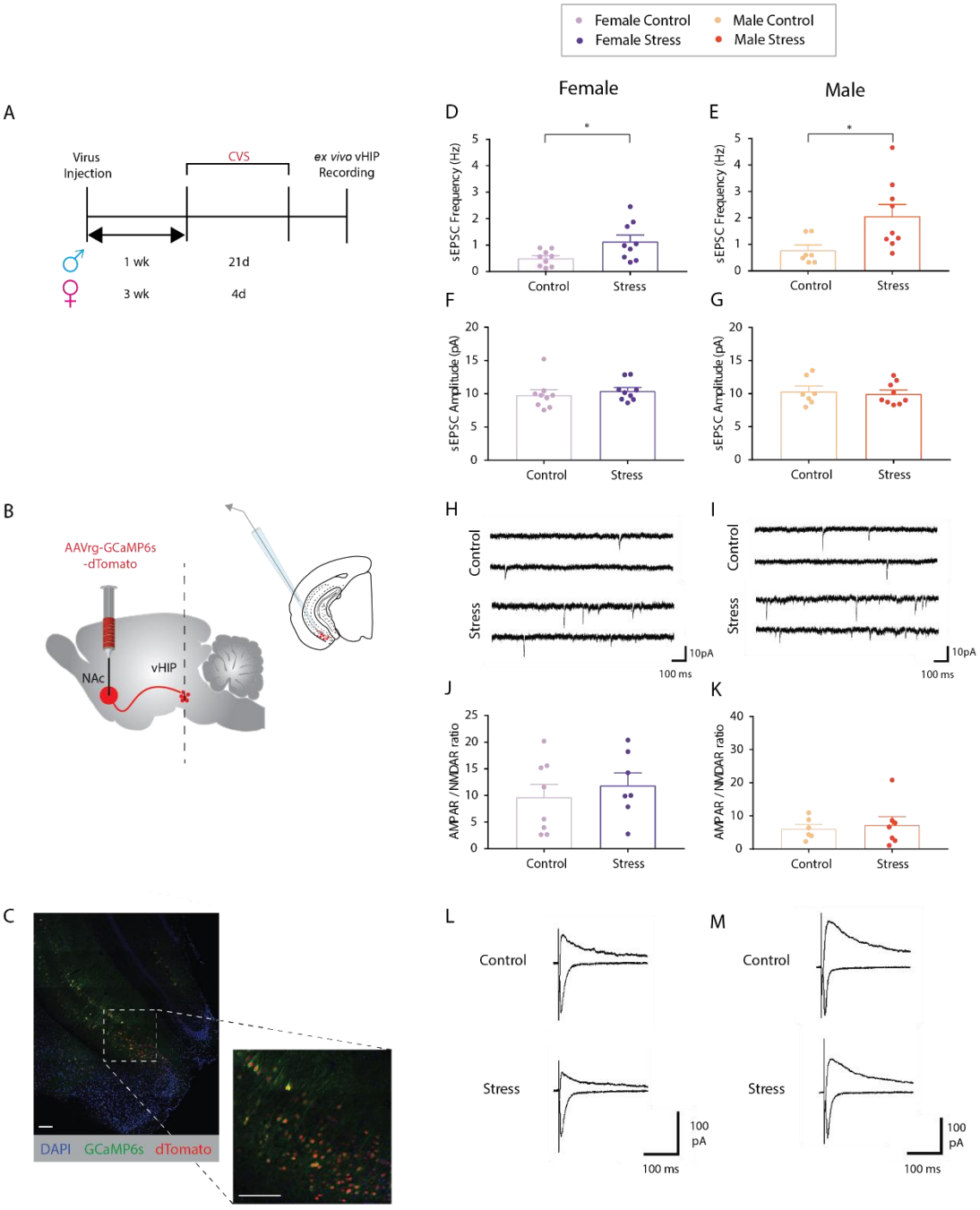

Figure 6. *Chronic variable stress (CVS) increases sEPSC frequency in both males and females. (a) Experimental timeline. (b) Schematic illustrating location of viral injection, retrograde expression and patch clamp recordings. (c) Immunohistochemistry image shows GCaMP6s-dTomato expression (scale bars represent 100 µm in both images). Both females and males show increases in sEPSC frequency (d; n=9,9, p=0.03, e; n=7,9, p=0.03) but not amplitude (f; n=9,9, p=0.52, g; n=7,9, p=0.71), and AMPAR:NMDAR ratio was also unchanged (j; n=10,7, p=0.52, k; n=6,7, p=0.71). Figures h,i,l,m show representative traces.* *p<0.05

Discussion

Identifying the neural mechanisms that shape individual differences in stress adaptation in both sexes is an essential foundation for developing novel strategies to redirect the course of stress-related psychiatric disorders in vulnerable individuals. Here we interrogated neural activity in the vHIP-NAc pathway, finding that baseline vHIP-NAc activity correlates with individual differences in both anxiety-like and social interaction behavior but not passive coping. Critically, we demonstrate that baseline neural activity differences are predictive of stress-induced alterations in these behavioral domains. Furthermore, we identify a pre-synaptic mechanism by which stress increases neural activity in this pathway to mediate stress-induced susceptibility in both sexes.

By probing *in vivo* neural activity across a range of behaviors we identified behavioral specificity in vHIP-NAc neural activity, revealing that properties of the neural signal relate to anxiety-like behavior and social interaction in both sexes, but not passive coping. Depression is a heterogeneous disorder marked by a wide range of symptoms. Our findings support that depression reflects dysfunction in distributed circuits that underlie specific behaviors or symptoms, rather than a unitary pathology (27). The vHIP has long been implicated in anxiety-like behavior, with lesions reducing stress reactivity and avoidance of anxiogenic environments (15-17, 28). Recent *in vivo* imaging and optogenetic manipulations identified a role specifically for vHIP projections to the hypothalamus in regulating avoidance of anxiogenic contexts in male mice (15). Here, we show that vHIP-NAc projections may encode elements of anxiety-like behavior in both sexes. Intriguingly, our data also implicate vHIP-NAc activity in social interaction in stress-naïve animals. Although activity in the vHIP-NAc pathway correlates with both social interaction and anxiety-like behavior, these behaviors do not correlate with each other (male: $F_{1,12}$=0.18, n=14, r=-0.12, p=0.68; female: $F_{1,11}$=0.22, n=13, r=-0.14, p=0.65) indicating that these relationships may represent distinct factors of stress vulnerability. Mice will optogenetically self-stimulate vHIP-NAc (24), suggesting this pathway may encode some element of reward or reinforcement. Consistent with this, social interaction, widely considered rewarding (23), associates with increased activity in this pathway in male and female mice. The association of vHIP-NAc neural activity with both increased social interaction and increased anxiogenic behavior

is at first surprising. We suggest the vHIP-NAc neural signal encodes a general property of salience rather than specifically reward or anxiety. This might also account for social preference data whereby vHIP-NAc activity correlates with increased interaction pre-stress yet also predicts decreased interaction time post-stress. In support of this, an intra-vHIP pharmacological manipulation that increases neuronal activity in NAc shell facilitates both sub-threshold contextual fear conditioning and conditioned place preference, suggesting that communication between vHIP and NAc may exert a valence-independent regulation of emotional salience (29). In this light, previous findings that vHIP-NAc supports reward may be reinterpreted as evidence that this pathway encodes salience. Indeed, mice will robustly work for salient stimuli with no intrinsic motivational value (30). Thus, rather than encoding specific behaviors or information about valence, the vHIP may communicate information about context or stimulus salience to the NAc to modulate behavioral output. Electrophysiological studies demonstrate that vHIP can gate other glutamatergic projections to NAc, inducing a bi-stable state in medium spiny neurons that may facilitate processing of other inputs (31-33), providing a circuit mechanism for the vHIP to exert modulatory control of motivated behavior. Thus, we would not necessarily expect the relationship between pre-stress vHIP-NAc activity and pre-stress social interaction behavior to be the same as the predictive relationship, or the relationship with anxiety-like behavior. However, it is clear that vHIP-NAc neural activity under basal conditions is telling of an animal's vulnerability.

A fundamental unanswered question is what drives the initial divergence such that apparently similar individuals respond differently to the same stress. That is, do pre-existing differences *prior* to stress render some individuals vulnerable to stress-induced pathology? Identifying these specific mechanistic risk factors creates the potential for precise interventions targeted to vulnerable individuals to treat the underlying dysfunction before additional pathological consequences accrue. This is an especially relevant treatment goal in depression given that increasing chronicity is accompanied by ever-increasing treatment-resistance (2). In this context, there has been a recent push to identify factors that predispose to susceptibility (5, 7, 8, 34). Here, we find that anxiety-like behavior prior to stress predicts post stress-anxiety-like behavior, consistent with previous work (8, 35). However, neither post-stress passive coping nor social

interaction deficits were predicted by pre-stress behavior. Importantly, we show that vHIP-NAc neural activity predicts both anxiety-like and social interaction behavior, suggesting that differences in neural processing of anxiogenic and rewarding stimuli predict future susceptibility and may influence individual stress adaptation. Our findings suggest that certain neural and behavioral abnormalities observed *following* stress, may actually define pre-existing vulnerability states that *precede* stress.

Our findings add to accumulating evidence linking anxiety and vulnerability to stress-induced depression-like behavior (8, 35-37). Rodents with elevated anxiety-like behavior at baseline are more vulnerable to chronic stress (38). Such trait anxiety also links to a variety of factors associated with stress vulnerability, including enhanced HPA-axis activation by acute stress (39, 40), social rank (36) and metabolic changes (36, 41). In humans, anxiety is the most frequently co-occurring symptom with depression, contributing to increased severity and recurrence. Trait anxiety also contributes to neuroticism (vulnerability to negative emotions), a trait with significant heritability which mediates the largest genetic risk for depression (42) , associating with variation in many genes (43, 44), including some associated with anxiety in rodents (39, 40, 45, 46). Standard tests of anxiety-like behavior probe an animal's willingness to engage potential threat and may reveal pre-existing abnormalities in circuit function and hormonal signaling that in turn mediate individual differences in exploration, cognitive processing and, ultimately, adaptation to stress and aversive experiences to mediate risk for depression-like behavior.

The distal causes of pre-existing differences in behavior and neural activity associated with differential stress adaptation remain to be fully explored. Individual differences in early life experience may play a role. Maternal care exerts a robust and enduring influence on the hippocampus such that the relative amount of licking and grooming in the first week of life associates with individual differences in hippocampal synaptic plasticity and excitability, morphology, glucocorticoid and mineralocorticoid expression levels and hippocampus-dependent fear learning in adulthood (47-49). Several of these factors associate with future susceptibility to chronic stress (8). Furthermore, rodents characterized as dominant versus subordinate, exhibit increased anxiety-like behavior at baseline and increased susceptibility after

stress (36). Levels of certain metabolites in the NAc are lower in subordinate animals at baseline but increased by stress specifically in these lower rank animals. Acetyl-L-carnitine (LAC), an endogenous compound with promising anti-depressant effects that supports energy metabolism, partially abolishes vulnerability in dominant animals (41). These and other environmental factors may contribute to differential vulnerability. Alternatively, individual differences may arise through compounding of stochastic differences during development. Ultimately, whatever the source, uncovering mechanisms of pre-existing vulnerability is a critical step in developing strategies for early intervention in at-risk populations.

Beyond identifying pre-existing differences in the vHIP-NAc pathway, we show that subsequent exposure to stress that is sufficient to increase anxiety- and depressive-like behavior (4d CVS females, 21d males) robustly increases vHIP-NAc activity in both sexes. Stress increased vHIP-NAc activity *in vivo* across a range of behaviors. Interestingly, while increased vHIP-NAc activity was observed in both males and females in the anxiogenic context of an open field and during tail suspension, in a test of social behavior, increased neural activity was observed only in female mice and only while actively interacting with a social target, suggesting that behavioral context regulates stress-induced alterations in neural activity within this pathway. To probe the mechanism of this *in vivo* stress-induced increased neural activity, we examined pre- and post-synaptic function in vHIP-NAc projecting neurons and found that stress increased sEPSC frequency, but not amplitude, and, also did not alter AMPA: NMDA ratio. This points to a pre-synaptic mechanism of increased input to these projection neurons and suggests it will be important to probe upstream to further resolve the circuit-level origin of stress-induced alterations in vHIP-NAc signaling. Previous work has found a role for the vHIP-NAc pathway in stress-induced susceptibility in males (18) and females (19). Increased vHIP-NAc neuron excitability in female mice may account for increased female susceptibility to stress-induced sucrose preference deficits. Here we demonstrate that, while females are initially more vulnerable to developing stress-induced depressive- and anxiety-like behavior, once evidenced, behavioral susceptibility is accompanied by similar increases in neural activity in this pathway in both sexes. These findings extend the mounting body of work implicating enhanced activity in vHIP, and specifically vHIP-NAc, in stress susceptibility by identifying both pre-existing differences

and stress-induced alterations in stress susceptibility in both sexes (7, 8, 18, 19, 34). Potentially, interventions designed to target this mechanism could be effective in treating and even protecting against stress-induced pathology in both sexes.

We used a longitudinal design to probe predictive relationships. A question that may arise is whether changes in behavior and neural activity are stress-induced or result from habituation or repeated behavioral testing. The fact that *ex vivo* electrophysiology in mice not exposed to behavioral testing revealed increases in sEPSC frequency in stressed compared to unstressed control animals argues against such a possibility. Increased sEPSC frequency measured *ex vivo* is consistent with *in vivo* activity changes revealed by fiber photometry, suggesting that stress increased neural vHIP-NAc activity. Behavioral and neural adaptations after stress were similar in both sexes, despite significant differences in experimental design (4d vs 21d between pre- and post-test) further arguing against a non-specific phenomenon due to repeated testing which would be expected to vary with time elapsed since testing. Our experimental design employed differing lengths of stress in males and females to induce comparable behavioral susceptibility, given known sex differences in effects of stress. While acute vs chronic stress differentially impacts neural circuit activity in male rats (50), this phenomenon is unlikely to account for sex-specific increases in *in vivo* activity during social interaction observed during comparable behavior. Furthermore, despite behavior-specific differences in neural changes, *ex vivo* electrophysiology revealed similar stress-induced neural adaptations in both sexes. Future research could explicitly examine how systematically varying stress chronicity impacts neural circuit function in both males and females.

Employing a longitudinal pathway-specific *in vivo* recording approach combined with targeted *ex vivo* interrogation of synaptic function we shed new light on the role of the vHIP-NAc pathway in specific stress-related behaviors and how this signal is modified by stress in males and females. Furthermore, we established that this pathway is not only active in these behavioral contexts, but the degree of activity is predictive of individual differences in adaptation to future stress. Effective treatments for depression remain limited. Inherent heterogeneities of the disorder with different individuals exhibiting different symptoms and varying responses to treatment, coupled

with the emergence of treatment resistance across recurrent episodes represent major obstacles. Identifying specific mechanisms underlying these behavioral abnormalities at early time points even preceding emergence of the full disorder will open the door not only to targeted treatments, but also to new strategies for prevention.

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Supplemental Materials

Supplemental Methods

Animals

7-week-old male and female C57BL/6J mice were obtained from Jackson Laboratories and maintained on a 12-h light-dark cycle (lights on at 7:00AM) at 22-25^{o}C group-housed with 2-4 same-sex cage-mates with *ad libitum* access to food and water for one week prior to start of manipulations. All experimental manipulations occurred during the light cycle. All experiments were conducted in accordance with guidelines of McGill University's Comparative Medicine and Animal Resources Center and approved by the McGill Animal Care Committee.

Stereotaxic Surgeries

Stereotaxic surgery was performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia. To achieve projection-specific GCaMP7f expression in glutamatergic vHIP NAc-projecting cells, 0.4µl pGP-AAVrg-syn-jGCaMP7f-WPRE virus (122) (7×10^{-12}/ml; Addgene) was infused into the NAc (A/P: +1.3, M/L: +0.60, D/V: -4.9) at a rate of 0.1µl per min, before raising the needle to D/V: -4.7 and infusing a further 0.3µl virus, allowed to diffuse for 10 min before withdrawing the needle. Chronically implantable optic fibers (Doric Lenses) with 200µm core and 0.48 NA threaded through ceramic ferrules were implanted above the ventral subiculum of the vHIP (A/P: -3.40, M/L: +3.00, D/V: -4.75). Recordings began minimum 4 weeks after surgery to allow sufficient time for stable and robust retrograde virus expression in vHIP. For electrophysiology experiments, animals were injected with a retrograding GCaMP6s containing a dTomato tag (AAVrg-hSyn1-GCaMP6s-P2A-nis-dTomato) in order to visually identify NAc-projecting cells in the vHIP. pGP-AAVrg-syn-jGCaMP7f-WPRE was a gift from Douglas Kim & GENIE project (122) (Addgene plasmid #104488; Addgene viral prep #104488-AAVrg). AAV-hSyn1-GCaMP6s-P2A-nis-dTomato was a gift from Jonathan Ting (Addgene plasmid #51084; Addgene viral prep #51084-AAVrg).

Chronic Variable Stress (CVS)

CVS was performed as previously described (48). Three stressors were administered as follows: 100 random foot shocks (0.45mA/1sec) over 1hr (administered in same-sex groups of 10 mice), 1hr tail suspension, 1hr restraint inside a 50-mL falcon tube (with holes for air circulation) in the home cage. One stressor was administered daily at a variable time between 7AM and 4PM, following the order foot shock, tail suspension, restraint for the length of the protocol. Males and females were subjected to 21 and 4 days of CVS respectively, with stressors presented in the same order across dayas to induce comparable phenotypes of stress-induced behavioral change in tests of anxiety- and depressive-like behavior. The sex-specific CVS protocols (different lengths for males and females) were determined based on pilot data obtained in our laboratory establishing the efficacy of the selected durations in each sex.

Behavioral Assessments

Mice were assessed on standard tests for depressive and anxiety-like behavior before and again after CVS. One test was conducted per day between the hours of 8AM and 2PM, with males and females tested on different days. Equipment was cleaned between testing sessions.

Open Field Test (OFT). Mice were placed in the center of an open arena (44cm x 44cm) made of white matte acrylic under red light and allowed to explore for 5 mins. Video tracking software (EthoVision XT 13, Noldus) recorded time in center (defined as 27.5cm x 27.5cm central zone) and periphery as well as total locomotion.

Social preference test (SP). Social interaction behavior was assessed in a two-stage test, with each stage lasting 5 min. In the habituation stage, mice, single housed overnight before testing, explored an arena (44cm × 44 cm) containing 4 Plexiglas dividers, splitting the arena into three equal areas; the two outer areas containing upturned pencil cups. In the second test stage, a novel age- and sex-matched C57BL/6J mouse (social target) was placed under one cup and mice again explored freely. The area containing the mouse was defined as the social area, the area with the empty cup the non-social area, and the middle the neutral area. Time spent in each area was recorded by video tracking software (EthoVision XT 13, Noldus). An experimenter

blind to experimental conditions annotated nose-to-grid events (NTG), where the mouse interacted directly with the cup/social target, with timestamps recorded in EthoVision.

Tail Suspension Test (TST). Mice were suspended by the tail from a metal shelf with a piece of lab tape for 5 mins. Video tracking software (EthoVision XT 13, Noldus) assessed immobility.

In Vivo Fiber Photometry

To measure calcium-associated changes in fluorescence in real time, recordings were made from vHIP NAc-projecting cells during behavioral tests both before and after stress. Samples were collected at a frequency of 12 KHz using Doric Studios hardware and software. Briefly, two light emitting diodes at 490 nm (GCaMP stimulation wavelength) and 405 nm (control for artifactual fluorescence) were coupled to a 200µm 0.48 N.A. optical fiber (Doric). The emission light was collected by the same optical fiber, passed through a GFP filter and focused onto a photodetector where the two output signals (raw-from the 490nm stimulation wavelength and control- from the 405nm wavelength) were separated based on modulation frequency. Recordings were coupled to the start of behavioral analysis by interfacing Doric Studios software with EthoVision. Data were extracted and analyzed using custom-written scripts in Matlab R2019b (The MathWorks) (215). To normalize the data, the control channel was fitted to the raw. The fitted control was then subtracted from the raw trace. The resultant trace was divided by the fitted control giving the ΔF/F. From here, the analysis diverged based on the behavioral test:

Open Field Test/Tail Suspension Test. Peak amplitude and frequency were analyzed across the entire test. Prior to peak detection, data were high pass filtered. High amplitude events (local maxima two MAD above the median) were filtered out and the median of the resultant trace calculated; The peak detection threshold was set at three times this median. Peak amplitude was determined by reference to the corrected fluorescence, calculated by subtracting the rolling minimum from the raw fluorescence. To be included in analyses, an animal must have had detectable peaks in at least one of the recorded conditions, affirming that recordings without detectable peaks were not attributable to technical limitations (e.g. poor viral

expression, fiber placement). Recordings with no detectable peaks (i.e., no increases in fluorescence reliably above baseline) were included in analysis of peak frequency but were not considered in the analysis of average peak amplitude. Note that, this resulted in instances of varying sample sizes between analyses of peak frequency and peak amplitude.

Social Preference. Traces were converted to Z-score. Peak frequency was calculated using the same procedure as above. Only peaks that occurred during social interaction events (NTG epochs) were considered. Mean Z-score was calculated by taking an average of the signal during these NTG bouts.

Ex Vivo Electrophysiology

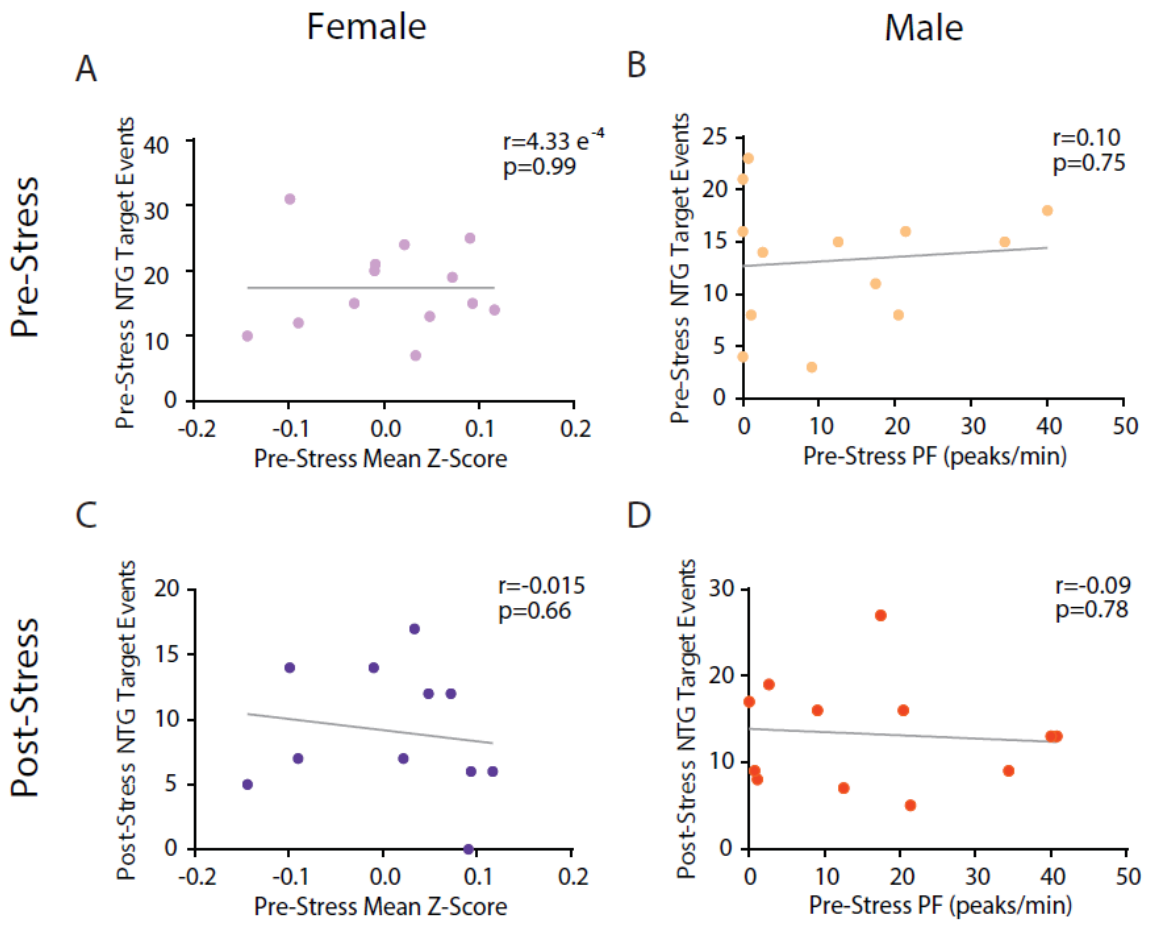
Slice preparation. Mice were deeply anesthetized by isoflurane and then perfused transcardially with 25-30 ml, carbogenated room-temperature NMDG artificial cerebrospinal fluid (aCSF; in mM: 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2{\cdot}4H_2O$, and 10 $MgSO_4{\cdot}7H_2O$ [pH 7.3–7.4, ~310 mOsmol/L]). Coronal slices (200 μm) were cut in carbogenated room-temperature NMDG aCSF with a Leica VT1200s vibratome. Slices were reactivated at 32-34 °C in NMDG aCSF for around 10 min then transferred to carbogenated room-temperature HEPES holding aCSF (in mM: 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2{\cdot}4H_2O$, 2 $MgSO_4{\cdot}7H_2O$ [pH 7.3-7.4, ~310 mOsmol/L]) until recording.

Whole-cell recordings. All recordings were performed at room temperature. During recording, brain slices were transferred to the recording chamber and were perfused with normal aCSF (in mM: 128 NaCl, 10 glucose, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 2 $MgCl_2{\cdot}6H_2O$, 3 KCl, 2 $CaCl_2{\cdot}4H_2O$ [pH 7.3-7.4, ~310 mOsmol/L]). Recordings were made from d-tomato positive cells in the ventral subiculum sub-field of the vHIP to target NAc-projecting neurons. Spontaneous or evoked excitatory postsynaptic currents (EPSCs) were recorded in the whole-cell mode using patch pipettes (3-5 MΩ) containing (mM) 130 Cs-methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 5 TEA-Cl, 4 Mg-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, 1 QX-314 (pH 7.25, ~290 mOsmol/L). Picrotoxin (50 μM, *Sigma*) was used in all recordings to block all $GABA_A$ receptor-mediated

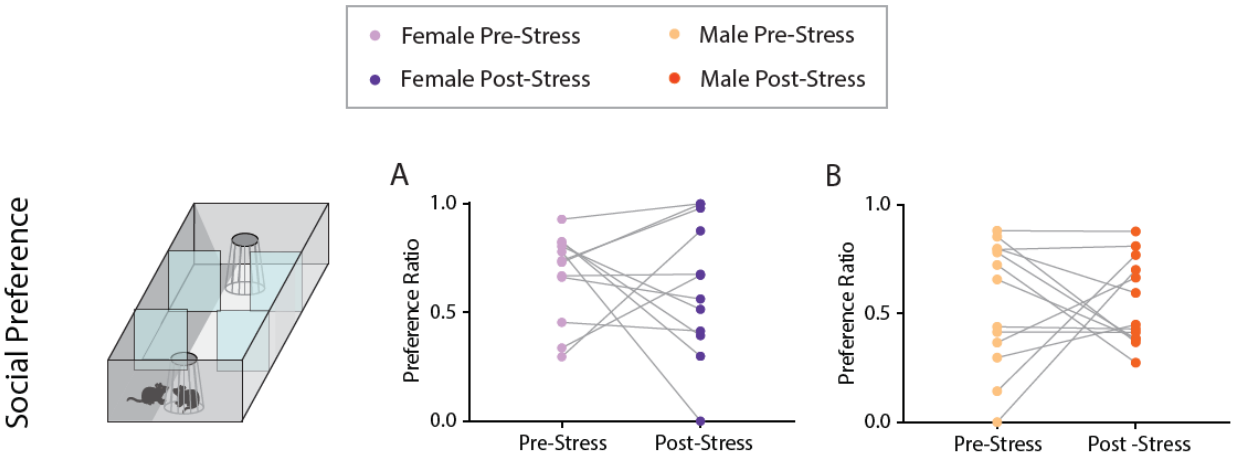
inhibitory synaptic transmission. Spontaneous EPSCs were recorded for at least 5 minutes while voltage-clamped at -60 mV. To probe AMPAR/NMDAR ratios, evoked EPSCs were generated by constant current pulses (0.08ms) at a frequency of 0.1 Hz through a bipolar electrode (*FHC*) placed in the ventral subiculum pyramidal layer. The AMPAR-mediated current (peak of evoked EPSC) was isolated by voltage-clamping the neuron at -60 mV and the NMDAR-mediated current was measured during voltage-clamp at +40 mV, by analyzing the peak 150ms after the stimulation artifact, when AMPAR-mediated current has already returned to baseline. Only recordings with access resistance stable at less than 20 MΩ over the entire recording period were included in analyses. Synaptic responses were amplified and digitized by Multiclamp 700B and Digidata 1550B respectively (*Molecular Devices*) (2 kHz low-pass Bessel filter and 10 kHz digitization) and stored in a PC for offline analysis using Clampfit (*Axon*) or MiniAnalysis (Synaptosoft).

Chemicals. All chemicals were purchased from Sigma except NMDG and HEPES, purchased from Fisher and QX-314 from Alomone Labs.

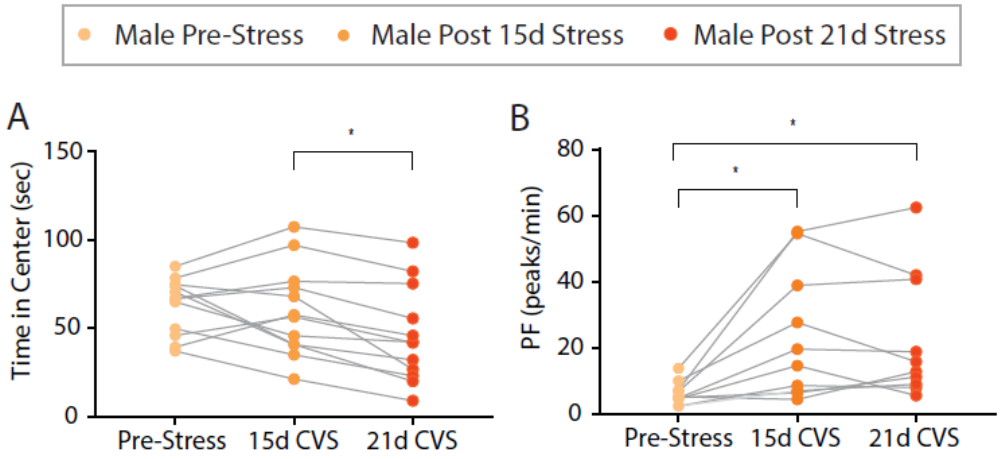
Statistics. Statistical analyses were performed using GraphPad Prism 7. Grubb's test was used to identify and exclude statistical outliers. Paired t-tests were used to compare pre- and post-stress measures; non-parametric Wilcoxon matched-pairs signed rank test was used when data sets did not meet the assumptions of normality. Linear regression was used to probe predictive relationships.



Supplemental Figure S1. *There is no relationship between pre-stress mean z-score during bouts of social interaction and number of social interaction bouts before stress in females (a; $F_{1,11}$=0.2.07e-5, n=13, r=-0.4.33e-4, p=0.99). Similarly, in males, there is no relationship between pre-stress peak frequency during SI bouts and the number of these bouts before stress (b; $F_{1,11}$=0.11, n=13, r=-0.10, p=0.75). There is no predictive relationship between mean Z-score during SI bouts and number of these bouts in females (c; $F_{1,9}$=0.2.07, n=11, r=-0.15, p=0.66) or peak frequency and bout number in males (d; $F_{1,10}$=0.083, n=12, r=-0.09, p=0.78) respectively.*



Supplemental Figure S2: *Pre- vs post-stress social preference ratio did not differ in either females (n=12, p=0.64) or males (n=13, p=0.84; Wilcoxon matched pairs signed ranked test).*



Supplemental Figure S3. *15d Sub-chronic variable stress in males increases vHIP-NAc activity but not anxiety-like behavior. (a; $F_{2,11}$=5.75, n=12, p=0.02; post-hoc: p=0.87) Males do not show increased time in center of an open field following 15 days of stress, but show a significant increase at the 21d time point. (b; $F_{2,11}$=8.86, n=10, p<0.01; post-hoc: p=0.03) Peak frequency is increased following 15d of stress compared to baseline but is not further increased after 21d stress (p=0.90). *p<0.05*

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Framing the Questions: Chapter 4

The previous chapter defines a role for the vHIP-NAc pathway in depressive and anxiety-like behavior in females as well as males and shows that both neural activity and anxiety-like behavior are predictive of future susceptibility. I also identified a stress induced increase in neural activity driven by a presynaptic mechanism.

The vHIP is one of many projections to the NAc, but it has important interactions with the PFC to drive NAc firing. Glutamatergic afferents have been shown across a wide body of literature to drive reward and motivated behavior, yet they also seem to play an important role in stress susceptibility, begging the question: what are these projections encoding and how do they influence behavior? Given stress involves chronic exposure to aversive stimuli, understanding how these projections encode aversive experiences is essential to our understanding of stress induced pathologies.

In this chapter, I interrogate how aversive experiences are encoded by both PFC and vHIP projections to the NAc. I look to investigate (1) how these projections encode aversive vs neutral cues (2) how they discriminate between them and (3) how this regulates ongoing behavior.

CHAPTER 4

Sexually dimorphic neural encoding of threat discrimination in accumbal afferents drives suppression of reward behavior

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Abstract

Learning to predict threat is essential but equally important yet often overlooked is learning about the absence of threat. Interrogating neural activity in two nucleus accumbens afferents in male and female mice during aversive and neutral cues reveals sex-specific encoding of cue discrimination and cue-mediated suppression of reward behavior. Sexual dimorphisms in the neural bases of threat discrimination may reflect sex differences in behavioral strategy relevant to differential psychiatric risk.

Main Text

The importance of learning to recognize and respond to impending threat is widely appreciated but the related process of recognizing when threat is absent is commonly overlooked. Threat inhibits appetitive behavior, e.g. food seeking, avoiding risk, but by learning when threat is not imminent, an animal can pursue essential goals in periods of relative safety. Neural mechanisms discriminating aversive and neutral events underpin adaptive behavior and are disrupted in psychopathology [1-3]. The nucleus accumbens (NAc) integrates diverse inputs [4-6], balancing threat and reward to orchestrate motivated behavior [6-9]. Glutamatergic projections from the ventral hippocampus (vHip) and medial prefrontal cortex (mPFC) to NAc are implicated in reward processing [4] and adaptation to chronic stress [10,11]. How these pathways integrate aversive information to modulate behavior is not fully understood, and, in females, largely unstudied, despite known sex-differences in stress-related psychopathologies [12,13]. Here, we examined how mPFC and vHip projections to NAc medial shell(mPFC-NAc, vHip-NAc), a major target for both vHIP and PFC afferents, encode aversive experiences to guide behavioral responding to threat.

To probe pathway-specific neural encoding of aversive cues in mPFC-NAc and vHip-NAc, we injected retrograding AAV-GCaMP7f in NAc and implanted optic fibers in vHip and mPFC to record Ca^{2+}-associated fluorescence while male and female mice encountered cue-shock (CS+) and cue-no outcome (CS-) pairings (Figure 1a,b). Males and females learned cue-shock associations by mid-training (Figure 1c,d), increasing freezing during the CS+. Cue discrimination increased across training (Figure 1e). Marginal sex differences in CS- mediated behavior emerged in late-training: CS- suppressed freezing in females, but not males (Figure 1c,d). Freezing suppression ratios confirmed that, relative to baseline, CS+ increased freezing in males and females but CS- suppressed freezing below baseline only in females (Figure 1f-i), suggesting here females may learn the CS- as a safety signal.

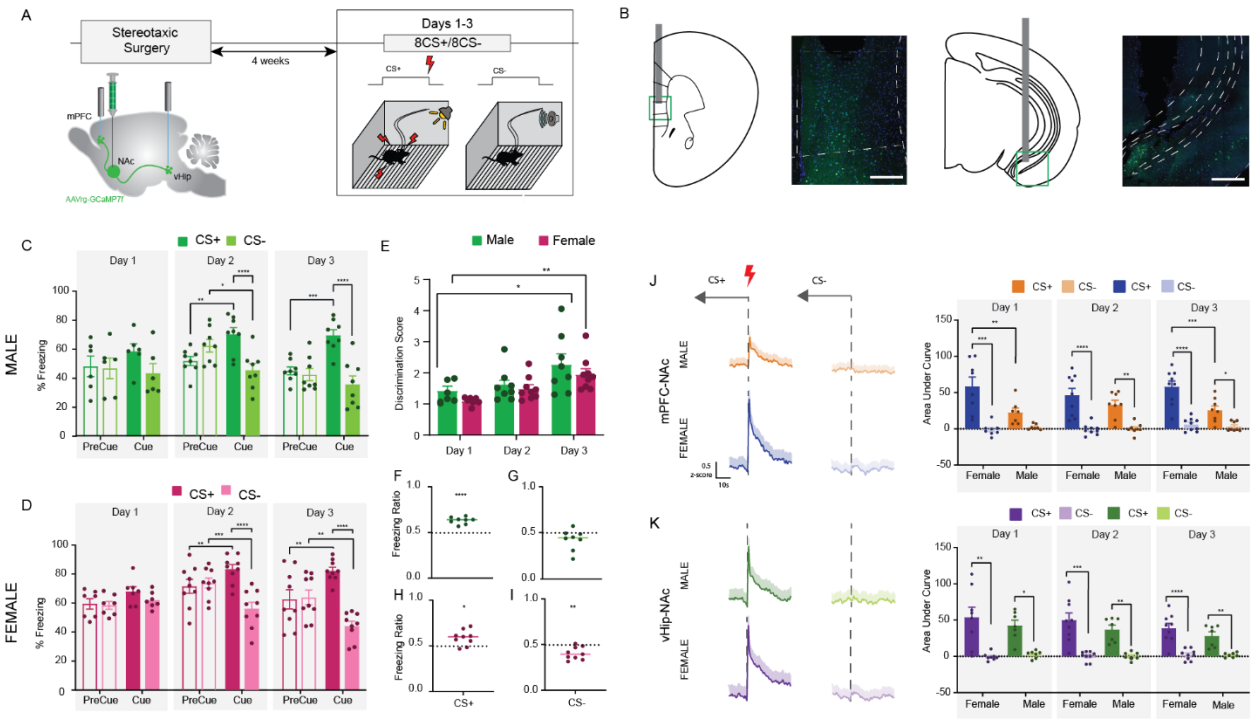
A
Stereotaxic Surgery
4 weeks
Days 1-3
8CS+/8CS-
CS+
CS-
mPFC
NAc
vHip
AAVrg-GCaMP7f
B
C
CS+ CS-
Day 1
Day 2
Day 3
% Freezing
PreCue
Cue
MALE
D
FEMALE
E
Male Female
Discrimination Score
F
Freezing Ratio
G
H
I
J
mPFC-NAc
vHip-NAc
z-score
10s
K
Area Under Curve
Female
Male

***Figure 1**. Male and female mice acquire discriminative Pavlovian conditioning. (A) Experimental timeline of surgery and discriminative Pavlovian conditioning. Male and female mice were injected with a retrograding GCaMP7f into the NAc and implanted with fibers above the vHIP and mPFC to image simultaneous projection-specific neural activity in both pathways (Scale bars represent 250um) (B). Following recovery and time for viral expression, mice were exposed daily to 8 CS+ cues co-terminating with footshock and 8 CS- cues with no outcome for 3 days. (C) In early-training (day 1), males showed a main effect of cue type on freezing ($F_{(1, 5)} = 10.32$, n=6, p=0.02). By mid-training (day 2), males froze more to the CS+ than to CS- ($F_{(1, 7)} = 33.47$, n=8, p=0.0007, post hocs: p=0.0014) and freezing increased from pre-cue for CS+ (p=0.0074) and decreased for CS- cue (p=0.011). At late-training (day 3) males froze more to the CS+ than CS- ($F_{(1, 7)} = 52.08$, n=8, p=0.0002, post hoc: p<0.0001) and relative to pre-cue, freezing increased during CS+ but was not altered by CS- (p=0.0002). (D) On training day 1, female freezing did not differ by cue type ($F_{(1, 6)} = 1.425$, n=7, p<0.05). In mid-training, females showed behavioral discrimination, freezing more to CS+ than CS- ($F_{(1, 8)} = 85.30$, n=9, p<0.0001, post hocs: p<0.0001) and, relative to the pre-cue period, increasing freezing to CS+ (p=0.0001) while decreasing freezing to CS- (p=0.0015). At late-training, females continue to discriminate, freezing more to CS+ than CS- ($F_{(1,8)} = 54.91$, n=9, p<0.0001, post hocs: p<0.0001) with CS+ and CS- exerting opposing modulation of freezing relative to pre-cue (p=0.0016). (E) Cue discrimination (% freezing CS+/ % freezing CS-) increased across training in both males and females ($F_{(2,41)} = 9.47$, n=8,9, p<0.0004, post hocs: p=0.0004, p=0.017). Freezing ratio (% freezing during cue/ % freezing during cue + % freezing during pre-cue) on day 3, showed that CS+ significantly increased freezing from pre-cue in males (n=8, p<0.0001) (F) and females (n=9,p=0.012) (H), but CS- significantly suppressed freezing in females (n=9, p=0.003)(I) but not males (n=8, p<0.05) (G). Footshock following CS+ increased neural activity in males and females compared to CS- no outcome in (J) mPFC-NAc with females exhibiting larger mPFC-NAc pathway response compared to males (Day 1: $F_{(1, 14)} = 8.23$, n=8,8, p=0.012, post hocs: p_F<0.0001, p_M=0.12, p_{MvF}=0.0019; Day 2: $F_{cue\ (1, 13)} = 56.74$, n=8,8 p<0.0001; $F_{cuexSex\ (1, 13)} = 2.83$, n=8,8 p<0.11post hocs: p_F<0.0001, p_M=0.002, p_{MvF}=0.09; Day3: ($F_{(1, 15)} = 9.47$, n=8,9, p=0.0077, post hocs: p_F<0.0001, p_M=0.01, p_{MvF}=0.0004) and (K) vHIP-NAc projections (Day 1: $F_{(1, 14)} = 26.46$, n=9,7, p=0.0001, post hocs: p_F=0.0014, p_M=0.017; Day 2: $F_{(1, 14)} = 43.44$, n=9,7, p<0.0001, post hocs: p_F=0.0001, p_M=0.0044; Day3: ($F_{(1, 14)} = 50.19$, n=9,7, p<0.0001, post hocs: p_F<0.0001, p_M=0.0029).*

To understand how threat is encoded, we then examined shock-associated changes in neural activity. Shock increased activity in both pathways (Figure 1j,k), with larger increases in mPFC-NAc in females than males, indicating augmented pathway-specific aversive processing. We next examined CS+ and CS- discrimination encoding (Figure 2a-d). Systematically contrasting CS+ and CS- elicited neural activity using a Generalized Additive Model (Figure 2e) identified two epochs maximally encoding cue identity: 1sec at cue onset and 8sec pre outcome (Figure 2f-i), the focus of subsequent analyses. Sex and pathway-specific neural signals emerged across training; activity at cue onset in mPFC-NAc discriminates cue type in females but not males, and in vHip-NAc, in males but not females. In mPFC-NAc in males, a CS- peak emerges in mid-training, with equivalent CS+ and CS- peaks in late-training (Figure 2j,n). In mPFC-NAc in females, CS+ and CS- peaks are similar in early-training with CS+ exceeding CS- peak in late-training (Figure 2k,o). In vHip-NAc, in males, similar CS+ and CS- peaks in early-training resolve to only a CS+ peak in late-training (Figure 2l,p), while in females, CS+ and CS- elicit similar peaks throughout training (Figure 2m,q). We also observed CS+ specific suppression in the pre-outcome period in both pathways and sexes across training (Figure S1).

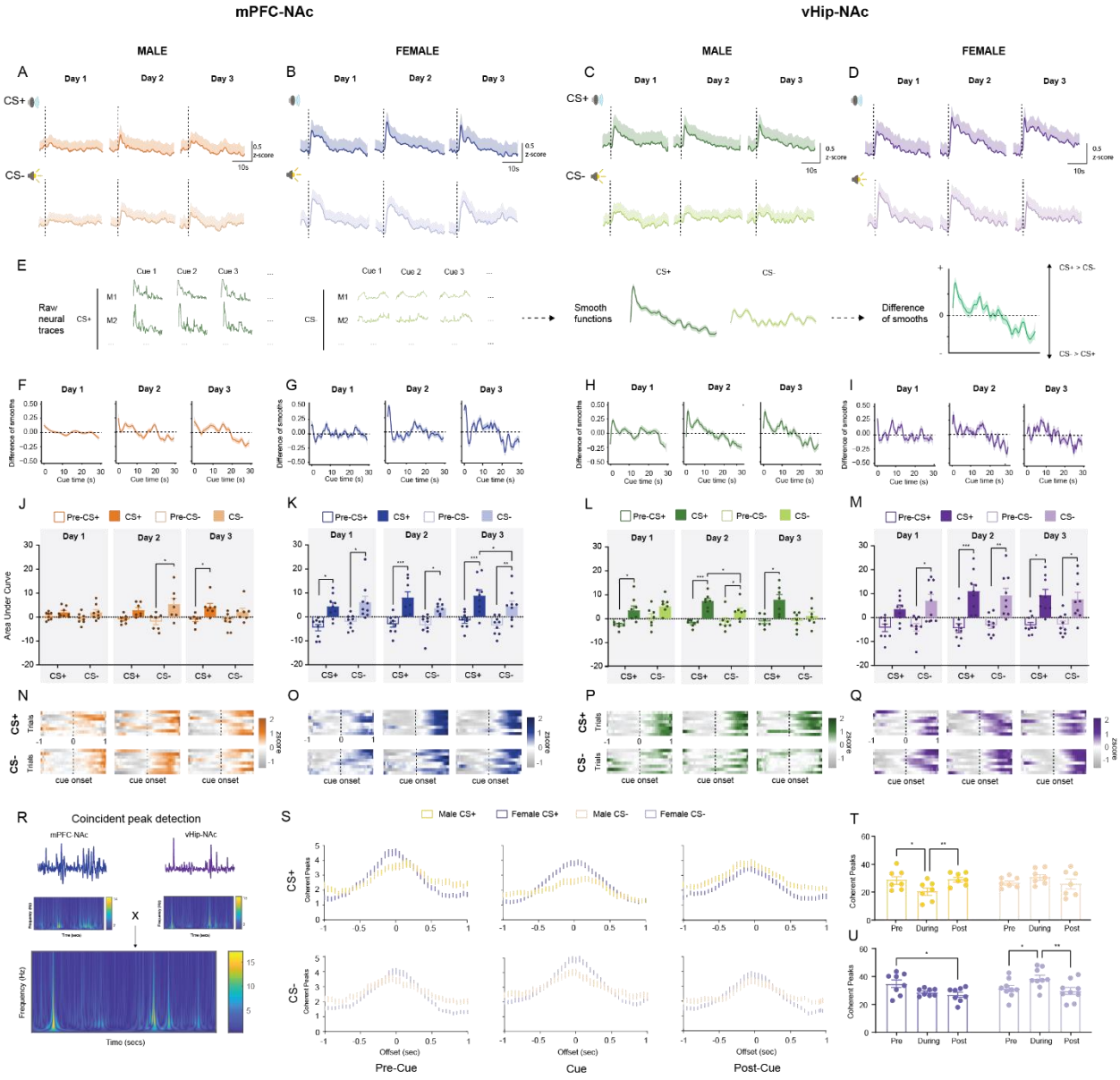
mPFC-NAc
vHip-NAc
MALE
FEMALE
MALE
FEMALE
A
B
C
D
Day 1
Day 2
Day 3
CS+
CS-
0.5 z-score
10s
E
Raw neural traces
Cue 1
Cue 2
Cue 3
M1
M2
Smooth functions
Difference of smooths
CS+ > CS-
CS- > CS+
F
G
H
I
Difference of smooths
Cue time (s)
J
K
L
M
Pre-CS+
CS+
Pre-CS-
CS-
Area Under Curve
N
O
P
Q
Trials
cue onset
zscore
R
Coincident peak detection
mPFC-NAc
vHip-NAc
Frequency (Hz)
Time (secs)
S
Male CS+
Female CS+
Male CS-
Female CS-
Coherent Peaks
Offset (sec)
Pre-Cue
Cue
Post-Cue
T
U
Pre
During
Post

Figure 2. *Sex-specific neural encoding of aversive and non-reinforced cues. (A-D) Average time-locked cue-elicited neural activity in mPFC-NAc and vHip-NAc shows peaks for CS+ and CS-. (e) Generalized Additive Modeling (GAM) uses a sum of smooth functions to model contributions of a fixed variable, cue type, to variation in time series of neural activity recorded during individual cue presentations nested within individual animals, accounting for animal ID as a random variable. To probe differences in CS+ and CS- elicited neural activity across the cue, the difference function of the two smooth functions is calculated, with large non-zero values indicating epochs of maximum difference. (f-i) GAM revealed differences in CS+ and CS- elicited neural activity that emerge across training and identified 2 periods of maximal difference: 1 sec at cue onset and 8 sec preceding cue termination. (j) Further analysis of neural activity at cue onset revealed that in early-training, activity in mPFC-NAc in males was not altered ($F_{(1,6)} = 15.44$, n=7, p=0.15), in mid-training there was an increase to CS-, but not CS+ ($F_{(1,6)} = 20.11$,n=7, p=0.004, post hocs: p=0.029) and, during late-training, to CS+ with a trending increase to CS- ($F_{(1,6)} = 10.58$,n=7, p=0.017, post hocs: p_{CS+}=0.013, p_{CS-}=0.08) (Figure 3a). (k) In females, PFC-NAc activity increased at cue onset for both cue types in early training ($F_{(1,8)} = 24.25$, n=9, p=0.0012, post hocs: $_{CS+}$=0.011, p_{CS-}=0.017), and in mid-training, ($F_{(1,8)} = 26.92$,n=9, p=0.0008, post hocs: p_{CS+}<0.0004, $p_{cs-=}$0.012), with increased activity to CS+ relative to CS- in late training ($F_{(1, 8)} = 22.5$,n=9, p=0.0015, post hocs: p_{cue}<0.04, $_{pre-cue}$=0.34) (p=0.036). In contrast, in the vHIP-NAc, (l) in males activity increased at cue onset to both cues in early training ($_{(1,6)} = 17.06$, n=7, p=0.006, post hocs: p_{CS+}=0.02, p_{CS-}=0.06) and mid-training ($F_{(1,6)} = 58.66$,n=7, p=0.0003, post hocs: p_{CS+}=0.0004, p_{cs-}=0.021) but activity to CS+ was significantly greater than to CS- in mid-training (p=0.025) and by late-training activity was only increased to CS+ ($F_{(1,6)} = 13.16$,n=7, p=0.01, post hocs: p_{CS+}=0.02). Females (m) show increased activity to both CS+ and CS- without discrimination throughout all three days (Figure 4b) (Day 1: $F_{(1,8)} = 23.17$,n=9, p=0.0013, post hocs: p_{CS+}=0.06, $p_{cs-=}$0.012; Day 2: $F_{(1,8)} = 30.65$,n=9, p=0.0005, post hocs: p_{CS+}=0.0007, $p_{cs-=}$0.0028;Day 3: $F_{(1,8)} = 23.92$,n=9, p=0.0012, post hocs: p_{CS+}=0.016,$p_{cs-=}$0.037). (n-q) Heatmaps illustrating trial by trial fluorescence changes at CS+ and CS- onset in representative animals. (r) Schematic illustrating the use of cross wavelet transform to quantify coherence between two fiber photometry time series recorded in mPFC-NAc and vHip-NAc of an individual animal. The cross-wavelet transform quantifies coherence between the two signals, with warmer colors indicating higher absolute values, to identify significant peaks in coherence (s). Probing coherence across a range of offsets reveals maximum coherent peaks at a 0 sec offset indicating synchronous signals. (t) In males, this synchrony is modulated by aversive cues ($F_{(2, 12)} = 9.35$, n=7, p=0.004), with a decrease in number of coherent peaks during the CS+ (p=0.02) which returns to higher baseline synchrony during the post-cue period (p=0.01). (u) In females, there is a trending decrease in coherent peaks during the CS+ ($F_{(2, 13)} = 6.309$, n=9, p=0.012, post-hoc:0.08) which is sustained into the post-cue period (p=0.02). In females, but not in males, the CS- exerts an opposing modulation, with an increase in coherent peaks from pre-cue to cue (p=0.02) that returns to baseline in the post-cue period (p=0.004).*

Time-locked neural activity in both mPFC-NAc and vHip-NAc encoded aversive events and cues predicting these events but discriminating non-threat cues was sex- and pathway-specific. We reasoned that if indeed these pathways carry distinct information between males and females, cue identity should be preferentially recoverable from neural activity in one pathway for each sex. A k-nearest neighbors classifier using late-training cue onset activity achieved reliable classification in females with mPFC-NAc but chance levels with vHip-NAc (Figure S2b,d). Conversely in males, vHip-NAc classification was reliable and mPFC-NAc at chance (Figure S2a,c). Using the full 30sec cue-elicited neural activity increased classifier accuracy across all predictors, however the sex-bias remained with mPFC-NAc outperforming vHip-NAc in females and vHip-NAc outperforming mPFC-NAc in males (Figure S2e-h) confirming sex-specificity in neural discrimination of cues.

mPFC and vHip inputs to NAc are highly convergent [14]. While examining each pathway in isolation suggested one pathway predominates in each sex, we reasoned that coordinated activity across pathways may also carry information and examined synchrony using wavelet signal decomposition to identify coherent peaks in paired mPFC-NAc and vHip-NAc recordings (Figure 2q). Exploring temporal lag/lead identified maximal coherence at zero offset (Figure 2r) with modulation by cue-type and period. In males, synchrony dropped during CS+ before returning to baseline in the post-cue period (Figure 2s), while in females reduced synchrony during CS+ persisted. Strikingly, in females, but not males, CS- exerted opposing modulation, increasing cue-induced synchrony (Figure 2t). Reduced synchrony may signal a pending aversive event and increased synchrony, resolution of threat or, safety. Persistent CS+ induced reduced synchrony in females may indicate sustained threat perception, defining a background threat-level against which increased CS-induced synchrony can signal safety.

We then asked if neural encoding of cue identity drives freezing, a species-specific fear/threat response. Linear mixed effects regression revealed that cue-onset neural activity explains surprisingly little variation in freezing (Table S1, Figure S3). Models integrating both mPFC-NAc and vHip-NAc did not significantly improve prediction. mPFC-NAc and vHip-NAc encode shock-predicting cues, but do not predict freezing, suggesting they do not mediate this behavior.

Analyzing neural activity-behavior relationships indicated that information carried in mPFC-NAc and vHip-NAc does not drive freezing, raising the question of the behavioral significance of cue information in these pathways. Given the role of NAc in integrating cortico-limbic information to regulate motivated behavior [4,15-17], we hypothesized that mPFC-NAc and vHip-NAc representations of threat and non-threat cues might specifically modulate reward behavior. To test this, we developed a conditioned suppression paradigm wherein mice learn to suppress rewarded lever pressing during a shock-predicting CS+ (but not CS-) and then chemogenetically inhibited mPFC-NAc or vHip-NAc using an intersectional cre-dependent viral strategy injecting retrograding AAV-cre into NAc and AAV-DIO-hM4Di into mPFC or vHip (Figure 3a-c). Upon stable lever pressing, mice progressed to discriminative fear conditioning with continued reward access. C21 injected prior to a test with active levers and non-reinforced cues induced pathway-specific inhibition (Figure 3c). Inhibiting mPFC-NAc had no effect in males; both hM4Di-DREADD and mCherry-controls suppressed lever pressing to CS+ and not CS- (Figure 3e). In females, mCherry-controls suppressed more to CS+ than CS-, while mPFC-NAc hM4Di-DREADD mice did not discriminate (Figure 3g). With vHip-NAc manipulations, hM4Di DREADD males showed less suppression to CS+ than mCherry-controls and neither group showed suppression to CS- (Figure 3i). In females, both hM4Di-DREADD mice and mCherry-controls similarly suppressed lever pressing during CS+ and not CS- (Figure 3k). As predicted from the limited behavioral variability explained from neural data (Figure S3), freezing was not altered by inhibition of either pathway, with all animals discriminating cues (Figure 3 f,h,j,l). This behavioral dissociation demonstrates that pathway-specific inhibition leaves cue learning intact while impairing how these threat and non-threat cues modulate reward seeking.

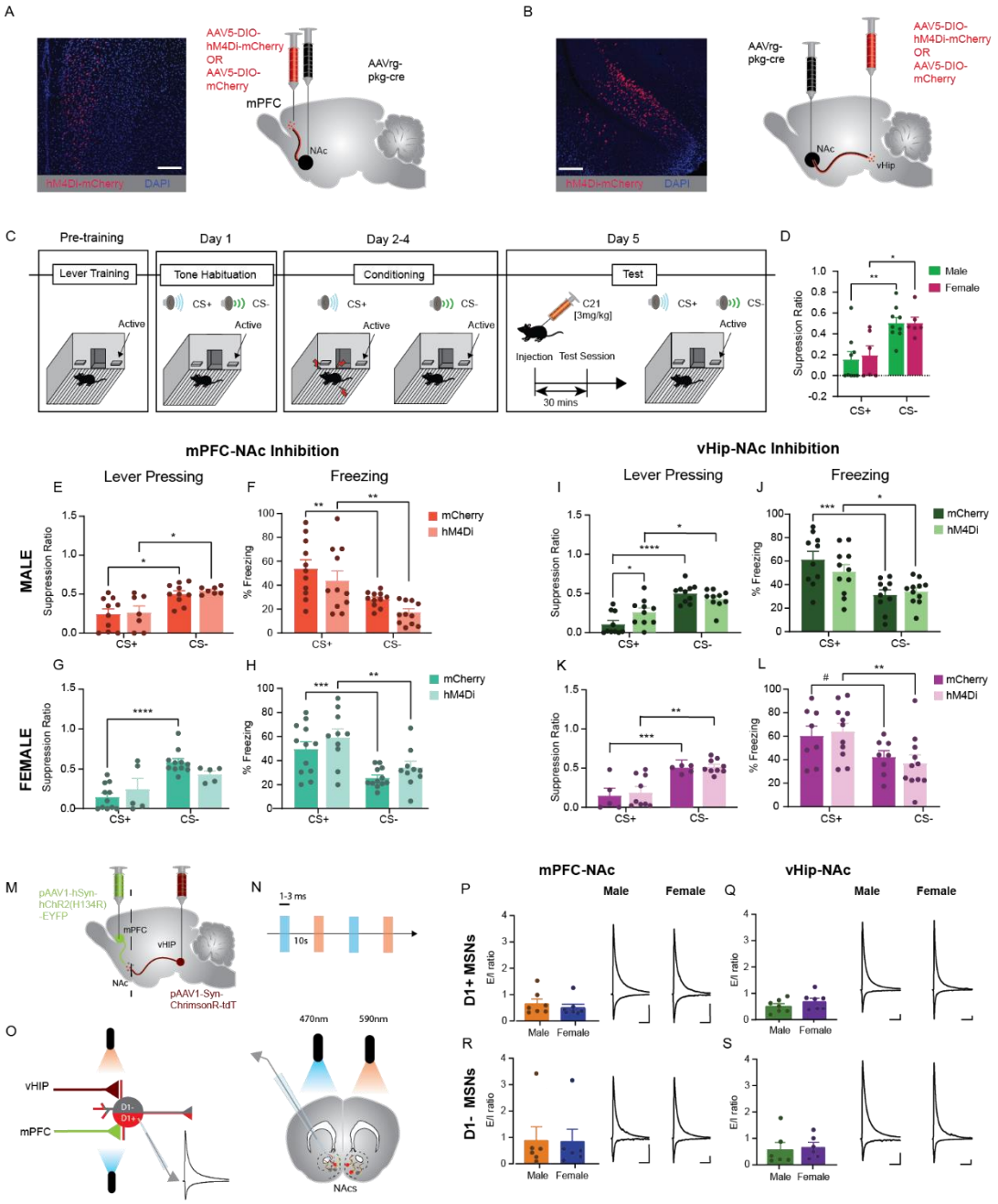
A
AAV5-DIO-hM4Di-mCherry OR AAV5-DIO-mCherry
AAVrg-pkg-cre
mPFC
NAc
hM4Di-mCherry
DAPI
B
AAVrg-pkg-cre
AAV5-DIO-hM4Di-mCherry OR AAV5-DIO-mCherry
NAc
vHip
C
Pre-training
Day 1
Day 2-4
Day 5
Lever Training
Tone Habituation
Conditioning
Test
Active
CS+
CS-
C21 [3mg/kg]
Injection
Test Session
30 mins
D
Male
Female
Supression Ratio
CS+
CS-
mPFC-NAc Inhibition
vHip-NAc Inhibition
Lever Pressing
Freezing
E
F
G
H
I
J
K
L
MALE
FEMALE
Suppression Ratio
% Freezing
mCherry
hM4Di
M
pAAV1-hSyn-hChR2(H134R)-EYFP
mPFC
vHIP
NAc
pAAV1-Syn-ChrimsonR-tdT
N
1-3 ms
10s
O
vHIP
mPFC
D1-
D1+
470nm
590nm
NAcs
P
Q
R
S
mPFC-NAc
vHip-NAc
Male
Female
D1+ MSNs
D1- MSNs
E/I ratio

Figure 3. *Sexually dimorphic control of reward seeking behavior by accumbal afferents is not due to differences in circuit wiring. An intersectional cre-dependent viral strategy targeted an inhibitory DREADD to either the (A) mPFC-NAc or (B) vHIP-NAc pathway (Scale bars represent 200um). (C) Mice were trained in a conditioned suppression paradigm to press a lever for chocolate milk reward prior to a 3-day Pavlovian discriminative fear conditioning paradigm. On a test day, C21 was injected to activate pathway-specific DREADD inhibition and mice were exposed to CS+ and CS- tones without further footshock while lever pressing for reward. (D) Behavioral piloting showed no sex differences in conditioned suppression; both male and female mice showed greater suppression of lever pressing during CS+ versus CS-, indicated by smaller suppression ratios ($F_{(1,13)}$ = 28.77, n=9,6, p=0.0001, post hocs: p_M=0.0012, p_F=0.012). (E) mPFC-NAc hM4Di-DREADD males showed no differences in suppression ratio ($F_{(1,15)}$ = 18.44,n=10,7, p=0.0006, post hocs: p_{mCh}=0.012, p_{hM4Di}=0.021) compared to mCherry-control males during either cue, and both groups discriminated CS+ and CS-. (F) mPFC-NAc inhibition in males did not impact freezing ($F_{(1,20)}$ = 26.13,n=11,11, p<0.0001, post hocs: p_{mCh}=0.004, p_{hM4Di}=0.004). (G) While female mCherry-control mice suppressed lever pressing more during CS+ than CS-, mPFC-NAc hM4Di-DREADD females did not ($F_{(1,14)}$ = 24.20,n=11,5, p=0.0002, post hocs: p_{mCh}<0.0001, p_{hM4Di}=0.18). (H) mPFC-NAc inhibition also did not modulate freezing in females with both groups freezing more to CS+ than CS- ($F_{(1,20)}$ = 35.12,n=12,10, p<0.0001, post hocs: p_{mCh}=0.0007, p_{hM4Di}=0.0012). (I) vHIP-NAc hM4Di-DREADD males and mCherry-controls discriminated CS+ and CS-, exhibiting smaller suppression ratios (increased suppression of lever pressing) to CS+ than CS- ($F_{(1,18)}$ = 42.2,n=12,10, p<0.0001, post hocs: p_{mCh}<0.0001, p_{hM4Di}=0.021). However, (I) vHIP-NAc hM4Di-DREADD males showed less CS+ mediated suppression than mCherry-controls ($F_{(1,18)}$ = 6.103, n=12,10, p=0.024, post hocs: p=0.039. (J) vHip-NAc inhibition did not alter cue discrimination assessed by freezing ($F_{(11,,18)}$ = 26.12, n=12,11, p=0.0005, post hocs: p_{mCh}<0.0001, p_{hM4Di}=0.03). (K) Female vHip-NAc hM4Di-DREADD and mCherry-controls both discriminated CS+ and CS- with no group differences in suppression ratio ($F_{(1,12)}$ = 54.36, n=5,9, p<0.0001, post hocs: p_{mCh}=0.0077, p_{hM4Di}=0.0009). (L)There were also no group differences in freezing during the CS+ cue ($F_{(1,17)}$ = 22.96, n=8,11, p=0.0004, post hocs: p_{mCh}=0.067, p_{hM4Di}=0.0017). (M) To probe circuit connectivity, animals were bilaterally injected with AAV-ChR2 and AAV-ChrimsonR in mPFC and vHIP, respectively (M). 470nm and 590nm light (3ms) was alternated to stimulate mPFC and vHIP terminals, respectively, during patch clamp recordings of optical EPSCs and IPSCs in D1+ and D1- cells in the NAc medial shell (N,O). No differences were observed in oEPSC/oIPSC ratio of D1-MSNs evoked by either mPFC (n=7, p=0.46) (N) or vHIP (n=7, p=0.26) (O) or D2-MSNs evoked by either mPFC (n=6, p=0.94) (P) or vHIP (n=6, p=0.79) (Q) (Scale bars represent 100ms on the x-axis and 100pA on the y-axis).*

vHip-NAc inhibition attenuated conditioned suppression in males but not females whereas mPFC-NAc inhibition impaired conditioned suppression and cue discrimination in females but not males. mPFC and vHip inputs converge in NAc medial shell to regulate medium spiny neuron (MSN) firing [18,19]. Different effects of pathway-specific inhibition between males and females could arise from sex differences in behavioral strategy mediating differential recruitment of similarly wired circuits, or alternatively, sex differences in circuit connectivity [20,21]. We therefore probed possible sex differences in synaptic drive onto the two populations of NAc MSNs, Drd1-enriched (D1- MSNs) and Drd2-enriched (D2-MSNs; largely Drd1-negative). We injected D1-tdTomato mice with pAAV1-hSyn-hChR2(H134R)-EYFP in mPFC and pAAV1-Syn-ChrimsonR-tdT in vHip (Figure 3m), prepared acute NAc slices and recorded D1+ and D1- (putative D2+) MSNs (Figure 3n,o). Optogenetically-evoked EPSCs (oEPSCs) and IPSCs (oIPSCs) were successfully evoked from both inputs in all recorded post-synaptic cells (alternating blue/ orange light) (Figure 3n,o) confirming widespread single-cell input convergence. There were no sex differences in oEPSC/oIPSC ratio for mPFC or vHip in D1+ or D1- MSNs (Figure 3p-r) confirming similar drive in males and females on both cell-types from both pathways. This strongly suggests that sex differences in pathway-specific behavioral control do not result from underlying differential circuit connectivity but rather from differential recruitment of similarly wired circuits in males and females, consistent with the sex-specific neural encoding revealed by fiber photometry (Figure 2).

Integration of reward and aversion is central to adaptive behavior and dysregulated in various psychopathologies [22]. We find that vHip-NAc and mPFC-NAc encode aversive cues and discriminate these from neutral cues to modulate reward behavior under threat. Surprisingly, despite similar behavior and circuit connectivity, we identify a sexual dimorphism in the neural encoding of threat discrimination that may indicate sex-specific strategies in the face of threat. Modest differences in freezing and alterations in inter-pathway synchrony suggest that females, but not males, process a non-reinforced cue as a safety signal, which could contribute to differential pathway recruitment. Chronic stress induces sex- and pathway-specific neural, transcriptional and morphological changes [23,24] and sex-specificity of neural encoding may contribute to sex differences in vulnerability to stress related disorders. Here we identify

mechanisms of threat processing and behavioral integration, identifying unexpected sex-specificity that may yield insight into mechanisms of stress-induced behavioral dysregulation and sex differences in psychiatric risk.

Methods

Animals

Mice were maintained on a 12-h light-dark cycle (lights on at 7:00AM) at 22-25^{0}C group-housed with 2-3 same-sex cage-mates with *ad libitum* access to food and water. All experimental manipulations occurred during the light cycle, in accordance with guidelines of McGill University's Comparative Medicine and Animal Resources Center and approved by the McGill Animal Care Committee.

Neural Recordings*:* D1-cre mice were used with an initial plan to simultaneously interrogate activity in the NAc, but due to technical difficulties, this data was discarded. D1-cre heterozygote mice bread on C57BL/6J background were obtained from Jackson laboratories and bred against D1-cre wild type mice. Pups were weaned at post-natal day 21 and housed with same-sex litter mates and heterozygote mice were separated 4 weeks post-weaning and housed with 2-3 same sex littermates.

Chemogenetic/optogenetic manipulations: 7-week-old male and female C57BL/6J mice were obtained from Jackson Laboratories and habituated to the colony room one week prior to start of manipulations. Mice were food restricted to 85% of their free-feeding body weight during experimentation.

Ex vivo electrophysiology: Male and female D1-tdTomato BAC transgenic mice (B6.Cg-Tg(Drd1a-tdTomato)6Calak/J) initially obtained from Jackson Laboratories were bred at the Comparative Medicine and Animal Resources Centre (CMARC) at McGill University.

Surgeries

Stereotaxic surgery was performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia. To achieve projection-specific GCaMP7f expression in glutamatergic NAc-projecting cells, 0.5µl pGP-AAVrg-syn-jGCaMP7f-WPRE virus [25] (1.85× 10^{13}GC/ml; Addgene) was infused into the NAc (A/P: +1.3, M/L: +/-0.60, D/V: -4.9) at a rate of 0.1µl per min, before raising the needle to D/V: -4.7 and infusing a further 0.5µl virus, allowed to diffuse for 10 min before withdrawing the

needle. Chronically implantable optic fibers (Neurophotometrics) with 200µm core and 0.37 NA threaded through ceramic ferrules were implanted above the ventral subiculum of the vHip (A/P: -3.40, M/L: +/-3.00, D/V: -4.75) and infralimbic mPFC (A/P: -0.3, M/L: +/-1.90, D/V: -2.80). Recordings began minimum 4 weeks after surgery to allow sufficient time for stable and robust retrograde virus expression.

To achieve projection specific expression of inhibitory designer receptors exclusively activated by designer drugs (DREADD) for chemogenetic manipulation, 0.5 µl of AAV-rg-pkg-cre diluted with sterile PBS at a ratio of 1:4 was bilaterally injected into the NAc (AP:+1.30, ML: +/- 1.81, D/V: -4.43, 15^{o} angle) and 0.5 µl of AAV5-hSyn-DIO-hM4D(Gi)-mCherry or AAV5-hSyn-DIO-mCherry was bilaterally injected into either the mPFC (AP:1.7, ML: +/- 0.75, D/V: -2.5, 12^{o} angle) or vHip (AP:-3.40, ML: +/- 3.95, D/V: -4.17, 12^{o} angle). Coordinates were adjusted to facilitate simultaneous bilateral injections targeting the same subregions as for fiber photometry. Manipulations began a minimum of 4 weeks after surgery to allow sufficient time for stable and robust retrograde virus expression.

For ex vivo electrophysiology experiments, stereotaxic injections were conducted on P56 – P77 mice. AAV vectors expressing ChrimsonR (pAAV1-Syn-ChrimsonR-tdT; Addgene) or channelrhodopsin-2 (hChR2) (pAAV1-hSyn-hChR2(H134R)-EYFP; Addgene) were used for independent optical stimulation of glutamatergic afferents in the NAc shell from the ventral hippocampus (vHip) and the infralimbic mPFC, respectively. Virus was bilaterally infused 0.5 µl pAAV1-hSyn-hChR2(H134R)-EYFP at a rate of 0.1 µl / min into infralimbic mPFC and 0.5 µl pAAV1-Syn-ChrimsonR-tdT into vHip.

Apparatus

Behavioral experiments were performed in standard MED Associates operant boxes (15.24 x 13.34 x 12.7 cm) enclosed in sound attenuating chambers outfitted with a programmable audio generator and a house light to deliver cues, shock-enabled grid floors and two retractable levers either side of a food port for delivering liquid chocolate milk reward (Nesquick). Food ports were

closed off during Pavlovian fear conditioning. Data was collected and protocol was run using MED-PC software. Behavior was recorded using Raspberry Pi cameras for offline analysis.

Pavlovian Fear Conditioning

Following a 120s habituation period, mice were exposed to 8, 30s presentations each of an auditory cue (2000Hz, 63dB) and a visual cue (house light). One cue served as a conditioned threat cue (CS+) and co-terminated with a 0.5s, 0.5mA shock, while the other as an unconditioned cue (CS-) with no outcome; cue identity was fully counterbalanced. Cues were randomly presented and were followed by a variable intertrial interval (ITI) averaging 90s.

Freezing ratio (FR) was calculated with the following formula:

$$FR = \frac{F_{cue}}{\left(F_{cue} + F_{precue}\right)}$$

Where F is the percent time freezing during the cue and F is the percent time freezing during an equivalent pre-cue period. A freezing ratio of 0.5 indicates equal freezing during cue and pre-cue. FR greater than 0.5 indicates increased freezing during the cue while an FR less than 0.5 indicates a reduction in freezing.

Discrimination score (DS) was calculated using the equation below:

$$DS = \frac{F_{CS+}}{F_{CS-}}$$

Where F_{CS+} and F_{CS-} are the percent time freezing during the CS+ and CS- cues respectively.

Frame Independent Projected Fiber Photometry

To measure calcium-associated changes in fluorescence in real time, recordings were made from vHip-NAc and mPFC NAc-projecting cells during discriminative Pavlovian fear conditioning. Samples were collected at a frequency of 20 Hz using Neurophotometrics hardware through Bonsai and FlyCap software. Recordings were coupled to the start of behavioral analysis by interfacing Bonsai with MED-PC using a custom DAQ box (Neurophotometrics). Data were extracted and analyzed using custom-written scripts in Matlab R2019a (The MathWorks) [26]. To

normalize the data, the control channel (415nm) was fitted to the raw (470nm). The fitted control was then subtracted from the raw trace. The resultant trace was divided by the fitted control giving the ΔF/F and converted to a z-score. The subsequent trace was detrended to remove bleaching artifacts [27].

Cross Wavelet Transform

To quantify the degree of coherence between vHIP-NAc and PFC-NAc pathways, we employed a cross wavelet transform (XWT) using the Python package PyCWT [28]. Briefly, a continuous wavelet transform (CWT), using the Mexican hat wavelet, was applied to the neural traces from both regions to detect peaks in activity in three separate windows: a pre-cue period ranging from 29 secs before cue onset to 2 secs before cue onset, a cue period ranging from cue onset to 3 secs before cue offset and a post-cue period ranging from 2 secs following cue offset to 29 secs following cue offset. The period around cue offset was excluded to remove the very large increases in activity following footshock. Having calculated the CWT's, identification of synchronous events was performed using the XWT (product of two CWTs). At each time point, the maximum value of the XWT over frequency range 0.5Hz to 1.0 Hz was found. For the resulting waveform, peaks with a significance value exceeding 0.9 [28], and were the product of two positive CWT values, were classed as synchronous events. This process was also performed for time offsets ranging from -1 sec to +1 sec around 0, to confirm that the highest degree of coherence was at time offset of 0 (zero offset indicates peaks that are synchronous between the two pathways). We compared the number of synchronous peaks (within a 0.1sec window) during pre-cue, cue and post-cue periods.

Conditioned Suppression

Mice were presented with 2 extended levers, with active lever presses reinforced by a liquid reward (Nesquik chocolate milk, diluted with water at a ratio of 2:1) and inactive lever presses with no outcome. Mice started on a fixed ratio 1 (FR1) delivery schedule where 1 lever press resulted in 1 liquid reward up to a maximum of 50 rewards. Criteria was met when mice achieved 20 lever presses on the correct lever at 80% accuracy for 3 consecutive days. Mice then progressed through random ratio (RR) training with 20% (RR5), 10% (RR10) and then 5% (RR20)

of active lever presses rewarded, remaining at each stage of RR training until meeting criteria of 20 rewards obtained with 80% accuracy for 3 consecutive days.

Following RR20, mice were trained on a modified Pavlovian fear conditioning with continued reinforced RR20 reinforced lever pressing. Conditioning consisted of 6, 30sec presentations each of two tones (A; 10000Hz, 72 dB; B: 2000Hz, 63dB) counterbalanced as either CS+ or CS- cues; CS+ cue co-terminated in a 0.5mA, 0.5sec shock, CS- no outcome. Following three days of training, conditioned suppression was tested under fear extinction conditions with levers continuing to be reinforced. Mice performing less than 100 lever presses during test were excluded from analysis. Custom scripts in combination with EZ track software was used to measure freezing behavior.

Suppression ratio (SR) was calculated with the following formula:

$$SR = \frac{LP_{cue}}{\left(LP_{cue} + LP_{pre-cue}\right)}$$

Where LP_{cue} is the number of lever presses during the cue and $LP_{pre\text{-}cue}$ is the number of lever presses during an equivalent pre-cue period. A suppression ratio of 0 indicates complete suppression and a ratio of 0.5 indicates no suppression.

Chemogenetic Manipulations

Compound 21(C21) (Hello Bio) was dissolved in 0.9% NaCl and stored in 2mL vials at -20^{o}C. A new vial was thawed before use every day before testing. 30 mins before the start of the test session, mice received an intraperitoneal injection of Compound 21 (C21) (3mg/kg) to activate inhibitory DREADDs.

Ex vivo patch clamp electrophysiology

Brain slice preparation: Mice were deeply anesthetized. Transcardial perfusion was performed with 20-25 ml of ice-chilled carbogenated NMDG artificial cerebrospinal fluid (ACSF) (containing in mM: 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2 \cdot 4H_2O$ and 10 $MgSO_4 \cdot 7H_2O$; pH: 7.3–7.4; osmolality: 300-310 mOsmol/kg). Brain slices (200 μm) were prepared in ice-chilled carbogenated NMDG aCSF by a vibratome (Lecia VT 1200S). After slice preparation, all brain slices were recovery in 32–34 °C carbogenated NMDG ACSF for 10 min. Then, brain slices were transferred into room-temperature carbogenated HEPES holding aCSF (containing in mM: 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2 \cdot 4H_2O$ and 2 $MgSO_4 \cdot 7H_2O$; pH: 7.3–7.4; osmolality: 300-310 mOsmol/kg). Brain slices were kept in HEPES holding ACSF before recording.

Recordings: Whole-cell recordings were performed in room-temperature carbogenated ACSF (containing in mM: 128 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 24 $NaHCO_3$ and 10 Glucose, pH 7.2; osmolality: 300-310 mOsmol/kg). Patch pipettes were filled by cesium-methanesulfonate based internal solution (containing in mM: 130 Cs-methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 5 TEA-Cl, 4 Mg-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine and 1 QX-314; pH 7.2; osmolality: 290-300 mOsmol/kg) for voltage-clamp recordings. D1+ medium spiny neurons (MSNs) and D1- MSNs were patched to record excitatory / inhibitory (E/I) ratio. MSNs were held at -70 mV to measure α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated currents in the present of *N*-methyl-D-aspartate receptor (NMDAR) antagonist, 3-(2-carboxypiperazin-4-yl) propyl-1phosphonic acid (CPP, 10 mM; HelloBio). Inhibitory postsynaptic currents (IPSCs) were recorded at AMPAR reversal potential (CPP, 10 mM) at +20 mV. Each patched MSNs (D1+ or D1-), E/I ratio were recorded by the activation of afferents from the IL and the vHipp. Optically evoked EPSCs and IPSCs were obtained every 10secs. Two different wavelengths of lights from a LED system (DC4100, Thorlabs) were used to activate afferent fibers from the IL (wavelength: 470 nm) and the vHipp (wavelength: 590 nm) alternatively. A pilot study established optimal parameters for independent pathway stimulation. Light pulse width (1-3 ms) and light intensity (150 pA) for both wavelengths of light were held constant for each neuron. This yielded stable

EPSCs (50 – 2000 pA) at -70 mV. All signals were amplified and digitized by Multiclamp 700B (Molecular Device) and Digidata 1550B (Molecular Device) respectively. Series and access resistance were monitored during the experiments and signals were bessel filtered at 2 kHz.

Statistics.

Inferential statistical analyses were performed using GraphPad Prism 7. Grubb's test was used to identify and exclude statistical outliers. Paired t-tests and two-way repeated measures ANOVAs were used where appropriate.

Linear mixed effects regressions were run using custom R scripts with neural activity at cue onset as a fixed effect, an arbitrary categorical variable assigned to each animal to account for inter-individual variability as a random effect and freezing as the dependent variable. An ANOVA was run to compare models using either vHip-NAc, mPFC-NAc or both vHip-NAc and mPFC-NAc activity at cue onset as a fixed variable and inter-animal variability and a random variable to a null model which includes only inter-animal variability as a predicting variable. Chi squared and p-values for the ANOVA are shown in table S1. Marginal R^2 (R^2m) represent the variability explained by fixed effects while conditional R^2 (R^2c) represents the variance explained by the entire model.

Generalized additive models (GAMs)) were generated using the "bam" function in custom R scripts with an arbitrary categorical variable assigned to each animal to account for inter-individual variability as a random effect and cue type as a fixed effect. We generated a set of smooth functions to capture contributions of cue type to variation in neural activity and took the difference of CS+ and CS- functions to find periods of maximal difference in neural encoding.

K Nearest Neighbor classifier was run using custom python scripts and KNeighborsTimeSeriesClassifier from tslearn library. Time series containing delta FF from either mPFC-NAc recordings or vHip-NAc recordings from either the first second of the cue or the entire cue period were used as input for the classifier and labeled based on cue type. Number of nearest neighbors (K) was made a hyperparameter.

Acknowledgements

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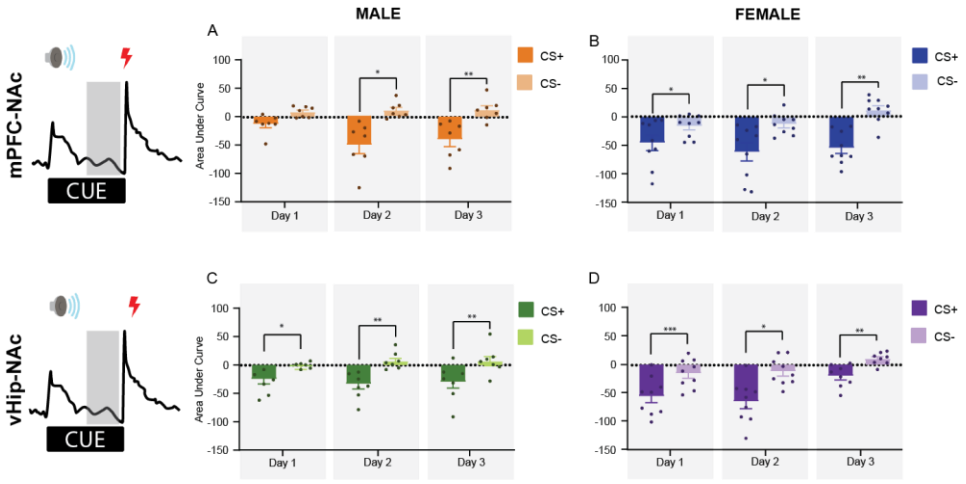
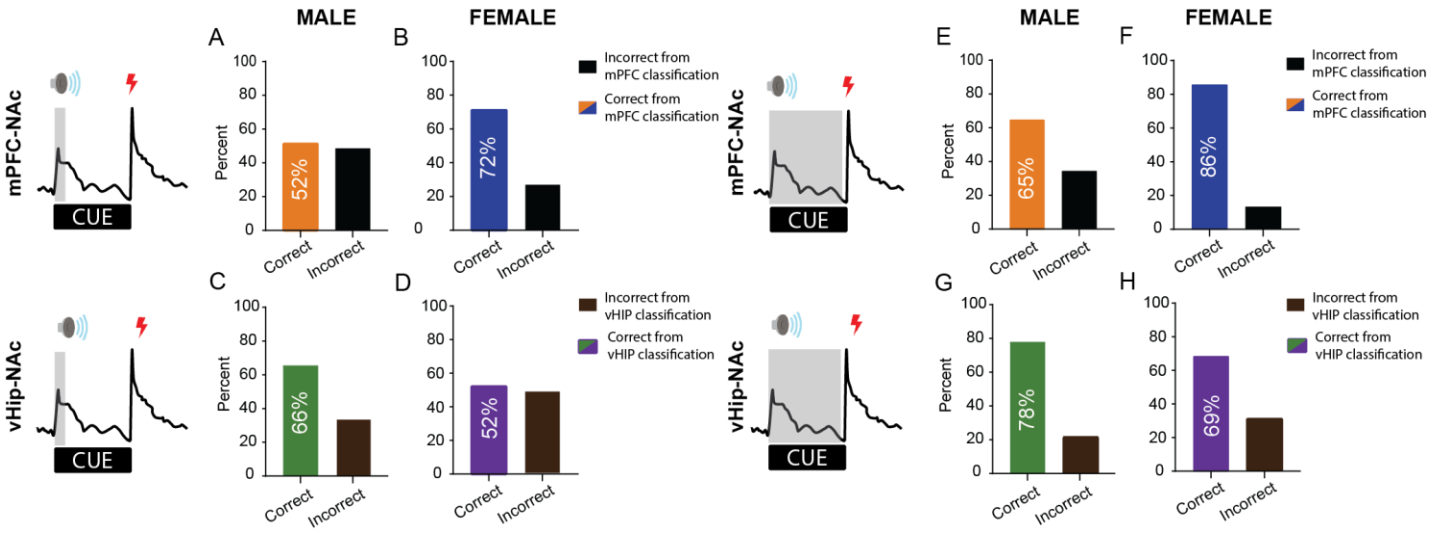
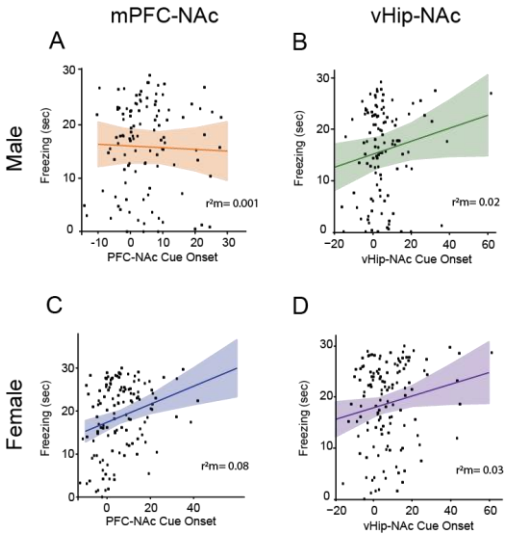
Supplemental Materials



Supplemental Figure 1. *CS+ mediates pre-outcome suppression in neural activity. In females neural activity in both mPFC- NAc (B) (Day 1: n=9, p=0.02; Day 2: n=9, p=0.04; Day 3: n=9, p=0.0016) and vHIP-NAc (D) (Day 1: n=9 p=0.0002; Day 2: n=9 p=0.019; Day 3: n=9, p=0.034) is suppressed during the final 8 sec of the CS+ compared to CS- across all days of training. In males, a similar trend is observed in mPFC-NAc (A) (n=8, p=0.068) and with a significant difference in vHIP-NAc (C)(n=6,p=0.01) in early-training, that is significant in mid- (mPFC-NAc: n=8, p=0.027 vHIP: n=7, p=0.0035) and late-training (mPFC-NAc: n=7, p=0.0041 vHIP: n=7, p=0.02).*



Supplemental Figure 2. *Accumbal afferents encode cue type in a sex-specific manner. Using a K-Nearest Neighbor's (KNN) classifier, cue identity was predicted from neural activity during the first 1 sec (A-D) or entirety (E-H) of the cue. (B) mPFC-NAc activity classified cue identity in females with 72% accuracy (B), but vHIP-NAc at chance levels (52%) (D). Conversely in males, classification with vHIP-NAc was reliable (A) (68%) but at chance with mPFC-NAc (52%) (C). Using the full 30 sec of cue-elicited neural activity increased classifier accuracy across all predictors, however a sex-bias remained with mPFC-NAc (85%) outperforming vHIP-NAc (69%) in females (F,H)) and the converse in males, with 65% accuracy using mPFC-NAc and 78% accuracy using vHIP-NAc (E,G).*



Supplemental Figure 3. *Accumbal afferent activity explains little variability in freezing behavior. A linear mixed effects regression assessed the relationship between freezing and neural activity while controlling for inter-individual variability. In males, mPFC-NAc activity accounts for less than 1% (A) of variance in freezing, while vHIP-NAc activity at cue onset accounts for 2.5% (B). In females, mPFC-NAc activity accounts for 8% of variance (C) while vHIP-NAc activity accounts for 3% (D).*

	Fixed variable	AIC	BIC	Log(Lik)	deviance	R^2m	R^2c	ChiSq	P
Female	None	903.26	911.8	-448.6	897.26	NA	0		
	vHIP Cue Onset	901.36	912.8	-446.7	893.36	0.03	0.04	9.079	0.05 *
	mPFC Cue Onset	894.15	905.55	-443.1	8886.2	0.08	0.1	11.118	0.0009*
	mPFC+vHIP Cue Onset	895.82	910.08	-442.91	885.82	0.08	0.1	11.441	0.003*
Male	None	794.62	802.77	-394.31	788.62	NA	0.15		
	vHIP Cue Onset	793.23	804.10	-392.61	785.23	0.03	0.18	0.1598	0.69
	mPFC Cue Onset	796.46	807.33	-394.23	788.46	0.001	0.15	3.3891	0.06
	mPFC+vHIP Cue Onset	792.52	806.12	-391.26	782.52	0.06	0.20	6.0909	0.05 *

Table 1. *Relationship between neural activity at cue onset and freezing behavior. ChiSq and p values are for comparisons of model with no fixed variable (just random variable) and a model with the indicated variable as a fixed variable as well as a random variable (see methods for detail). In females, vHIP-NAc activity at cue onset and mPFC-NAc activity at cue onset significantly improve a null model an explain 3% and 8% of variance, respectively. In males, neither vHIP-NAc or mPFC-NAc activity at cue onset improve a null model and explain 0.1% and 3% of variance, respectively.*

Chapter 5

Discussion

Stress is a major risk factor for depression, but most who encounter stress do not go onto develop the disorder indicating that there is some additional vulnerability factor that remains to be understood (13, 14). Using pre-clinical stress models for depression (48, 49), I aimed to identify how this differential vulnerability is encoded in neural circuits previously shown to be involved in behavioral adaptation to stress and then dissect how these circuits integrate aversive information to shape behavior.

5.1 Probing Vulnerability

Current treatments for depression fail to adequately treat the disorder in up to 50% of people (10). Even among those for whom treatment is effective, depression will often return, with each episode further increasing the risk for a subsequent episode (10), making prevention an important goal in developing new treatments. Stress can trigger depression, but there is considerable variability in how individuals respond, with some developing the disorder and others developing resilience (13). Previous studies have generally focused on the mechanisms of stress induced behavioral dysregulation, identifying differences between mice that are susceptible or resilient after chronic stress (92, 129, 132). Although this has provided valuable insights into the mechanisms of susceptibility, withing such experiments it is impossible to disentangle stress-induced changes from pre-existing mechanisms of vulnerability. In chapter 2, I present one of the first pre-clinical studies examining circuit mechanisms of vulnerability(205, 206)(204, 205)(204, 205). Using novel analyses for in vivo calcium imaging data, I identified, for the first time, differences in cell-type specific neural activity that were associated with behavioral adaptation to stress, finding increased activity in D1-MSNs at baseline and during social interaction in future resilient animals (207). I also identified differences in how D1-MSNs encode social stimuli, with resilient but not susceptible mice differentiating engagement with a social stimuli and entry to a corner zone. Our work was consistent with previous findings identifying D1-MSN activity as being pro-resilient following stress (132) but suggests that some of these differences between resilient and susceptible mice identified after stress, may be present before and could constitute mechanisms of vulnerability. Since I published this work, a number of other groups have elaborated other potential vulnerability markers, including brain region–specific metabolic profiles (208, 209), neural activity (207, 210, 211) and pre-existing behavioral

alterations (208, 209, 212) that associate with susceptibility to future stress in rodents. Identifying biomarkers that both are translatable to the human condition and represent targetable mechanisms continues to be an important research goal.

The nucleus accumbens has been a consistent focus for identifying mechanisms of vulnerability. McCullough *et al.* (211) looked at the mechanisms underlying sleep disturbances seen with CSDS. Systematically examining the effects of chemogenetic manipulations of D1- and D2-MSNs, they showed that inhibiting D1-MSNs and exciting D2-MSNs recapitulates the effects of CSDS on sleep, while mice in which D1-MSN activity was increased showed partially corrected sleep and D2-MSN inhibition had no effect. However, these manipulations had no impact on depressive- and anxiety-like behavior in stress-naïve animals. Rather, exciting D1-MSNs before stress increased resilience to later defeat, while inhibition led to susceptibility after a sub-chronic defeat, showing that D1-MSN activity modulates vulnerability to future stress. Furthermore, although social interaction and anxiety-like behavior were not altered, sleep disturbances were observed, a phenomenon typically associated with susceptibility itself. These findings align with our own, in suggesting that neural dysregulations associated with susceptibility after stress may actually be present before stress, not only contributing to future vulnerability but also manifesting in subtle yet observable behavioral alterations. Recent work by Radwan *et al.* (213) also identified pre-existing sleep disturbances in susceptible mice, showing that before CSDS, future susceptible mice sleep more than future resilient mice, and also exhibit disruptions in their sleep cycle which accurately predicted susceptibility to a future defeat. In humans, sleep disturbances have been shown to precede the emergence of depression and are predictive of recurrences, potentially making them a useful biomarker for depression vulnerability (214). The findings of McCullough *et al.* (211) replicate our findings from chapter 2, linking D1-MSN activity to future resilience (207), but extend it to sleep, establishing a causal link between a precursor to susceptibility, decreased D1-MSN activity, and a potential target for preventative therapeutics, sleep disturbance.

Mounting evidence points to other subtle behavioral biomarkers of susceptibility associated with latent vulnerability, rather than a truly “hidden” phenotype that emerges only after stress. Larrieu *et al.* (208) found that social rank was predictive of stress susceptibility and correlated

with individual differences in anxiety like-behavior before stress, with dominant males exhibiting increased anxiety-like behavior compared with their subordinate counterparts and increased vulnerability to defeat. To determine if a metabolic profile might explain these differences in stress response, they examined metabolite patterns in the NAc associated with vulnerability, defining a factor accounting for the majority of the variance in the NAc metabolic profile. Dominant animals showed higher levels of this factor in the absence of stress compared with their lower-rank (subordinate) cage mates, leading the authors to hypothesize that resilient mice are better able to handle the metabolic load caused by stress owing to specific metabolite profiles. Follow-up work by the same lab found that treatment with acetyl-L-carnitine during stress, rescued many stress-induced behavioral deficits (209). Critically, they showed that the pre-existing differences in metabolic profiles in the NAc associate with a clear behavioral phenotype that is present before stress and identified a targetable mechanism for drug treatment.

A number of other studies confirm that anxiety-like behavior in rodents is predictive of future stress susceptibility. Nasca et al (212) showed that anxiety-like behavior in a light dark box correlated with impaired glutamatergic signaling in the hippocampus and increased IL-6 release, both metrics that have been associated with vulnerability to stress (205, 206). Consistent with this, in chapter 3, I show that increased anxiety-like behavior correlates with increased vHIP-NAc activity as mice explore their environment and that both of these metrics predict future stress susceptibility (210). In humans, anxiety and depression are highly co-morbid with anxiety also predicting higher risk of recurrence. Trait anxiety also contributes to neuroticism (vulnerability to negative emotions), a prominent risk factor for depression (215), making this a potentially translatable biomarker for depression vulnerability. That being said, this is not reported in all studies. McCullough et al did not find differences in anxiety-like behavior by stimulating D1- and D2-MSNs in a standard OFT, perhaps pointing to circuit specificity of this behavior (only dysregulations in certain regions will increase anxiety-like behavior). Other papers simply do not look into this biomarker (207, 216).

Building upon previous literature which had identified cell-type specific activity in the NAc as a key regulator of susceptibility (132), I established that pre-existing differences in NAc neural

activity predict future behavioral adaptation to stress, providing one of the first demonstrations of a neural signature of vulnerability (207). There is now considerable evidence establishing that pre-existing dysregulations influence an individual's response to stress, ultimately identifying both behavioral and neural predictors for susceptibility, some with considerable translatability to clinical depression (208, 211, 212). Given poor treatment outcomes and high rates of recurrence of depression, it is of particular value to identify vulnerable individuals before the emergence of the disorder in the hope that the disorder might be prevented instead of merely treated. Findings from my research and subsequent work of other groups holds promise for developing clinical biomarkers with targetable mechanisms for individualized treatment.

5.2 A discussion on the inclusion of females in pre-clinical research

One major risk factor contributing to increased vulnerability to depression is sex. A wide body of literature has shown female mice to be susceptible to shorter lengths of stress compared to males, which maps onto the human condition where women are twice as likely to develop the disorder compared to men (7). This is particularly concerning given the majority of research in animal models for depression has exclusively used males. Indeed, many papers have relied on CSDS (48, 131, 188, 207), a paradigm that is difficult to translate to females (40, 45). Most of our knowledge of basic science, behavior and circuit function comes from males and when females have been included, experimental designs often rely on behaviors and assumptions validated in males. Results can be misleading given ample evidence that females exhibit different behavioral strategies (55, 217, 218), such as more active escape responses during shock predicting cues (56). The few stress studies which have included both males and females provide compelling evidence of sex differences in stress susceptibility. Following a 21-d CVS, there is evidence of sex-specific gene transcription networks across the NAc, vHIP and PFC (ventromedial PFC) (50, 51, 219) as well as functional and morphologically distinct alterations in the PrL-NAc in males and females (53). Importantly, females are susceptible to a 6d-SCVS (49, 206), while males are resilient and this may be due in part to a pro-resilient effect of testosterone in males (200) as well as differences in expression levels of certain genes (51).

The widespread bias in pre-clinical research is fueled, in part, by the idea that females are "more complex" or "more difficult" to study due to cyclical fluctuations in estrous cycle which may impact behavior and other metrics (220). In reality, males also display hormonal variability with testosterone exhibiting circadian fluctuations (221) and considerable inter-individual differences between cage mates. For example dominant animals have significantly higher levels of testosterone compared to their subordinate cage mates (222). Testosterone is well known to lead to variability in behavior (200), and yet, studies including males do not quantify levels of this hormone. This brings up an important distinction between studying sex differences and studying both sexes (223). The former refers to an area of research that aims to explain why/ how males and females differ. The latter aims to study the same phenomenon in both sexes, being aware that males and females may behave differently, or achieve similar behaviors through different mechanisms. The difference between sex differences and studying both sexes also informs experimental design. To study sex differences, identical paradigms should be applied to both in an attempt to reveal behavioral differences and their underlying mechanisms. However, studying similar phenomena in both sexes can require use of different paradigms. Williams et al (200) used a 6-d SCVS to investigate differential vulnerability, finding that testosterone leads to decreased excitability in vHIP-NAc and protects against susceptibility, leaving males resilient and females susceptible to a sub-chronic stress. In chapter 4, I use identical protocols in both sexes to understand how threat is encoded, finding ultimately that this is different in males and females. In chapter 3, I took into account sex differences in vulnerability and designed a sex-specific protocol to induce similar behavioral phenotypes in males and females, ultimately identifying similar mechanisms of vulnerability and susceptibility (210). To this point, research looking into the source of sex differences or impact of hormones on behavior should include hormone levels as a variable. In this case, estrous cycle as well as testosterone levels should be noted (220). However, in work studying both sexes which makes no conclusions about why sex differences are present or the potential impact of gonadal, neither testosterone nor estrous cycle are relevant variables. My work aims to study mechanisms of encoding in both sexes to comprehensively understand neural processing, without a primary focus on the source of potential sex differences. Ultimately, females represent 50% of the population and are twice as likely to get depression

compared to males; including females in basic research is essential. However, care needs to be taken to use appropriate protocols and tests that investigate or account for sex differences and that allow us to make conclusions in both sexes rather than studying females in a manner biased by a male-centric literature.

Similarly, it is important to study both sexes thoroughly before making an assumption that there are no sex differences. In chapter 5, I identify sex differences in the neural discrimination of aversive and neutral cues, with PFC-NAc discriminating in females and vHIP-NAc discriminating in males. Interestingly, males and females appear similar across many metrics. They both show similar behavioral discrimination of aversive and neutral cues and encode aversive information in both pathways, showing increased PFC- and vHIP-NAc activity in response to both shock and CS+ onset and a decrease in activity in anticipation of shock. It is only when examining neural discrimination of aversive and neutral cues that I identified a sex-specific divergence in neural encoding. This difference was confirmed with chemogenetic inhibition of either vHIP- or PFC-NAc during a conditioned suppression task, which led to a suppression of lever pressing in males and females, respectively. Other papers have failed to report sex differences in fear conditioning paradigms, combining males and females for neural analyses following similar behavioral results. Pignatelli et al, for example, (189) combined males and females without exploring sex differences in neural encoding of aversive cues. Furthermore, instead of doing a between subjects' study where mice learn a cue shock association in the presence of a CS- cue, they have a tone control group that never gets exposed to shock, thus potentially masking the sex differences found in our paradigm. Our findings highlight the importance of rigorously studying both sexes and disaggregating data as sex difference may be present at any level.

5.3 Influence of NAc afferents on behavior and stress response

Early chapters focused on identifying neural signatures of vulnerability in males and females. However, finding how accumbal circuity seemed to predict behavior and be modulated by stress, one question that emerged was how these pathways encode aversive information was integrated to influence behavior and whether this was similar in males and females given sex differences I observe in stress vulnerability. The NAc is considered a limbic motor interface, integrating

information from various projections to modulate behavioral output. The PFC and vHIP, among other glutamatergic afferents, send excitatory inputs to the NAc to drive firing in MSNs, which have low excitability and require significant excitatory drive to initiate action potentials. Information from these projections is thought to have significant influence on behavior through their interaction with each other. The question is what do these pathways encode and how do they influence behavior?

Previous literature suggests multiple, sometimes conflicting roles for these pathways. Both IL- and vHIP-NAc projections can drive motivated behavior, with stimulation supporting lever pressing and conditioned place preference (141), implicating these projections in reward. However, they are also implicated in encoding aversive information. vHIP-NAc displays increased activity in response to aversive stimuli and cues, contributing to footshock-induced negative affective states (189), as well as driving chronic stress-induced social avoidance behavior in males (92). In chapter 4, I found increases in vHIP-NAc activity following stress as well as finding that baseline vHIP-NAc activity predicts anxiety-like behavior and future stress vulnerability (210). IL-NAc also encodes conditioned aversive cues (224) but drives social interaction behavior following stress (92). In chapter 5, I find that both projections encode aversive stimuli and the cues that predict them, but discriminate in a sex specific manner, ultimately driving the suppression of reward seeking behavior to aversive cues. In line with this, increased vHIP-NAc behavior also predicts decreased anxiety-like behavior, measured by increased avoidance behavior in an open field. How then do these projections encode both aversion and reward? I hypothesize that distinct populations of cells contained in these projections carry information about the aversive or rewarding nature of the environment. Evidence in these and other pathways points to functionally distinct populations of cells in the same region. In the PrL-NAc, an adjacent region, a small population of projecting cells encoded the aversive nature of the environment; stimulation of these isolated cells, but not the whole population, drove avoidance behavior (100). Similarly, a specific population of IL-NAc projections receive input from the BLA and encode aversive stimuli and cues (224). Likely, certain cells project from the vHIP and PFC, providing input to drive goal directed/approach behavior while, others may carry information about threat that serves to reduce it. In the case of stress, there are neural dysregulations that impact this signaling,

ultimately leading to impaired approach/avoidance and motivated behavior (53, 189, 210). Following chronic stress, I see an increase in neural activity in the vHIP-NAc pathway, driven by increased input to the cell. This pathway likely carries contextual information in order to influence how an animal engages with environmental stimuli and stress-induced hyperactivity in this pathway likely leads to dysregulated behavior, such as social avoidance, anhedonia and anxiety-like behavior. Importantly, I find a non-significant relationship between vHIP-NAc activity and immobility in a TST, indicating that passive coping is not regulated by this pathway. In line with this, I confirm freezing to an aversive cue is also not regulated by vHIP or PFC-NAc, indicating that perhaps these pathways influence active approach/ avoidance responses in face of varying stimuli. However, more work will be needed to look into these hypotheses. In particular, my work relied on fiber photometry which is a powerful technique for measuring in vivo neural activity, but is limited in that it measures a bulk population level signal. Thus, functional specificity of specific cell subpopulations would have been obscured. In order to probe this hypothesis, it is necessary to use single cell imaging techniques such as miniendoscopes or two photon imaging in order to identify populations of cells that are responsive to aversive stimuli as well as functional tagging of neurons to drive/inhibit activity in these cells specifically (100, 101). Furthermore, it is possible that these pathways carry a salience signal, while input from other regions may carry information about valence in a similar way. But again, these hypotheses require additional testing.

5.4 Limitations and Future Directions

This thesis has presented novel data about how vulnerability to stress was predicted by pre-existing differences in neural activity and how this signal related to behavior. I also presented work clearly showing the importance of studying both sexes in basic research. However, there are technical and methodological limitations as well as open questions that require further investigation.

An obvious limitation is the exclusive use of males in chapter 2. We, like previous literature, used CSDS as a model for depression in order to get defined populations of resilient and susceptible mice, thus limiting ourselves to one sex. WDS, although translatable to females, does not produce

as severe of a stress phenotype compared to CSDS, thus making it difficult to define resilient and susceptible populations, although some groups are looking to do this with alternative behavioral tests (46). CSDS on the other hand produces a bimodal stress population, with resilience and susceptibility being associated with distinct neural adaptations that differ from control; this is yet to be thoroughly explored in either sex with WDS. Future work should certainly look into the role of these cells in driving vulnerability in females as well, perhaps in a way that doesn't rely on defined phenotypes (as in chapter 3).

In chapter 5, I use chemogenetic inhibition to look at the role of vHIP and PFC-NAc pathways on the suppression of reward seeking behavior to aversive cues, finding that suppression of these pathways inhibits this suppression in a sex specific manner. Ideally, I would also have presented the effect of an excitatory manipulation to determine if behavior could be induced by pathway-specific stimulation of neural activity. In other experiments not shown in this thesis, I optogenetically stimulated this pathway over the first 10s of the CS+ following a conditioned suppression task with a subthreshold Pavlovian fear conditioning paradigm in which animals show mild discrimination and low levels of freezing. I hypothesized that this would lead to an increase in suppression to the CS+ cue in a sex specific manner, however I saw no effect. Previous literature provides an explanation for this. PrL-NAc projections have also been shown to encode aversive information, although excitation of the pathway has no effect on reward seeking. Suppression of reward seeking was only driven by stimulating a functionally defined population of cells that respond to the shocks (100). This provides evidence, in a different circuit, of functionally specific parallel population in the same projection that likely serve different functions. Future work using more precise, activity-based targeting techniques could look into this phenomenon.

Future work should explore the source of pre-existing differences in circuit mechanisms of vulnerability. Our work and others have shown pre-existing differences in behavior (208, 210, 212), circuit activity (207, 210, 211) and molecular mechanisms (205, 208) drive differences in stress susceptibility. However, little is known about where/ when these differences emerge. Perturbations in early life have been shown to drive differences in stress susceptibility without influencing pre-stress behavior (225). ELS is thought to prime the brain, leading to long lasting

changes in circuit function that leave an animal vulnerable to stress in adulthood; this is what's known as a two-hit stress model where initial disruption need a second "hit" or stressor to drive widespread behavioral abnormalities (226, 227). But how do these differences emerge in the absence of major disruptions such as ELS? Uncontrollable variability in early-life experience may play a role. Maternal care in the first week of life, specifically levels of licking and grooming, lead to differences in hippocampal synaptic and neuronal properties (228-230), having a profound effect on fear learning in adulthood. Several of these properties have been associated with susceptibility to chronic stress (212). Hormones may also influence vulnerability. Testosterone has been shown to confer resilience to a 6-day SCVS by reducing vHIP-NAc excitability, providing an explanation for increased vulnerability in females (200). Examining levels of testosterone and other hormones may shed light on individual differences in vulnerability as well. Alternatively, compounding of stochastic differences during development may drive differences in behavior.

In these chapters, I have presented various instances of sex differences in behavior as well as neural encoding. My goal was to comprehensively probe mechanisms of aversive encoding and stress vulnerability in both sexes. Although it is beyond the scope of this work, one interesting question that remains is the source of these sex by pathway interactions. Hormone driven sex differences have been described in the development and pruning in several brain regions, including the mPFC and vHIP (231) and many of these same regions are associated with cognitive functioning in adulthood (232, 233). Furthermore, PFC disruptions in adolescence lead to impaired behavioral inhibition in adulthood in males (234), making developmental differences a good candidate to explain these sex differences in the neural control of behavior. Although, more work needs to be done to look at how sex differences in development relate to the functional role of these regions in fear conditioning and stress susceptibility in adulthood (231). Another potential explanation could be that males and females employ different strategies in the face of threat, thus recruiting different pathways. Evidence suggests that females exhibit different behavioral responses to predator odors (235, 236) and to shock predicting cues (56). Indeed, I find modest differences in safety signaling, with females freezing less to CS- compare to baseline. This may explain differential neural recruitment in response to threat.

Finally, I investigated how accumbal afferents integrate aversive information in to probe the basic function of these pathways. Stress is a bombardment of aversive stimuli. Understanding how aversive stimuli are encoded and discriminated from neutral stimuli in a controlled paradigm can give insight into the neural mechanisms regulating stress adaptation. However, I are aware that individual differences in this encoding could be an important element of stress vulnerability. Future work should examine the relationship between individual differences in encoding of aversive stimuli and stress vulnerability. For example, Pignatelli et al used fear conditioning as a stress paradigm, finding that it led to anhedonia and passive coping behavior. Perhaps the amplitude of neural response to shock or degree of neural discrimination may be predictive of the susceptibility. In our data, females show increased response to shock in their PFC-NAc, which could potentially perhaps contribute to increased vulnerability to stress.

Chapter 6

Summary and Conclusions

This thesis began with a simple question, that, at the time, had gone unexplored in the literature: how do pre-existing differences in neural activity influence one's susceptibility to a future stress? I developed and employed a novel analysis method to quantify peaks in calcium fluctuations from populations of neurons allowing me to identify, for the first time, differences in cell-type specific activity in the nucleus accumbens that associated with behavioral phenotype following chronic stress. This result was consistent with previous work identifying a role for these cell-subtypes in behavioral adaptations to stress (132) but suggested that mechanisms of susceptibility were actually present before stress and represented mechanisms of vulnerability. Since then, there has been a push to uncover how pre-existing differences may pre-dispose individuals to being susceptible. In follow-up work, I used a similar design to identify signatures of vulnerability in projections to the NAc from the vHIP, a pathway that had been previously implicated in susceptibility, effectively extending my original finding to an upstream pathway and expanding to females as well as males. Using a longitudinal design, I tracked stress-induced changes in neural activity, finding increases in vHIP-NAc activity following stress that map on to a pre-synaptic mechanism. Although many papers have identified differences in glutamate release probability (92) and excitability (200) in susceptible vs resilient animals, none, to our knowledge had confirmed this in vivo up until now. I also identify behavioral specificity in the signal finding that it predicts social interaction and anxiety -like behavior but not passive coping. Expanding our focus to also include the PFC-NAc projections, a pathway that has important interactions with the vHIP-NAc, I examined how these circuits encode aversive information to influence behavior. As stress involves chronic exposure to aversive information, this is central to understanding behavioral adaptation to stress. I find that both pathways encode aversive information in both sexes, while discrimination is pathway and sex specific. Surprisingly, I find that this pathway doesn't drive classical fear behavior but drives suppression of reward seeking behavior to aversive cues. This work contributes to the sparse literature involving females, identifying important sex differences in neural encoding of discrimination.

Taken together, these studies demonstrate that individual differences in neural activity in specific pathways determine how animals interact and engage with their environment. These same pathways integrate aversive information to influence behavioral output and are sensitive to

chronic stress. Individual differences in the activity of these pathways, as well as certain behaviors, are predictive of future stress susceptibility. Although speculative, these differences in neural activity may emerge from small variations in rearing and early life that ultimately predispose some individuals to susceptibility. This work has potential implications for the treatment and prevention of depression. Finally, our data has made clear that pre-existing differences in neural activity contribute to vulnerability, essentially showing that the way circuits encode various stimuli to drive behavior is telling of how they will respond to stress and making it clear that dysregulations that have been associated with susceptibility may be present before stress and contribute to vulnerability. It follows that following stress, susceptible mice and depressed humans, who present with a wide range of neural dysregulations that significantly impact behavior, will be more at risk for recurrence. Thus, it is wise to prevent the disorder before the emergence of these severe symptoms when neural dysregulations are limited and behavioral deficits, subtle, rather than waiting to treat a full-blown disorder.

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