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Viral injection of RNA polymerase II-interacting protein RPRD2 in the nucleus accumbens induces anxiety-like behavior in mice

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A thesis in partial fulfillment of the requirements for the degree of Master's in Science in Anatomy and Neurobiology at Virginia Commonwealth University

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List of Abbreviations

B-Actin: Beta-Actin

CSDS: Chronic Social Defeat Stress

CID: CTD Interaction Domain

CTD: C-Terminal Domain (of RNA Polymerase II)

EPM: Elevated Plus Maze

GFP: Green Fluorescent Protein

HIV: Human Immunodeficiency Virus

HSV: Herpes Simplex Virus

IZ: Interaction Zone

NAc: Nucleus Accumbens

Pol II: RNA Polymerase II

pSer5: Phosphorylated Serine 5

RPRD2: Regulation of Nuclear Pre-mRNA Domain Containing Protein 2

SEM: Standard Error of Mean

Ser5: Serine 5 on the CTD of Pol II

SI: Social Interaction

TBP: TATA binding protein

Abstract

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Anxiety and its related disorders have become increasingly prevalent as more awareness and acceptance of mental illnesses have come to fruition, especially in the light of the ongoing COVID-19 pandemic. Anxiety can affect anyone regardless of their age, sex, or social status and is currently the most commonly diagnosed mental illness worldwide (Bandelow & Michaelis, 2015). While there are several effective treatments available, the underlying brain mechanisms that cause anxiety are still largely unknown and further research continues to piece together the complex pathophysiology behind this disease. The use of laboratory animal models, such as mice, to induce and observe anxiety-like behaviors are an effective research method to determine specific molecular mechanisms that can influence anxiety. These studies may help to identify more effective treatments and improve the understanding of complex mental illnesses.

Our laboratory previously identified increased levels of protein expression of RPRD2 in the nucleus accumbens (NAc) of mice that were resilient to chronic social defeat stress (Hamilton et al., 2020). Known as regulation of nuclear pre-mRNA domain containing protein 2, this protein is largely unstudied and its mechanism and function remain incompletely understood. RPRD2 is a member of the RPRD family of proteins, which are known to downregulate transcription through interaction with the c-terminal domain of RNA polymerase II (Ali et al., 2019; Ramani et al., 2021). This thesis project began by exploring the specific effects of RPRD2 in the NAc of C57BL/6J mice, and while the initial hypothesis was that RPRD2 overexpression would make mice resilient to social defeat stress, it was subsequently found that there is a relationship between RPRD2 expression and anxiety-like behaviors.

To study this protein, a viral vector was created by inserting cDNA encoding RPRD2 into a destination vector using a LR clonase reaction. By packaging this plasmid within herpes simplex virus (HSV) and injecting mice with this viral RPRD2 in the NAc, it was observed that there was an increase in anxiety-like behavior such as avoiding the open arms of the elevated plus maze. These behaviors were noted regardless of whether these mice previously experienced chronic social defeat stress. However, the viral RPRD2 did not have any significant effects on the social behaviors of mice in the social interaction test, even when the mice were exposed to an accelerated chronic social defeat stress paradigm. This thesis proposed a potential mechanism for RPRD2 through binding to the c-terminal domain of RNA polymerase II. Once RPRD2 binds, it is thought to pause the progression of transcription to the elongation phase by dephosphorylating Serine 5. While more research must be performed to confirm this mechanism, the data from this thesis suggests that the injection of HSV-RPRD2 in the NAc increases anxiety-like behavior in

mice. The results of this study will help to better understand one potential mechanism of anxiety as well as provide more insight to the *in vivo* function of the protein RPRD2.

Introduction

Generalized Anxiety Disorders

Mental illnesses have a high level of prevalence in the United States, with millions of people affected each year. The most commonly diagnosed mental illnesses are anxiety disorders that include generalized anxiety disorder (GAD), social anxiety disorder (SAD), and specific phobias (Bandelow & Michaelis, 2015). Anxiety disorders are commonly comorbid with other mood disorders such as major depression or bipolar and are often seen in individuals who have chronic medical illnesses (Hidalgo & Sheehan, 2012). It is estimated that a third of the population will be diagnosed with an anxiety disorder in their lifetime, with roughly twice as many women being diagnosed than men (Bandelow & Michaelis, 2015). While most anxiety disorders will be diagnosed by the age of 20, GAD is more prevalent in middle-age with an average onset at the age of 31 (Wittchen & Hoyer, 2001; Bandelow & Michaelis, 2015). As the negative stigma associated with mental health is decreasing, these numbers may increase as people feel more comfortable seeking help and having honest conversations about their symptoms.

Acute episodes of anxiety can be beneficial to human performance and productivity, but the presence of chronic anxiety can have disabling effects. GAD is characterized by symptoms such as enduring worrying, restlessness, and excessive anxiety that persists but is not linked to stressful events (Wittchen & Hoyer, 2001). GAD and other anxiety disorders can also be associated with physical symptoms such as sweating, increased heart rate, and fatigue. High levels of anxiety can impact a person's ability to think clearly, finish tasks, and respond appropriately, leading to problems in relationships, employment, and overall health (Brahmbhatt

et al., 2021). Anxiety disorders are also commonly misdiagnosed due to the variability of symptoms shown and the comorbidities present (Brahmbhatt et al., 2021; Bandelow & Michaelis, 2015). There are currently several effective treatments for anxiety such as cognitive behavioral therapy (CBT), selective serotonin reuptake inhibitors (SSRIs), benzodiazepines, and several others that can relieve the symptoms of anxiety (Brahmbhatt et al., 2021). The average remission rate of patients across all treatments is estimated at roughly 51 percent, meaning that about half of people seeking treatment for anxiety do not experience long-term relief (Springer et al., 2018). Despite the high rate of people diagnosed with anxiety disorders, the underlying mechanisms are still unclear and require further research to improve the long-term effectiveness of treatments to GAD and other anxiety disorders.

Animal Models of Anxiety

The high disease burden of anxiety has necessitated the development of multiple animal models useful for the study of anxiety disorders. These models are created through conflict that create approach-avoidance behaviors and are beneficial to the discovery of the mechanisms of anxiety and the clinical effects of drugs (Campos et al., 2013). One of the more common subjects for these tests are rodents, although other animals can be used effectively. For the purposes of this thesis project, the focus will be on social defeat, the social interaction test, the elevated plus maze, and the open field test.

Chronic social defeat stress (CSDS) is a paradigm where a novel male C57B/6J mouse is introduced into the home cage of an aggressor male CD-1 mouse. This animal model of prolonged social stress has aided in understanding underlying mechanisms for disorders such as

anxiety or depression (Golden et al., 2011). This model takes a C57BL/6J mouse and repeatedly exposes it to an aggressor CD-1 mouse. While this physical exposure only takes place for about ten minutes, the mice are continuously exposed to sensory stress by being placed in a cage with a CD-1 aggressor present but separated by a perforated divider (Golden et al., 2011). This experience is repeated once a day for ten days, however there are multiple modifications to this experiment that remain effective in causing mice to be "defeated" such as the accelerated CSDS. The bouts of social defeat experienced by the C57BL/6J mouse leads to anxious and depressive behavior in a majority of mice.

The social interaction (SI) test can be used to measure social behavior in mice after they experience CSDS. The SI test measures for social deficits and anxiety-like behaviors in mice and is thought to mimic the state of anxiety similar to that experienced in GAD (File & Seth, 2003). C57BL/6J mice will be placed into an open field arena for 2.5 minutes, then have another 2.5 minutes in the same field with a novel CD-1 aggressor mouse present in a cage. Mice will be defined as susceptible if they spend less time in the interaction zone, the zone near the CD-1 mouse, when the CD-1 is present in comparison to when it is absent. Mice will be defined as resilient if they spend more time in the interaction zone when the CD-1 mouse is present (Golden et al., 2011). Susceptible and resilient mice are associated with social interaction (SI) ratios which are calculated by dividing the time spent in the interaction zone when the CD-1 is present by the time spent in the interaction zone when the CD-1 is absent. SI ratios less than 1 are used to define the mouse as susceptible and SI ratios greater than 1 are resilient mice (Golden et al., 2011). The zones are defined by digital animal tracking technology (Ethovision XT) and include corner zones, the interaction zone, and a center zone.

The elevated plus maze (EPM) is used to measure innate anxiety in mice. This test is based on rodents' instinctive tendency to explore their environment and the natural avoidance of bright, open, and elevated spaces (Campos et al., 2013). The EPM is also used to measure anxiety-like behavior in mice and has been found to have a strong predictive validity in defining brain regions and mechanisms involved in anxiety (Walf & Frye, 2007). This apparatus is raised above the ground and consists of four "arms," with two enclosed arms that are opposed perpendicular to two open arms. Mice that spend greater amounts of time in the closed arms are thought to be more anxious or stressed than mice who visit the open arms of the EPM (Kraeuter et al., 2019). This test does not require any prior conditioning and is effective in measuring anxiety-like behavior in mice.

The open field test is another test that is utilized to measure anxiety-like behavior in rodents. If a mouse spends less time in the center of the open field, they are deemed to be more anxious (Walz et al., 2016). This is associated with a behavior known as thigmotaxis, which is defined as the tendency of mice to stay close to the walls of an open space when exploring. Thigmotaxis and decreased time in the center are well-established markers of increased anxiety-like behavior in rodents (Walz et al., 2016). These tests have a high effectiveness in recreating anxiety-like behavior in mice that are useful in determining mechanisms of action and pharmacological agents.

The Nucleus Accumbens

The nucleus accumbens (NAc) is commonly referred to as the reward circuit of the brain due to its interconnectedness with the limbic system. The NAc can be divided into two sections

anatomically, the core in the center and the outer shell. These sections are chemically and molecularly diverse from each other and are thought to have different functions; the core involved in learning and purposeful action selection during goal-directed behaviors, while the shell is involved with emotional and motivational responses (Baik, 2020). Although the NAc is known for its involvement in the reward circuit, the NAc is also involved in behavioral responses to both aversive and rewarding stimuli. In a human study, fMRI was used in individuals with high levels of anxiety, and it showed an increase of activation when the stressor could be actively avoided by performing a behavior (Levita et al., 2012). However, there is a decrease in activation of this brain region when the individual can passively avoid a stressor by omitting a behavior, showing the influence of NAc in influencing motivated behaviors (Levita et al., 2012).

The NAc are largely composed of GABAergic medium spiny neurons (MSNs) that express dopamine D1 receptors or D2 receptors (Baik, 2020). Since the NAc is a critical component of the reward circuitry in the brain, it plays a key role in regulating anxiety (Xiao et al., 2020). The NAc receives input from limbic systems such as the amygdala, hippocampus, and prefrontal cortex and responds to stress signals (Li et al., 2021). While D1 and D2 MSNs make up a majority of the NAc, studies have shown that parvalbumin interneurons in the NAc have a major effect on anxiety-like behaviors (Xiao et al., 2020). However, due to the complexity of anxiety, there are likely many factors that result in this behavior and should be analyzed to provide more effective treatments. Anxiety is the most prevalent mental disorder, and avoidance behavior is a central symptom that contributes to further perpetuating the anxious state (Xiao et al., 2020).

Disruption in the NAc has been linked to depressive behaviors. A study on rats who experienced high levels of anxiety showed that the NAc had decreased arborization, shorter

dendrites, and lower spine density (Gebara et al., 2021). Their MSNs also had reduced excitatory inputs and lower connectivity between cells (Gebara et al., 2021). A balance between the activity of D1-MSNs and D2-MSNs is thought to be crucial for normal behaviors, and the disruption of this is thought to be involved in the pathophysiology of major depressive disorder, OCD, and bipolar disorder (Baik, 2020). Deep-brain stimulation of the NAc can alleviate symptoms in treatment resistant OCD patients and addiction patients, showing its importance in the mechanisms of these diseases (Levita et al., 2012). In addition, studies have shown that molecular changes in the cells caused by chronic stress or high levels of anxiety can be reversed through antidepressant treatment (Gebara et al., 2021). The NAc plays a key role in the pathophysiology of anxious and depressed individuals and targeting of this brain region can regulate these behaviors.

RNA Polymerase II Function

RNA Polymerase II (Pol II) is an important component of transcription in all eukaryotic cells. Pol II is an enzyme that catalyzes and directs the synthesis of messenger RNA (mRNA) from a DNA template (Cramer, 2004). The transcription cycle has three main phases: initiation, elongation, and termination. In order for initiation to occur, five general transcription factors TFIIB, -D, -E, -F, and -H are required to recognize the promoter region to unwind the DNA and form the "transcription bubble" **(Figure 14A)** (Liu et al., 2013). These transcription factors bind to the TBP and form a transcription initiation complex with Pol II. Following the initiation of transcription, Pol II continues to move along the template to elongate the transcript one nucleotide at a time from 5' to 3' **(Figure 14A)** (Corden, 2021). The enzyme can maintain the bubble, translocate along the template DNA, synthesize RNA, and proofread the nascent RNA

independently (Cramer, 2004). Once Pol II reaches the 3' end of the transcript, it releases the completed transcript and terminates transcription. Each step of transcription can be influenced by proteins that interact with the C-terminus of the largest subunit (Corden, 2021).

Pol II has a mass over 500 kDa and consists of twelve subunits that have unique interactions and composition (Woychik & Young, 1990). Of the twelve subunits, the first three (Rpb1, Rpb2, and Rpb3) are the largest. A number of different proteins can interact with this enzyme, especially the carboxy-terminal end of one subunit, to regulate its activity and production of the type and quantity of RNA transcripts formed in response to a cell's requirements (Woychik & Young, 1990). The C-terminal domain (CTD) protrudes off Pol II subunit Rpb1 and consists of 52 heptapeptide repeats with the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Cramer, 2004). Various signals known as post-translational modifications (PTM) can phosphorylate or dephosphorylate heptapeptide repeats of the CTD which correspond to different parts of the transcription cycle such as initiation, elongation, and termination (Ramani et al., 2021). PTM of the CTD are crucial to coordinating and organizing the events of transcription and proper gene expression.

Patterns of CTD phosphorylation regulate a specific interaction with RNA processing factors; for example, recycling of Pol II after termination requires dephosphorylation of the CTD (Cramer, 2004). The phosphorylation of Serine-5 (Ser5) is required for Pol II to be released from the promoter region and progress to the elongation phase of transcription (Clark, 2010). Ser5 phosphorylation peaks around the transcription start site and is gradually removed by phosphatases as Pol II elongates (Hsin & Manley, 2012). Proteins that interact with the CTD can be involved in various transcriptional steps, demonstrating that this region of Pol II is crucial in

regulating the cycle. Loss of the CTD results in decreased efficiency and or the loss of all processing steps (Clark, 2010).

Regulation of Nuclear Pre-mRNA Domain Containing Protein 2

Regulation of cellular processes such as gene expression is critical to the function and survival of the cell. Pol II is the enzyme responsible for transcribing all protein-coding regions of DNA. Once Pol II is recruited to the gene promoter, different factors can affect the rate at which RNA is transcribed. There is a rate-limiting step found in higher eukaryotes known as promoter-proximal pausing that is associated with specific phosphorylation events at the CTD of Pol II (Winczura et al., 2021). There are many factors that interact with the CTD to affect transcription, to include the RPRD (Regulation of Nuclear Pre-mRNA Domain) family of proteins (RPRD1A, RPRD1B, and RPRD2). RPRD1A and RPRD1B contain similar structures and coiled-coil domains that facilitate their interaction with the CTD (Winczura et al., 2021). Proteins containing CTD-interacting domains (CID) such as RPRDs have an affinity for lysine residues that are acetylated (i.e., K7ac) (Ali et al., 2019). This interaction promotes Ser5 dephosphorylation, which pauses transcription by preventing Pol II from moving into the elongation phase of transcription (Ali et al., 2019; Ramani et al., 2021). This process is thought to be the main mechanism of RPRDs to downregulate transcription.

While RPRD1A and RPRD1B are known paralogs, their functions are quite different (Winczura et al., 2021). RPRD1B is associated with cell proliferation and tumors, while increased expression of RPRD1A slows cell growth and suppresses tumor growth (Winczura et al., 2021). RPRD2 is much larger than its counterparts with little known about its function. RPRD2 is also known as REAF, or RNA-associated early-stage antiviral factor. In comparison to

its counterparts, RPRD2 has serine- and proline-rich domains, with the latter region becoming hyperphosphorylated during M phase (Ni et al., 2011). RPRD family proteins are all involved in transcription, but RPRD2 may have a specific role in microRNA biogenesis. RPRD2 is regulated by Interleukin-32a (IL-32a), an anti-inflammatory cytokine. IL-32a can regulate RPRD2 to suppress miRNA synthesis when the cells are experiencing inflammation (Son et al., 2017).

RPRD2 has become prominent in human immunodeficiency virus (HIV) research. This protein can restrict HIV replication and limit integration of the virus into the genome (Gibbons et al., 2020). Research has shown that RPRD2 acts early post-entry of HIV and can block the virus during or following initiation of reverse transcription, before the virus can enter the nucleus (Marno et al., 2014). However, HIV type 1 contains an accessory protein known as Vpr that targets RPRD2 for degradation to overcome this restriction and successfully infect cells (Gibbons et al., 2020). If the level of RPRD2 in a cell decreases, then the cell is more susceptible to infection by HIV. RPRD2 can interact with both cellular and viral nucleic acids and the protein has the capability to target incoming viral RNA or genomes (Marno et al., 2014). While the exact mechanism in this interaction is not yet fully understood, it is important to note that RPRD2 can bind to the CTD. The data from these studies (Gibbons et al., 2020, Marno et al., 2014) demonstrate that RPRD2 is an active component in restricting HIV from infecting the cell and a potential treatment may be increasing levels of RPRD2 in cells to overcome the effects of Vpr.

RPRD2 and its Role in Anxiety

In a previous study from the Hamilton group, higher levels of RPRD2 were found in the NAc, ventral hippocampus, and medial prefrontal cortex of mice that were resilient to CSDS (Hamilton et al., 2020). This led to the question of whether RPRD2 played a role in stress-related behaviors. While there is knowledge that RPRD2 functions by influencing Ser5 dephosphorylation to pause transcription initiation (Ali et al., 2019; Ramani et al., 2021), there are no current studies that focus on the relationship between this protein and stress-related behaviors.

Given the preliminary data from Dr. Hamilton's research (2020), the hypothesis was that higher levels of the protein RPRD2 in the NAc would protect against stress-induced behavioral adaptations, including social deficits and anxiety-like behaviors. However, this hypothesis was rejected based on the observation within this thesis that viral injection of RPRD2 did not improve stress-induced social deficits and actually precipitated a worsening of anxiety-like behaviors. The increase in anxiety behaviors occurred regardless of exposure to CSDS and was dependent on the injection of viral RPRD2. These data below will improve the understanding of the function of the protein RPRD2 and propose one potential brain mechanism for the onset of anxiety disorders. Thus, one can conclude that elevated levels of RPRD2 in the NAc observed in resilient C57/B6 mice (Hamilton et al., 2020) is likely not driving resilient behaviors and is being compensated for by other brain mechanisms in these behaviorally resilient animals. Further understanding of the mechanisms for anxiety disorders will improve the treatment available by targeting specific factors that contribute to its onset. In addition, this data will contribute to the knowledge of the functionality of RPRD2.

Materials and Methods

Animal Husbandry

This study used adult male C57BL/6J mice aged 8-11 weeks of age and 6-month-old CD-1 retired male breeders (CD-1 aggressors) from Jackson Laboratories. All animals were housed in a temperature (22-25°C) and humidity-controlled colony room in static cages. C57BL/6J mice were group housed with five to a cage and CD-1 aggressor mice were housed individually. The room was kept on a 12-hour light/dark cycle with lights on at 7 a.m. and lights off at 7 p.m. Mice were provided standard food and water that was freely available. All studies were conducted in accordance with the Institutional Animal Care and Use Committee of Virginia Commonwealth University and National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all relevant guidelines and regulations were followed.

Viral Vectors

RPRD2 was inserted into the p1005 plasmid using an LR reaction. The ThermoFisher Scientific gateway LR Clonase II cloning protocol from the ThermoFisher Scientific website was followed and the Gateway LR Clonase II Enzyme mix kit was used (catalog number 11791-020 and 11971-100). RPRD2 was confirmed to be inserted correctly via sanger sequencing (GENEWIZ; New Jersey) **(Figure 1)**. Colonies containing RPRD2 were chosen, and a miniprep (catalog number 27106) and maxiprep (catalog number 12163) were performed following the kit protocol from QIAGEN to amplify the bacteria for viral packaging into the herpes simplex virus (HSV). Samples were then shipped to the Gene Delivery Technology Core at Massachusetts

General Hospital for viral packaging. Once packaged, aliquots were made and stored in -80 degrees Celsius to be used in viral gene transfer through stereotaxic surgery.

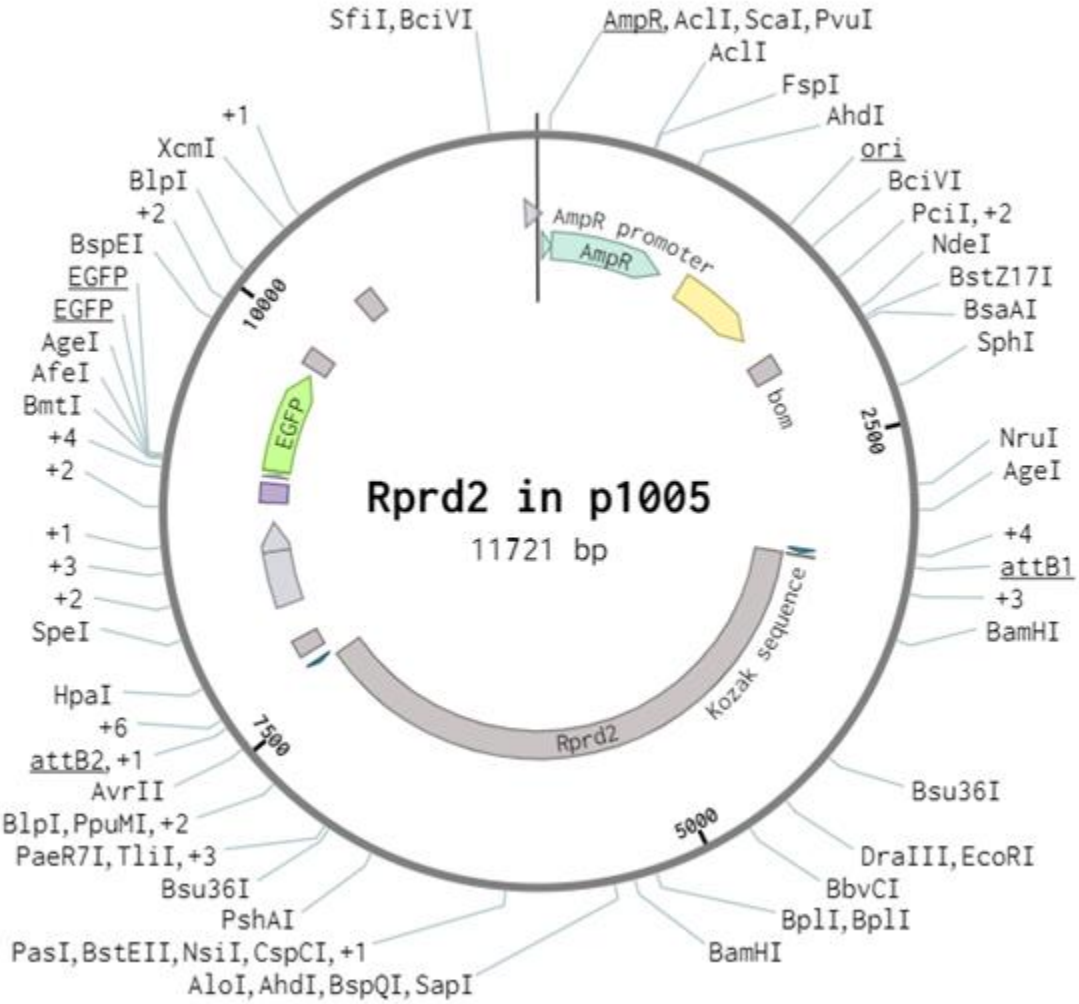

Rprd2 in p1005
11721 bp
SfiI,BciVI
AmpR,AclI,ScaI,PvuI
AclI
FspI
AhdI
ori
BciVI
PciI,+2
NdeI
BstZ17I
BsaAI
SphI
NruI
AgeI
+4
attB1
+3
BamHI
Bsu36I
DraIII,EcoRI
BbvCI
BplI,BplI
BamHI
AloI,AhdI,BspQI,SapI
PasI,BstEII,NsiI,CspCI,+1
PshAI
Bsu36I
PaeR7I,TliI,+3
BlpI,PpuMI,+2
AvrII
attB2,+1
+6
HpaI
SpeI
+2
+3
+1
BmtI
AfeI
AgeI
EGFP
EGFP
BspEI
+2
BlpI
XcmI
+1
AmpR promoter
AmpR
EGFP
bom
Rprd2
Kozak sequence
10000
2500
5000
7500

Figure 1: *RPRD2 inserted into p1005 plasmid.* Gateway cloning used an LR reaction to insert RPRD2 into a p1005 destination vector flanked by attB sites. This DNA clone was confirmed via sanger sequencing and packaged into a viral vector to be used in stereotaxic surgery.

Stereotaxic Surgery

Stereotaxic surgeries targeting the NAc were performed. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) dissolved in sterile water. Mice were positioned in a stereotaxic instrument (Kopf model 942) and the skull surface was exposed. The NAc was targeted bilaterally (Bregma: anterior-posterior: +1.6, medial-lateral: +1.5, dorsal-ventral: -4.4) at 10° from the midline. Hamilton syringes (5 µl; catalog number 84851) were used to infuse a total of 1.0 µl of virus. The virus was delivered at a rate of 0.1 µl per minute, followed by a five-minute break to allow the virus to diffuse in the tissue. In all experiments, proper NAc targeting was confirmed via tissue analysis of the needle track and GFP expression. After surgery, the mice were allowed to recover for 24 hours prior to the accelerated CSDS. Mice in the group without CSDS had three days to recover before behavior analysis. Mice were monitored for any signs of distress such as lack of grooming, weight loss, and altered locomotion. Surgeries were completed with aid from Joseph Picone, Natalie Truby, and Gabriella Silva, trained graduate students in the Hamilton Lab.

Chronic Social Defeat Stress

Mice were then divided into three different groups. The first group of C57BL/6J mice (n=20) experienced a five-day accelerated CSDS **(Figure 2B)**, the second group (n=20) experienced a six-day accelerated CSDS **(Figure 2C)**, and the last group (n=20) did not experience CSDS **(Figure 2A)**. Two different lengths of CSDS were used in the effort to precipitate a stronger defeat experience in the hopes of visualizing a significant difference in resilience levels for mice injected with HSV-RPRD2. For each group, half were injected (n=10)

with viral RPRD2 into the NAc and the other half (n=10) were injected with viral GFP as a non-functional control.

The experiment utilized an accelerated CSDS protocol **(Figure 3A)**. CD-1 retired breeder mice were screened for aggressive behavior for three-minute intervals over three days. CD-1 mice that consistently physically dominated male C57BL/6J screener mice were used as aggressors for the study. C57BL/6J mice were exposed for five or six consecutive days to an aggressive novel CD-1 retired breeder for 11 minutes twice a day, with one encounter in the morning and one in the afternoon to allow mice time to recover after each encounter. In each defeat session, the male C57BL/6J mouse would be placed in the home cage of a CD-1 aggressor mouse to be aggressed upon. With every exposure, the C57BL/6J mouse encountered a novel CD-1 aggressor. After the defeat, C57BL/6J mice were then separated from the aggressor by a perforated divider within the same cage to maintain constant contact and sensory stress with the CD-1 aggressor present. After the final defeat took place, C57BL/6J mice were moved to individually housed cages for 24 hours. Two mice (One HSV-GFP, one HSV-RPRD2) from the five day CSDS group **(Figure 2B)** and four (Three HSV-GFP, one HSV-RPRD2) mice from the six day CSDS group **(Figure 2C)** received injuries that necessitated their removal from the defeat protocol and were not analyzed in the behavior study.

Behavior Analysis

Twenty-four hours after the final defeat (or 3 days after surgery for the group that did not experience CSDS) mice were tested in the SI, EPM and the open field tests. Group 1 **(Figure 2B)** tested 18 mice (HSV-RPRD2 n=9, HSV-GFP n=9), group 2 **(Figure 2C)** tested 16 mice

(HSV-RPRD2 n=9, HSV-GFP n=7), and group 3 **(Figure 2A)** tested 20 mice (HSV-RPRD2 n=10, HSV-GFP n=10). These tests took place on the same day due to the short expression period of HSV. SI **(Figure 3B)** took place by first allowing mice to habituate and explore an open arena with a wire mesh cage (interaction zone) centered against one wall for 2.5 minutes. Data acquired from the first 2.5 minute period without the CD-1 aggressor present was used to measure thigmotaxis data as the open field test. Then, the mouse returned to the arena for another 2.5 minutes with a novel CD-1 present in the wire mesh cage. This CD-1 aggressor was not used in CSDS to determine consistent SI for all C57BL/6J mice. The arena was wiped clean with 70% EtOH before a new C57BL/6J mouse was placed in the arena. Data were then analyzed as time spent in the interaction zone without the aggressor compared to time spent within the interaction zone with the aggressor present. This data were then calculated into SI ratios by taking the time the C57BL/6J mouse spent in the interaction zone with the CD-1 mouse present and dividing it by the time spent in the interaction zone with the CD-1 absent (SI ratio = time in IZ target present / time in IZ target absent). Total distance moved by the C57BL/6J mouse was recorded and evaluated when the target was absent.

Mice were then tested in an EPM to measure non-social related anxiety. EPM testing was performed about 20-30 minutes after SI. The EPM apparatus **(Figure 3C)** is constructed of black Plexiglass and consists of two open arms and two closed arms connected by a central platform (10 x 10 cm). Open and closed arms are perpendicular to each other and are 30 cm in length and 5 cm in width. Alternating arms are enclosed with opaque walls that are 40 cm in height. The other arms that have no walls have raised edges that are about 1 cm to ensure that the mouse does not fall off the maze while exploring. The maze is elevated 60 cm off the floor. C57BL/6J mice were placed in the center of the maze and their exploration was tracked for five minutes.

The platform of the EPM was wiped clean with 70% EtOH after each trial prior to a new C57BL/6J mouse entering the maze. All behavior tests took place in a red-light room, where no defeats took place, and were monitored and recorded by Ethovision XT (Noldus Information Technologies; Leesburg, VA). Behavior analysis was performed with the help of Claire Atiyeh, a master's student in the Hamilton Lab.

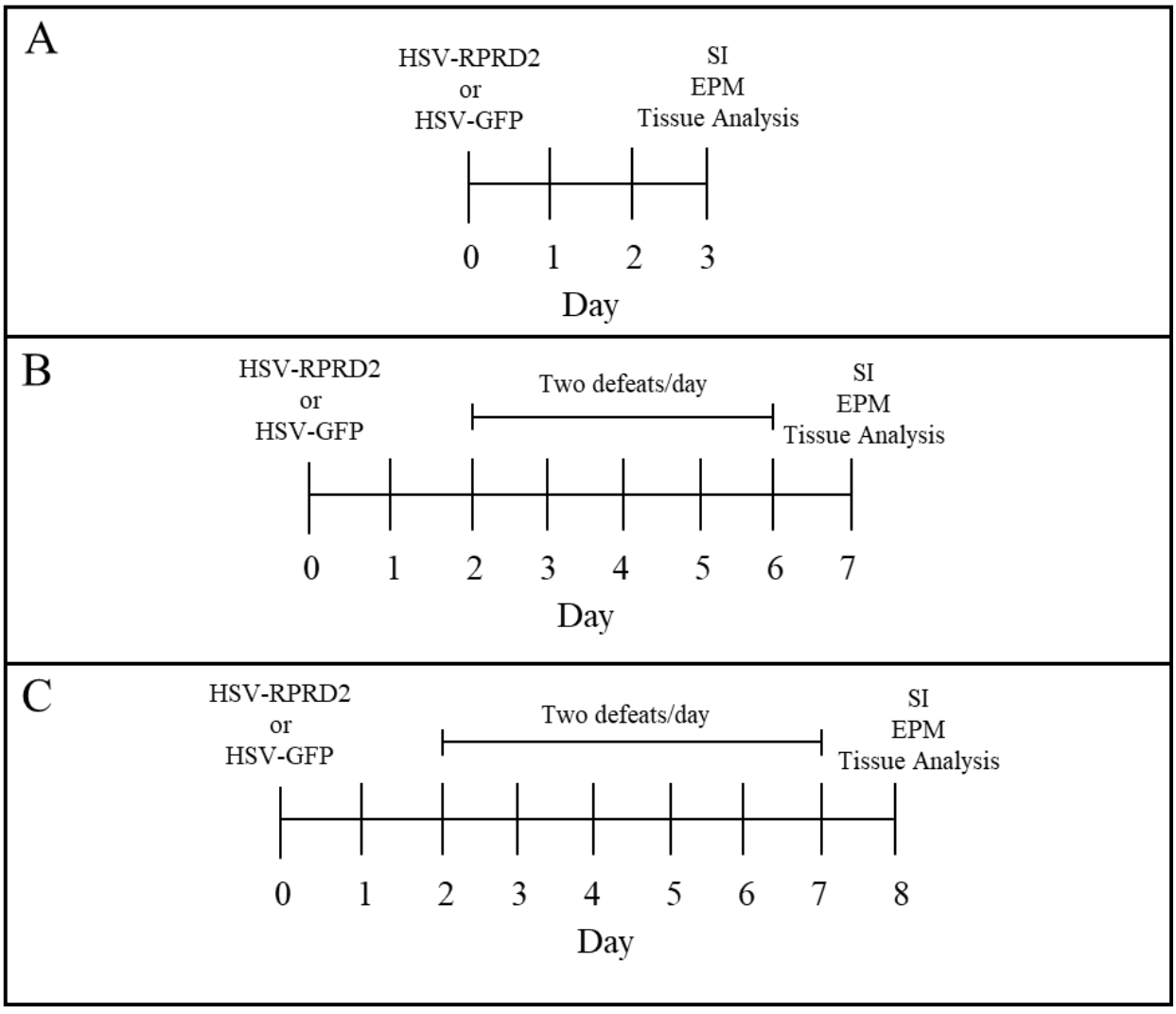
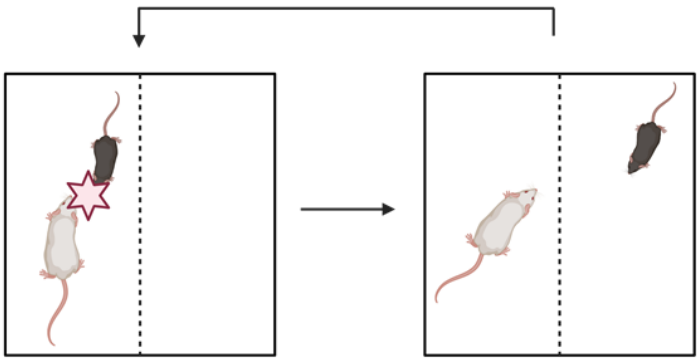

A
HSV-RPRD2
or
HSV-GFP
SI
EPM
Tissue Analysis
0 1 2 3
Day
B
HSV-RPRD2
or
HSV-GFP
Two defeats/day
SI
EPM
Tissue Analysis
0 1 2 3 4 5 6 7
Day
C
HSV-RPRD2
or
HSV-GFP
Two defeats/day
SI
EPM
Tissue Analysis
0 1 2 3 4 5 6 7 8
Day

Figure 2: *Overview of study.* A) Group one did not experience CSDS. Three days after stereotaxic surgery mice were tested for behavior and sacrificed; n=10 HSV-RPRD2 and n=10 HSV-GFP mice. B) Group two experienced a five-day accelerated CSDS with two defeats per day for 10 minutes each; n=9 HSV-RPRD2 and n=9 HSV-GFP. Behavior analysis occurred 24 hours after the final defeat and the animals were sacrificed to collect tissue. C) Group three experienced a six-day accelerated CSDS with two defeats per day for 11 minutes each; n=9 HSV-RPRD2 and n=7 HSV-GFP. Behavior analysis occurred 24 hours after the final defeat and the animals were sacrificed to collect tissue.

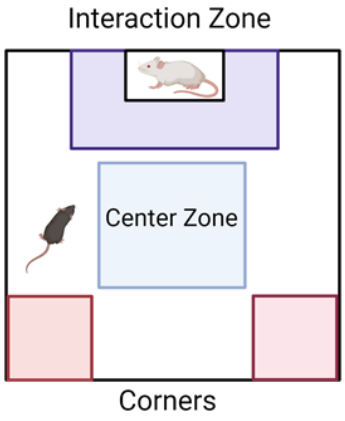
A



Physical Stress
(10 min 2x/day)

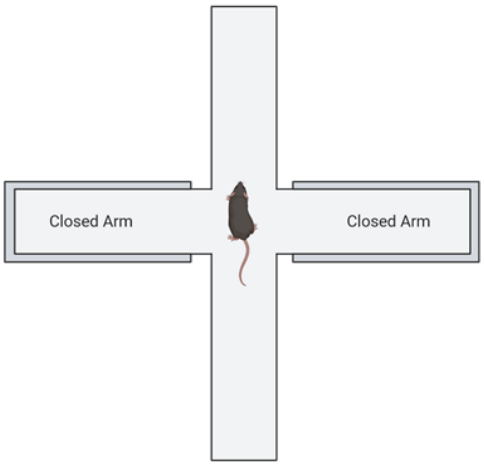
Sensory Stress
(24 hr)

B



Social Interaction (5 minutes)

C



Elevated Plus Maze (5 minutes)

Figure 3: *CSDS, SI and EPM.* A) Accelerated CSDS paradigm. C57BL/6J mice are placed in the home cage of a CD-1 aggressor twice daily for 10 minutes each. After the defeat period, they are placed on the other side of the cage separated by a perforated divider. B) The SI test gives C57BL/6J mice 2.5 minutes to explore the open field by themselves, and 2.5 minutes to explore with a CD-1 aggressor present in a cage in the interaction zone. Time spent in each zone is recorded by Ethovision XT. Center zone data taken from the first 2.5 minutes was used to measure thigmotaxis in C57BL/6J mice. C) C57BL/6J mice explore the EPM for a five minute period. The time spent in the closed arms of the maze is recorded by Ethovision XT. *Created using BioRender.com.*

Tissue Extraction

Half an hour after behavioral testing, NAc tissue was collected from rapidly cervical dislocated and decapitated animals without anesthesia. Brains were removed one at a time and promptly sectioned into 1 mm coronal slices using a brain matrix. Bilateral tissue punches from the NAc (12 gauge; internal diameter, 2.16 mm) were frozen on dry ice and immediately stored at -80°C. The anatomically correct presence of GFP in the NAc was confirmed visually through dissection and mice with weak or incorrect expression were excluded from the results. One mouse (HSV-GFP) was excluded from the first group **(Figure 2B)** for faint expression, two mice (both HSV-RPRD2) were excluded from the second group **(Figure 2C)** for undetectable expression, and five mice (four HSV-RPRD2, one HSV-GFP) were excluded from the third group **(Figure 2A)** for faint expression. Tissue extraction and sacrificing of the mice was performed with help from Joseph Picone, Gabriella Silva, Claire Atiyeh and Natalie Truby, trained graduate students in the Hamilton Lab.

Western Blotting

Western blots were performed from tissue samples of mice that did not experience CSDS **(Figure 2A)** and the five day accelerated CSDS **(Figure 2B)** groups. Protein was extracted from bilateral NAc punches and was suspended in a RIPA sample and 2x Laemmli buffer. Next, 15 µl of each sample and 10 µl of the well standards (Precision Plus Protein Unstained Standards and the Precision Plus Protein Kaleidoscope, BIO-RAD) were run on a TGX (Tris-Glycine eXtended) stain free gel (BIO-RAD; catalog number 5678094) at 100 V for 2 hours. The protein

was then transferred to a membrane using Trans Blot Turbo Packs (BIO-RAD; catalog number 1704157) in the Trans-Blot Turbo Transfer System (BIO-RAD) for 10 minutes.

The membrane was blocked in 5% milk in TBST for 1 hour before incubating with RPRD2 primary antibody and 3% BSA overnight at room temperature on a rocking platform. The primary antibody for RPRD2 was made using a 1:2000/2.5% BSA dilution (17.5 µl and 35 ml) with the primary Ab10363, Goat pAb to RPRD2 50 ug (1mg/mL) (Abcam). The following morning, the membrane was washed with TBST with extra Tween 20 and incubated with the secondary antibody and 2.5% milk with TBST. Secondary antibodies for RPRD2 were made using 5 µl of Dnk pAb to Goat IgG 500 ug 2mg/ml and 50 ml of 2.5 % milk in TBST. After five more washes on the rocking platform, the membrane was exposed to Clarity ECL substrate (BIO-RAD; catalog number 170-5060) and developed in the ChemiDoc MP instrument. Using the Image Lab Software, RPRD2 band intensity could be quantified and normalized to the presence of Beta-Actin in the lane.

This process was repeated to visualize the band intensity of GFP, pSer5 and RNA Pol II in the membrane and that data was normalized to the total protein level. Primary antibodies for GFP were made with the dilution 1:10000/2.5% BSA with the primary mouse mAB (Proteintech). Primary antibodies for pSer5 and Pol II were made with the dilution of 1:1000/2.5% BSA (35 µl and 35 ml) with the primary Rb pAb to RNA polymerase II CTD repeat YSPTSPS (pSer5) and Rb pAb to RNA polymerase II CTD repeat YSPTSPS, respectively (Abcam). After the primary antibody was added, the membrane was placed on a rocking platform in the cold room (40 C) overnight. The membrane was washed three times with TBST, then the secondary antibody was added to the membrane. Secondary antibodies for GFP were made with 5 µl of anti-mouse-HRP with 50 ml of 2.5% milk in TBST. Secondary antibodies for

pSer5 and RNA Pol II were made with 5 µl of anti-rabbit-HRP with 50 ml of 2.5% milk in TBST. After one hour, the membranes were washed with TBST and could be developed in the ChemiDoc instrument. RNA Pol II and GFP bands were then quantified through normalization of the total protein in the band to Beta-Actin. pSer5 bands were quantified and normalized to the expression of RNA Pol II in the membrane. Western blot and its analysis were performed with the guidance of Xiaohong Cui, the laboratory technician, and the help of Claire Atiyeh.

Statistical Analysis

Statistics were performed in Prism version 9 for Windows (GraphPad Software, La Jolla, CA). Unpaired *t*-tests were used for the comparison of groups that received HSV-RPRD2 or HSV-GFP in the analysis of the SI, EPM and open field tests and Western blots. Two-way ANOVAs were performed to analyze the effects of the two independent variables, viral injection and exposure to CSDS, on the behavioral data. A Pearson's correlation coefficient test was performed to analyze the strength of relationship between RPRD2 expression and pSer5 expression and time spent in the open arms of the EPM. Outliers are identified by using the robust regression and outlier removal (ROUT) test and are excluded from the study when applicable. Samples that did not express GFP upon tissue analysis are also excluded from the study. Significance is reported at $p<0.05$.

Results

Injection of HSV-RPRD2 to the NAc does not influence social behavior.

In the SI test, unpaired *t*-tests showed no difference between mice who were injected with HSV-RPRD2 and those that were injected with HSV-GFP in individual groups ($p>0.05$). There was no difference in the number of resilient mice or the number of susceptible mice between the two groups. The SI ratios were calculated by dividing the time that the C57BL/6J mouse spent in the interaction zone while the target was present by the time spent in the interaction zone while the target was absent. A two-way ANOVA found no statistical significance between mice who were exposed to CSDS or not (stressed or unstressed) or in mice who received HSV-RPRD2 in comparison to HSV-GFP ($p=0.8837$; $p=0.9085$) **(Figure 4)**. No differences were seen between groups that experienced accelerated CSDS or the group that did not experience CSDS **(Figure 4)**. There was no significant interaction between these two variables. In addition, the injection of HSV-RPRD2 did not impact the SI ratio significantly from the control groups. From these data, it can be concluded that HSV-RPRD2 had no significant effect on social behavior as tested in this task, regardless of the influence of CSDS.

HSV-RPRD2 shows no significant effect in time spent in the center of the open field test.

Mice who were injected with HSV-RPRD2 in the NAc spent a similar time in the center of the field as the control group **(Figure 5A)**. A two-way ANOVA analyzed that this data is not significant between mice injected with HSV-RPRD2 and mice injected with HSV-GFP ($p=0.1484$). CSDS does not appear to have an effect on this behavior based on this data ($p=0.2318$). The interaction between these two variables was not significant. Distance traveled in

the open field was similar between all mice, regardless of viral injection or CSDS (p=0.3735; p=0.3992) **(Figure 5B)**. Mice that spend increased time in the center show a tendency known as thigmotaxis, or staying near the walls in an open field, which is an indicator of increased anxiety-like behavior. This data is not significant, it cannot be concluded that HSV-RPRD2 in the NAc had an influence on the behavior of mice to avoid the center of the field.

EPM test shows significant difference in time spent in open arms, but not distance traveled.

A two-way ANOVA revealed that mice injected with HSV-RPRD2 in the NAc spent significantly (p=0.0332) less time exploring the open arms of the maze in comparison to the control group **(Figure 6A)**. Mice that experienced stress (CSDS) also spent significantly less time in the open arms of the EPM than the unstressed group (p=0.0265). The interaction between these two terms was not significant. The distance traveled by the mice in both the RPRD2 group and the GFP group was not significant (p=0.0849) and was not influenced by exposure to CSDS (p=0.1327) **(Figure 6B)**. The injection of HSV-RPRD2 does not affect the total movement of mice in the field. The data from the EPM shows that mice injected with HSV-RPRD2 in the NAc spend significantly less time in the open arms of the EPM, indicating greater anxiety-like behavior. Since this phenotype was observed even in mice that had not previously experienced CSDS, it can be inferred that RPRD2 overexpression in the NAc is singularly sufficient to manifest anxiety-like behaviors.

Western blot analysis of RPRD2, pSer5, and Pol II data.

Western blot analysis was performed on two of the subject groups: the group that had no CSDS and the group that had a 5 day accelerated CSDS. Normalization of Western blot data used Image Lab Software (BIO-RAD) to quantify the bands of RPRD2, Ser5, RNA Pol II, or GFP and divide it by the quantified band of the loading control B-Actin (molecular weight=42 kDa). Normalization makes it possible to accurately analyze the protein levels within the membrane and minimizes any variances between samples that may be present.

RPRD2 has a molecular weight of about 220 kDa as seen on the membrane of Western blots from the tissue samples of the mice (**Figure 7A)**. Normalization of RPRD2 to the control B-Actin shows no difference in expression of RPRD2 in mice that received HSV-RPRD2 in comparison to the control group **(Figure 7B)**. An unpaired *t*-test shows that this data is not significantly different (p=0.2494), but this data may be affected by external factors such as accuracy of NAc microdissection, *in vivo* regulation of RPRD2 protein levels, and the HSV-mediated overexpression of RPRD2 being restricted to neurons in the NAc. However, future studies should be performed to determine that levels of RPRD2 are higher in the membrane of mice that receive the viral vector and different methods may be required to get more accurate results.

The molecular weight of RNA Pol II pSer5 is about 250 kDa and is observed on the membranes of the Western blot **(Figure 9A)**. Normalization of RNA Pol II pSer5 to RNA Pol II reveals no difference in expression of pSer5 in the NAc of mice who received HSV-RPRD2 **(Figure 9B)**. An unpaired *t*-test reveals that this data is not significant (p=0.5679). A decrease in pSer5 expression in mice injected with HSV-RPRD2 would allude to a potential mechanism for RPRD2, but no conclusions can be drawn based on the preliminary data from this thesis. However, further studies would be required to provide more clarification.

Pol II has a molecular weight of about 250 kDa on the membrane of the Western blot **(Figure 10A)**. The normalization of Pol II to B-Actin shows no difference between the two subject groups. An unpaired *t*-test shows that this data is not significant (p=0.4665) **(Figure 10B)**. Mice injected with HSV-RPRD2 in the NAc do not have significantly different levels of Pol II in comparison to the control group.

GFP has a molecular weight of about 27 kDa on the membrane of the Western blot **(Figure 12A)**. The normalization of GFP to B-Actin shows no difference between the two subject groups injected with either HSV-RPRD2 or HSV-GFP. An unpaired *t*-test shows that this data is not significant (p=0.5328) **(Figure 12B)**. This graph confirms the presence of the virus in the NAc tissue and shows no significantly different levels of GFP present between the two groups.

Correlation graphs between RPRD2 expression and anxiety behavior shows no significant relationship.

A correlation graph between the two variables RPRD2 expression normalized to B-Actin and anxiety-like behavior, as shown by time spent in the open arms of the EPM, shows no significant relationship **(Figure 8)**. A Pearson correlation coefficient was found to be -0.03417 and the p value was calculated to be not significant (p=0.8577). The simple linear regression of the graph shows the equation is y=-1.048*X+32.92. The data indicates no significant correlation between the levels of RPRD2 present in the NAc and the anxiety-like behavior of time spent in the open arms of the EPM.

Correlation graphs between RPRD2 expression and pSer5 expression show no significant relationship.

A correlation graph between the two variables RPRD2 expression and pSer5 expression, both normalized to B-Actin, shows no significant relationship **(Figure 11)**. A Pearson correlation coefficient was found to be 0.05162 and the p value was calculated to be not significant (p=0.7827). The simple linear regression of the graph shows the equation is y=0.06618*X+1.09. This data indicates that there is no significant correlation between the levels of RPRD2 present in the NAc and the levels of pSer5 present in the membrane.

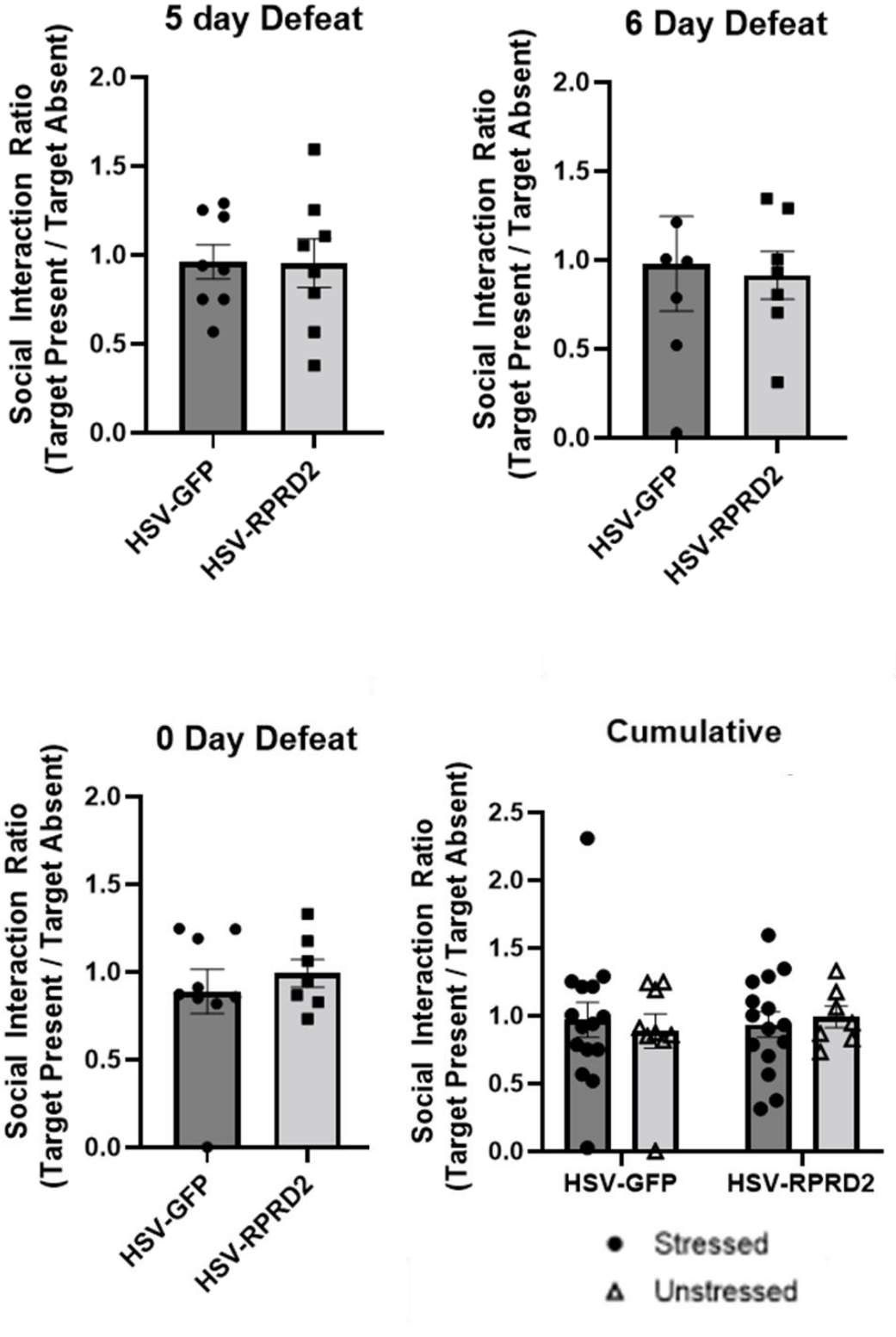

5 day Defeat
Social Interaction Ratio
(Target Present / Target Absent)
2.0
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2
6 Day Defeat
Social Interaction Ratio
(Target Present / Target Absent)
2.0
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2
0 Day Defeat
Social Interaction Ratio
(Target Present / Target Absent)
2.0
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2
Cumulative
Social Interaction Ratio
(Target Present / Target Absent)
2.5
2.0
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2
Stressed
Unstressed

Figure 4: *HSV-RPRD2 does not influence social behavior, regardless of CSDS.* No significant difference was observed between social interaction ratios of HSV-GFP and HSV-RPRD2, irrespective of whether groups experienced CSDS. SI ratio greater than 1 indicates a resilient mouse and SI ratio less than 1 indicates a susceptible mouse. Each group had roughly equal amounts of resilient and susceptible mice, indicating that HSV-RPRD2 had no effect on social behavior. Data is from 5 day CSDS, 6 Day CSDS, and 0 day CSDS **(Figure 2).** HSV-GFP n=23; HSV-RPRD2 n=22. One HSV-GFP mouse was identified as an outlier (5 day CSDS, ROUT Q = 1%) and excluded. Symbols represent mean ± SEM.

A

B

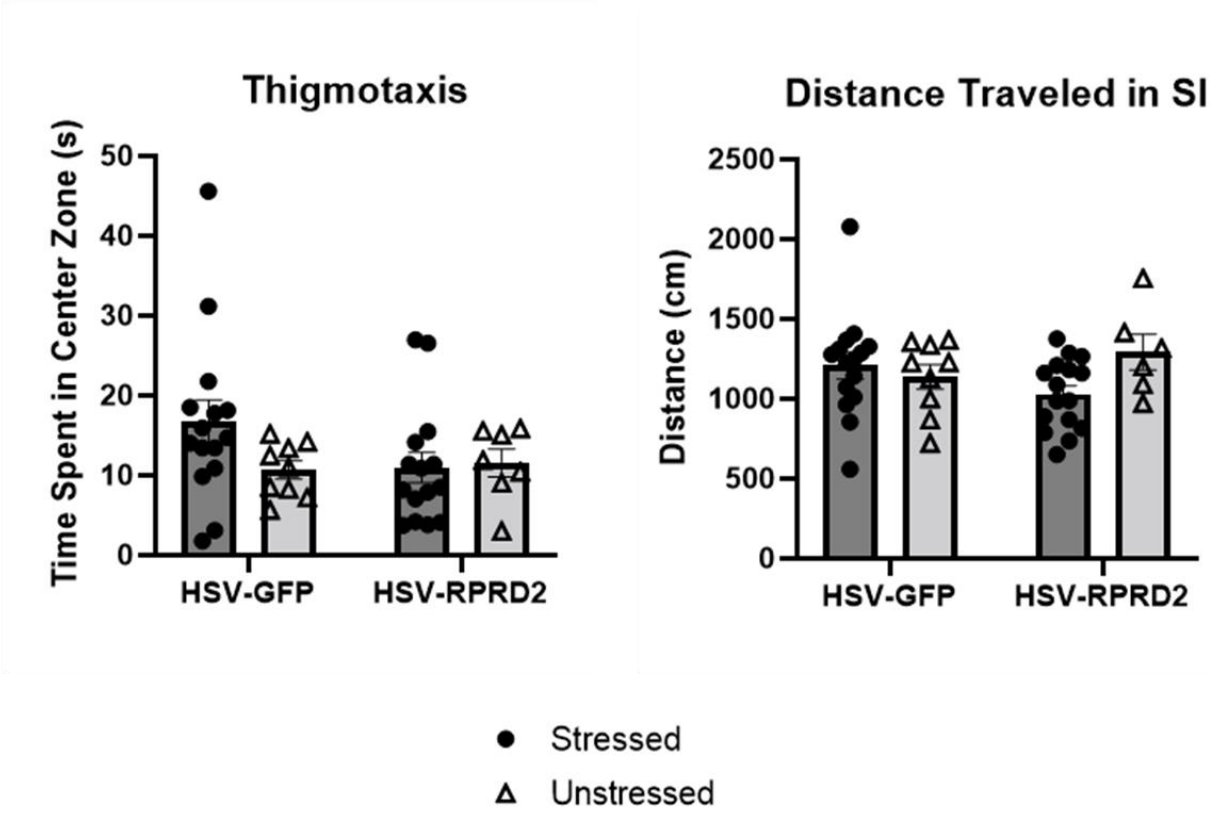

Thigmotaxis
Time Spent in Center Zone (s)
50
40
30
20
10
0
HSV-GFP
HSV-RPRD2
Distance Traveled in SI
Distance (cm)
2500
2000
1500
1000
500
0
HSV-GFP
HSV-RPRD2
Stressed
Unstressed

Figure 5: *HSV-RPRD2 shows no significant effect in time spent in the center of the open field test.* A) The time spent in the center of the field of mice injected with HSV-RPRD2 is not significantly different from the control group HSV-GFP. This behavior is not influenced by the experience of CSDS. B) Distance traveled in the open field is not modified by HSV-RPRD2 injection. Data is from 5 day CSDS, 6 Day CSDS, and 0 day CSDS **(Figure 2).** HSV-GFP n=24; HSV-RPRD2 n=22. Symbols represent mean ± SEM.

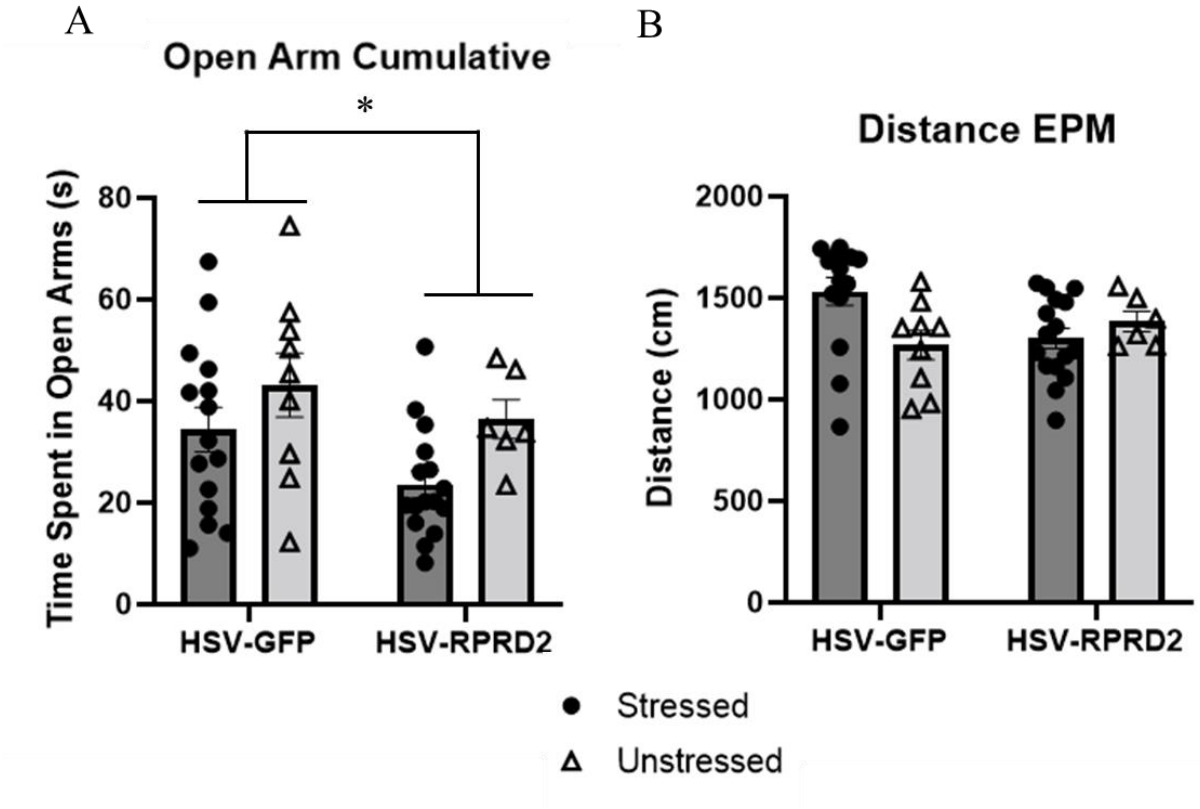
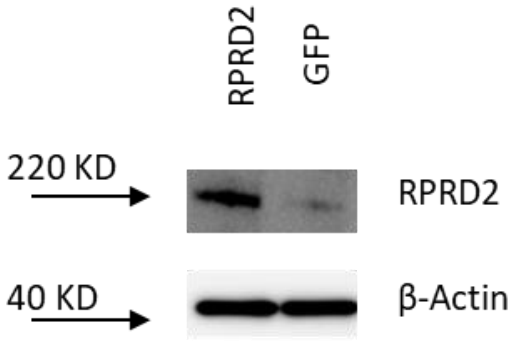

A
Open Arm Cumulative
*
Time Spent in Open Arms (s)
80
60
40
20
0
HSV-GFP
HSV-RPRD2
B
Distance EPM
Distance (cm)
2000
1500
1000
500
0
HSV-GFP
HSV-RPRD2
Stressed
Unstressed

Figure 6: *HSV-RPRD2 spent significantly less time in the open arms of the EPM, but not distance traveled.* A) HSV-RPRD2 injected mice spend significantly less time on the open arms than the control group (p=0.0332). Mice who were stressed through CSDS spent significantly less time on the open arms (p=0.0265), though the interaction between these terms was not significant. B) No significant difference in distance traveled on the EPM between groups was found to be influenced by viral injection of RPRD2 or GFP or exposure to CSDS (stressed or unstressed) (p=0.0849; p=0.1327). Data is from 5 day CSDS, 6 Day CSDS, and 0 day CSDS **(Figure 2).** HSV-GFP n=24; HSV-RPRD2 n=22. Asterisks indicate effects with $p<0.05$. Symbols represent mean ± SEM.

A


RPRD2
GFP
220 KD
RPRD2
40 KD
β-Actin

B

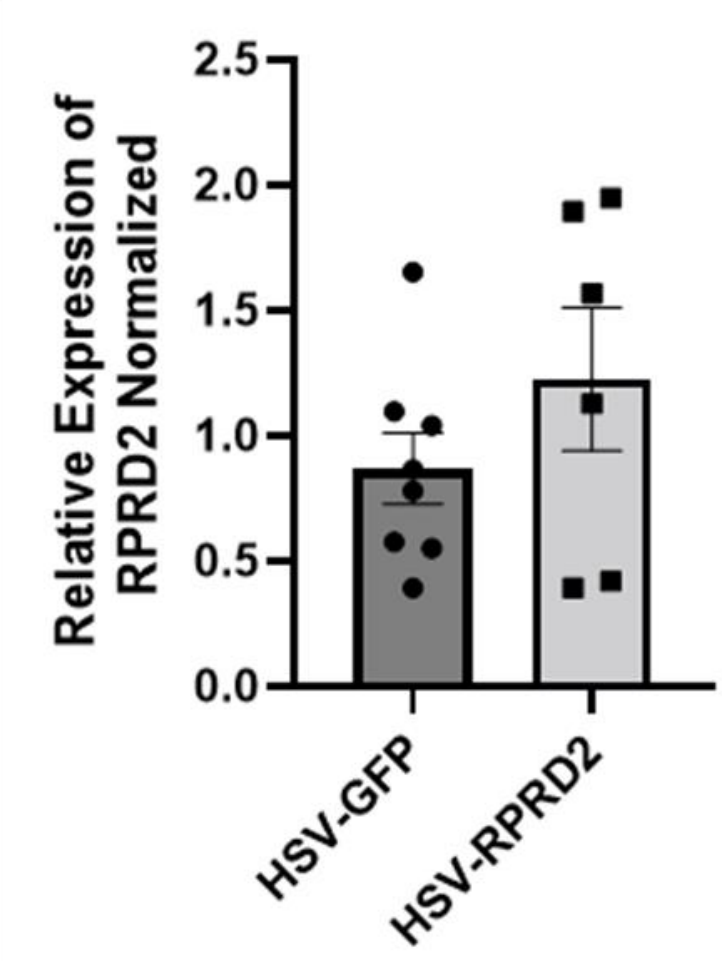

Relative Expression of RPRD2 Normalized
2.5
2.0
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2

Figure 7: *RPRD2 Western blot data.* A) Western blot for RPRD2 on membrane and B-Actin. B) Relative expression of RPRD2 normalized to B-Actin shows increased RPRD2 in the NAc has a trend of increased RPRD2 expression in the membrane (p=0.2494). Data is from 0 day CSDS **(Figure 2).** HSV-GFP n=8; HSV-RPRD2 n=6. One HSV-GFP mouse was identified as an outlier (0 day CSDS, ROUT Q = 1%) and excluded. Symbols represent mean ± SEM.

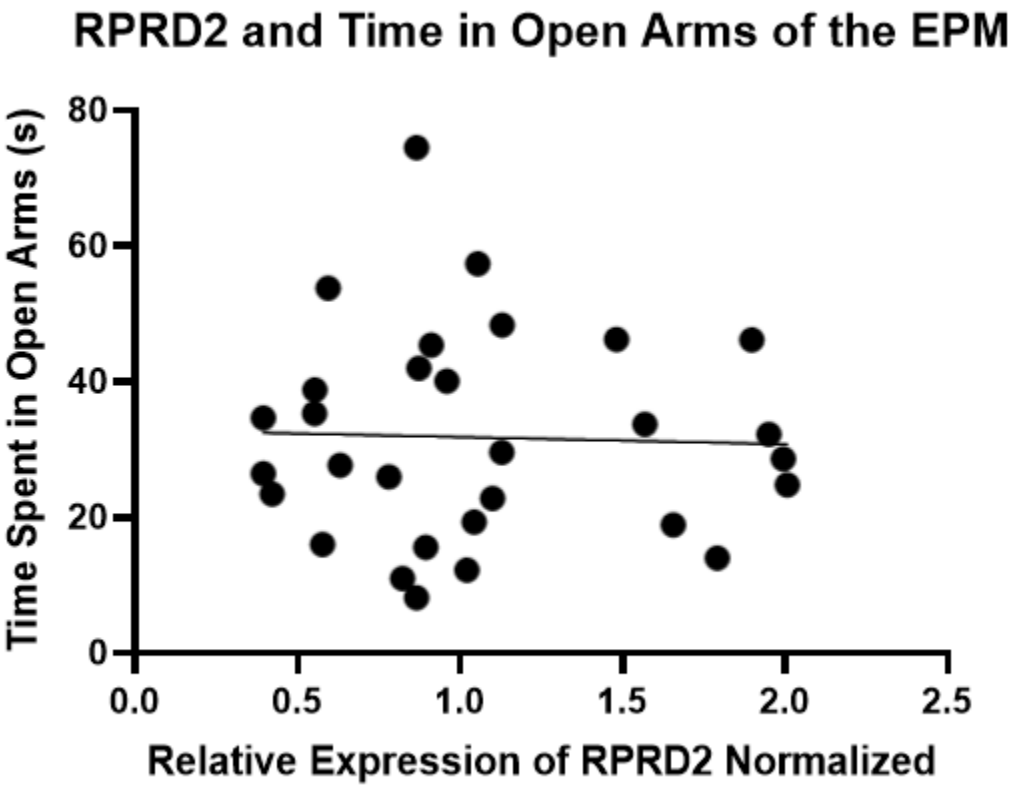
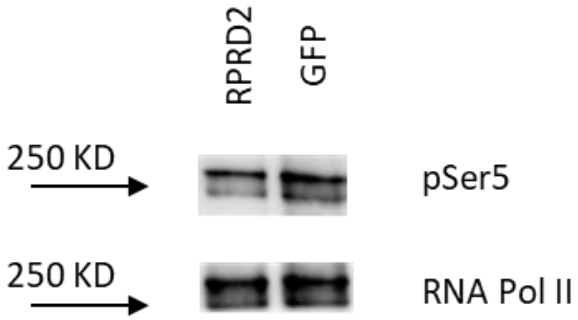

RPRD2 and Time in Open Arms of the EPM
Time Spent in Open Arms (s)
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
Relative Expression of RPRD2 Normalized

Figure 8: *Correlation between level of RPRD2 expression and time spent in the open arms of the EPM shows no significant relationship.* Correlation graph of RPRD2 expression normalized to B-Actin compared to the time the mouse spent in the open arms of the EPM shows no relationship between the two variables. Pearson's correlation of RPRD2 to time in the open arms of the EPM = -0.03471. Data is from 5 day CSDS and 0 day CSDS **(Figure 2).** n=31. Line represents simple linear regression.

A


RPRD2
GFP
250 KD
pSer5
250 KD
RNA Pol II

B

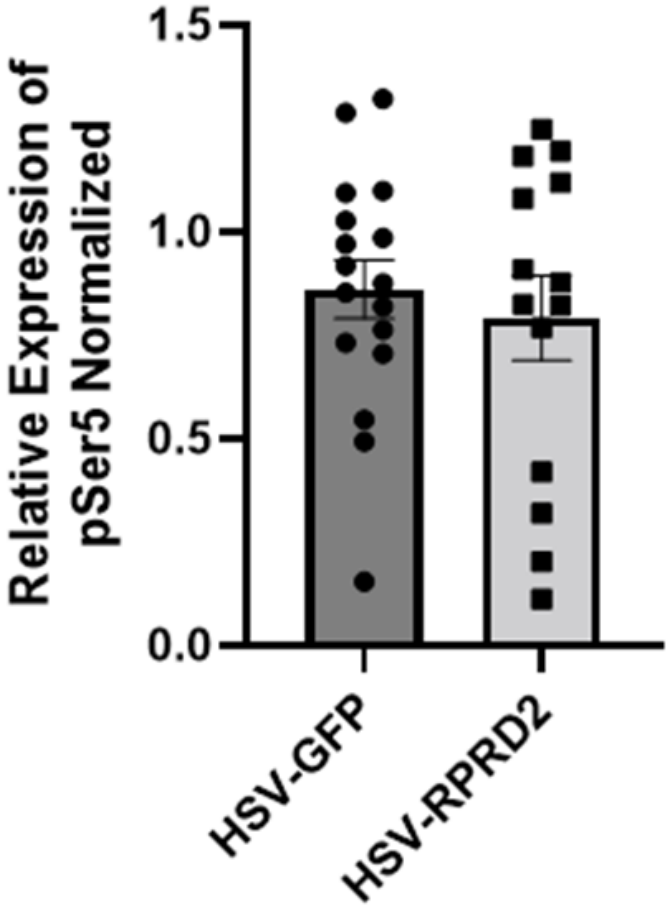
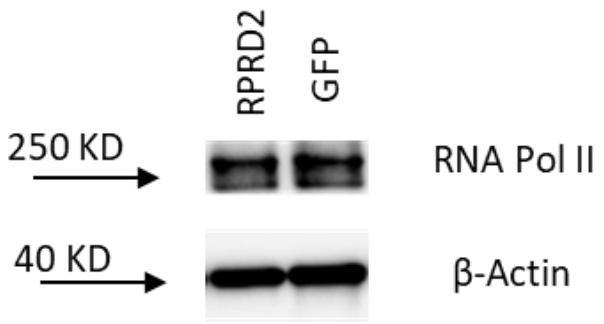

Relative Expression of pSer5 Normalized
1.5
1.0
0.5
0.0
HSV-GFP
HSV-RPRD2

Figure 9: *Pol II pSer5 Western blot data.* A) Western blot for Pol II pSer5 on membrane and RNA Pol II. B) Relative expression of Pol II pSer5 normalized to RNA Pol II shows no difference in Pol II pSer5 expression in the membrane (p=0.5679). Data is from 5 day CSDS and 0 day CSDS **(Figure 2).** HSV-GFP n=17; HSV-RPRD2 n=15. Symbols represent mean ± SEM.

A


RPRD2
GFP
250 KD
RNA Pol II
40 KD
β-Actin

B

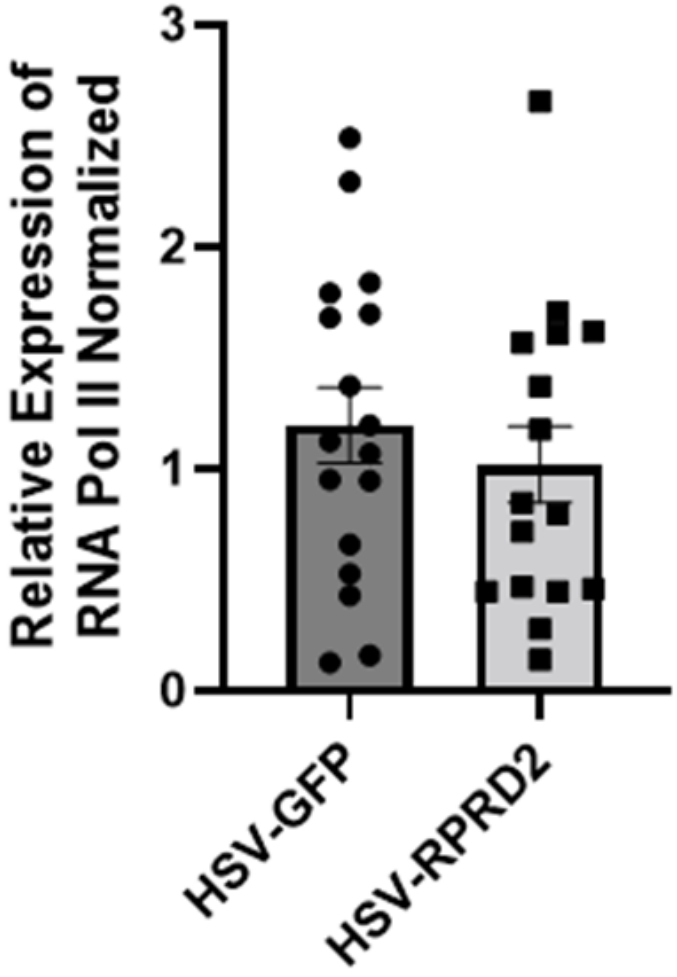

Relative Expression of RNA Pol II Normalized
3
2
1
0
HSV-GFP
HSV-RPRD2

Figure 10: *RNA Pol II Western blot data.* A) Western blot for RNA Pol II on membrane and B-Actin. B) Relative expression of RNA Pol II normalized to B-Actin shows no significant difference between groups. Data is from 5 day CSDS and 0 day CSDS **(Figure 2).** HSV-GFP n=17; HSV-RPRD2 n=15. Symbols represent mean ± SEM.

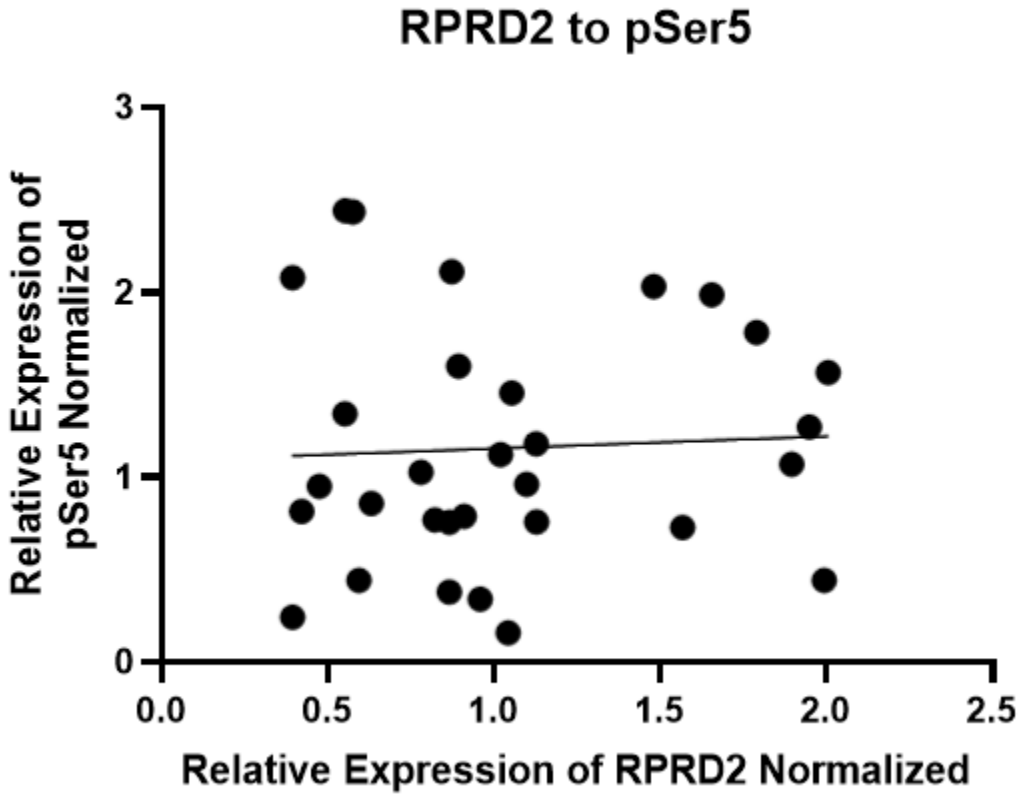
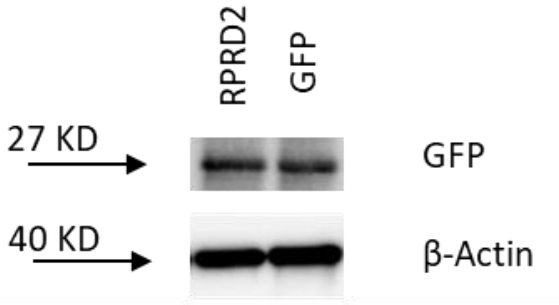

RPRD2 to pSer5
Relative Expression of pSer5 Normalized
Relative Expression of RPRD2 Normalized
0
1
2
3
0.0
0.5
1.0
1.5
2.0
2.5

Figure 11: *Correlation of expression of RPRD2 to expression of pSer5 shows no significant relationship*. Correlation graph of RPRD2 expression normalized to B-Actin compared to the pSer5 expression normalized to B-Actin shows no relationship between the two variables. Pearson's correlation of RPRD2 to pSer5 = 0.065162. Data is from 5 day CSDS and 0 day CSDS **(Figure 2).** n=31. Line represents simple linear regression.

A


RPRD2
GFP
27 KD
GFP
40 KD
β-Actin

B

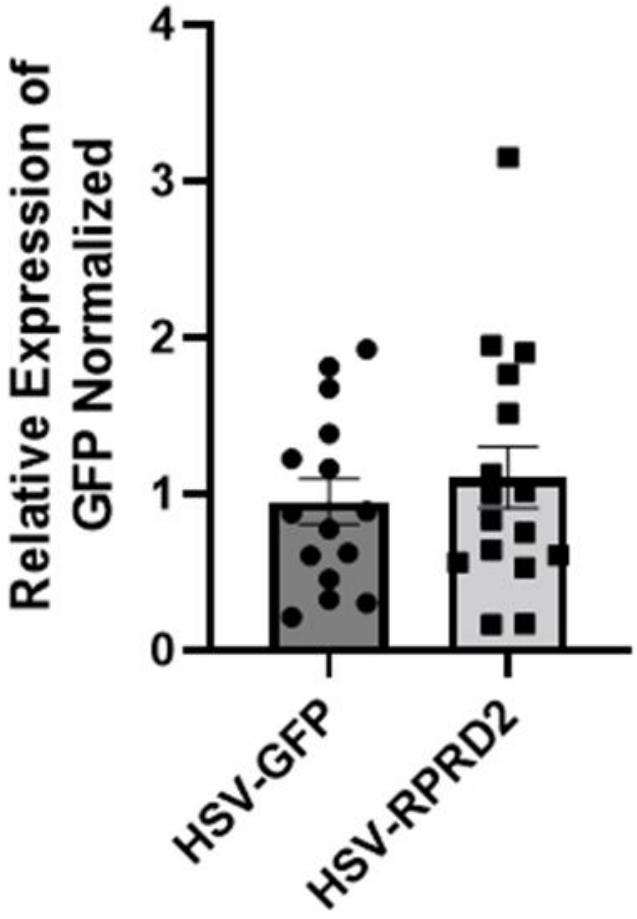

Relative Expression of GFP Normalized
4
3
2
1
0
HSV-GFP
HSV-RPRD2

Figure 12: *GFP Western blot data.* A) Representative Western blot for GFP on membrane and B-Actin. B) Relative expression of GFP normalized to B-Actin shows no significant difference between groups. Data is from 5 day CSDS and 0 day CSDS **(Figure 2).** HSV-GFP n=16; HSV-RPRD2 n=15. One HSV-GFP mouse was identified as an outlier (5 day CSDS, ROUT Q=1%) and excluded from this figure. Symbols represent mean ± SEM.

Discussion

Research focused on the potential mechanisms of anxiety disorders are needed for the discovery of improved therapies and treatments. Due to the increased rate of diagnosis of these disorders all around the world, it is imperative to pursue further knowledge of the complex intricacies of anxiety disorders. While RPRD2 has limited research, its potential interaction with the CTD of Pol II and its effect in resilient mice from previous studies led to this thesis to observe its specific effects (Hamilton et al., 2020; Ali et al., 2019; Ramani et al., 2021). While the initial hypothesis of this study anticipated increased expression of RPRD2 would result in mice resilient to CSDS, the results of this study demonstrate that injection of HSV-RPRD2 in the NAc induces anxiety-like behavior in mice. Whether similar mechanisms occur in humans suffering from anxiety disorders remains to be seen.

Injection of HSV-RPRD2 into the NAc did not impact social behavior

The original hypothesis of this thesis project was that higher levels of RPRD2 would induce resilience in mice exposed to CSDS and would have an SI ratio greater than one, based on previous results from the lab (Hamilton et al., 2020). However, the data from the behavior analysis of the mice following an accelerated CSDS consistently revealed no difference between the group that received HSV-RPRD2 and the group that received HSV-GFP, in terms of social metrics **(Figure 4)**. There were equal amounts of mice with an SI ratio greater than one and an SI ratio less than one in both groups, showing that the original hypothesis must be rejected. In addition, the mice who did not experience CSDS were not more susceptible than the other

groups, which may signify that the accelerated CSDS protocol did not stress the C57BL/6J mice enough.

There are no current studies that focus on the relationship between RPRD2 and social behavior, but the data from this project leads us to conclude that viral RPRD2 injection into the NAc has no influence on social behavior in mice regardless of CSDS. The protein likely does not play a role in the influence or reinforcement of social behavior in mice. Other compensatory mechanisms were likely responsible for the increase in levels of RPRD2 seen in the NAc in the preliminary data from our group (Hamilton et al., 2020). The injection of HSV-RPRD2 consistently had no effect on the SI ratio of C57BL/6J mice and is not affected by CSDS differently than mice injected with HSV-GFP.

HSV-RPRD2 induces anxiety-like behavior in mice

The injection of HSV-RPRD2 to the NAc induced anxiety-like behaviors in mice. Mice that received HSV-RPRD2 spent significantly less time in the open arms of the EPM **(Figure 6A)**, signifying increased anxiety-like in these animals in comparison to the HSV-GFP group (Kraeuter et al., 2019; Campos et al., 2013). An interesting note from the study is that after mice were single-housed after CSDS, there were four mice who received HSV-RPRD2 who did not make their "beds," while all other mice did (data not shown). This may be an additional marker of anxiety in this group of mice. Further data and repeated studies may find consistent results with this study.

The NAc is thought to be a major part of the pathophysiology of multiple mental illnesses due to its role in the reward processing of the brain. The NAc is critical in influencing motivated

behaviors, including those to avoid potential stressors or negative stimuli (Levita et al., 2012). Increased levels of RPRD2 in the NAc may work to increase the animal's aversion to anxiety-provoking stimuli such as an open, elevated space in the EPM. While the stereotaxic surgery performed in this project did not specifically target the shell or the core, the injection of HSV-RPRD2 likely causes a disruption or imbalance in the NAc that alters the processing of motivation.

Effects of RPRD2 on female mice

This study observed the effects of viral injection of HSV-RPRD2 in the NAc of only male C57BL/6J mice. This project utilized male mice to determine the effects of increased expression of RPRD2 within the NAc on accelerated CSDS because male CD-1 aggressors will not defeat female mice without modifications to either the female mouse or the CD-1 (Franco et al., 2022). Since results found the onset of anxiety-like behaviors in unstressed adult males injected with virla RPRD2, this study could be replicated in adult female mice to determine if RPRD2 has comparable effects on both sexes. If this study were repeated with female mice, similar results of an increase in anxious behavior after RPRD2 injection into the NAc would be anticipated. The hypothesis for that study would be that increased levels of RPRD2 in the NAc would increase anxiety-like behaviors different in female than male mice, such as decreased time in the open arms and thigmotaxis in female C57BL/6J mice.

Since females are significantly more likely to be affected by anxiety in human subjects (Bandelow & Michaelis, 2015), female C57BL/6J mice may experience more pronounced anxiety-like behaviors than the male subjects. However, this protein currently has no established

effects in female mice since research on RPRD2 is so limited as of date. Future studies that use female mice will be imperative to the development of anti-anxiety treatments that target RPRD2, especially if these studies yield significant data. Research on both male and female mice can allow for a better understanding of RPRD2 and is crucial to learning the mechanism of action for this protein and anxiety behaviors.

Limitations of HSV-RPRD2 expression in the NAc

From the Western blots that were analyzed, there was no significant difference in RPRD2 levels between the two groups **(Figure 7B)**. The group injected with HSV-RPRD2 did not have a significantly higher presence of the protein RPRD2 in the PVDF membrane than the control group that just received HSV-GFP. From the tool that was created **(Figure 1)** and the anxiety-like behavior in mice who received the RPRD2 virus seen in the EPM, it is possible that other factors are at play that account for the insignificant difference in expression of RPRD2 between the two subject groups of mice.

HSVs are effective in targeting neurons and are a non-genome inserting virus that can package large plasmids. However, it has a very short expression period of roughly three days, and after this period, HSV cannot be identified reliably within the neurons (Lachmann, 2004). This may contribute to the lower expression of HSV-RPRD2 recorded in **Figure 7**. In addition, the dispersion of RPRD2 and the ambiguity of the punches from the brain tissue may have led to less accurate data in Western blot analysis. While Western blots are extremely accurate in detecting proteins present in tissue, the data may not have reached significance due to the repression of the virus, the dispersion of RPRD2 throughout the brain region, and the size of the

punches taken from the NAc. HSVs specifically target neurons, but it is unknown which cell type in the NAc is driving this behavioral response. Further studies should be conducted to confirm an overexpression of the presence of RPRD2 in the NAc. In addition, future studies should be performed to confirm the specificity of the RPRD2 antibody used in this thesis to confirm its validity.

Limitations of Targeting the NAc in Stereotaxic Surgery

The stereotaxic surgery performed in this thesis project did not specifically target the shell or the core of the NAc. As previously mentioned, the shell and the core are molecularly diverse from each other and have different functions with the shell more focused on emotional and motivational responses and the core involved in learning and goal-directed behaviors (Baik, 2020). The core and the shell have considerable differences in connectivity. Dorsal regions of the medial prefrontal cortex innervate the core while the shell receives afferents from ventral regions of the medial prefrontal cortex (Feja et al., 2014). In addition, the core sends efferent signals to the basal ganglia while the shell projects to subcortical limbic structures (Feja et al., 2014). The differences in connectivity leads to the functional dichotomy of these two regions that cause distinct and unique responses to anxiety provoking stimuli.

Since this study did not specifically target the core or shell of the NAc, it is unknown which region of the NAc is driving the anxiety-like behavior seen in mice injected with viral RPRD2. In addition, since the stereotaxic surgery was non-specific, it may have resulted in a bimodal distribution of the virus throughout the NAc. This may provide another reason that there was a high degree of variance in the behaviors observed in the C57BL/6J mice as well as the

variation in Western blot analysis of expression levels of HSV-RPRD2 in the NAc tissue. Future studies should observe the behavior induced by overexpression of RPRD2 while focusing on targeting an individual region of the NAc.

Proposed mechanism of action for RPRD2

Based on the knowledge of the RPRD family of proteins, another aim of this thesis project was to propose a potential mechanism for how this protein can influence anxiety-behavior. Prior studies have shown that RPRD family proteins contain CIDs that can influence transcription, so this thesis focused on RPRD2 and its influence on Pol II Ser5 (Ali et al., 2019). To do so, the level of Pol II and pSer5 was analyzed through Western blots of tissue from mice injected with HSV-RPRD2 and HSV-GFP and compared **(Figures 9 and 10)**. The data showed no significant difference in the level of pSer5 in the NAc of mice who received HSV-RPRD2 **(Figure 9)**. On the basis of the data collected and prior knowledge of the protein, the mechanism that upregulated RPRD2 lowers the levels of pSer5 in the PVDF membrane could not be validated.

RPRD2 binds to the CTD of Pol II to influence Ser5 phosphorylation **(Figure 13)**. The protein then dephosphorylates, which causes transcription to pause **(Figure 14B)**. Pol II is unable to progress into the elongation phase of transcription until Ser5 can be re-phosphorylated and RPRD2 is removed from the CTD. The interaction with the phosphorylation of the CTD is a rate-limiting step that is critical in an accurate transcription and cell survival (Winczura et al., 2021). This thesis was hoping to support previous research (Ali et al., 2019) that suggests that

proteins from the RPRD family can dephosphorylate the CTD and downregulate transcription, but no conclusions can be drawn from the data recorded from this study.

It is important to note that the limitations previously mentioned affected the expression of pSer5 and Pol II in the NAc tissue. HSVs infect neurons, but they are short lasting and typically only express in the membrane for three days. It is difficult to determine if the samples were taken at the proper time that RPRD2 would have an effect on pSer5. Future studies should consider more specific targeting in the brain and other measures that can be used to determine protein expression such as IHC or PCR. The data performed in this study was not significant, but future studies would likely confirm this mechanism.

Conclusions and Future Directions

This thesis project determined that RPRD2 injected into the NAc significantly increases anxiety-like behavior in C57BL/6J mice, regardless of exposure to CSDS. Injection of viral RPRD2 increased anxiety-like behaviors such as avoidance of the open arms of the EPM in comparison to mice who received the control HSV-GFP. RPRD2 did not have any effect on social behavior and did not impact the SI ratio of mice that experienced an accelerated CSDS. The increased anxiety-like behaviors observed may occur through RPRD2's interaction with the CTD of Pol II, where it binds to pSer5 and dephosphorylates it. This prevents Pol II from progressing into the elongation phase of transcription until RPRD2 can be removed and Ser5 can be phosphorylated again.

The current understanding is that RPRD2 induces anxiety-like behavior through pausing transcription by dephosphorylating Ser5 on the CTD of Pol II in the NAc. While prior studies

from this group (Hamilton et al., 2020) showed that RPRD2 was higher in the NAc of animals resilient to CSDS, the data from this thesis indicates that other brain mechanisms are likely responsible for this effect and not the protein of interest. Mice injected with HSV-RPRD2 displayed greater anxiety-like behaviors than mice injected with HSV-GFP across the behavioral data that was analyzed. The study of this protein can lead to more effective anti-anxiety treatments through confirming its mechanisms in the brain. The development of new treatments that target this mechanism should reduce levels of RPRD2 in the brain in order to reduce symptoms of anxiety. However, significant research must be performed prior in furtherance of gaining a more comprehensive understanding of this protein and its role in anxiety behaviors.

The protein of interest from this study, RPRD2, currently has very limited research. There are no current studies that link this protein to organismal behaviors. Future studies should reinforce that RPRD2 has an effect on anxiety-like behaviors and further explore its relationship with depression, social behaviors, and stress. Since females are more affected by anxiety disorders than males, it would be advantageous to study the effects of RPRD2 on female C57BL/6J mice. This study focused solely on the NAc, and other brain regions may be worthwhile to study. Further projects should also confirm a significant increase in the levels of RPRD2 in tissue after viral injection, perhaps through immunohistochemistry (IHC) instead of Western blot analysis. IHC can detect the exact location of a target protein within a tissue sample, which may be beneficial in determining shell versus core expression of HSV-RPRD2 (Kaliyappan et al., 2012). Additional studies should also confirm the mechanism of action for RPRD2 with the ultimate goal of developing a treatment to reduce anxiety behaviors.

In conclusion, this thesis provides a novel perspective that RPRD2 induces anxiety-like behavior in male C57BL/6J mice when injected in the NAc. The accelerated CSDS paradigm did

not have any apparent effects on mice injected with this virus and social behaviors were not significantly impacted. The Western blot analysis of brain tissue from the NAc hinted at a potential mechanism for RPRD2 to induce anxiety-like behaviors by pausing transcription, but no significant data was recorded. While there are currently no studies that have researched a relationship with RPRD2 and anxiety behaviors, future studies should focus on this protein to better understand its effects and its mechanisms in order to produce more effective, long-lasting treatments for anxiety disorders.

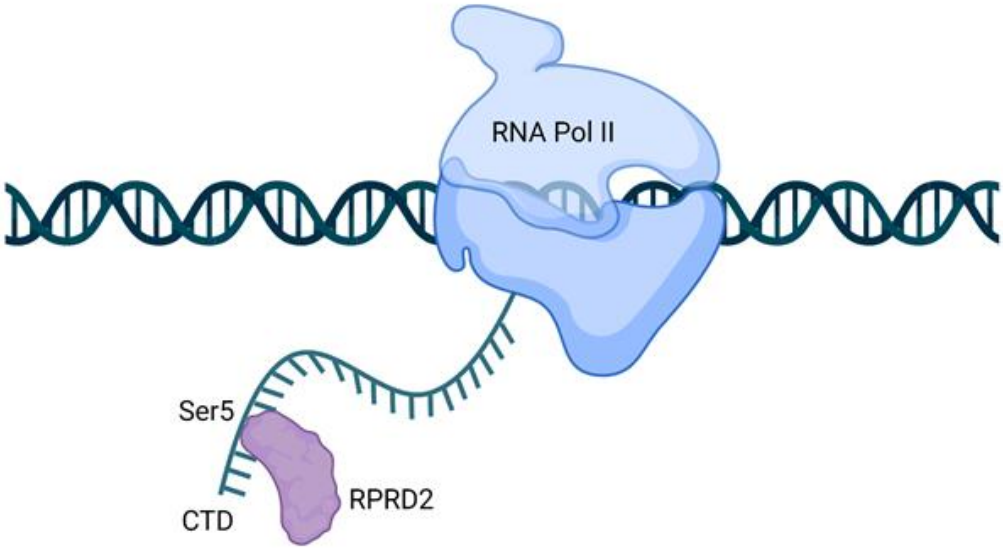
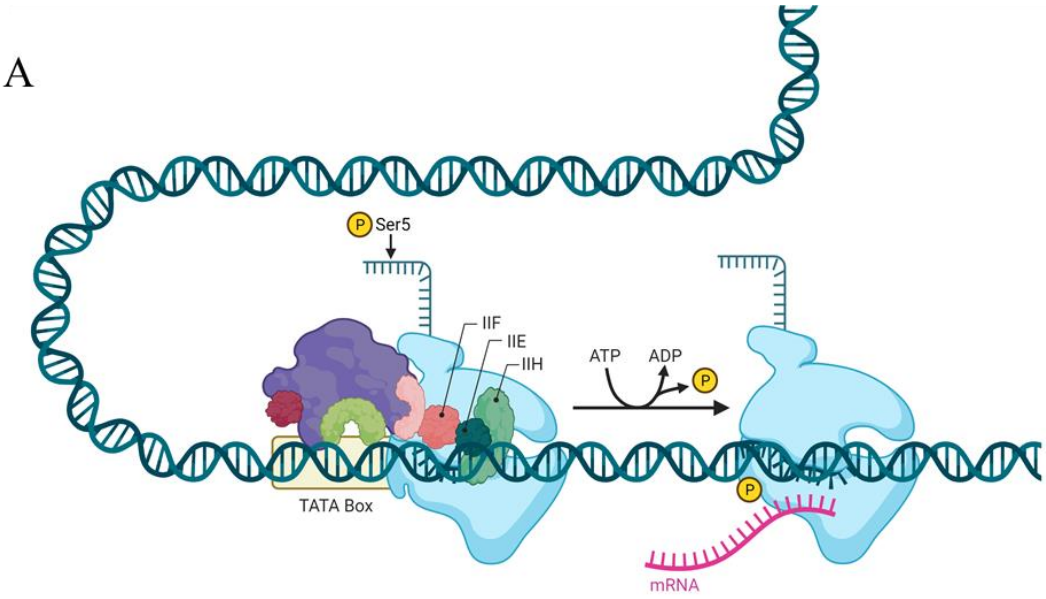
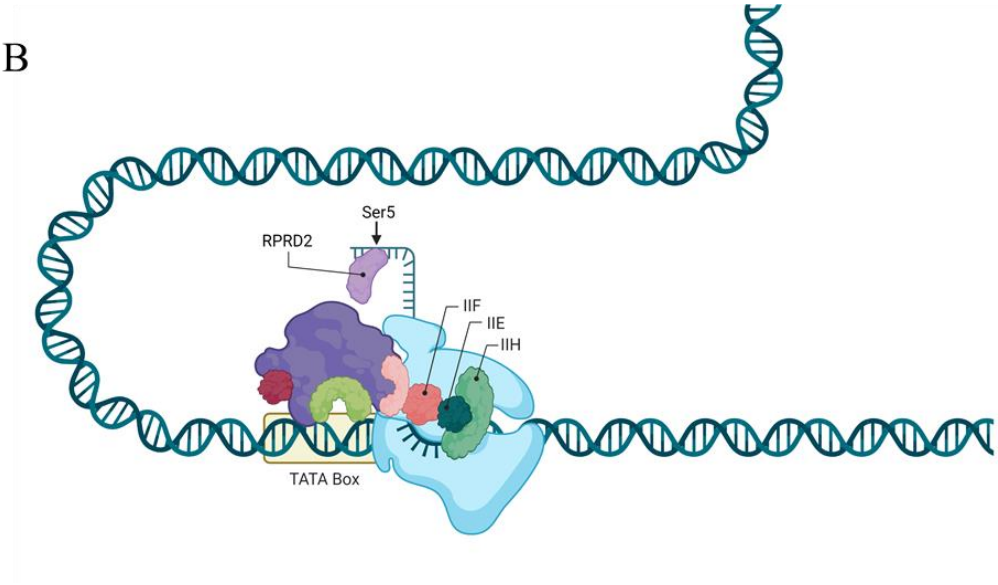

RNA Pol II
Ser5
CTD
RPRD2

Figure 13: *Proposed mechanism of RPRD2.* RPRD2 binds to a phosphorylated Ser 5 on the CTD of RNA Pol II. RPRD2 then dephosphorylates Ser5 and pauses transcription by preventing RNA Pol II from moving forward. *Created with BioRender.com.*

A


P Ser5
IIF
IIE
IIH
ATP
ADP
P
TATA Box
P
mRNA

B


Ser5
RPRD2
IIF
IIE
IIH
TATA Box

C

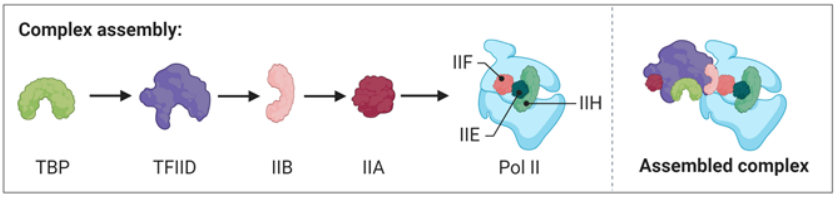

Complex assembly:
TBP
TFIID
IIB
IIA
IIF
IIH
IIE
Pol II
Assembled complex

Figure 14: *RPRD2 pauses transcription*. A) TFII factor -D recognizes the promoter region of the DNA and binds to the TBP to form a transcription-initiation complex. Transcription can be initiated, and Pol II progresses into the elongation phase and produces mRNA. B) RPRD2 binds to pSer5 on the CTD of Pol II, dephosphorylates it, and prevents Pol II from progressing into elongation phase. C) Key of protein factors in the transcription complex. *Created with BioRender.com*.

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