The Mechanisms of Norepinephrine Signalling in Developing Resilience to Trauma-type Stress in Mice

by

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ABSTRACT

Stress-related psychiatric disorders, such as depression, are characterized by dysregulated neurobiological responses to chronic stress, but the mechanisms that drive resilience or susceptibility to these conditions remain poorly understood. This thesis explores the role of norepinephrine in modulating stress responses, specifically in the development of resilience to trauma-type stress. Using the learned helplessness paradigm in mice, we investigated the neurobiological differences between resilient, susceptible, and control groups, with a focus on c-Fos expression as a marker of neuronal activation. Preliminary heatmaps of qualitative data identified elevated levels of c-Fos expression in the primary and secondary motor cortex, the bed nucleus of the stria terminalis (BNST), and the prelimbic cortex (PL) of the prefrontal cortex following exposure to the learned helplessness paradigm.

Our study did not find significant differences in c-Fos expression between resilient, susceptible, and control mice, which may be due to the limited sample size. However, we did observe significant differences between VMAT2^{DBHcre} KO mice and wild-type mice. In the VMAT2 knockout, the normal process of norepinephrine packaging into synaptic vesicles is disrupted and norepinephrine could not be released into the synaptic cleft. Specifically, wild-type mice showed elevated c-Fos expression across the three target brain regions compared to the KO mice. This observation demonstrates the pivotal role of NE transmission in leveraging the effects of stress in the learned-helplessness paradigm, and the necessity for a better understanding of its role.

Overall, this research contributes to the growing understanding of how chronic stress alters brain function and behavior, emphasizing the critical role of norepinephrine in stress resilience. By investigating both neurobiological mechanisms and behavioral outcomes, these findings offer insights that could inform future therapeutic approaches for treating depression and other stress-related disorders.

RÉSUMÉ

Les troubles psychiatriques liés au stress, comme la dépression, sont caractérisés par une dérégulation des réponses neurobiologiques au stress chronique, mais les mécanismes qui déterminent la résilience ou la susceptibilité à ces conditions restent mal compris. Cette thèse explore le rôle de la norépinéphrine dans la modulation des réponses au stress, en particulier dans le développement de la résilience au stress de type traumatique. En utilisant le paradigme de l'impuissance apprise chez la souris, nous avons étudié les différences neurobiologiques entre les groupes résilients, sensibles et de témoins, en mettant l'accent sur l'expression de c-Fos comme marqueur de l'activation neuronale. Les cartes thermiques préliminaires des données qualitatives ont identifié des niveaux élevés d'expression de c-Fos dans le cortex moteur primaire et secondaire, le noyau du stria terminalis (BNST) et le cortex prélimbique (PL) du cortex préfrontal après l'exposition au paradigme de l'impuissance apprise.

Notre étude n'a pas mis en évidence de différences significatives dans l'expression de c-Fos entre les souris résilientes, sensibles et témoins, ce qui peut être dû à la taille limitée de l'échantillon. Cependant, nous avons observé des différences significatives entre les souris VMAT2^{DBHcre} KO et les souris de type sauvage. Chez les souris VMAT2 knock-out, le processus normal de conditionnement de la noradrénaline dans les vésicules synaptiques est perturbé et la noradrénaline ne peut pas être libérée dans la fente synaptique. Plus précisément, les souris de type sauvage présentent une expression élevée de c-Fos dans les trois régions cérébrales cibles par rapport aux

souris KO. Cette observation démontre le rôle central de la transmission de la NE dans l'optimisation des effets du stress dans le paradigme de l'impuissance apprise, et la nécessité de mieux comprendre son rôle.

Dans l'ensemble, cette recherche contribue à une meilleure compréhension de la manière dont le stress chronique altère les fonctions cérébrales et le comportement, en soulignant le rôle critique de la norépinéphrine dans la résilience au stress. En étudiant à la fois les mécanismes neurobiologiques et les résultats comportementaux, ces résultats offrent des perspectives qui pourraient éclairer les futures approches thérapeutiques pour traiter la dépression et d'autres troubles liés au stress.

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Finally, I extend my heartfelt thanks to my families and friends for their patience, understanding, and support throughout this research journey.

CONTRIBUTION OF AUTHORS

Dr. Giros and Dr. Bairachnaya conceptualized the project and contributed to the development of the experimental design. During the first year of my master's program, I collaborated with Dr. Bairachnaya on various behavioral experiments for her study, which helped familiarize me with the lab and experimental protocols. For this study, I independently scheduled and conducted all the learned helplessness experiments, with occasional assistance from Dr. Bairachnaya as needed. Dr. Bairachnaya and PhD student Eric Daroczi assisted in the mouse brain perfusions and extractions post learned helplessness experiment. I was responsible for slicing the extracted brains using the cryostat, performing immunohistochemistry on all brain slices, and capturing brain images using a fluorescence microscope. Dr. Bairachnaya provided guidance on data analysis, and both Dr. Giros and Dr. Bairachnaya offered valuable feedback on the writing of my master's thesis.

ADDITIONAL STUDENT CONTRIBUTIONS

As a side project during my master's degree, I contributed to a series of experiments investigating the effects of FTX-101, a drug under development that modulates the Plexin A1/Neuropilin 1 receptor complex. FTX-101 is being developed for clinical applications aimed at repairing the myelin sheath. Our experiments were designed to ensure that the drug does not produce any deleterious behavioral effects. I participated in all stages of the experiment, which were conducted in collaboration with Maryia Bairachnaya. The appendix provides a comprehensive overview of the experimental methods used, including the open field test, the elevated plus maze, the novel object recognition test (evaluated for both short- and long-term memory), and the rotarod test to assess motor coordination. Furthermore, the appendix details the results obtained from these experiments and discusses their implications within the broader context of the study (see Appendix B, p.50).

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LIST OF ABBREVIATIONS

AF555	Alexa fluor 555
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
c-Fos	Cellular fos
CRH	Corticotropin-releasing hormone
CSDS	Chronic social defeat stress
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DBH	Dopamine beta-hydroxylase
KO	Knockout
LC	Locus coeruleus
LH	Learned helplessness
M1	Primary motor cortex
M2	Secondary motor cortex
MC	Motor cortex
NE	Norepinephrine
NGS	Normal goat serum
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween detergent

PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI	Piriform cortex
PL	Prelimbic cortex
TD1	Test day 1
TD10	Test day 10
VMAT2	Vesicular monoamine transporter 2
$VMAT2^{DBHcre}$ KO	Vesicular monoamine transporter 2 gene knockout in dopamine beta-hydroxylase expressing neurons
WT	Wild-type

1. INTRODUCTION

Depression is one of the most prevalent and debilitating mental health disorders worldwide, affecting millions of individuals across diverse age groups, cultures, and socioeconomic backgrounds. Symptoms of depression range from pervasive sadness, anhedonia, and feelings of hopelessness and worthlessness to severe cases marked by chronic suicidal ideations[1]. While depression and other anxiety/mood disorders are separate diagnoses, they frequently co-occur and share overlapping symptomatology and underlying neurobiological mechanisms. The symptoms of depression can profoundly impact various domains of life, including relationships, work, and physical health, leading to significant impairment and diminished quality of life for those affected[1,2]. Considering their pervasive impact, understanding the underlying mechanisms of major depressive disorder is critical for developing effective interventions, treatments, and improving outcomes for individuals struggling with mental illness.

The concept of learned helplessness in humans, coined by psychologist Martin Seligman in the late 1960s, highlights a coping deficit in escape or avoidance following exposure to uncontrollable stressors[3]. It suggests that an event's negativity isn't inherent but rather arises from an individual's cognitive interpretations of the situation[3]. While some individuals may develop depressive disorders under similar circumstances, others demonstrate resilience and are able to evade such outcomes. Resilience, the capacity to rebound from adversity and avert adverse changes, stands in contrast to vulnerability[3,4]. From a therapeutic perspective, understanding the mechanisms underlying resilience-

building in response to stress could form the cornerstone of interventions targeting psychopathologies associated with stress and depression. By understanding these processes, therapeutic strategies can be devised to bolster resilience and mitigate the impact of stressors, offering new avenues for effective treatment and prevention of depression-related disorders.

A similar heterogeneity of resilience and vulnerability has been observed in mouse models that are subjected to the learned helplessness (LH) paradigm, a stress-related animal model of depression-like behavior[5]. Although originally used as a test for helplessness, the inescapable and uncontrollable foot shocks that act as a stressor lead to a state of despondency and hopelessness that are similarly observed in patients with depression, providing valuable insights into the psychological mechanisms underlying human depression[5,6]. Central to both concepts is the experience of a perceived lack of control or agency over one's circumstances. In the learned helplessness paradigm, mice subjected to uncontrollable aversive stimuli demonstrate a pattern of passive resignation, characterized by diminished motivation to escape, or avoid adverse outcomes[3,5,7]. Similarly, individuals grappling with depression often exhibit a pervasive sense of hopelessness and helplessness, feeling powerless to alter their situation or alleviate their emotional distress. Emotionally and behaviorally, both learned helplessness and depression engender a profound sense of despair, sadness, and emotional numbness that leads to withdrawal from social interactions and reduced engagement in everyday activities[5]. As such, the convergence of behavioral and psychological traits between

mouse models of learned helplessness and human depression underscores the translational value of animal research in elucidating the intricate mechanisms underlying depression.

The lack of control over aversive stimuli in the learned helplessness paradigm leads to various effects on behavior. In terms of locomotion, animals subjected to uncontrollable shocks exhibit reduced activity levels afterward and react passively to stress[5]. Consequently, difficulties in escaping have been attributed to declines in locomotion, and the term "learned inactivity" has been introduced to describe this phenomenon[3]. Furthermore, a study by Gellner et al. imaged dendritic spines of layer V neurons of the primary motor cortex at certain time intervals after chronic social defeat stress (CSDS)[8]. CSDS is an animal model used to study the effects of prolonged social stress, where a subject animal is repeatedly exposed to a dominant conspecific, leading to social defeat and stress-related behavioral changes analogous to social withdrawal and anhedonia. They found that stress typically resulted in a notable decrease in spine density in the motor cortex (MC), and the changes in spine dynamics varied based on stress phenotype. Two days after CSDS, both resilient and susceptible mice exhibited a significant decline in spine density compared to control mice. However, by the eleventh-day post-CSDS, spine density had returned to normal levels in resilient mice but remained unchanged in susceptible mice[8]. Another study conducted by Salomons et al. used structural MRI to assess gray and white matter in individuals with chronic pain, a group particularly susceptible to helplessness due to prolonged exposure to poorly controlled pain

stressors[9]. Their findings revealed that the degree of self-reported helplessness was associated with cortical thickness in the supplementary motor area and midcingulate cortex, regions involved in the cognitive aspects of motor behavior[9]. These results suggest that structural features of brain regions associated with motor planning and function may predispose certain individuals to helplessness when faced with uncontrollable stressors, such as chronic pain. This perspective may also help explain why some mice exhibit escape behavior in the learned helplessness paradigm while others remain susceptible.

Another brain region that has shown to be affected in chronic stress and depression is the bed nucleus of the stria terminalis (BNST)[10]. The BNST modulates stress responses and releases neurotransmitters to the hypothalamus, and other limbic structures, and the brainstem. Studies indicate that BNST circuits play a crucial role in regulating fear responses to unpredictable threats, which may contribute to the development and manifestation of anxiety[11]. Furthermore, chronic stress paradigms have been shown to induce structural and functional changes in the BNST, leading to maladaptive stress responses and depressive-like behaviors[11-14]. For instance, in a study by Kim S. et al., stimulating the oval nucleus of the BNST increased both behavioural and physiological measures of anxiety, suggesting an anxiogenic role for the oval nucleus of the BNST, and were consistent with the results obtained by modulating the entire BNST[13]. And in the context of uncontrollable stress in animal experiments, a study by Schulz et al., found that rats remain immobile longer in the second of two swim tests, characteristic of learned

helplessness. Results revealed that, compared to wild-type mice, BNST-lesioned animals displayed immobility significantly earlier and for longer durations in the second swim test. Rats with BNST lesions also showed significantly reduced escape behavior in the form of fewer numbers of jumps and dives compared to controls[14]. These findings highlight the BNST's involvement in mediating stress and anxiety, as well as its broader implications for understanding the neural basis of depression.

Research has demonstrated that stress significantly increases c-Fos expression throughout the prefrontal cortex (PFC) in rats, including subregions such as the prelimbic cortex (PL)[15,16]. Acute restraint stress has been shown to elevate Per1 and c-Fos mRNA expression across PFC subregions, with a pronounced effect in the PL. Interestingly, male rats displayed significantly higher levels of stress-induced c-Fos mRNA compared to females, suggesting potential sex-specific differences in stress responses within the PFC[15]. In a related study, Uliana et al. examined the long-term effects of PL lesions during adolescence on anxiety-like behaviors and vulnerability to learned helplessness[16]. Their findings revealed that disrupting the PL during this critical developmental period led to heightened anxiety responses and an increased proportion of helpless behavior in adulthood. Specifically, rats with adolescent PL lesions exhibited a significant delay in escape latency and a greater number of escape failures during behavioral tests compared to saline-injected controls[16]. These results highlight the crucial role of the PL in shaping stress resilience and adaptive responses and underscores the importance of the PL in

modulating stress-related behaviors and its potential influence on the long-term outcomes of stress exposure.

A study by Kim Y. et al. on whole-brain mapping of neuronal activity in mice with learned helplessness revealed that the locus coeruleus (LC) was the only subcortical region with significantly elevated cellular Fos (c-Fos) expression in helpless compared to resilient mice[16]. This finding aligns with the role of corticotropin-releasing hormone (CRH), a key mediator of the stress response released by the hypothalamus, in activating LC neurons during stress. In turn, these activated LC neurons synthesize and release norepinephrine (NE)[17]. McCall et al. further explored this pathway using optogenetics, circuit tracing, and behavioral analysis. They demonstrated that CRH released by the central amygdala induces high-frequency neuronal activity in the LC. This activity triggers the synaptic release of norepinephrine, which subsequently leads to stress-induced anxiety and aversion[18]. Norepinephrine, synthesized in the LC, plays a critical role in modulating an individual's response to stress and adversity. Dysregulation of norepinephrine signaling is believed to contribute to the maladaptive responses observed in learned helplessness paradigms, underscoring its importance in shaping stress resilience and coping mechanisms[19,20]. Supporting this, recent findings from our lab showed that norepinephrine-depleted mice exhibited significantly faster and more consistent escape behavior compared to wild-type mice in the learned helplessness test, reflecting traits characteristic of a resilient phenotype. This further emphasizes the crucial role of norepinephrine signaling in determining behavioral responses to stress.

Indeed, previous studies have given rise to the monoaminergic hypothesis of depression, which posits that depression was associated with decreased levels of norepinephrine[21-23]. On the contrary, other studies have shown that excitation of the LC and increase in noradrenergic activity is implicated in the enhanced manifestations of anxiety, stress, and fear responses and contributes to the etiology of stress-induced depression[21]. And several animal studies have illustrated the positive relationship between stress and the upregulation of released norepinephrine[21,24,26]. This paradoxical role of norepinephrine between its therapeutic effect and stress response thus complicates our understanding of the etiology of depression. In an experiment where they used c-Fos activation to look at the activation of noradrenergic neurons in mice after being exposed to tailshocks, the groups that received 50 and 100 shocks had significantly higher degrees of activation of noradrenergic neurons than the 0 and 10 shock groups[26]. Ultimately, the intricate interplay between norepinephrine signaling and stress responses of learned helplessness underscores the multifaceted nature of stress-induced depression. Highlighting the complexity of their underlying mechanisms and the need for further research in discovering novel therapeutic interventions targeting monoaminergic pathways.

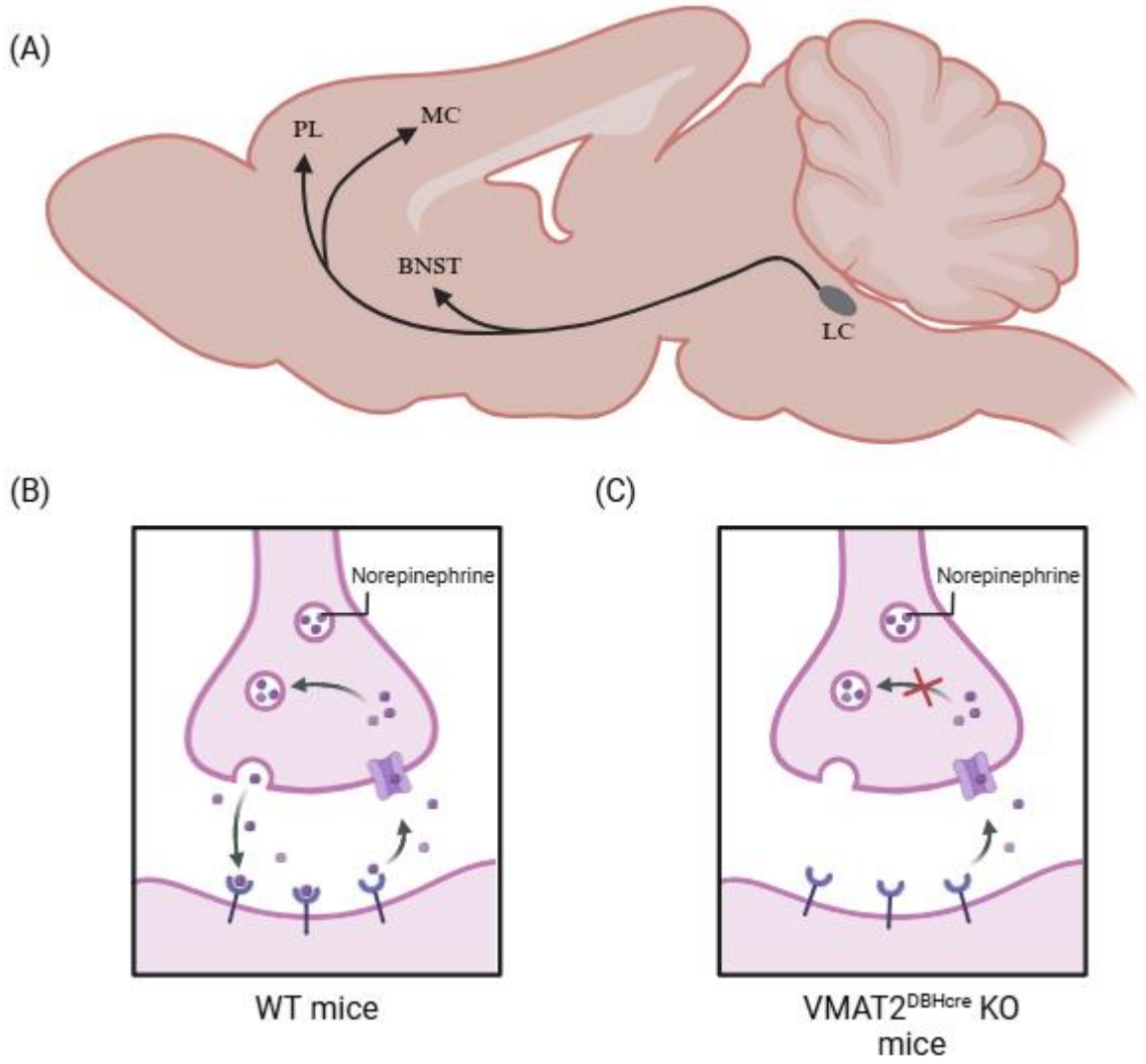

Figure 1. (A) Sagittal cross-section of a mouse brain illustrating the anatomical locations of the locus coeruleus (LC), bed nucleus of the stria terminalis (BNST), prelimbic cortex of the medial prefrontal cortex (PL), and motor cortex (MC). Arrows indicate the synaptic norepinephrine pathways originating from the locus coeruleus to the targeted brain regions. (B) Schematic representation of the synaptic cleft in wild-type (WT) mice, demonstrating the release of norepinephrine into the synaptic cleft, subsequent reuptake into the presynaptic neuron, and its repackaging into synaptic vesicles. (C) Schematic representation of the synaptic cleft in VMAT2^{DBHcre} KO mice, showing how the knockout disrupts the normal packaging of norepinephrine into synaptic vesicles. Hence, preventing the release of norepinephrine into the synaptic cleft.

In this investigation, our objective is to investigate alterations in mouse brain activity within the motor cortex (MC), bed nucleus of the stria terminalis (BNST), and the prelimbic cortex of the medial prefrontal cortex (PL) following exposure to the learned helplessness paradigm (Figure 1A). Many neurological and psychiatric disorders can be linked to dysfunction of monoaminergic systems. Although the origin of monoaminergic dysfunction varies, manipulation of vesicular function could be a useful target for modulating monoamine dysregulation[27,28]. For purposes of this study, we will focus on norepinephrine packaging by vesicular monoamine transporter 2 (VMAT2), an essential protein for monoaminergic neurotransmission, from the presynaptic cytoplasm into synaptic vesicles (Figure 1B). VMAT2 relies on the proton gradient generated by ATPase across the vesicular membrane and the transport of chloride into the vesicle. The high concentration of intravesicular protons allows for the exchange of two protons for each molecule of neurotransmitter[27]. To study the dysregulation of fear behaviour in mice, Branco et al. conducted a study using transgenic mice engineered to express either 5% (VMAT2-LO mice) or 200% (VMAT2-HI mice) of the normal wild-type levels of VMAT2 protein. Their findings revealed that VMAT2-LO mice exhibited significantly reduced VMAT2 protein levels in the hippocampus and amygdala, diminished monoaminergic vesicular storage capacity in the striatum and frontal cortex, and lower extracellular concentrations of dopamine and norepinephrine compared to wild-type mice. Additionally, VMAT2-LO mice displayed heightened cued and contextual fear responses, impaired fear habituation, difficulty distinguishing between threat and safety cues, altered startle responses, and behaviors indicative of an anxiogenic-like phenotype[28].

Our study includes both wild-type (WT) mice and genetically modified mice engineered with a norepinephrine (NE) depletion achieved with the knockout of the vesicular monoamine transporter 2 (VMAT2) gene in dopamine beta-hydroxylase (DBH)-expressing neurons ($VMAT2^{DBHcre}$ KO). This genetic modification disrupts the ability of noradrenergic neurons in the locus coeruleus to package norepinephrine into synaptic vesicles, effectively impairing normal NE signaling (Figure 1C)[29]. By analyzing immunohistochemical staining for c-Fos, a marker of neuronal activation, we aim to map the activation patterns within key brain regions in resilient and susceptible mice (both WT and KO) exposed to inescapable foot shocks. These findings will be compared to neural circuits in control mice that have not been exposed to stressors. Given the role of norepinephrine in stress responses, we hypothesize that $VMAT2^{DBHcre}$ KO mice will have a significant reduction in both the total number and relative proportion of c-Fos-positive cells within the targeted brain regions compared to WT mice. Furthermore, we predict that mice classified as susceptible to chronic stress following exposure to the learned helplessness paradigm will exhibit higher levels of c-Fos expression in these regions compared to both resilient and control mice. With the results of this study, we aim to deepen our understanding of how NE depletion affects stress-induced neural activity and the mechanisms underlying susceptibility and resilience to chronic stress.

The extensive impact of chronic stress and depression on individuals' lives highlights the critical need for a deeper understanding of the mechanisms driving these debilitating conditions. These disorders not only affect mental health but also have long-term

implications for physical health, interpersonal relationships, and overall quality of life. By observing learned helplessness paradigms and examining the neurobiological pathways associated with these phenomena, we can unravel the intricate relationship between stress, resilience, and the norepinephrine signaling pathways that play a central role in these conditions. The exploration of these pathways offers valuable insights into how specific brain regions contribute to maladaptive responses to stress and the development of depressive symptoms. Furthermore, this knowledge opens avenues for targeted interventions, particularly by modulating norepinephrine activity through pharmaceutical approaches. Reducing excessive norepinephrine signaling in key brain regions may help mitigate the adverse effects of chronic stress and promote more adaptive responses. Continued research in this area holds significant potential for advancing therapeutic strategies aimed at relieving the burden of mental illness. Innovative treatments targeting the neurobiological underpinnings of depression could improve resilience, enhance coping mechanisms, and ultimately improve mental health and well-being for individuals affected by these pervasive conditions.

2. MATERIALS & METHODS

2.1 Animals

A total of 19 adult male mice with vesicular monoamine transporter 2 (VMAT2) knockout in dopamine beta-hydroxylase (DBH) neurons ($VMAT2^{DBHcre}$ KO) and 42 adult male WT mice were used. The mice were group-housed (2-5/cage) before the learned helplessness (LH) paradigm and single-housed after their first training phase in the LH paradigm. Mice were fed ad libitum and maintained in the same room under a 12h light-dark cycle at constant temperature (24 °C). Animal housing, breeding, and care were operated in accordance with the Canadian Council on Animal Care guidelines (CCAC), and all methods were approved by the Animal Care Committee from the Douglas Institute Research Center (protocol number 7179). Mice were assigned to experimental groups based on their genotype.

2.2 Learned Helplessness Paradigm

Both mice genotypes were subjected to the learned helplessness paradigm. The decision to employ the learned helplessness paradigm as opposed to the chronic social defeat stress (CSDS) test for modeling depression stemmed from our specific research objectives. Our aim was to elucidate the neurobiological underpinnings of chronic stress and stress-induced depression and anxiety, particularly in contexts devoid of social influence from other mice. Unlike the CSDS test, which primarily assesses social avoidance behaviors, our focus centered on exploring cognitive processes such as the perception of control and discerning individual variations in stress susceptibility. By

utilizing the learned helplessness paradigm (Figure 2), we sought to delve deeper into the psychological mechanisms underlying stress responses and resilience, thus providing valuable insights into the multifaceted nature of depression etiology.

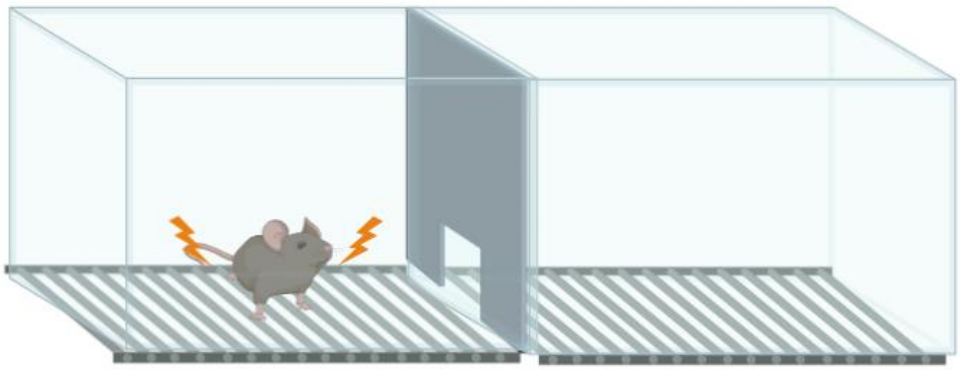

Figure 2. Learned helplessness apparatus. Comprised of two equally sized compartments, measuring 30.7 cm × 33.3 cm × 56.2 cm, separated by a wall with a central open door that slides up and down from the floor. The floor is made of stainless-steel grids (4 mm in diameter with 13 mm spacing), through which footshocks are administered.

The protocol is divided into two phases: the training and testing phase. During the training phase, depression-like symptoms will be induced in the mice by confining them to one compartment for 30 minutes, where they are given 120 random and inescapable foot shocks at an intensity of 0.25mA. After allowing the mice a recovery period (24h) from the training phase, the mice then enter test day 1 (TD1). The mouse is given a brief acclimatization period of 3 minutes where it can explore both compartments. Following the acclimatization period, mice were exposed to 20 trials of unpredictable but escapable foot shock delivered at an intensity of 0.25mA. The footshocks cease when the mouse crosses to the other compartment, a failure to cross results in an escape failure. If the mouse fails to escape, the footshocks terminate after 30 seconds, before

the next trial begins. The session duration ranged from 15 to 20 minutes, varying based on the number of successful escapes and the time it took for each mouse to escape. For each shock, the latency to escape, along with the number of successful escapes and failures, was recorded. Escape behavior in response to foot shocks serves as a measure of resilience: susceptible (helpless) mice exhibited longer escape latencies and more failures, while resilient (non-helpless) mice demonstrated shorter escape latencies and fewer failures.

Using MATLAB software, classification of mice into resilient and susceptible phenotypes was based on a *k*-means cluster analysis (k=2) using latency to escape and number of failures as parameters. This analysis categorized the mice into two groups—resilient (non-helpless) and susceptible (helpless)—for the training and test day.

2.3 Perfusion

Three hours after test day 1 (TD1) of the learned helplessness (LH) paradigm, mice underwent anesthesia induction utilizing isoflurane gas, ensuring a consistent and controlled sedation protocol. Subsequently, transcardial perfusion was conducted through the left ventricle using a prepared solution of 1X phosphate-buffered saline (PBS) to preserve tissue integrity while effectively flushing out blood contaminants. This initial perfusion step was immediately followed by a subsequent perfusion with 4% paraformaldehyde (PFA) solution in PBS, aimed at robustly fixating brain tissue specimens to enable precise histological analyses. Post-perfusion, the brain specimens

were extracted and transferred to specialized tubes containing PFA solution, where they were stored for a duration of 24 hours to ensure thorough fixation. After the fixation period, dehydration of the brain specimens was achieved via immersion in a cryoprotectant solution comprising 30% sucrose in PBS, designed to shield the specimens from structural damage during the freezing process. Finally, the prepared brain specimens were dry-frozen at a maintained temperature of -80°C, ensuring optimal preservation of tissue integrity for subsequent histological and immunohistochemical analyses.

2.4 Immunohistochemistry

Whole brain coronal sections (40 µm thickness) of KO (n=10) and WT (n=10) adult mice were then cut on a cryostat (Leica) and stored in wells of 1X PBS solution until further processing. The free-floating sections were washed for 2 x 5 min in 1X PBS. The slices were then incubated in permeabilization buffer solution with 1% normal goat serum (NGS) and 2.5% Triton X-100 for 2 x 10 min. Sections were blocked for 1 h in blocking solution with 5% NGS and 10% bovine serum albumin (BSA). After this period, the slices were incubated for 46 h at 4°C with primary polyclonal rabbit antibody sc-52 (anti-c-Fos 1:250, Santa Cruz Biotechnology). Afterwards, sections were washed for 2 x 10 min in phosphate-buffered saline with Tween detergent (PBST) and incubated with secondary goat antibodies Alexa Fluor 555 (goat anti-rabbit IgG 1:500, ThermoFisher Scientific) for 2 h at room temperature. After washing for 2 x 10 min in PBST solution, the sections were mounted onto slides (Fisherbrand™, ThermoFisher Scientific) and cover slipped with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Invitrogen™, ThermoFisher Scientific). Several anticipated challenges are expected to arise during this stage of the

experimental process, primarily concerning the delicate handling and preservation of brain sections for subsequent imaging and analysis. Brain tissue can be prone to damage during the perfusion procedure and during the transfer of free-floating brain sections between wells during subsequent immunohistochemical. Given the critical importance of preserving the structural integrity of these brain regions for accurate analysis, attention to technique will be paramount in mitigating these challenges and ensuring the integrity of our experimental outcomes.

2.5 Image Capture

Images were acquired utilizing a ZEISS ApoTome microscope, leveraging distinct blue (DAPI) fluorescence filters to ensure precise visualization and capture of cellular fluorescence signals. To optimize image quality and resolution, a Z-stacking was used, enabling acquisition of consecutive sections at 10 µm intervals. All fluorescent images were then processed using the orthographic projection function before commencing the analysis. Identical camera and microscope settings were used throughout capturing of images and analysis of the slides.

2.6 Heatmap Generation

Digital images of whole brain sections were analyzed using QuPath software to generate a density heatmap, which served as preliminary qualitative data for comparing c-Fos expression in different brain regions between KO and WT mice. The "Cell Detection" function in QuPath was utilized with the following parameters (requested pixel size: 0.32

μm; background radius: 6 μm; sigma: 3 μm; minimum area: 20 μm²; maximum area: 400 μm²; threshold: 100). Subsequently, the "Train Object Classifier" function was employed to train the software to detect and label cells. Cells expressing both DAPI and AF555 were identified as c-Fos positive, while cells expressing only DAPI were identified as c-Fos negative. Finally, the "Density Map" function in QuPath (density radius: 150), was used to create preliminary heatmaps. These heatmaps served to display and highlight target brain regions for further quantification.

2.7 Quantitative c-Fos Image Analysis

Digital images were analyzed using QuPath software to ascertain the c-Fos cell count within specific brain regions delineated in the imaged sections. Initially, the total cell count was computed by identifying and quantifying the DAPI-stained cells present in the images using the “Cell Detection” function described in the previous section. Subsequently, the number of active c-Fos expressing cells within the designated brain regions was determined by instructing the software to identify DAPI-stained cells exhibiting concomitant Alexa Fluor 555 fluorescence. The ratio of double-labeled c-Fos expressing cells to the total cell count within a given brain region was then calculated, facilitating comparisons across mice of identical or divergent genotypes. This analytical approach enabled a precise assessment of c-Fos expression dynamics and its potential modulation across experimental groups.

2.8 Statistical Analysis

Unpaired t-tests were conducted to compare the percentage of c-Fos-expressing cells between KO and WT mice prior to exposure to the learned helplessness paradigm. Additionally, a one-way ANOVA was performed to assess differences in c-Fos expression among resilient, susceptible, and control mice across both genotypes.

3. RESULTS

3.1. Learned Helplessness

In our investigation, we sought to determine the association between mouse phenotype, after exposure to the learned helplessness paradigm 1 day after training phase, and the genotype of the mice. Mice exhibiting an escape rate exceeding 50% were classified as resilient, while those with an escape rate falling below 50% were deemed susceptible. Our analysis revealed noteworthy disparities between the two genotypes. Although the percentage of resilient VMAT2^{DBHcre} KO mice, with a resilience rate of 42.1%, was higher than wild-type mice which had a 28.6% likelihood of resilience, the data was not statistically significant.

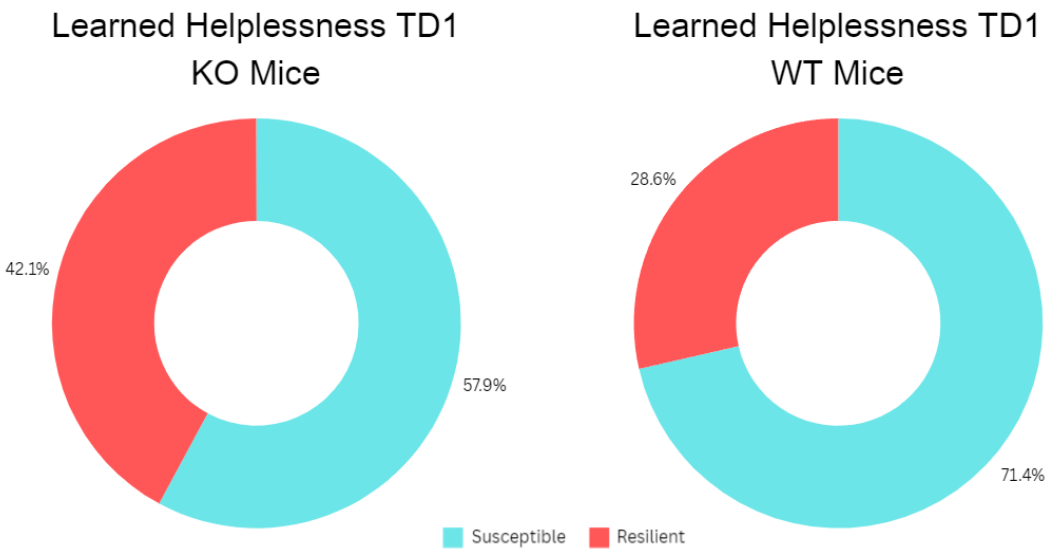

Figure 3. Percentage of VMAT2^{DBHcre} KO (n = 19) and WT adult male mice (n = 42) that are resilient and susceptible to the learned helplessness paradigm 1 day after training phase. Resilient and susceptible phenotype was recorded as nominal data. Results show that there is no statistical difference between the percentage of resilient KO mice (42.1%) and the percentage of resilient WT mice (28.6%) after TD1 (Chi-square test, p=0.454).

3.2 Preliminary c-Fos Heatmap

To identify the specific brain regions for further analysis, a comprehensive whole-brain heatmap analysis was conducted using QuPath software on distinct coronal brain sections. This approach allowed for the visualization of c-Fos expression patterns across the brain, enabling a qualitative assessment of regions exhibiting heightened c-Fos levels in wild-type mice relative to VMAT2^{DBHcre} KO mice during the learned helplessness paradigm (Fig. 4). Darkened regions on the heatmap correspond to lower levels of c-Fos expression, indicating diminished neuronal activity in those specific brain areas, whereas brighter regions signify heightened neuronal activity and increased c-Fos expression. Consistent with our initial hypothesis, the observed disparity in c-Fos expression levels between experimental groups suggests a corresponding difference in neuronal activity. Specifically, KO mice exhibited decreased levels of c-Fos compared to their WT counterparts in certain brain regions. However, visual assessment alone proved challenging in discerning significant differences in c-Fos expression between distinct phenotype groups within both the KO and WT mice, underscoring the need for quantitative analysis to ascertain subtle changes in neural activation patterns. Given the various brain regions, our focus was directed towards forebrain structures for the purposes of this investigation. Based on initial observations, we have identified the primary (M1) and secondary motor cortex (M2), the bed nucleus of the stria terminalis (BNST), and the prelimbic cortex of the prefrontal cortex (PL) as primary areas of interest (Fig. 4A, B, C, D). Additionally, the piriform cortex was selected as a control region characterized by consistently high c-Fos expression levels across genotypes, thereby serving to ensure that any alterations in c-Fos expression within specific regions were not confounded by global changes in c-Fos expression levels (Fig. 4E, F).

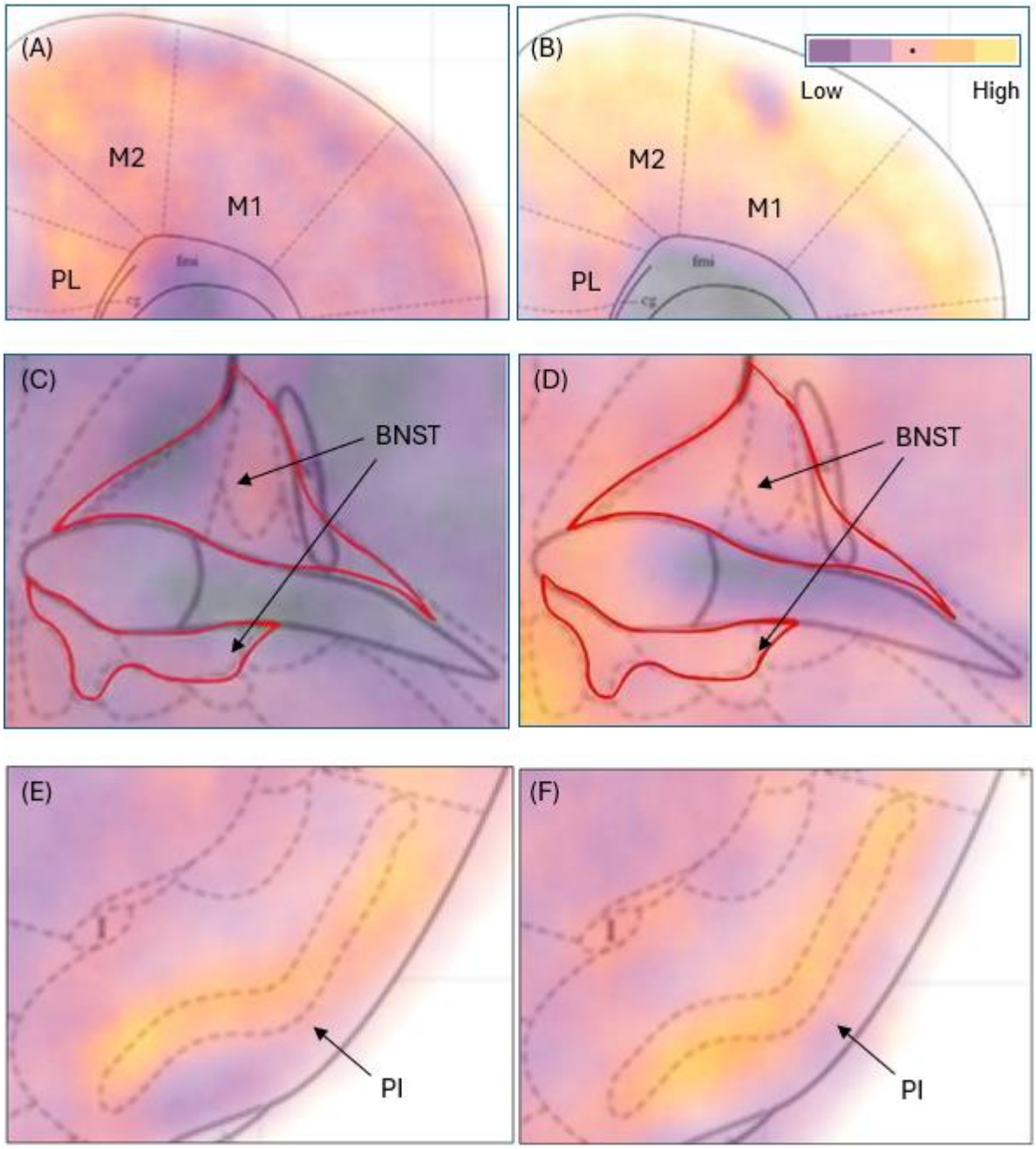

Figure 4. Levels of c-Fos expression in the forebrain in response to the learned helplessness paradigm. Decreased c-Fos expression in the primary (M1) and secondary motor cortex (M2), and the prelimbic cortex of the prefrontal cortex (PL) in VMAT2^{DBHcre} KO mice (A) compared to WT mice (B). Decreased c-Fos expression in the bed nucleus of the stria terminalis (BNST) in VMAT2^{DBHcre} KO mice (C) compared to WT mice (D). No change in c-Fos expression in the piriform cortex (PI) in VMAT2^{DBHcre} KO mice (E) compared to WT mice (F).

3.3 c-Fos Analysis

Following the identification of the target brain regions as outlined in the preceding section, we used the QuPath software to conduct quantitative analyses (see Appendix A, p. 49). Specifically, we calculated the ratio of c-Fos labeled cells expressing Alexa Fluor 555 to the total number of cells expressing DAPI within each region of interest. This method facilitated the precise quantification of c-Fos expression percentages across the examined brain sections, enabling a comprehensive assessment of c-Fos distribution. Our objective was to discern any significant differences in c-Fos expression among different phenotypes within each genotype, namely VMAT2^{DBHcre} KO and wild type (WT). Figure 5 presents the mean data for the percentage of c-Fos expression in VMAT2^{DBHcre} KO mice across resilient, susceptible, and control phenotypes within the target brain regions. Subsequently, we conducted a one-way ANOVA analysis for each notable brain region, and the resulting p-values indicated that none of the brain regions in VMAT2^{DBHcre} KO mice exhibited statistically significant differences across the phenotype groups. Specifically, comparable percentages of c-Fos labeled cells were observed in resilient, susceptible, and control mice, suggesting uniformity in c-Fos expression levels across these phenotypes.

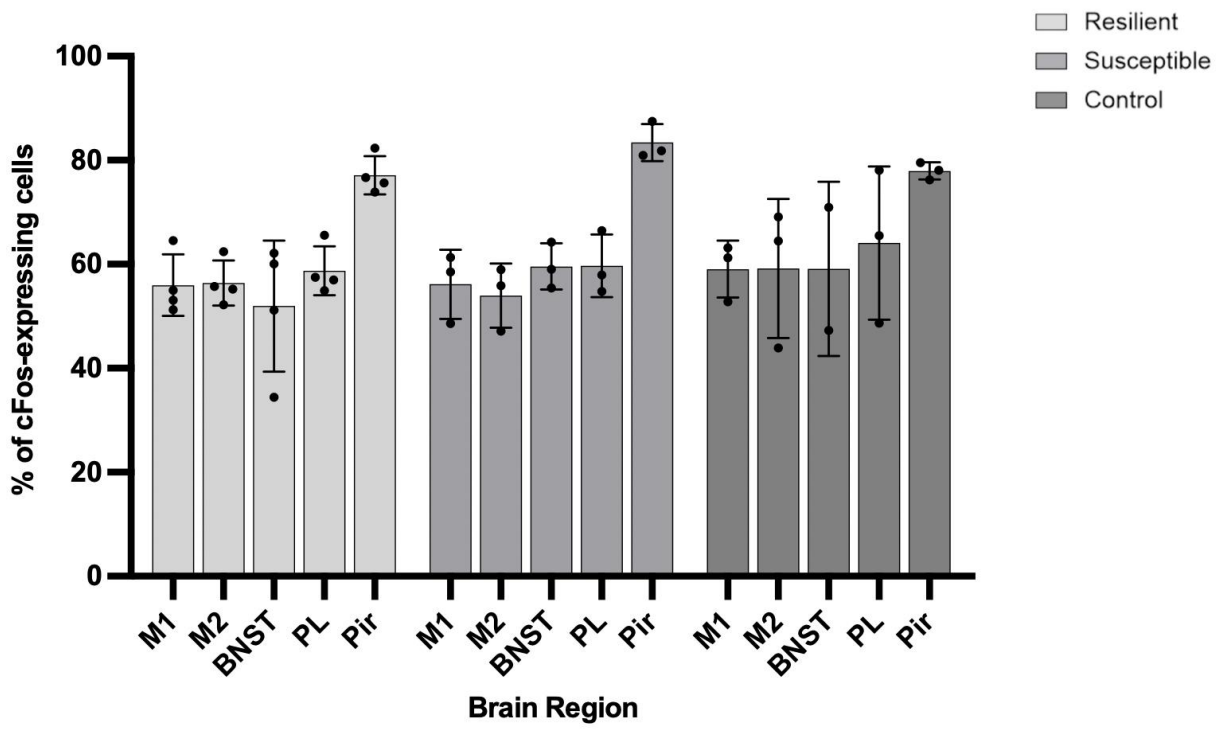

Figure 5. Percentage of c-Fos labeled cells to total number of cells in VMAT2^{DBHcre} KO mice (resilient, susceptible, and control) in different brain regions. Note: n = 4 for resilient; n = 3 for susceptible; n = 3 for control. One-way ANOVA for all brain regions across mouse groups were statistically insignificant p>0.05. Follow-up post-hoc analysis showed no further significance.

Figure 6 presents the mean data pertaining to the percentage of c-Fos expression in wild type (WT) mice across resilient, susceptible, and control phenotypes within the target brain regions. Following a parallel approach, we conducted a one-way ANOVA analysis for each notable brain region in WT mice. The resulting p-values demonstrated that, akin to the findings in VMAT2^{DBHcre} KO mice, none of the brain regions in WT mice exhibited statistically significant differences across the phenotype groups. Thus, consistent with the observations in the VMAT2^{DBHcre} KO mice, the c-Fos expression levels remained uniform across resilient, susceptible, and control phenotypes in WT mice.

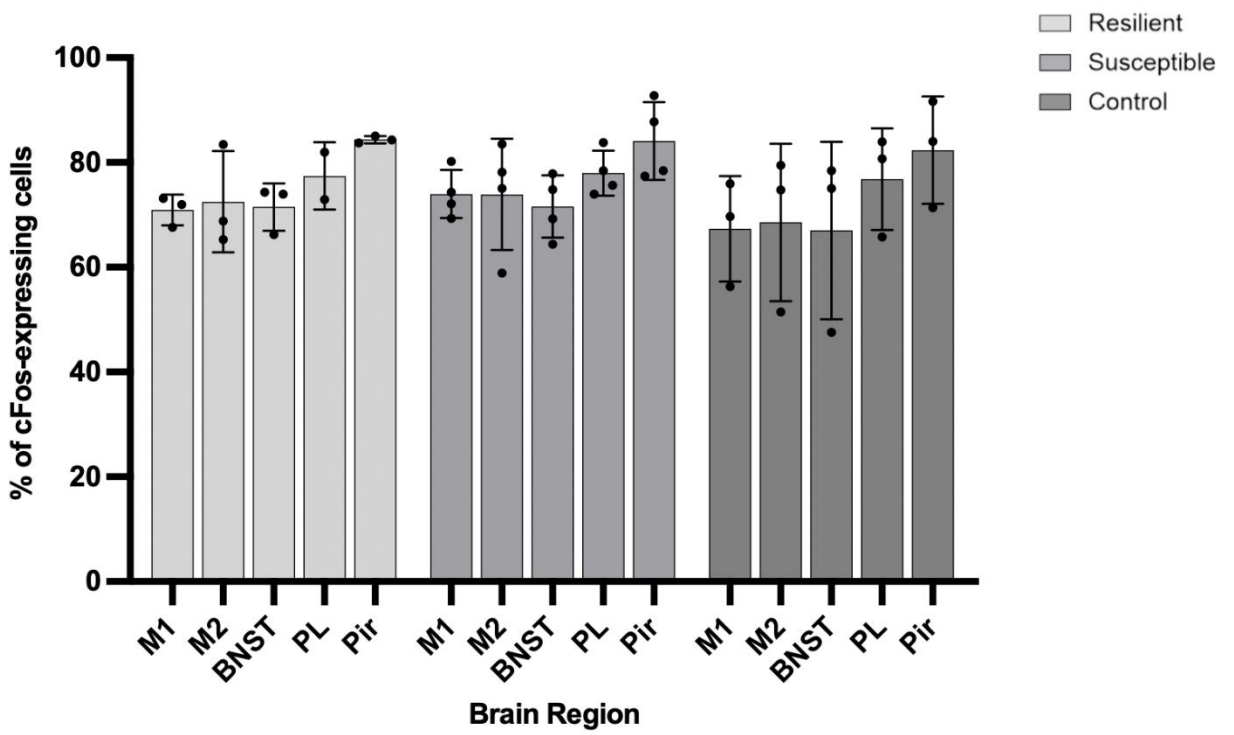

Figure 6. Percentage of c-Fos labeled cells to total number of cells in WT mice (resilient, susceptible, and control) in different brain regions. Note: *n* = 3 for resilient; *n* = 4 for susceptible; *n* = 3 for control. One-way ANOVA for all brain regions across mouse groups were statistically insignificant $p>0.05$. Follow-up post-hoc analysis showed no further significance.

To ascertain potential differences in c-Fos expression between VMAT2^{DBHcre} KO mice and WT mice, we conducted a comparative analysis of the percentage of c-Fos labeled cells in both genotypes (Figure 7). Because we found no differences between resilient and vulnerable mice in both genotypes, all mice from the same genotype are pooled together for this analysis. Our control region, the piriform cortex, exhibited no statistically significant difference between the two genotypes, suggesting a lack of uniform c-Fos increase across the entire brain section. Notably, in accordance with our hypothesis, we observed a significant reduction in c-Fos expression levels in key brain regions, including the primary and secondary motor cortex, the bed nucleus of the stria terminalis, and the prelimbic cortex of the prefrontal cortex, in VMAT2^{DBHcre} KO mice compared to WT mice.

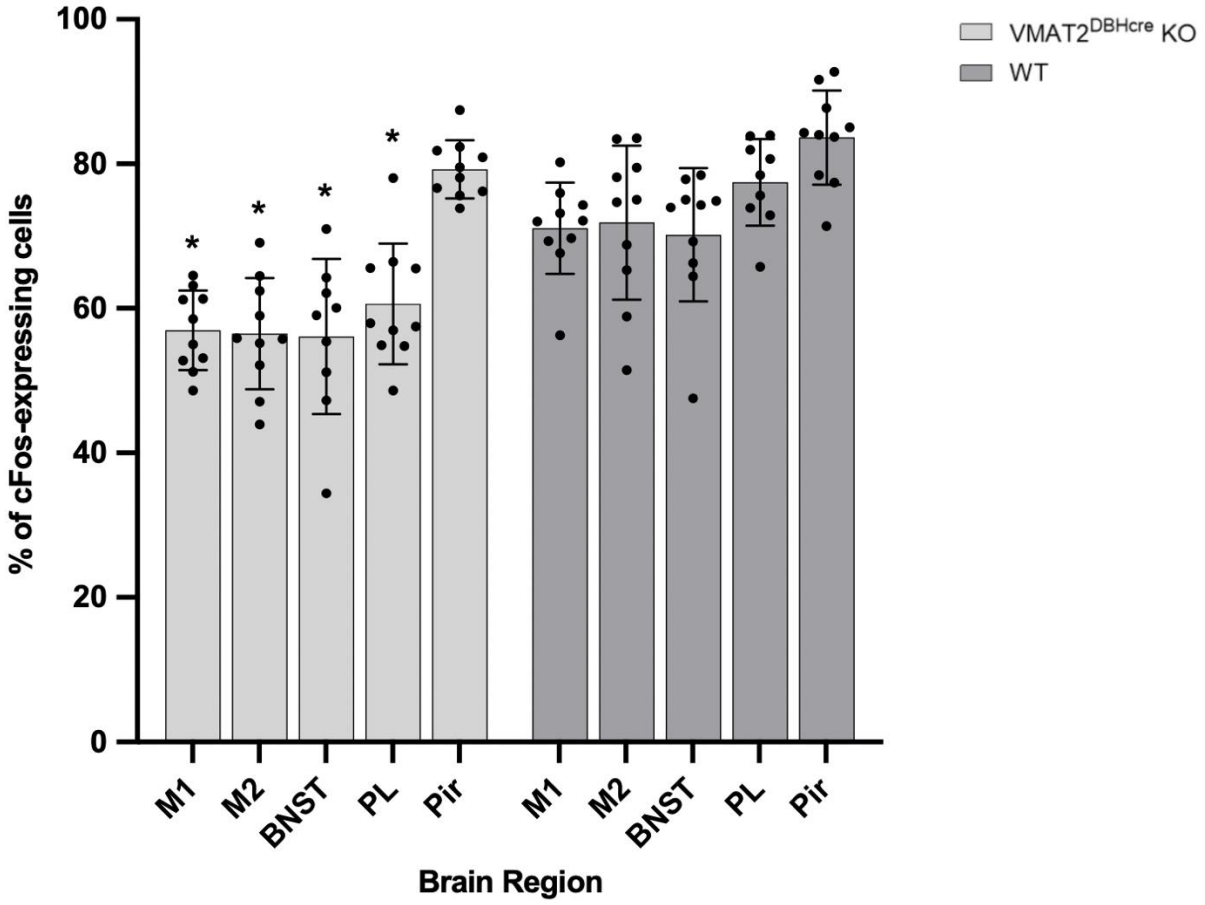

Figure 7. Percentage of c-Fos labeled cells to total number of cells in both mice genotypes in different brain regions. Note: *n* = 10 for VMAT2^{DBHcre} KO mice; *n* = 10 for WT mice. One-way ANOVA for all brain regions across mouse groups were statistically significant $p<0.01$, with the exception of the control brain region (Pir) which was not statistically significant $p>0.05$.

4. DISCUSSION

When confronted with stress, individuals often exhibit distinct behavioral responses, with some displaying resilience while others are susceptible and prone to developing mood disorders. Despite its clinical significance, the neural mechanisms underlying such varied responses remain incompletely understood. One extensively utilized animal model for investigating the neural alterations associated with stress-induced depressive-like behavior in mice is the learned helplessness paradigm. The concept of learned helplessness refers to a deficit in escape or avoidance behavior following exposure to uncontrollable stress. It is widely recognized as a depression-like coping deficit in adverse situations that are potentially avoidable[3,5,6]. In alignment with our findings and other studies, we observe that mice exhibit increased resilience to uncontrollable stress during the test phase compared to the training phase[30,31]. During the training phase, animals are exposed to uncontrollable stress, learning that their actions have no influence over the outcome. This induces a state of learned helplessness, characterized by a reduction in effort and activity when confronted with stressors. This behavior mirrors how chronic stress in humans can foster feelings of powerlessness and a decline in motivation.

As a result, during the testing phase, mice that are susceptible to learned helplessness fail to respond appropriately, even when provided with an opportunity to escape or mitigate the stressor. However, resilient mice, which do not develop helplessness, actively engage in escape behaviors and avoid the stressor effectively. This pattern of resilience and susceptibility in mice closely parallels human experiences. In humans, learned

helplessness manifests as a psychological state where individuals believe they lack the ability to alter or improve their circumstances, even when control is possible. Such perceptions contribute to the development of depression, where individuals feel trapped in their conditions and are unmotivated to make efforts to alleviate stress.

Norepinephrine, a neurotransmitter produced and released by the locus coeruleus, plays a crucial role in modulating fear and stress responses in the body, and elevated levels of norepinephrine have been previously linked to stress-induced depression. During periods of stress, increased norepinephrine activity can heighten arousal and vigilance, which may initially help individuals cope with challenging situations[16,32]. However, prolonged, or excessive activation of the norepinephrine system due to chronic stress can lead to dysregulation in the brain, contributing to the development of depressive symptoms[32]. As a result, our objective was to elucidate how changes in norepinephrine activity in VMAT2^{DBHcre} KO compared to wild type mice could possibly affect the response following exposure to the learned helplessness paradigm. To achieve this, we initially conducted a preliminary qualitative heatmap analysis on whole brain sections, identifying three specific target brain regions for further investigation: the primary and secondary motor cortex, the bed nucleus of the stria terminalis, and the prelimbic cortex of the medial prefrontal cortex.

In the present study, using heatmaps showing c-Fos expression across the whole brain section, we observed that there was lower c-Fos expression, and by extension lower NE-dependent neuronal activity, in the target brain regions in VMAT2^{DBHcre} KO mice than in

WT mice. This observation is consistent with previous studies where the aforementioned brain regions have been shown to be affected by chronic stress and depression. Escape and avoidance impairments are characteristic features of the learned helplessness paradigm. Susceptible animals exposed to uncontrollable stressors, such as inescapable foot shocks, demonstrate alterations in motor activity[3,5,33]. Our observations during learned helplessness experiments corroborate these findings, revealing diminished locomotor activity, characterized by impaired escape and avoidance responses and a passive reaction to aversive stimuli. The bed nucleus of the stria terminalis plays a crucial role in regulating various aspects of stress and anxiety responses[34]. As part of the extended amygdala, the BNST is involved in processing and integrating emotional information related to threat perception. It receives inputs from various brain regions involved in stress and anxiety, including the amygdala, hippocampus, and prefrontal cortex, and it sends outputs to regions involved in fear and stress responses[14,35]. And finally, the prelimbic cortex also holds significance in stress processing. Chronic stress can lead to structural and functional changes in the prelimbic cortex, contributing to maladaptive behaviors, including deficits impaired fear extinction of the continuous uncontrollable footshocks[16,34]. Each of these regions is innervated by noradrenergic neurons projecting from the locus coeruleus, the primary site of norepinephrine synthesis and release[36]. The accumulating evidence highlighting the impact of NE dysfunction and alterations in these brain regions underscores the urgency for targeted research aimed at developing therapeutic interventions for stress-induced depression.

Further quantification of c-Fos expression in our study on the motor cortex, bed nucleus of the stria terminalis, and prelimbic cortex aimed to deepen our understanding of how the depletion of the VMAT2 gene influenced norepinephrine levels and whether this knockout impacted norepinephrine activity across resilient, susceptible, and control mice. Our findings reveal that VMAT2^{DBHcre} KO mice show a higher chance of being resilient compared to WT mice 1 day after training phase following exposure to the learned helplessness paradigm. We also see a significant decrease in c-Fos expression in VMAT2^{DBHcre} KO mice compared to WT mice across all three targeted brain regions. The VMAT2 gene encodes a crucial protein involved in the packaging and transport of monoamine neurotransmitters, including dopamine (DA), norepinephrine, and serotonin, from the cytoplasm into synaptic vesicles. These synaptic vesicles serve as storage compartments for neurotransmitters, releasing them into the synaptic cleft upon nerve impulse stimulation. DBH neurons, characterized by the presence of dopamine beta-hydroxylase, are a specific neuronal subtype responsible for synthesizing and releasing norepinephrine. Within these neurons, the enzyme dopamine beta-hydroxylase allows the conversion of dopamine into norepinephrine.

Consequently, a VMAT2 knockout specifically targeting DBH neurons disrupts the standard process of norepinephrine packaging into synaptic vesicles. This disruption ultimately leads to diminished NE activity, stemming from the impaired storage and release of NE into the synaptic cleft. Considering the diminished levels of norepinephrine in VMAT2^{DBHcre} KO mice, coupled with the concurrent decrease in c-Fos expression within

specific brain regions in these animals, we suggest that the depletion of norepinephrine may contribute to the reduced expression of c-Fos observed in this study. Ultimately, the reduced firing rate of noradrenergic neurons diminishes fear responses within the motor cortex, BNST, and PL, potentially shifting mice from a susceptible phenotype towards resilience following exposure to uncontrollable stressors. Our findings demonstrate that these brain regions play a role in stress responses due to their norepinephrine (NE) afferents, as shown by the significant reduction in c-Fos activation following the learned helplessness paradigm in KO mice. This insight highlights the intricate relationship between norepinephrine dynamics and stress-induced behavioral responses, presenting a potential therapeutic avenue for pharmaceutical interventions aimed at inhibiting VMAT2 in humans as a treatment for stress-induced depression. However, it is also important to consider the potential limitations of targeting VMAT2 pharmacologically or even in further studies. VMAT2 is not exclusive to norepinephrine neurons; it also regulates the packaging and release of other key neurotransmitters, such as dopamine and serotonin[27,37]. As a result, drugs designed to inhibit VMAT2 may lack the specificity needed to target NE pathways alone. Therefore, further research is necessary to assess the therapeutic viability, selectivity, and broader neurochemical effects of VMAT2 inhibition in response to chronic stress.

Ultimately, our investigation did not reveal any significant differences in c-Fos expression between resilient, susceptible, and control mice within the same genotype in the brain regions analyzed. This outcome may be attributable to the limited sample size, with a

minimum of three mice per group. Future studies should aim to increase the sample size, as this may help uncover significant differences in c-Fos expression. Additionally, our learned helplessness paradigm assessed the mice one day after the training phase (TD1). Retesting the mice at a later time, such as ten days post-training (TD10), and comparing the data between the training phase, TD1, and TD10, could provide further insights. The extended interval between tests would allow the mice more time to adapt to chronic stress, potentially increasing or diminishing the resilient and susceptible phenotypes.

This aligns with recent findings from our lab, which showed that on TD10, the percentage of resilient male mice in the KO group was significantly higher than that in the WT group, as compared to TD1 mice (Figure 8A)[29]. This approach could also uncover variations in c-Fos expression among resilient, susceptible, and control mice within the same genotype. Additionally, the data indicates that WT females exhibit a higher percentage of resilient individuals compared to WT males across all test days (Figure 8C). These findings underscore the importance of considering sex differences in both mice and, by extension, humans when assessing resilience and individual responses to chronic stress.

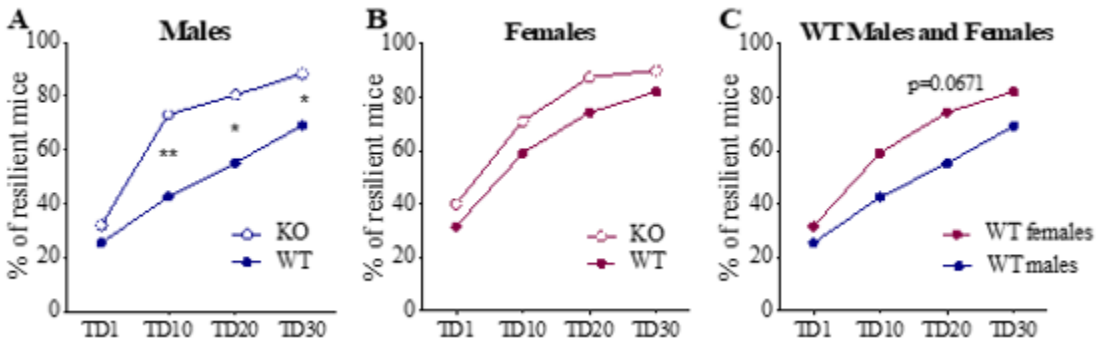

Figure 8. NE depletion facilitates extinction of helpless behaviour in the LH test in mice. (A) In males, starting TD10 the percentage of resilient mice in the KO group was significantly higher than in the WT group (Chi-square test, $p<0.01$). The difference between WT and KO remained significant on TD20 and TD30 (Chi-square test, $p<0.05$), indicating more prominent extinction of the susceptible phenotype over time in KOs. (B) Although KO females demonstrated higher percentage of resilient animals, the difference from WTs was not significant. (C) In WT females, percentage of resilient mice was higher than in WT males, showing a trend towards significance at TD20 (Chi-square test, $p=0.0671$).

There is also the possibility that c-Fos may not be a complete marker for norepinephrine activity during a stress response. As a marker, it is not completely cell-type specific, it detects whether a neuron is activated and expresses c-Fos. However, it does not indicate whether that neuron is excitatory or inhibitory, nor does it distinguish between neurons and glial cells, like astrocytes and microglia[38]. Furthermore, c-Fos is unable to identify the different types of stimuli occurring in target brain regions or differentiate between specific norepinephrine pathways pertaining to our specific study, limiting its ability to offer detailed insights on the role of NE circuits on chronic stress[38].

Future studies should aim to incorporate additional brain regions located more rostrally, such as the basolateral amygdala and the paraventricular nucleus of the hypothalamus, both of which are critically involved in stress and emotional regulation. Including these

regions could provide further insights into the role of norepinephrine signaling in stress resilience and susceptibility. Additionally, to deepen our understanding of noradrenergic activation in downstream targets of the locus coeruleus-norepinephrine (LC-NE) system, these studies could utilize retrovirus injection techniques in DBHcre mice. This approach would allow for tracing the monosynaptic inputs from the locus coeruleus to specific target regions of interest, such as the motor cortex, bed nucleus of the stria terminalis, basolateral amygdala, paraventricular nucleus of the hypothalamus, habenular nucleus etc., following exposure to the learned helplessness paradigm. By mapping these inputs, we could determine the specific pathways through which norepinephrine modulates stress responses and identify potential mechanisms of resilience and susceptibility. Moreover, this type of synaptic tracing could be paired with optogenetic or chemogenetic manipulation to selectively activate or inhibit the LC-NE circuit at chosen targets during behavioral testing. This would allow us to assess the functional role of norepinephrine in mediating behavioral outcomes associated with learned helplessness and potentially link these neural circuits to changes in c-Fos expression, providing a more dynamic and mechanistic understanding of the underlying processes.

Furthermore, while primarily known for its role as the brain's major source of norepinephrine, the locus coeruleus also produces and co-releases dopamine, which may contribute to stress responses and stress-induced depression. Dopaminergic signaling in target and other brain regions, such as the bed nucleus of the stria terminalis and the nucleus accumbens, may interact with NE neurotransmission to influence susceptibility

or resilience to chronic stress. Interestingly, a study found that NE and DA modulated stress responses in the BNST in opposite ways. An aversive stimulus inhibited dopamine while causing norepinephrine release, whereas a non-aversive stimulus activated dopaminergic signaling and inhibited norepinephrine[39]. A study by Willmore et al. showed that resilient mice showed increased DA activity in the nucleus accumbens when subjected to periods of chronic stress[40]. And decreased dopaminergic receptors in the caudate nucleus of helpless rats is shown to have motor deficits associated with LH behaviour[41]. Given the strong interplay between dopaminergic and noradrenergic systems, further research is needed to determine how LC-derived dopamine and norepinephrine influence one's behavior to chronic stress and determine how the relationship between the two neurotransmitters would affect stress-induced depression.

As mentioned earlier, drugs targeting VMAT2 in humans may not have the intended efficacy and specificity to decrease extracellular NE levels, further experiments can be conducted to target certain noradrenergic receptors. Noradrenergic receptors are G protein-coupled receptors that mediate the effects of NE in the central and peripheral nervous systems. They are divided into two main classes, α (alpha) receptors and β (beta) receptors. Increased α_1 and β receptor activity is often linked to heightened responses to stress and increased susceptibility to chronic stress and depression[42]. However, the α_2 receptor is an autoreceptor located on the presynapse that inhibits norepinephrine release and is often linked to adaptive responses to stress and can increase resilience during chronic stress. Increased and excessive release of NE in the synaptic cleft bind to

presynaptic α_2 receptors in a noradrenergic negative feedback mechanism to close Ca^{2+} channels on the presynapse to inhibit NE release in the synapse, ultimately lowering NE activity in the brain during chronic stress[42,43]. Given the role of α_2 receptors, further studies can investigate the therapeutic potential of α_2 receptors agonists, thus decreasing the release of norepinephrine into the synaptic cleft and preventing NE from binding to postsynaptic noradrenergic receptors.

Taken together, these proposed directions underscore the importance of expanding our understanding of the LC-NE system and its role in stress resilience and susceptibility. By incorporating additional brain regions and using advanced techniques to manipulate neuronal activity, future research can illuminate the specific pathways through which norepinephrine influences stress-related behaviors. Moreover, targeting noradrenergic receptors, particularly α_2 autoreceptors, offers a promising avenue for further studies, potentially leading to better treatments for stress-related disorders. In conclusion, further research in the role of norepinephrine signalling during chronic stress would significantly advance our knowledge of the neurobiological basis of susceptibility and resilience, and potentially allow us to target certain pathways in humans to develop therapeutic options for patients struggling with chronic stress and depression.

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6. APPENDICES

6.1 Appendix A

Table 1. Percentage of c-Fos labeled cells to total number of cells in VMAT2^{DBHcre} KO mice (resilient, susceptible, and control) in different brain regions.

	VMAT2^{DBHcre} KO						
	Resilient		Susceptible		Control		
Brain Region	*M*	*SD*	*M*	*SD*	*M*	*SD*	*p*
M1	55.98	5.92	56.15	6.66	59.07	5.51	0.776
M2	56.39	4.31	53.97	6.15	59.18	13.40	0.757
BNST	51.95	12.63	59.58	4.43	59.12	16.76	0.649
PL	58.75	4.70	59.72	6.02	64.07	14.76	0.738
Pir	77.11	3.66	83.40	3.55	77.95	1.67	0.080

Note: *n* = 4 for resilient; *n* = 3 for susceptible; *n* = 3 for control. One-way ANOVA for all brain regions across mouse groups were statistically insignificant *p*>0.05. Follow-up post-hoc analysis showed no further significance.

Table 2. Percentage of c-Fos labeled cells to total number of cells in WT mice (resilient, susceptible, and control) in different brain regions.

	WT						
	Resilient		Susceptible		Control		
Brain Region	*M*	*SD*	*M*	*SD*	*M*	*SD*	*p*
M1	70.93	2.92	73.99	4.62	67.33	10.05	0.434
M2	72.51	9.65	73.90	10.61	68.56	15.01	0.838
BNST	71.52	4.54	71.59	5.96	67.01	16.94	0.815
PL	77.44	6.40	77.96	4.33	76.82	9.70	0.977
Pir	84.37	0.13	84.09	7.42	82.36	10.24	0.935

Note: *n* = 3 for resilient; *n* = 4 for susceptible; *n* = 3 for control. One-way ANOVA for all brain regions across mouse groups were statistically insignificant *p*>0.05. Follow-up post-hoc analysis showed no further significance.

Table 3. Percentage of c-Fos labeled cells to total number of cells in both mice genotypes in different brain regions.

	VMAT2^{DBHcre} KO		WT		
Brain Region	*Mean*	*SD*	*Mean*	*SD*	*p*
M1	56.96	5.51	71.07	6.32	0.00005
M2	56.50	7.68	71.88	10.67	0.00164
BNST	56.09*	10.74	70.19	9.22	0.00675
PL	60.64	8.34	77.46*	6.00	0.00011
Pir	79.25	4.03	83.65	6.52	0.08591

Note: *n* = 10 for VMAT2^{DBHcre} KO mice; *n* = 10 for WT mice; **n* = 9.

6.2 Appendix B

FINAL STUDY REPORT

Find Therapeutics Report No. FTX-PHA-011

Characterization of the behavioral effects of FTX-101
administered daily by intraperitoneal injection to C57/BL6 mice
up to 4 consecutive weeks

Testing site:
Douglas Hospital Research Center
6875 Blv Lasalle
Montreal, QC, H4H 1R3

Sponsor:
Find Therapeutics
7171 Frederick-Banting
Saint-Laurent, QC, H4S 1Z9

Issue Date:
June 17, 2024

1. INTRODUCTION

The main goal of the study was to test the effect of FTX-101 on different behavioral paradigms to assess its effects on motor, cognitive, and mood-related dimensions using the following experimental models:

Open Field Test
Elevated Plus maze
Novel Object Recognition Test (short and long-term evaluation)
Rotarod evaluation of motor coordination

1.1 Experimental Design

FTX-101 was administered daily by intraperitoneal (IP) injection to C57/BL6 male and female mice for 4 consecutive weeks and its effects were assessed before Day 1 of dosing, then on Days 14 and 28 (± 3 days). Animals of the control group received the vehicle (phosphate buffered saline [PBS] 1X, pH 7.4).

Groups	**FTX-101 (mg/kg)**	**Treatment Regimen*** **	**Administration Route****	**Number of animals**	
				Males	*Females*
1	0*	Once daily for 28 days	IP	5	7
2	0.1	Once daily for 28 days	IP	5	7
3	2.5	Once daily for 28 days	IP	5	7

** Control animals were injected with the Vehicle (PBS 1X, pH 7.4)*
***Injection site alternated on the right and left side of the abdomen to minimize possible local irritation*
****Starting Day: April 20, 2024*

The dose volume administered to each animal including controls was 10 mL/kg.

Following dosing on Day 28 and the completion of the tests, the animals were euthanized with CO_2 and brains were extracted, flash-frozen and stored at -80°C for future use.

1.2 Justification for Selection of the Animal Model, Route of Administration, Species and Dose Levels

FTX-101 had previously been tested for its toxicity (up to 28 days) in CD1 mice and Sprague-Dawley rats (up to 15 mg/kg) and in Beagle dogs (up to 10 mg/kg). Daily subcutaneous injections of FTX-101, up to 28 days did not induce any toxicity, even at the highest dose.

In this study, FTX-101 was administered intraperitoneally once a day for 4 consecutive weeks at the doses indicated below:

- The dose of 0.1 mg/kg of FTX-101 was selected based on results from previous murine models (Cuprizone model for demyelination/remyelination and experimental

allergic encephalomyelitis/EAE models for neuroinflammation with demyelination). In these models, this dose was found to be pharmacologically active (acceleration of remyelination and decrease of the clinical score indicative of the severity of the neurological impairments induced by neuroinflammation associated with demyelination).

- The dose of 2.5 mg/kg of FTX-101 corresponds to the highest plasma exposure dose with non-observed adverse effects (NOAEL) obtained in rats exposed to FTX-101 for 4 consecutive weeks.

1.3 Study Schedule

Animal arrival date:	April 09, 2024
Experimental start date (date of first baseline test):	April 15, 2024
First treatment:	April 20, 2024
Last necropsy:	May 17, 2024
Study completion date:	Date the Study Director signs the final report

2. TEST ITEM INFORMATION

2.1 Test Item Action

The test item, FTX-101, is a 29-mer peptide that modulates the Plexin A1/Neuropilin 1 receptor complex. FTX-101 is currently being developed for the treatment of clinical indications requiring repair of the myelin sheath.

2.2 Characterization of Test and Control/Vehicle Items

Test Item:	Identity:	FTX-101
	Description:	White to off-white powder
	Batch No.:	BX1004312
	Retest Date:	July 21, 2024
	Purity (HPLC):	97.0%
	Water content (w/w):	1.32%
	API content (free base):	82.8% w/w
	Correction Factor:	1.21
	Storage Conditions:	Frozen (-20°C), protected from light
	Handling Precautions:	Standard laboratory precautions
	Supplier:	Cordon Pharma Brussels via Find Therapeutics

Correction factor: 1/API content = 1/0.828 = 1.21

Control/Vehicle Item:	Identity:	Phosphate buffered saline (PBS) 1X, pH 7.4
	Description:	Clear colorless liquid
	Storage Conditions:	Room temperature (15 to 30°C)/ Refrigerated (2 to 8°C) once opened
	Handling Precautions:	Standard laboratory precautions

2.3 Preparation of Test and Control/Vehicle Item Formulations

The control/vehicle item was used as supplied for dose administration to the control animals (Group 1) and for the preparation of the test item formulations (Groups 2 and 3).
The test item formulations were prepared once weekly, according to the instructions provided by the Sponsor and detailed below.

1. For Group 3 formulation, the appropriate amount (7.548 mg) of FTX-101 (using a correction factor of 1.21) was weighed and transferred into an appropriate container.
1. The appropriate volume of the control/vehicle item, PBS 1X pH 7.4 (~90% of the final volume), was measured and added to the container.
2. The formulation was mixed using a magnetic stir bar.
3. The pH of the formulation was measured and adjusted to pH 7.4 (± 0.1) using 1N NaOH. The final pH was recorded.
4. The formulation was brought to final volume (QS; 25 mL) with the control/vehicle item, PBS 1X pH 7.4 and further mixed, until complete dissolution. Final concentration of 0.250 mg/mL.
5. For Group 2 formulation, an appropriate volume (1 mL) of the formulation prepared for Group 3 was aliquoted and transferred into an appropriate container.
6. The formulation was brought to final volume (QS;) with the control/vehicle item, PBS 1X pH 7.4 and further mixed. Final concentration of 0.0100 mg/mL.
7. The test and control/vehicle formulations were stored refrigerated (2 to 8°C) when not used for dosing on the same day of preparation. When stored refrigerated, the test and control/vehicle formulations were allowed to equilibrate to room temperature for a minimum of 30 minutes prior to administration on days of dosing.
8. Upon completion of dosing, the remaining test and control/vehicle formulations were discarded.

3. TEST SYSTEM

Forty-two (42) C57/BL6 mice (18 males and 18 females, plus 3 male/3 female spares), seven to eight weeks old, were purchased from Charles River and were maintained in a specific pathogen-free barrier facility. At initiation of treatment, their body weights ranged from 20 to 23 g for males and 19 to 21 g for females.

3.1 Animal Care Committee

All experimental procedures were approved by the Douglas Hospital ethics committee, overseen by the Canadian Council for Animal Protection (Protocol #7179). The 3Rs (Replacement, Reduction, Refinement) rules were applied following the CCAC Canadian Council on Animal Care) directives.

3.2 Health Status

On arrival at Douglas Hospital, all animals were weighed and subjected to a visual inspection to ensure satisfactory health status. During habituation in the housing room, several males demonstrated aggressive behavior towards their cagemates, resulting in severe injuries and fight wounds. As a result, 9 males were excluded from the study and euthanized. All other animals were deemed suitable for use in the study.

3.3 Housing

Mice were held at no more than 4 mice per cage in ventilated cages with appropriate bedding material (Envigo, Corn cob 1/4", T.7097). The bedding was changed at appropriate intervals to maintain hygienic conditions. Following randomization, all bins were labeled to indicate study, group, animal and bin numbers, and sex.

3.4 Identification

A permanent marker on the tail uniquely identified each animal following arrival.

3.5 Room Environment

The housing conditions applied were the standard of the animal house at the Douglas Hospital, namely:

- Temperature: 23 ± 3^{O}C
- Humidity: 50 ± 10%
- Photoperiod: 12h light/12h dark
- HEPA filtered air
- 8 to 12 air exchanges per hour with no recirculation

3.6 Diet/Water

Water (tap in washed bottles) and food (Envigo, Teklad T.2018.15) were provided to the animals ad libitum, except during designated procedures such as those requiring removal of the animal from the home bin.

3.7 Acclimation

8-day acclimation period was allowed between receipt of the animals and the start of treatment to accustom the rats to the laboratory environment.

3.8 Allocation to Study Groups

During the acclimation period, 18 male and 18 female mice were assigned to their respective dose groups based on the mean baseline measurements in each behavioral test. Males and females were randomized separately.

3.9 Administration of the Test and Control/Vehicle Items

The test and control/vehicle items were administered by a single intraperitoneal injection using a hypodermic needle attached to as syringe at a dose volume of 10 mL/kg. The actual volume administered to each rat was calculated and adjusted based on the most recent practical body weight of each animal. Mice were weighed every other day until the end of the study.

4. TERMINATION AND SAMPLE COLLECTION

Mice were anesthetized with isoflurane, euthanized with CO_2, and immediately decapitated. Brains were extracted and flash-frozen by dipping in liquid nitrogen-cooled isopentane (2-methylbutane, Merck, Billerica, MA), and stored at −80°C until further use.

5. ARCHIVING

All data and specimens that were generated during this study at the Douglas Hospital, together with the original copy of the study plan and the final report, will be retained for approximately 1 year, at the Douglas Hospital. The archiving period will commence from the date of study finalization.

The Study Director agrees to give Find Therapeutics sufficient advance notification of any intended disposal of such materials after the 1-year holding period, to allow Find Therapeutics to secure alternative storage facilities.

6. RESULTS

In the current study the effect of FTX-101 was tested on several behavioral paradigms to assess its impact on motor, cognitive, and mood-related dimensions. Additionally, its potential side effects at high doses were evaluated. General locomotor activity level, anxiety level, and exploratory activity were assessed in the Open Field and the Elevated Plus Maze tests. Short- and long-term memory was evaluated in the novel object recognition test. Lastly, motor learning and coordination were assessed in the rotarod test.

Before the compound was administered, all animals were tested for baseline measurements in each behavioral test. This data was used to split the animals into treatment groups, ensuring that the mean values for the measured parameters did not differ between groups.

FTX-101 was administered daily by intraperitoneal (i.p.) injection to C57/BL6 mice for 4 consecutive weeks at doses of 0.1 mg/kg or 2.5 mg/kg. The control group was injected with the PBS 1X buffer, pH 7.4.

6.1 Open Field Test

Rodents exhibit a natural aversion to open and well-lit spaces. However, they also possess a willingness to explore perceived threatening stimuli. Reduced anxiety levels lead to increased exploratory behavior, while heightened anxiety levels result in reduced locomotion and a preference for remaining close to the walls of the arena (thigmotaxis). Open Field Test (OFT) also allows evaluation of the spontaneous locomotion of rodents and some stereotypic behavior.

A square open arena (45 x 45 x 50cm tall) made of opaque gray plastic was used. Mice were placed in the center of the arena at the beginning of the trial and allowed to freely explore the arena for 10 minutes.

The time spent in motion (speed ≥ 2 cm/s), total distance traveled, number of entries, and time spent in the central zone (defined as the middle square area 20 x 20cm of the arena) were measured.

In the open field test, general locomotor activity was measured as total distance moved during 10 minutes exploration of the apparatus. In males, repeated measurements (RM) two-way ANOVA didn't reveal any significant effect of treatment or treatment x time interaction. However, a significant effect of time was observed ($F(1, 12) = 11.57$, $p<0.01$). Tukey's multiple comparisons test showed that the total distance moved was significantly lower after 4 weeks of treatment with FTX-101 at the dose level of 0.1 mg/kg compared to 2 weeks of treatment (p adj.<0.01) (Fig.1A). In females, RM two-way ANOVA also didn't reveal any significant effect of treatment or treatment x time interaction, but a significant effect of time ($F(1, 18) = 34.12$, $p<0.001$). Tukey's multiple comparisons test showed a significant difference between 2 weeks and 4 weeks of treatment in control (p adj.<0.01), 0.1 mg/kg (p adj.<0.01) and 2.5 mg/kg (p adj.<0.05) (Fig.1D). These results indicate that both male and female mice showed significant decrease in locomotor activity over time, with specific differences noted between different time points, but the treatment did not appear to have any effect on these changes.

Anxiolytic effects of treatment were assessed by measuring time spent in the center zone of the Open Field. In males, RM two-way ANOVA didn't reveal any significant effect of treatment or treatment x time interaction. However, a significant effect of time was observed ($F(1, 12) = 9.905$, $p<0.01$). Tukey's multiple comparisons test showed significant decrease in time spent in center between 2 weeks and 4 weeks in control group (p adj.<0.01), and non-significant decrease in 2.5 mg/kg group (p adj.=0.059) (Fig.1B). In females, RM two-way ANOVA didn't reveal any significant effect of treatment or treatment x time interaction, but showed a significant effect of time ($F(1, 18) = 11.51$, $p<0.01$). Tukey's multiple comparisons test showed significant difference between 2 weeks and 4 weeks of treatment in the 0.1 mg/kg (p adj.<0.05) and 2.5 mg/kg treated groups (p adj.<0.05) (Fig.1E). Taken together, the results demonstrate that time spent in the center zone decreased significantly over time in both males and females, indicating increased anxiety levels or habituation to the apparatus, but this was not significantly affected by the treatment.

Exploratory activity was evaluated by measuring vertical activity. In males, RM two-way ANOVA didn't show any significant effect of treatment, time or treatment x time interaction (Fig.1C). In females, RM two-way ANOVA didn't reveal any significant effect of treatment or treatment x time interaction, but did show a significant effect of time ($F(1, 18) = 26.12$, $p<0.001$). Tukey's multiple comparisons test showed significant decrease in vertical activity between 2 weeks and 4 weeks in control group (p adj.<0.01), 2.5 mg/kg treated group (p adj. <0.01), and non-significant decrease in 0.1 mg/kg treated group (p adj. = 0.0512) (Fig.1F). These results show that vertical exploratory activity decreased significantly over time in females, but not in males, and this was not significantly influenced by the treatment.

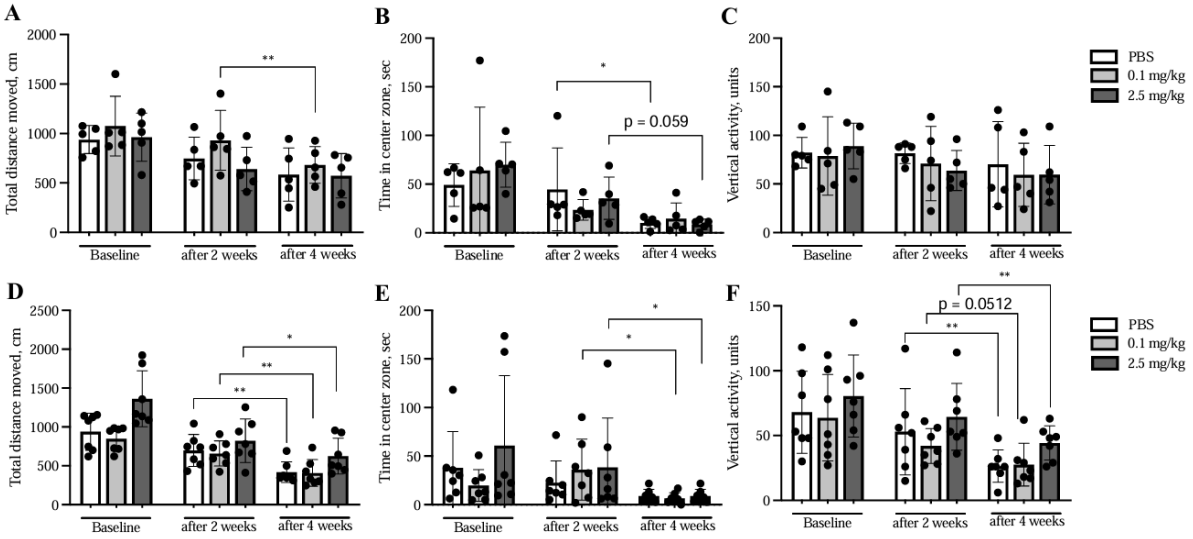

Figure 1. General locomotor activity in the Open Field Test. A-C: Performance in Open Field Test of male mice (n = 5 per group). A = Total distance moved during the 10 min test period. B = Percentage of time spent in the center zone. C = Vertical activity. D-F: Performance in Open Field Test of female mice (n = 7 per group). D = Total distance moved during the 10 min test period. E = Percentage of time spent in the center zone. F = Vertical activity. Data are expressed as mean ± SD. *p<0.01

6.2 Elevated Plus Maze

The Elevated Plus Maze (EPM) is a test measuring anxiety in laboratory animals, typically using rodents as a screening test for putative anxiolytic or anxiogenic compounds. The model is based on the test animal's aversion to open spaces and its tendency to be thigmotactic. In the EPM, this anxiety-like behavior is manifested by the animal spending more time in the closed arms.

The EPM is designed in the form of a cross with four branched arms, including 2 opposing open arms (30 x 5 cm) and 2 arms closed off by a dark wall (30 x 5 x 11 cm). The arms radiate from a central platform (5 cm^2), and the apparatus is 50 cm above the ground. Mice were placed on the central platform, facing an open arm, and were allowed to explore the maze for 5 minutes. An entry into the open or closed arm was defined when all four paws were inside the arm.

The percentage of time spent in the open arms, the number of entries into the opened and closed arms, and the time spent on the central platform were recorded as behavioral parameters.

In males and females, RM two-way ANOVA didn't show any significant effect of treatment, time or treatment x time interaction on the percentage of time spent in open arms (Fig. 2A,C). However, RM two-way ANOVA revealed a significant effect of time on the number of entries in males ($F(1, 12) = 12.65$, $p < 0.01$). Tukey's multiple comparisons test showed significant decrease in this parameter between the two treatment time points in the control group (p adj.<0.05) and 2.5 mg/kg treated group (p adj. <0.05) (Fig. 2B). In females, RM two-way ANOVA also revealed a significant effect of time ($F (1, 18) = 18.26$, $p<0.001$). Tukey's multiple comparisons test showed a significant decrease between the two treatment time points in control group (p adj.<0.05) and 2.5 mg/kg treated group (p adj. <0.01) (Fig. 2D). Taken together, these results show that the time spent in the open arms did not change significantly due to treatment, but the number of entries into the open arms decreased significantly over time in both males and females regardless of the treatment.

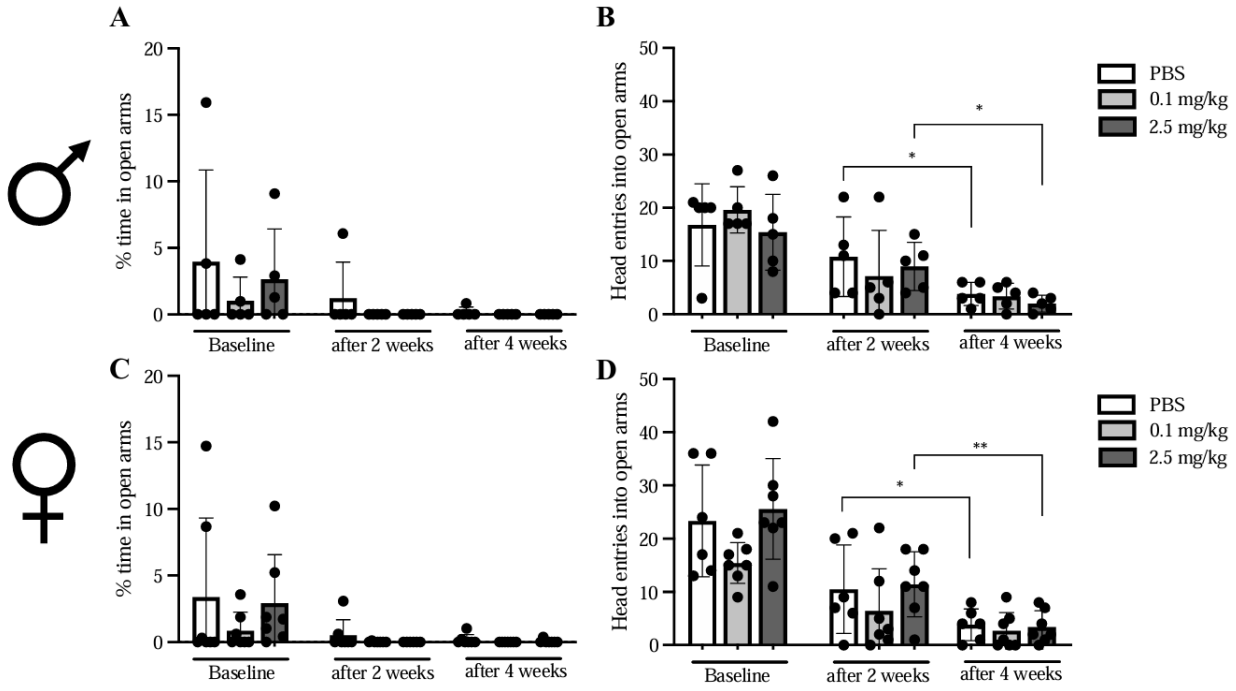

Figure 2. Elevated Plus Maze Test: Percentage of time spent in open arms for males (A) and females (C). Head entries into the open arms for males (B) and females (D). Data are expressed as mean ± SD. *p<0.01

6.3 Novel Object Recognition Test

The mice were tested in an Open Field box (45 x 45 x 50 cm). On the habituation day, mice were allowed to explore an empty arena for 5 min. On the training day, 24 hours later, the mice were allowed to explore two identical objects for 5 minutes. Short- and long-term memory was assessed in the novel object recognition test 4h and 24h, respectively, after the familiarization phase. The novel object discrimination index was calculated as the time spent exploring the novel object divided by the time spent exploring both objects, multiplied by 100. It should be noted that when a mouse spent less than 10 seconds exploring both objects, this mouse was excluded from further analysis. In males and females, RM two-way ANOVA didn't show any significant effect of treatment, time or treatment x time interaction on short- or long-term memory (Fig. 3). These results indicate that neither short-term nor long-term memory was significantly affected by the treatment.

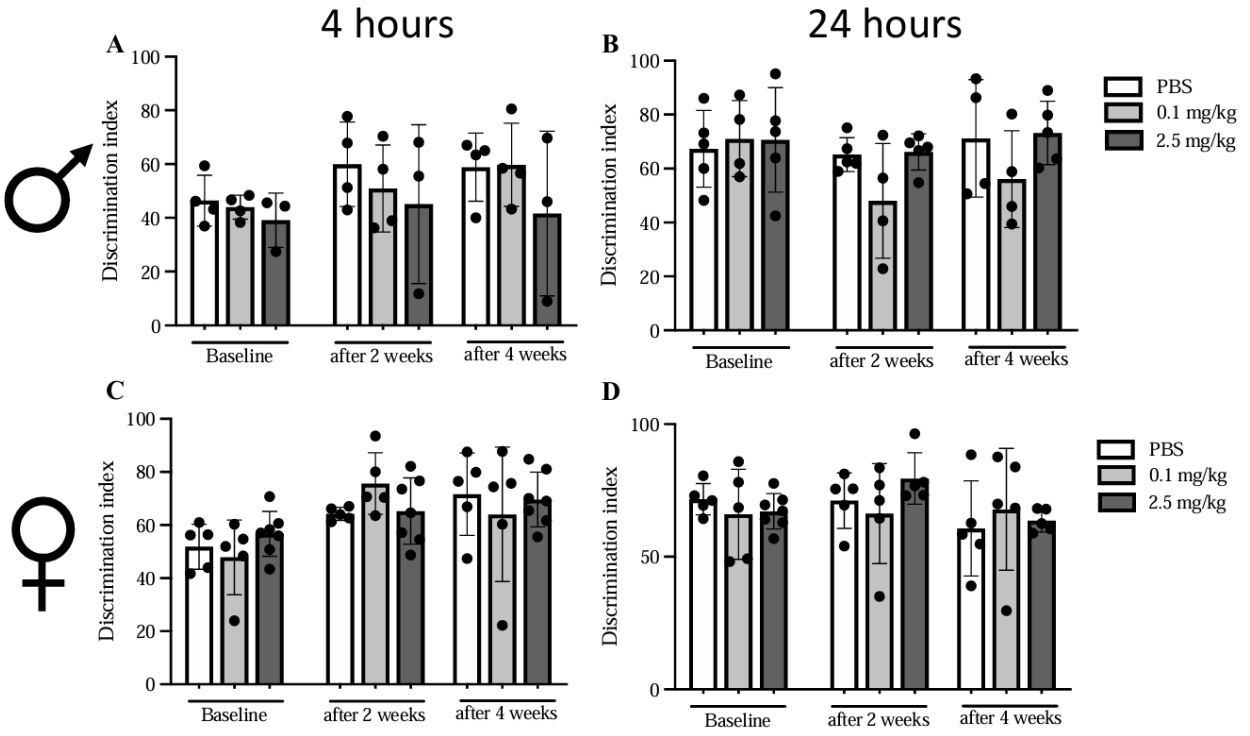

Figure 3. Novel object recognition test: short-term (A,C) and long-term (B,D) memory. Novel object discrimination index for males (A,B) and females (C,D). Data are expressed as mean ± SD.

6.4 Rotarod Test

In the rotarod test, rodents were assessed for motor coordination and balance using a rotating rod apparatus. After acclimation to the testing environment, rodents underwent a training session to familiarize themselves with the rotating rod. During testing, rodents were placed on the rotating rod, and the latency to fall was recorded. The speed of the rotarod was accelerated from 4 to 40 rpm over 5 minutes. Five trials were conducted with 15-minute rest periods in between to prevent fatigue. It was observed that FTX-101 at a dose of 2.5 mg/kg impaired rotarod performance in males after 4 weeks of treatment (Fig.4C). When comparing average latency to fall across all trials in males, RM two-way ANOVA didn't show any significant effect of treatment, time or treatment x time interaction (Fig. 4G). In females, RM two-way ANOVA revealed a significant effect of time ($F(1, 18) = 11.84$, $p<0.01$). Tukey's multiple comparisons test showed significant increase in this parameter between the two treatment time points in the control group (p adj.<0.01) and 0.1 mg/kg treated group (p adj. <0.05) (Fig. 4H). Taken together, the results show that motor learning and coordination were not significantly affected by the treatment overall, but the 2.5 mg/kg dose impaired performance in males. Females showed an improvement over time, independent of treatment.

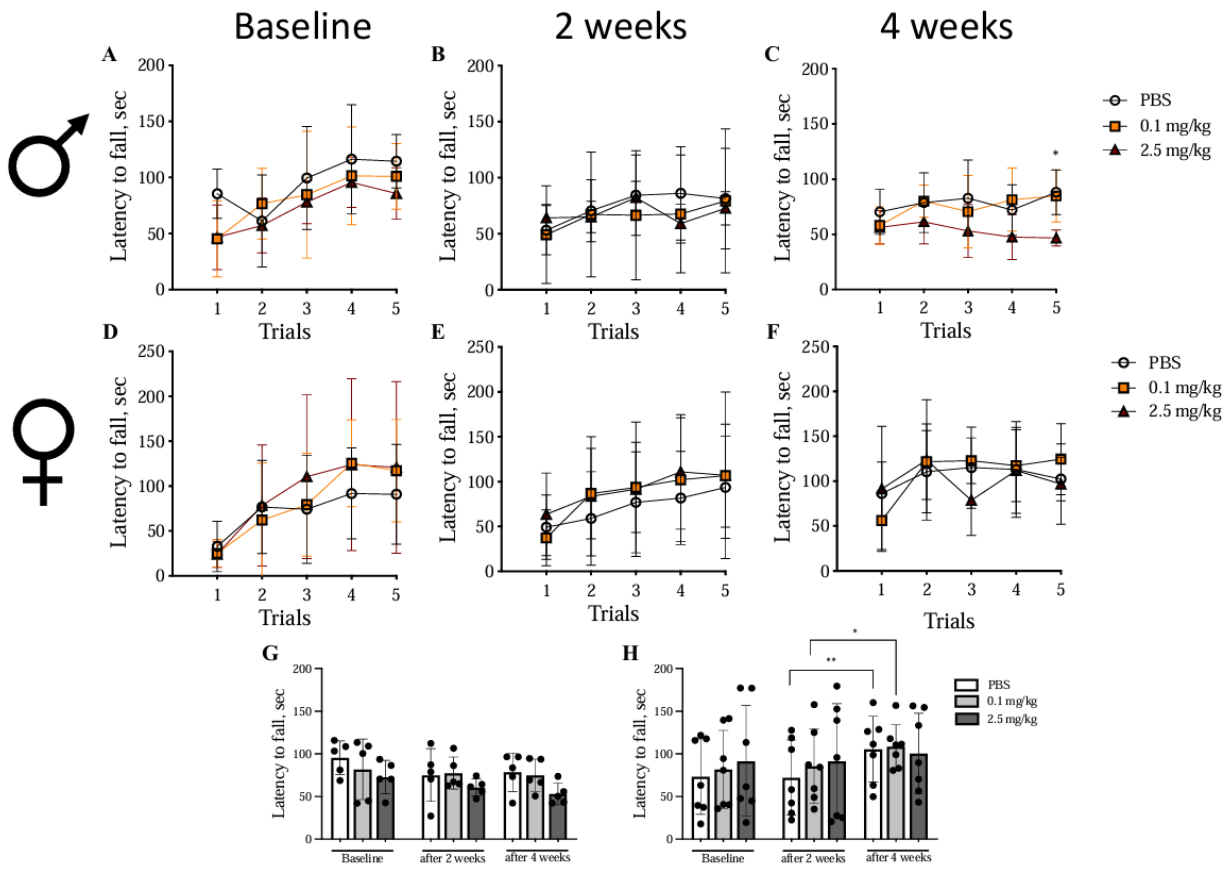

Figure 4. Time spent on the rod of the accelerating rotarod for every trial completed at baseline (A,D), 2 weeks of treatment (B,E) and 4 weeks of treatment (C,F) in males (A-C) and females (D-F). Data represent the mean of latency to fall per trial in seconds ± SD. Lower panels represent the average mean latency to fall in seconds ± SD of the all five trials of each training day.

7. SUMMARY AND CONCLUSION

Overall, compared to the vehicle-treated animals, the administration of FTX-101 at doses of 0.1 mg/kg and 2.5 mg/kg over a period of 4 weeks did not significantly affect general locomotor activity, anxiety levels, exploratory activity, or cognitive function in C57/BL6 male and female mice. The only notable effect was a gradual decrease in the rotarod performance observed in the males treated for 4 weeks with FTX-101 at 2.5 mg/kg. However, this was not the case in the female group treated at the same dose level or in the animals that received 0.1 mg/kg. In addition, the 2.5 mg/kg dose is the highest dose that we administer to rodents because of the saturation of the absorption. In humans, the proposed starting dose is appr. 50-fold lower compared to the 2.5 mg/kg in the rat.

The observed changes over time in various behavioral measures appear to be independent of the treatment with FTX-101, and suggest that some factors common to all groups (e.g. habituation to the apparatus, stress from injection itself) are responsible for the significant main effect of time.