

PTSD related Behavior, Schaffer Collateral Pathway Organization and Hippocampal
Transcript Expression in a Traf-2 and Nck Interacting Kinase (TNIK) -/- Mouse
Model

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Sincerely,

Steven Dash

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ABSTRACT

Title of Thesis/Dissertation: PTSD related Behavior, Schaffer Collateral Pathway Organization and Hippocampal Transcript Expression in a Traf-2 and Nck Interacting Kinase (TNIK) -/- Mouse

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Thesis directed by: Dr. Kwang Choi, Assistant Professor, Psychiatry

Post Traumatic Stress disorder (PTSD) is prevalent in both the military and general population. Symptoms can begin soon after the onset of trauma or at any time throughout the lifetime. Twin studies of those with PTSD reveal genetic susceptibility. This led to interest in identifying genetic risk factors for PTSD. Even large genome studies have not identified and confirmed genes linked to PTSD.

To study aspects of PTSD animal models can be used, which allow greater control over variables such as genetic background. Rodent fear conditioning, experimental induction of a fearful response to a cue or context, is used to model aspects of PTSD such as intrusive memories. Using fear memory as criteria for selective breeding, a mouse model was created where mice display high [fear susceptible (FS)] or low [fear resilient (FR)] levels of fear based on breeding background. Functional MRI of these mice revealed the hippocampus of FS mice showed higher baseline activity.

To investigate differences in neural activity, we performed RNA sequencing from hippocampal tissue of FS and FR mice. A number of transcripts were identified with known neuronal functions that were candidates for further study. One transcript with higher expression in the FS mice was Traf2 and Nck Interacting Kinase (*Tnik*). *Tnik* is an emerging psychiatric disease risk factor, and *Tnik* knockout in mice leads to a deficit in some tests of hippocampal dependent memory.

In order to study *Tnik* loss of function we obtained a *Tnik* ^{-/-} mouse (Pfizer) and elected to study the behavioral effects of *Tnik* ^{-/-}, how *Tnik* affects hippocampal structure and transcript expression. We hypothesized that *Tnik* ^{-/-} mice will display a deficit in spatial memory, differences in fear memory performance and alterations in the hippocampus that could underlie molecular and behavioral effects relevant to PTSD.

We confirmed deficits in spatial memory, deficits in both cued and contextual fear memory and a disruption of the Schaffer collateral pathway in the hippocampus of *Tnik* ^{-/-} mice, which is crucial to learning and memory. We also identified a number of transcripts differentially expressed between *Tnik* ^{-/-} and WT mice that could play a role in the differences we observed in these animals.

TABLE OF CONTENTS

TABLE OF CONTENTS.....	ix
LIST OF TABLES	xii
LIST OF FIGURES	xiii
Chapter 1: Literature Review	14
Post Traumatic Stress Disorder.....	14
Genetics of PTSD	16
Fear Conditioning as a Model to Study PTSD	17
Fear Susceptible and Fear Resilient Mice.....	18
The Hippocampus, Fear Memory and PTSD.....	18
Traf2 and Nck Interacting Kinase (TNIK) as a Gene of Interest for PTSD	19
TNIK and Synaptic Plasticity	22
TNIK and Wnt Signaling	23
Previous Work in <i>Tnik</i> Knockout (-/-) Animals	27
Summary and Hypothesis	28
Chapter 2: Methods.....	30
<i>Tnik</i> -/- Mice	30
Behavioral Tests.....	30
Timeline	30
Open Field.....	31
Y-Maze	31
Elevated Zero Maze	31
Fear Conditioning	32
Conditioning Phase	32
Contextual Fear Test	32
Cued Fear Test	32
Hot Plate.....	33
Data Analysis	33
CRISPR/Cas9 Experiments	33
Cell Culture	33
CRISPR/Cas9 Generation	34
Transfection	35
Hippocampal Structure Experiments	36
Perfusion and Tissue Fixation.....	36
Brain Slicing	36
Immunohistochemical Staining	36
Cresyl Violet Staining.....	37

RNA Sequencing	38
Hippocampal Isolation	38
RNA Isolation	39
Library Preparation and RNA Sequencing	39
RNA Quantity and Integrity	39
Library Integrity	40
Data Analysis	41
Chapter 3: Behavioral Phenotyping of <i>Tnik</i> ^{-/-} Mice	42
Introduction	42
Results	43
Open Field Testing Reveals Hyperactivity in <i>Tnik</i> ^{-/-} Mice	44
Fear Memory Performance	45
<i>Tnik</i> ^{-/-} Mice Show a Deficit in Contextual Fear Memory	45
<i>Tnik</i> ^{-/-} Mice Show a Deficit in Cued Fear Memory	46
<i>Tnik</i> ^{-/-} Mice Show a Spatial Memory Deficit in Y-Maze Testing	47
EZM Reveals Decreased Anxiety-Like Behavior in <i>Tnik</i> ^{-/-} Mice	48
Hot Plate Test	49
Behavioral Summary	49
Discussion	50
Spatial and Cued, Contextual Fear Memory Impaired in <i>Tnik</i> ^{-/-} Mice	50
Altered Anxiety-like Behavior in <i>Tnik</i> ^{-/-} Mice	51
Hyperactive Locomotor Behavior in <i>Tnik</i> ^{-/-} Mice	52
<i>Tnik</i> ^{-/-} Mice as an Animal Model of PTSD Symptoms	53
Chapter 4: The Role of <i>Tnik</i> in the Schaffer Collateral Pathway and Cell Survival	54
Introduction	54
<i>Tnik</i> and Hippocampal Neuronal Morphology	54
The Role of <i>Tnik</i> in Neurogenesis and Cell Survival	55
Summary and Hypothesis	56
Results	58
Disruption of the Schaffer Collateral Pathway in <i>Tnik</i> ^{-/-} Mice	58
Decrease in Dentate Gyrus Size in <i>Tnik</i> ^{-/-} Mice	61
Loss of Cell Viability in <i>Tnik</i> CRISPR/Cas9 Transfected Cells	61
Discussion	63
Loss of <i>Tnik</i> Function Leads to Hippocampal Specific Alterations	63
<i>Tnik</i> Loss of Function Leads to Loss of Cell Viability via GSK3 β	65
Chapter 5: Comparison of <i>Tnik</i> ^{-/-} and WT Hippocampal Transcript Expression	67
Introduction	67
Important Roles of <i>Tnik</i> in Neurological Structure and Function	67
<i>Tnik</i> and Hyperactive Locomotor Behavior	67
<i>Tnik</i> in Hippocampal Structure, Function and Spatial Memory	67
<i>Tnik</i> in Cell Proliferation and Survival	68

Summary	69
Results	70
Transcript Identification.....	70
GO Biological Process Analysis	74
Introduction.....	74
Hippocampal Structure, Function and Spatial Memory	74
Dopamine Signaling Related Transcripts	77
Cell Proliferation and Survival	78
Disease Risk Factors	80
Discussion	84
Dopamine Hypothesis of Schizophrenia and TNIK	84
TNIK and Alzheimer's Disease	86
Implications for PTSD	87
Chapter 6: General Discussion and Conclusions	89
Studying <i>Tnik</i> ^{-/-} as an Animal Model of PTSD Symptoms	89
Anxiety-Like Behavior in <i>Tnik</i> ^{-/-} Mice: Implications for PTSD.....	89
Fear and Spatial Memory Deficits in <i>Tnik</i> ^{-/-} Mice: Implications for PTSD	91
Hyperactivity in <i>Tnik</i> ^{-/-} Mice: Implications for PTSD.....	94
<i>Tnik</i> ^{-/-} Mice, FS Mice and PTSD	95
PTSD and Suicide Risk.....	97
REFERENCES	100

LIST OF TABLES

Table 1. Pilot RNAseq experiment results. Tissue was taken from the hippocampus of both FS and FR mice for comparison. Transcripts presented display at least a 2 fold change difference and had a significant Q-value < 0.1	21
Table 2. Cresyl Violet staining protocol	38
Table 3. RNA quantity and integrity	40
Table 4. Library fragment size information.	40
Table 3- Phenotypes in <i>Tnik</i> -/- Mice, FS Mice and PTSD.	53
Table 6. GO annotation pathway analysis of differentially expressed transcripts (Fold change= log(2) fold change). Negative fold change transcripts are upregulated in WT while normal fold change transcripts are upregulated in TNIK -/- mice (*= gene occurs in multiple pathways, bold transcripts are in WT or TNIK -/- top 25).	82
Table 7. Top 25 fold change (fold change= log(2) fold change)transcripts in TNIK -/- and WT mice presented with Q-value (adjusted p-value) and fold change relative to opposite condition.	83
Table 8- Phenotypes in <i>Tnik</i> -/- Mice, FS Mice and PTSD.	96

LIST OF FIGURES

Figure 1- TNIK Function Summary.	26
Figure 2- Behavioral Timeline.	30
Figure 3- Open Field Reveals Hyperactivity in <i>Tnik</i> ^{-/-} Mice.	44
Figure 4- Contextual Fear Test Shows Fear Memory Deficit in <i>Tnik</i> ^{-/-} Mice.	45
Figure 5- Cued Fear Test Shows Fear Memory Deficit in <i>Tnik</i> ^{-/-} Mice.	46
Figure 6- Y-Maze Reveals Spatial Memory Deficit in <i>Tnik</i> ^{-/-} Mice.	47
Figure 7- Elevated Zero Maze Shows Decreased Anxiety-Like Behavior in <i>Tnik</i> ^{-/-} Mice.	48
Figure 8- Hot Plate Reveals no Nociceptive Differences.	49
Figure 9- Schaffer Collateral Pathway.	57
Figure 10- MAP2 Staining of Hippocampus and Cortex.	60
Figure 11- Dentate Gyrus of <i>Tnik</i> ^{-/-} and WT Mice.	61
Figure 12- Cell Counts from <i>Tnik</i> ^{-/-} CRISPR/Cas9 Transfected Cells.	62
Figure 13- Hierarchical Clustering.	71
Figure 14- Volcano Plot of Transcripts.	72
Figure 15- Fragments Significant by Adjusted P-Values.	72
Figure 16- Heatmap of Top Transcripts.	73

Chapter 1: Literature Review

Post Traumatic Stress Disorder

The health and safety of service members in our nation's military is of the utmost importance during a time when there are numerous ongoing conflicts throughout the world. Post-traumatic stress disorder (PTSD) is a major health concern in both military and civilian populations with a serious impact on quality of life for those with PTSD and their families. Recent data in combat veterans from the Vietnam War through the current wars in Iraq and Afghanistan estimate the percentage of returning veterans experiencing PTSD symptoms to be as high as 17% (133). Other studies of Vietnam veterans report late onset of symptoms and estimate occurrence of lifetime PTSD following combat trauma to be much higher, indicating that current returning soldiers are most likely at an increased lifetime risk of PTSD (57, 79).

The DSM-V lists several symptom categories for PTSD diagnosis. The first is the presence of a traumatic event in a patient's past that was either directly experienced or witnessed, an indirect experience (death of a relative or close friend) or repeated exposure to qualifying events, usually a first responder to traumatic events (4). The second category is experiencing intruding memories or re-experiencing of the traumatic event, which includes nightmares, intrusive thoughts or memories and flashbacks (4). Third is the presence of avoidant symptoms, where the person avoids reminders of the event such as thoughts or feelings associated

with the event or avoids people, places or situations that are similar to the event. A new category emphasized in the DSM-V is negative alterations in mood or cognition including memory problems, negative thoughts about oneself or others, extreme guilt, horror, shame or sadness or reduced interest in previously enjoyable activities or isolation from others (4). The emphasis on this last category is in response to the recent prevalence of these symptoms in patients and the impact they have on quality of life (115, 164). Finally, patients with PTSD have to experience increased trauma-related arousal as defined by irritability or aggression, risky or destructive behavior hypervigilance, increased startle or difficulty concentrating or sleeping (4). At least one symptom from each category must be present and must also persist for at least one month to qualify for a diagnosis of PTSD.

These symptoms can be very intrusive in everyday life and may last for years following trauma, making returning to a normal life following combat more difficult for soldiers who develop PTSD. The latest information from the Study to Assess Risk and Resilience in Service members (STARRS) seeks to identify major mental health concerns facing the military and to help identify interventions that will help service members (155). One of the goals of the STARRS program, and PTSD research in general, is to investigate social, demographic and genetic factors that contribute to individual differences in response to trauma (115, 141, 164). Why some individuals develop PTSD following trauma while others remain resilient is critical to understanding this disorder. Identifying any factors linked to risk or resilience of developing PTSD in response to trauma is one of the next steps to understanding and treating this disorder.

Genetics of PTSD

The reason that certain individuals are at greater risk of developing PTSD following trauma while others are resilient is unknown. Underlying genetic causes that lead to risk or resilience of developing PTSD following trauma have been proposed (5). The most notable evidence of genetic susceptibility comes from twin studies of PTSD (138, 160). Studies of 4042 male twin pairs (2,224 monozygotic, 1,818 dizygotic) from the Vietnam War showed significant inheritance of risk of PTSD symptoms, ranging from 13% to 34%, of the re-experiencing, avoidance and arousal symptoms being genetically inherited (160). Non-combat related PTSD also appears to be inherited with 60% of high-risk trauma in a separate study of twins and non-twin siblings (male and female) being attributed to genetic inheritance (138).

The twin studies strongly suggest a genetic link to PTSD susceptibility, however efforts to identify specific genetic loci that are linked to risk or resilience of developing PTSD following trauma have thus far been unsuccessful. Genome wide association studies (GWAS) have been non-reproducible in identifying genes linked to PTSD symptoms and have led to no therapeutic targets or mechanistic understanding of PTSD pathology. These studies may not have had sufficient participation to identify genetic loci but there is also the issue of lacking a common genetic background, where random differences between individuals complicate analysis and make it more difficult to identify the specific, relevant genes. Another approach to identifying genes is to utilize animal models of certain aspects of PTSD, where animals can be bred on a common genetic background and specific genes

influencing specific behaviors can be identified. Using this approach it may be possible to identify genes in animal models that can later be confirmed in human studies.

Fear Conditioning as a Model to Study PTSD

PTSD is a difficult disorder to model, as it is not one individual process and involves multiple brain regions for different aspects of the disorder. One of the hallmark symptoms of PTSD is intrusive thoughts or memories in response to situations reminiscent of the initial trauma (4). The way fear memory is encoded and recalled is aberrant in PTSD, with patients showing heightened fear even to non-threatening situations because of a previously made association during trauma (4). Fear conditioning paradigms are one approach used to model aspects of PTSD in animals. By measuring rodents fear responses under varying conditions it is possible to investigate factors such as drug treatments that alter memory for fear. Fear conditioning has a number of variations including being cue or context based. Contextual fear conditioning involves placing rodents in a context for a training session and exposing them to a foot shock. For the test phase rodents are placed back in the same context and their fearful response to the context is measured. In cued fear conditioning a rodent is placed into a training chamber and exposed to a cue, usually an auditory tone, prior to receiving a foot shock. The rodent is then placed into a novel context and exposed to the cue (tone) and again fear memory is assessed. These paradigms are similar to the intrusive thoughts and memories seen in PTSD, as both induce a behavioral response and fear behavior to specific context or cues (5). By studying fear learning and memory expression in rodents we can

better understand the process of fear memory consolidation and retrieval, which will help identify mechanisms underlying the exaggerated fearful responses in PTSD patients.

Fear Susceptible and Fear Resilient Mice

Recently, an animal model was developed in which an intercross line of mice were selectively bred for several generations based on their performance on cued and contextual fear behaviors (125). C57/BL6 and DBA2J mouse lines, which display heightened and decreased fear memory respectively, were intercrossed and resulting mice were bred for several generations based on their performance on cued and contextual fear memory (106, 125). Mice that displayed high levels of freezing were bred together while animals that showed low levels of freezing were bred together. After several generations, this led to fear susceptible (FS) mice, and fear resilient (FR) mice (33). Utilizing these FR and FS mice several quantitative trait loci (QTL), or sections of DNA linked to a specific trait (in this case fear memory performance) were identified (125). These regions of DNA include genes identified in both the prefrontal cortex as well as the amygdala that correlate with the FS versus FR phenotypic differences in freezing behavior (26, 106). The genes identified include genes associated with mitochondrial function (MAOB, SLC25A14 and TUBB3) as well as cannabinoid receptor 1 (CBR1) all in the PFC (26, 106).

The Hippocampus, Fear Memory and PTSD

A study utilizing manganese enhanced magnetic resonance imaging (MRI), discovered several regions of differential baseline activity (106). The significant

differences were found in several regions of the hippocampus including the dorsal hippocampus and subfields *Cornu Ammonis 1 and 2* (CA1 and CA2). These regions of the hippocampus displayed higher levels of baseline locomotor activity, in the FS relative to those of the FR mice (106). Since the functional differences were observed in the hippocampus, it is important to understand the molecular mechanisms underlying the functional differences in the hippocampus that may contribute to the phenotypic differences between the FS and FR mice.

Interest in the hippocampus is supported by the relationship between declarative memory (memory for events, facts etc.) and hippocampal volume in some PTSD patients (82). A portion of PTSD patients have deficits in declarative memory and decreased hippocampal volume after suffering from PTSD symptoms for some time (159, 176). It is not yet clear, whether suffering from PTSD leads to hippocampal atrophy and declarative memory deficits or whether these deficits existed prior to the trauma and development of symptoms (159, 176). This means genetic differences between individuals that cause heightened activity in the hippocampus are good targets as genetic causes underlying risk to develop PTSD.

Traf2 and Nck Interacting Kinase (TNIK) as a Gene of Interest for PTSD

Following identification of the hippocampus as a region of interest in the behavioral differences between FS and FR mice we performed a pilot experiment comparing transcriptomes from hippocampal tissue from FS and FR mice. This data revealed a number of transcripts with differential expression between the FS and FR mice that have potential for further study (Table 1, unpublished). One of these genes, Traf2 and Nck interacting kinase (TNIK) is highly expressed in the FS

compared to the FR mice as identified by RNA sequencing and confirmed by qPCR.

There are a number of ways TNIK could be linked to the fear memory differences between FS and FR mice and the aspects of PTSD modeled by these mice.

Gene ID	Increased in	Fold Change	Q-Value
<i>Ccdc93</i>	FS	16.08	0.10
<i>Chn1</i>	FS	15.18	0.10
<i>Rit1</i>	FS	12.66	0.10
<i>Haghl</i>	FS	9.13	0.10
<i>Dab1</i>	FS	8.86	0.10
<i>Arhgap4</i>	FS	5.70	0.10
<i>Tnik</i>	FS	5.11	0.10
<i>Ak4</i>	FS	4.98	0.10
<i>Btrc</i>	FS	4.50	0.10
<i>Mavs</i>	FS	4.38	0.10
<i>Gm10653</i>	FS	4.31	0.10
<i>Pnoc</i>	FS	4.18	0.10
<i>Prss41</i>	FS	4.09	0.10
<i>Rbck1</i>	FS	3.88	0.10
<i>Tle3</i>	FS	3.77	0.10
<i>Npw</i>	FS	3.61	0.10
<i>Rnf146</i>	FS	3.51	0.10
<i>Fibp</i>	FS	3.26	0.10
<i>Eno3</i>	FS	2.94	0.10
<i>Clasp2</i>	FS	2.82	0.10
<i>N6amt1</i>	FS	2.64	0.10
<i>Dynll2</i>	FS	2.64	0.10
<i>Chrd</i>	FS	2.21	0.10
<i>Ecsit</i>	FS	2.18	0.10

Gene ID	Increased in	Fold Change	Q-Value
<i>Myh14</i>	FR	32.1	0.0002
<i>Zc3h11a</i>	FR	26.09	0.0002
<i>Lrrc57</i>	FR	25.66	0.0002
<i>Kit</i>	FR	23.09	0.0002
<i>Sorbs3</i>	FR	10.76	0.0002
<i>Scn3b</i>	FR	9.78	0.0002
<i>Med23</i>	FR	9.75	0.0002
<i>Ghitm</i>	FR	9.16	0.0002
<i>Rpgrr</i>	FR	7.06	0.0002
<i>H13</i>	FR	6.87	0.0002
<i>Fam149b</i>	FR	6.66	0.0002
<i>Stat3</i>	FR	6.17	0.0002
<i>Gm20735</i>	FR	6.15	0.0002
<i>Tmem183a</i>	FR	6.09	0.0002
<i>Lpgat1</i>	FR	5.02	0.0002
<i>Snora78</i>	FR	4.37	0.0002
<i>Srsf3</i>	FR	3.65	0.0002
<i>Wdr12</i>	FR	3.55	0.0002
<i>Ywhaz</i>	FR	3.53	0.0002
<i>Al854517</i>	FR	3.14	0.0002
<i>2-Mar</i>	FR	2.68	0.0002
<i>Eno3</i>	FR	2.63	0.0002
<i>Mir2861</i>	FR	2.31	0.10
<i>Repin1</i>	FR	1.98	0.10

Table 1. Pilot RNAseq experiment results. Tissue was taken from the hippocampus of both FS and FR mice for comparison. Transcripts presented display at least a 2 fold change difference and had a significant Q-value < 0.1.

TNIK and Synaptic Plasticity

TNIK interacts with disrupted in schizophrenia 1 (DISC1) in the post-synaptic density (PSD) in key brain regions associated with schizophrenia symptoms and progression (170). This includes the prefrontal cortex (PFC), as well as the pyramidal cell layer of the hippocampus, both of which show enriched TNIK in expression in neurons (28). DISC1 appears to inhibit TNIK activity, which subsequently destabilizes PSD proteins including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors, thereby decreasing AMPA receptor mediated currents (170). This would disrupt long-term potentiation (LTP), the process by which synapses are strengthened following activation (174). LTP is a complex process that is based on activity in a neuron, which leads to the strengthening of a synaptic connection and is the molecular basis for learning and memory. When *N*-Methyl-D-aspartate (NMDA) glutamate receptors are activated by glutamate across a synapse, more AMPA type glutamate receptors are incorporated into the membrane which strengthens the connection (174). TNIK expression in the PSD is required for the incorporation of AMPA receptors into the membrane of the PSD (28). Additionally, blocking TNIK expression *in vitro* stops this process leading to lower surface expression of AMPA receptors (28, 170). The localization of TNIK expression in neurons also implies that it is an important regulator of synaptic strength. Based on electron microscopy results, TNIK clusters in the PSD along the lateral edge of spines (16). It is in this area that the trafficking of AMPA receptors is known to occur, again highlighting the potentially important role of TNIK in this process (16, 28, 73). Differences in the way individuals encode and recall memories

may be aberrant in those with PTSD and this may be an important part of how PTSD symptoms develop.

TNIK has also been identified as a risk factor gene for intellectual disability (ID). ID is a generalized disability in adaptive functioning in conceptual, social and practical intelligence (6). This is due to roles in synaptic development and synaptic transmission during development, since this is when the symptoms first become apparent. The role TNIK plays in synapses appears to be very complex, as it is not only a mediator of signaling pathways in the CNS, but also plays a role directly phosphorylating proteins in the synapse and altering their function.

TNIK and Wnt Signaling

The original identification of TNIK as a disease risk factor was in cancer biology. Specifically, TNIK was found to be highly expressed in colorectal cancer and cancer of the intestinal epithelium (10,12). Increased Wnt signaling has also been observed in these cancers, and TNIK has been established to play a role in regulating the Wnt signaling pathway in epithelial cells of the intestine (26). TNIK directly interacts with TCF4, which is one of the primary Wnt transcription factors (149). TNIK acts as an activating kinase for TCF4, which in turn promotes intestinal and colorectal epithelial cells to remain in their stem cell phase. This leads to an overabundance of cell growth and cancer development (70, 103, 149). The activation of TCF4 via TNIK is dependent on TNIK phosphorylating TCF4, and can be blocked by truncating the TNIK kinase domain (149). Constitutively active TNIK leads to an increase in Wnt related genes that lead to cell survival and proliferation in cancer (103). These multiple functions of TNIK in cell survival and reorganization

have made it a prime target for repressive drugs in the treatment of treatment refractory colorectal cancer, with multiple kinase inhibitors being developed for this purpose (15, 70, 105, 149).

Interestingly, TCF4 also affects neural development. TCF4 interacts with the pro-neuronal transcription factor Math1, which is responsible for driving the creation of a neural progenitor population in the cerebellar rhombic lip (51). TCF4 and Math1 have a direct interaction that is required for proper cerebellar development (51, 171). Math1 is also important in the development of glutamatergic neurons as part of the sequence of proper glutamatergic neuronal differentiation (69). Likewise, TNK1 is expressed in glutamatergic neurons in the cerebellum, cortex and hippocampus (16). How TNK1 may interact with Math1 and TCF4 is unexplored, but TNK1 has an effect on neurogenesis as well. In a TNK1 knockout animal, impaired neurogenesis was observed in the dentate gyrus region of the hippocampus, showing these genes may all be involved in a similar pathway (28). Any role TNK1 plays in cell proliferation and survival in neural genes remains unknown, though this line of research has implications in disorders such as PTSD, where brain regions such as the hippocampus sometimes show atrophy (159, 176).

TNK1 interacts with GSK3 β which is also involved in Wnt signaling (Figure 1). Many of the functions of TNK1 appear to be dependent on downstream targets of GSK3 β , and the effect TNK1 has on neuronal process elongation and branching appear to be dependent on changes in GSK3 β activity levels (145). TNK1 phosphorylates and inactivates GSK3 β (28). GSK3 β has a very complex effect on neuronal process development, with inhibition of GSK3 β being required for the

initiation of axonal growth while also being required for branching of processes following elongation (30, 122, 179). The complicated role GSK3 β plays in neuronal development highlights why TNIK expression is so crucial in this process. As an inhibitor of GSK3 β , TNIK may play a role in the spatial and temporal control of GSK3 β and therefore an important role in the development of neuronal projections. This interaction may also involve DISC1, which is known to interact with both TNIK and GSK3 β , although if this interaction is mediated by DISC1 remains unknown (154, 170). A neuronal specific and brain region specific assessment of TNIK function in the CNS has not been performed. Gain or loss of function studies of brain regions where TNIK is known to have a powerful effect on neuronal function, development and behavior, such as the hippocampus, would help elucidate the ways TNIK may be contributing to disease states in which it is implicated (16, 28, 170).

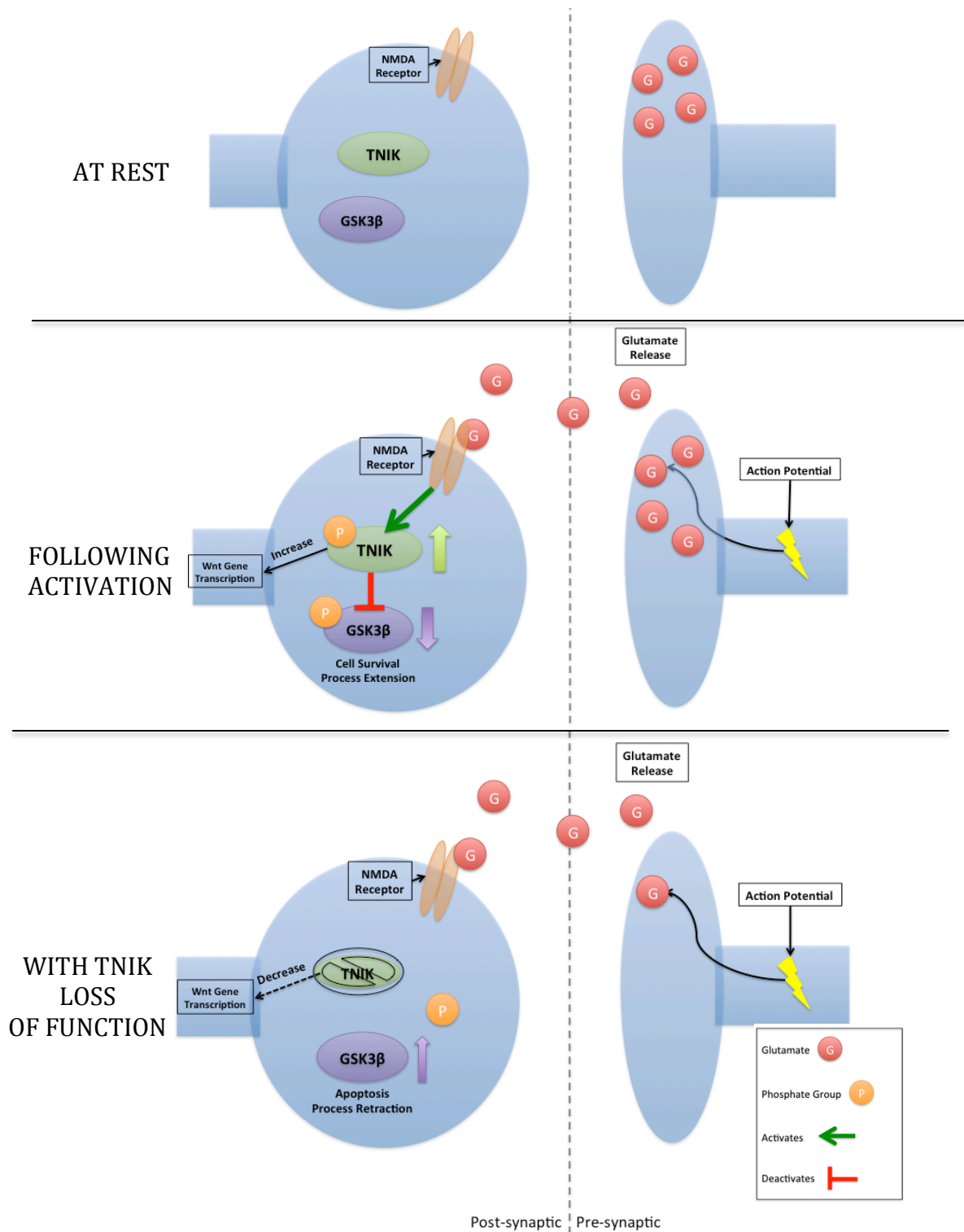


Figure 1- TNIK Function Summary.

Diagram summarizing TNIK function in neurons. TNIK is an important mediator in Wnt signaling, and is critical to the transcription of Wnt target genes (103). Following activation of NMDA receptors by glutamate, TNIK is phosphorylated and activated, leading to inhibition of GSK3β and activation of Wnt target genes and axon elongation via GSK3β (29). Loss of TNIK function leads to increased GSK3β activity, cell death, a decrease in neuronal process extension and a decrease in Wnt gene related gene transcription.

Previous Work in *Tnik* Knockout (-/-) Animals

Recently, *Tnik*, through research conducted in the previously mentioned *Tnik* knockout animal, has been identified as an important signaling molecule in hippocampal neurons (28). A study with these knockout mice has confirmed that *Tnik* is essential for the incorporation of AMPA receptors into the post-synaptic density *in vivo*, and absence of *Tnik* leads to a decrease in glutamatergic signaling (28). These mice display a deficit in learning object-location relationships, which is dependent on glutamatergic signaling (28). The knockout mice also display deficits in spatial learning and memory which are dependent on hippocampal neuronal function (28).

These effects may be dependent on the impaired neurogenesis displayed in the dentate gyrus but this connection has not yet been explored. *Tnik* expression is highly regionally specific to regions CA1 and the molecular cell layer of the dentate gyrus in the hippocampus. Within these regions *Tnik* is primarily expressed in excitatory glutamatergic neurons including pyramidal cells in CA1 and the dentate gyrus (16). These regions are important in the formation of memory as this is the region where place cells, cells activated by a specific context, were first described (109, 110, 117). Utilizing optogenetic approaches to express a light activated ion channel, channelrhodopsin-2 (ChR2), in activated neurons researchers have been able to show a “false” fear memory. Mice were fear conditioned with foot shocks, which activates certain engram-bearing neurons in CA1 and the dentate gyrus in the hippocampus. By activating these neurons during conditioning they also labeled them with ChR2 which becomes embedded in the surface of these neurons (128).

The mice were then placed in a new context and the ChR2 expressing neurons were light activated (128). When placed in a context in which they have never been animals displayed freezing behavior when the neurons in the dentate gyrus were light activated, thus showing a false fear of a novel context (128).

Since *Tnik* is expressed in these same engram encoding neurons in the dentate gyrus and *Tnik* $-/-$ animals show memory deficits this implies *Tnik* may be important for normal memory formation as well as implicated in disorders of abnormal memory and neuronal function (16, 28). It has been previously established that *Tnik* plays a role in neuronal process extension as well as neurogenesis (28, 73). Considering that *Tnik* is expressed not only in the dentate gyrus, where neurogenesis occurs, but also in CA1-CA3 it would be of interest to study if TNIK plays a role in the morphology of neurons in this pathway (16). Specifically, the pathway from CA3 to CA1, which is known as the Schaffer collateral pathway, and is crucial to learning and memory (10, 31, 157, 181). Inhibition of this pathway leads to disruptions in memory, and any disruption caused by gain or loss of TNIK function would have implications in mental disorders where spatial memory is affected, such as Alzheimer's disease (AD), schizophrenia, ADHD and PTSD (36, 38, 90, 153, 158). These evidence suggest that TNIK affects this crucial pathway for normal learning and memory of interest for a number of disorders.

Summary and Hypothesis

TNIK appears to be an important target to study for human disease for a variety of reasons. It is an emerging risk factor for a number of psychiatric disorders through its interaction with DISC1 including: schizophrenia, ADHD, intellectual

disability and major depressive disorder (MDD) (6, 28, 86, 170). This may be due to the role TNIK plays in neuronal process development and adult neurogenesis in the hippocampus (12, 28, 103). TNIK plays a role in cytoskeletal organization, as a mediator of the Wnt signaling pathway and inhibitor of GSK3 β , which plays a role in both neural development and function (28, 73, 103, 137).

Based on known functions of *Tnik* we hypothesize that *Tnik* loss of function alters fear memory performance, spatial memory and anxiety; impairs neuronal development and adult neurogenesis in the hippocampus; and regulates transcripts related to these behaviors and structures.

Chapter 2: Methods

Tnik ^{-/-} Mice

Tnik ^{-/-} mice were developed by the Pfizer Inc. (previously described) and were bred and maintained at the Charles River Laboratories International, Inc. prior to shipment to USUHS for behavioral experimentation (16). Briefly, exon 7 of *Tnik*, which contains 43 amino acids of the kinase domain, was removed by flanking exon 7 with loxP sites utilizing a targeting vector (16). This led to the generation of *Tnik* ^{Δ7} mice lacking exon 7 and a functional kinase domain (16). In total 35 male mice aged between 2 and 4 months were obtained with the following genotypes: 12 wild type, 12 heterozygotes and 11 homozygotes.

Behavioral Tests

Timeline



Figure 2- Behavioral Timeline.

Mice (*Tnik* ^{+/+}, *Tnik* ^{-/-} WT mice) were subjected to a battery of behavioral tests over a 12 day period. Tests were ordered from least to most invasive, to prevent aberrant results from over testing. Following behavior animals were either perfused for immunohistochemistry or sacrificed and dissected for RNAseq analysis.

Open Field

Animals were placed in the open field boxes (40 cm by 40 cm chamber) and allowed to move freely for half an hour (Stoelting Co., Woodale, IL, model no. 60100)(18). Animal movement was captured by a camera suspended above the chamber and recorded with Any-maze software (Stoelting Co, Woodale, IL). Total distance traveled and average speed were both recorded.

Y-Maze

The Y-maze consists of 3 arms, each 50 cm long, 11 cm wide and 10 cm wide separated by 120° and made of opaque Plexiglas (Stoelting Co., Woodale, IL, model no. 68014). Visual cues are positioned around the maze so mice can recognize the different arms. Mice are placed in the maze for two separate 5-minute trials separated by 4 hours. In the first trial one of the 3 arms is blocked so the mouse cannot enter it (3, 166). In the second trial mice are allowed to freely explore the maze and the time spent in each arm and frequency of entrances to each arm were measured using Any-Maze software.

Elevated Zero Maze

The elevated zero maze was used to measure anxiety-like behaviors. Mice were placed in a circular apparatus (50 cm diameter), which is separated into four equally sized quadrants (Stoelting Co., Woodale, IL, model no. 60190). The quadrants are either open armed or enclosed with walls. The open quadrants are across from one another with the closed arms separating them. Mice were placed in this maze for 5 minutes and their behavior was recorded using Any-Maze software

(18). The percentage of the total time spent in the open and closed arms were recorded.

Fear Conditioning

Conditioning Phase

Mice were placed in a conditioning chamber for training for 4 minutes prior to onset of the conditioned stimulus (tone)(Ugo Basile, Varese, Italy). The conditioned stimulus lasted for 30 seconds prior to the onset of the unconditioned stimulus (foot shock, 0.8 mA), which coincided with the last 2 seconds of the conditioned stimulus. The mice then remained in the chamber for an additional 30 seconds prior to onset of the next trial. Three pairings of tone and foot shock were performed (85, 106).

Contextual Fear Test

Mice were then tested 24 hours later for freezing behavior (lack of movement, measured by Any-Maze software) in a test phase that lasted 15 minutes in the same context as the conditioning phase. Time freezing was measured and percent of total time freezing was calculated (85, 106).

Cued Fear Test

Twenty-four hours after the contextual fear testing mice were placed in a novel context and presented with the cue (tone) from the conditioning phase. Freezing behavior was measured during ten, 30-second tones with a random inter-trial interval. Time freezing was measured and the percent of total time freezing was calculated (85, 106).

Hot Plate

Mice were placed onto a hot plate (60°C) until a hind paw response was recorded (Ugo Basile, Varese, Italy, model no. 35150-002). A hind paw response is considered licking or shaking of the hind paw or jumping to escape the apparatus. Time to response was recorded and mice were immediately removed following response or at a cutoff time of one minute.

Data Analysis

The majority of statistical analysis for the behavioral data was performed by one-way ANOVA in Graphpad Prism, using Dunnett's multiple comparisons test for posthoc analysis to compare genotypes. This analysis strategy was used for all measures in OF, Y-maze and Hot Plate.

Fear conditioning and EZM data were analyzed using analysis of covariance (ANCOVA). Freezing percentage was analyzed for genotype and open field distance was included as a covariate since baseline activity can impact freezing behavior in rodents. This approach allowed us to investigate both genotype and baseline activity in relation to freezing percentage between the genotypes.

CRISPR/Cas9 Experiments

Cell Culture

Experiments were performed using P19 mouse embryonic carcinoma cells obtained from ATCC. P19 cells were maintained in MEM α (Thermo Fisher Scientific) containing 10% FBS and penicillin/streptomycin and were passaged 3 times a week at a 1:4 ratio when cells exceeded 80% confluence.

CRISPR/Cas9 Generation

Guide RNAs (gRNAs) for CRISPR/Cas9 plasmids were generated on CHOPCHOP (chopchop.cbu.uib.no) targeting the *Tnik* promoter region. Single stranded nucleotides for creating a double stranded gRNA were then ordered from Integrated DNA technologies (www.idtdna.com). Two separate plasmids were used to generate 2 plasmids with the gRNA sequence targeting *Tnik*. The first was pSpCas9 (BB)2A-Puro and the second was pSpCas9(BB)-2A-GFP (130, 131).

gRNAs were annealed in a thermocycler at 37°C for 30 minutes followed by 5 minutes at 95°C and a 5°C/min minute ramp down (130). Once gRNAs were annealed we performed a digestion/ligation by combining 1 µg of the plasmid to: 1 µl of gRNA, 1 µl of T4 DNA ligase (New England Biosciences, NEB), 1 µl BbsI (NEB), 1 µl T4 DNA ligase buffer with ATP and ddH₂O to a final volume of 20 µl. Samples were then run in the thermocycler for one hour at 37°C.

The unverified plasmids were then transformed into NEB 5-alpha Competent *E. coli* (NEB). 1 µl of plasmid DNA was combined with 250 µl LB broth with 20 µl of competent cells and placed on ice for 20 minutes. This was followed by one minute of heat shock in a bead bath at 37°C for 1 minute and 2 minutes back on ice. 250 µl of SOC media was then added and the mixture was placed in a shaking incubator at 37°C for 45 minutes. This mixture was then streaked onto a 10 cm LB agar dish with ampicillin and placed in an incubator overnight.

Individual colonies of bacteria were selected from the plates the following day and grown in LB broth with ampicillin overnight. Plasmid DNA was isolated

from the samples following the protocol for the DNeasy Mini Spin Kit (QIAGEN) and sent out for sequencing to confirm incorporation of the gRNA into the plasmid.

Transfection

Cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific). Cells were plated and grown until they approached 50% confluency. Cells were simultaneously transfected with 2 ug of both the GFP and Puromycin selective plasmids. The plasmid DNA was added to 250 μ l of Opti-MEM serum free media along with 10 μ l of P3000 reagent. In a separate tube 5 μ l of Lipofectamine 3000 reagent was added to 125 μ l of Opti-MEM media and incubated for 5 minutes. These 2 mixtures are then combined and incubated for 20 minutes at room temperature. The combination of Lipofectamine and plasmid DNA was then added drop wise to the wells to be transfected.

Cells were checked 24 hours later for fluorescence to confirm transfection. 48 hours after transfection cell were treated with puromycin to promote selectivity of transfected cells. Cells were then taken and a serial dilution was performed in a 96 well plate, starting with 4,000 cells in the first well and diluted 1:10 down the first column of wells then again at 1:10 across all rows of cells. Individual colonies were identified and confirmed for fluorescence. These cells were then grown and transferred into a 35 mm culture dish before being grown in a T25 flask.

Hippocampal Structure Experiments

Perfusion and Tissue Fixation

One week following behavioral experiments, mice were given an intraperitoneal injection of ketamine/xylazine as anesthesia before an incision was made in the midsection, the rib cage was cut and retracted to access the heart. Mice were then transcardially perfused with cold PBS followed by 4% paraformaldehyde until the body became rigid to preserve the brain. Brains were removed from the skull, cryoprotected by soaking in a 30% sucrose solution until the brains dropped to the bottom of the solution and frozen in powdered dry ice and stored at -80°C.

Brain Slicing

Brains were embedded in Polyfreeze Tissue Freezing Medium (Sigma-Aldrich) and cut into 20 μ M coronal sections on a cryostat. Brain sections were kept beginning at -2.0mm from Bregma. Slices were mounted on Superfrost Plus microscope slides (Thermofisher Scientific) at 4 slices per slide. Sections were kept in a non-serial order to yield multiple slides with 4 similar sections. 12 slides per animal were kept for immunohistochemistry and Cresyl violet staining.

Immunohistochemical Staining

Slices were rehydrated with PBS for 10 minutes. They were then placed in ammonium chloride (50 mM) for 1 hour at room temperature. Slides were covered in 10% normal goat serum (NGS, Sigma-Aldrich) for one hour. They were then washed 3 times with PBS. They were then incubated in 90 percent PBST (PBS with 0.1% Tween) with a primary antibody for MAP2 (Cell Signalling #4542) at 1:2000

dilution overnight at 4°C. Slides were then again washed with PBS 3 times for 5 minutes. Secondary antibodies were diluted 1:1000 in PBST. The secondary used was goat anti-rabbit IgG H&L (Alexa Fluor 488). The secondary antibody was added to the slides and left for 1 hour at room temperature. Slides were then washed twice with PBS for 5 minutes. A coverslip was added using VECTASHIELD HardSet antifade mounting medium, which contains DAPI (Vector Labs). Slides were then left laying flat in a drawer overnight then transferred to 4°C for long term storage.

Four WT and 4 *Tnik* ^{-/-} mice were stained and imaged from each group (4 sections per mouse). Z-stacked images from sections stained for MAP2 and DAPI were taken under a Zeiss 700 confocal laser-scanning microscope (40x magnification). Z-stacked images were collapsed into a single 2D image for analysis with 8 images (2 per mouse per group) taken and analyzed per group from both the CA1 region of the hippocampus and cortex. To quantify the observed differences between the 2 groups we used the Directionality plugin in FIJI (ImageJ) (140). Dispersion values obtained from analyzing these images were compared with a Student's *t*-test to compare the means obtained from each group.

Cresyl Violet Staining

Slides were removed from storage at -80 °C and allowed to thaw and dry for one hour prior to staining. Slides were moved from one solution to the next according to the following protocol:

Following Cresyl violet staining sections were cover slipped using Permount Mounting Medium (Thermo Fisher Scientific). Slides were imaged under a light

microscope at 20X magnification and images were taken with a camera of the dentate gyrus for analysis.

Solution	Time
100% Ethanol	20 minutes
100% Ethanol	5 minutes
100% Ethanol	3 minutes
dH ₂ O	3 minutes
Cresyl Violet	1 minute
95% Ethanol + 10% Ascetic Acid	Until sections are a medium blue color
100% Ethanol	2 minutes
100% Ethanol	2 minutes
Xylene	2 minutes
Xylene	2 minutes

Table 2. Cresyl Violet staining protocol

RNA Sequencing

Hippocampal Isolation

Mice were given an intraperitoneal injection of ketamine/xylazine (1 mg/kg) as anesthesia before their heads were removed below the neck with a pair of scissors and placed on a dissecting platform over ice. A scalpel was used to cut the scalp, which was then peeled back and skulls were cut down the midline with scissors and pulled back with a rongeur to expose the brain. Brains were then removed with a surgical spatula and placed on an ice plate prior to isolation of the hippocampus. The hippocampus was identified, following peeling back the cortex, and scooped out and quickly moved to 500 μ L cold QIAzol and minced with scissors (Qiagen) before being sonicated three times at maximum intensity for 30 seconds. Samples were stored at -80 °C prior to RNA isolation.

RNA Isolation

RNA was first extracted from QIAzol (Qiagen) by adding an equal volume of chloroform, centrifuging at 4 °C for 15 minutes and removal of the upper aqueous phase for input into the Qiagen RNeasy Mini Kit for in silica based purification of total RNA ideal for sequencing (127). Total RNA was isolated, with an enrichment for mRNA (<200 bp RNA excluded), from the product of the chloroform extraction according to the manufacturers instructions, including the optional DNase digestion step to eliminate as much DNA as possible and promote purity of RNA samples prior to library preparation (127). RNA samples were quantified on the Qubit system (Thermo Fisher Scientific) and integrity of RNA samples was assessed using the Fragment Analyzer Automated CE System (Advanced Analytical).

Library Preparation and RNA Sequencing

Libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina) according to the manufacturers' instructions with an input of 500 ng of RNA (74). Libraries were assessed for integrity using the Fragment Analyzer Automated CE System (Advanced Analytical) prior to sequencing. Libraries were clustered at 2.8 pM and sequenced on the Illumina NextSeq500 (Illumina) using the 150-cycle high output kit in accordance with all manufacturers' instructions.

RNA Quantity and Integrity

Following RNA isolation, samples were loaded and analyzed using the Fragment Analyzer Automated CE System (Advanced Analytical), to identify any

degradation of samples prior to sequencing. Quantity and integrity of RNA is reported in the table below, with RQN (RNA Integrity Number) being acceptable at or above 7 for sequencing purposes.

Sample ID	Genotype	Total RNA conc. (ng/ μ L)	RQN(RIN)
154-1	WT	609.2	7.3
154-2	WT	577.7	7
189-1	WT	858.1	8.1
189-2	WT	908.4	6.7
152-1	HET	953.6	7.2
152-2	HET	829.3	6.9
194-1	HET	974.8	7.1
194-2	HET	803.8	8.1
150	HOM	995.6	7
157	HOM	704.0	8.4
202	HOM	760.8	9
250	HOM	896.7	8.9

Table 3. RNA quantity and integrity.

Library Integrity

To ensure library concentrations were within parameters for sequencing they were analyzed on the Fragment Analyzer Automated CE System (Advanced Analytical). Peak library size is reported. None of the libraries showed a peak at 129 bp, which would indicate the presence of self-bound primer that would interfere with sequencing.

Sample ID	Genotype	Library Peak Size (bp)
154-1	WT	308
154-2	WT	305
189-1	WT	287
189-2	WT	307
152-1	HET	286
152-2	HET	286
194-1	HET	295
194-2	HET	317
150	HOM	291
157	HOM	296
202	HOM	319
250	HOM	323

Table 4. Library fragment size information.

Data Analysis

Data were analyzed using the TopHat alignment and Cufflinks assembly &DE v1.0 App set of tools in BaseSpace from Illumina. First, per-sample reads (FASTQ) were filtered and trimmed using Bowtie (v. 0.12.9). Reads were then aligned to the reference genome using TopHat2 (v. 2.0.7) and variant calling was performed using the Isaac Variant Caller (v. 2.0.5). Finally quantification and differential expression was performed using Cufflinks (v. 2.1.1).

Chapter 3: Behavioral Phenotyping of *Tnfrsf25*^{-/-} Mice

Introduction

Post-traumatic stress disorder (PTSD) is a major health concern in both military and civilian populations. Identifying factors linked to risk or resilience of developing PTSD in response to trauma is one of the next steps to understanding and treating this disorder, and genetics are proposed to be one underlying factor(5). The most notable evidence of genetic susceptibility comes from twin studies of PTSD in both military and civilian populations showing high inheritance of risk (138, 160). Results from genome wide association studies (GWAS) have been non-reproducible in identifying genes linked to PTSD. One way to identify candidate genes is to utilize animal models of certain aspects of PTSD, where animals from a common genetic background can be selectively bred to identify genes related to behavioral phenotypes .

One of the hallmark symptoms of PTSD is intrusive thoughts or memories in response to situations reminiscent of the initial trauma (4). To model this aspect of PTSD in animals, researchers use classical fear conditioning. FS and FR mice were developed to study genetics of fear memory and a functional imaging study in these mice revealed baseline activity differences in the hippocampus (CA1 and CA2)(106).

After identification of the hippocampus as a region of interest we performed next generation, whole transcriptome sequencing of hippocampal tissue from FS and FR mice. This approach revealed a number of transcripts with differential expression between the FS and FR mice that have potential for further study (Table

1). Using functional annotation analysis, we identified a subset of genes, which are enriched in the CNS, affecting neuronal morphology, synaptic activity and memory performance. One of these genes, Traf2 and Nck interacting kinase (TNIK) was more highly expressed in the FS compared to the FR mice as identified by RNA sequencing and confirmed by qPCR.

With the identification of differential *Tnik* expression in mouse line divergently bred based on fear memory performance, we decided to investigate fear memory differences in *Tnik* ^{-/-} mice. We hypothesized that *Tnik* ^{-/-} mice would display a deficit in fear learning on fear conditioning tests. We also sought to investigate other behaviors in *Tnik* ^{-/-} mice including spatial memory to investigate any generalized memory deficits, anxiety to determine if *Tnik* loss of function causes anxiety-like behavior differences and baseline locomotor activity and nociception as possible confounds to the other behavioral assays. The goal of this study was to determine if *Tnik* contributes to behavioral phenotypic differences between FS and FR mice and may be a candidate gene for PTSD.

Results

We performed a battery of behavioral tests to determine how *Tnik* loss of function affects behaviors related to the mouse line where *Tnik* was identified (FS vs. FR mice) and the disorder those mice were intended to study, PTSD (33, 106). We performed OF as a measure of baseline activity, cued and contextual fear conditioning to determine if *Tnik* ^{-/-} leads to a fear memory deficit, Y-maze to assess any generalized spatial memory deficit in *Tnik* ^{-/-} mice, the EZM to measure anxiety-like behavior and the hot plate to measure nociception.

Open Field Testing Reveals Hyperactivity in *Tnik* $-/-$ Mice

Open field testing was performed as a measure of baseline locomotor activity for comparison between genotypes, as activity levels can be a confounding variable for other behavioral experiments. *Tnik* $-/-$ mice displayed hyperactive locomotor activity evidenced by a greater distance traveled and a greater average speed than the WT and *Tnik* $+/-$ mice (Figure 3A-B). Time course data showed *Tnik* $-/-$ mice traveled a greater distance during all segments but the first (Figure 3D). There was no significant difference in center time between the genotypes (Figure 3C). Center time is oftentimes used as a measure of anxiety-like behavior in mice, with less anxious animals spending more time in the center (18).

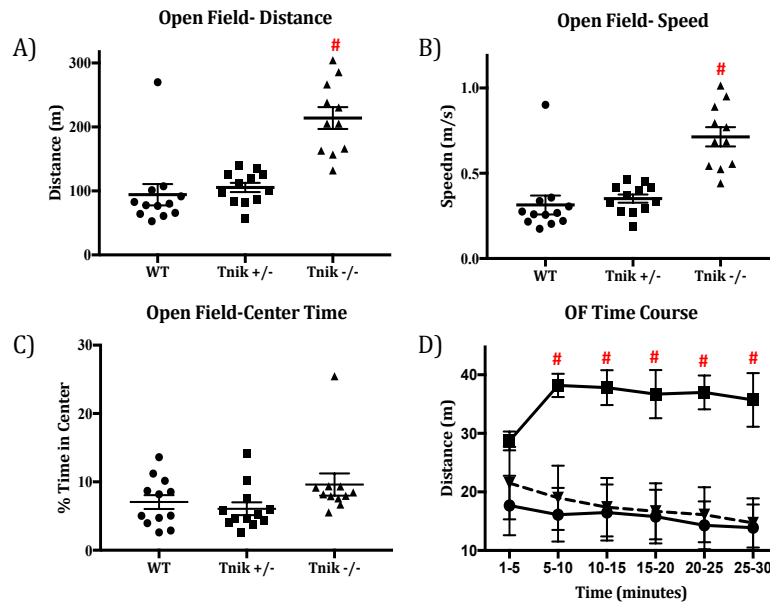


Figure 3- Open Field Reveals Hyperactivity in *Tnik* $-/-$ Mice.

Open field testing in *Tnik* $-/-$ mice traveled farther (A) [F(2,32)=21.03, $p<0.0001$], one-way ANOVA with Dunnett's multiple comparisons test WT vs. *Tnik* $+/-$, $p=0.7975$, WT vs *Tnik* $-/-$, $p<0.0001$] and faster (B) [F(2,32)=21.04, $p<0.0001$, one-way ANOVA with Dunnett's multiple comparisons test]. There were no differences in center time between groups (C) [F(2,32)=2.244, $p<0.0001$, one-way ANOVA with Dunnett's multiple comparisons test, WT vs. *Tnik* $+/-$, $p=0.7973$, WT vs *Tnik* $-/-$, $p<0.0001$]. Mice per group were as follows: WT=12, *Tnik* $+/-$ =12 and *Tnik* $-/-$ =11. All graphs display values $\pm$ SEM. (D) Time course data of OF distance traveled broken down into 5-minute blocks. *Tnik* $-/-$ mice spent significantly more time traveling during each 5-minute block relative to WT mice. (#= significant at $p<0.05$)

Fear Memory Performance

Tnik ^{-/-} Mice Show a Deficit in Contextual Fear Memory

One way ANCOVA revealed genotype had a significant effect on freezing percentage while open field activity did not explain the variation observed [$F(2,32)=4.525$, $p=0.019$](Figure 4A-C). Type III sum of squares revealed that *Tnik* ^{-/-} mice froze less than both WT and *Tnik* ^{+/-} mice (adjusted p for genotype=0.005)(Figure 4C). OF activity did not significantly impact the freezing percentage on contextual fear behavior (adjusted p for OF activity= 0.25)(Figure 4C).

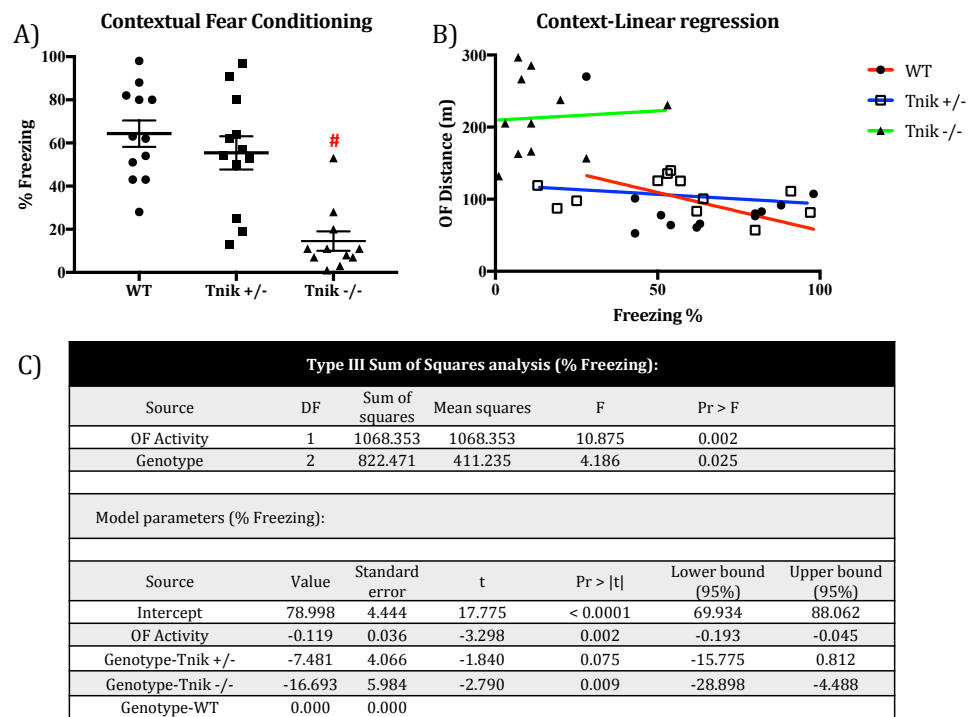


Figure 4- Contextual Fear Test Shows Fear Memory Deficit in *Tnik* ^{-/-} Mice.

Tnik ^{-/-} mice display less freezing on the contextual fear test (A, C)[$F(2,32)=4.525$, adjusted $p=0.019$, one way ANCOVA with type III sum of squares). There was no effect of OF distance on contextual fear freezing levels (B, C)[$F(2,32)=1.35$, adjusted $p=0.254$, one way ANCOVA with type III sum of squares] revealing baseline activity not to be a covariate. Mice per group were as follows: WT=12, *Tnik* ^{+/-}=12 and *Tnik* ^{-/-}=11. All graphs display values $\pm$ SEM. (#= significant at $p<0.05$)

Tnik -/- Mice Show a Deficit in Cued Fear Memory

One way ANCOVA on genotype with open field activity as a covariate indicated a significant effect of both on the freezing percentage [OF-F(2,32)=10.88, $p=0.002$, Genotype-F(2,32)=4.2, $p=0.025$](Figure 5A-C). Type III sum of squares revealed that *Tnik* -/- mice froze less than both WT and *Tnik* +/- mice (adjusted p for genotype=0.025)(Figure 5C). OF activity also significantly affected the results (adjusted p of OF activity= 0.002)(Figure 5C).

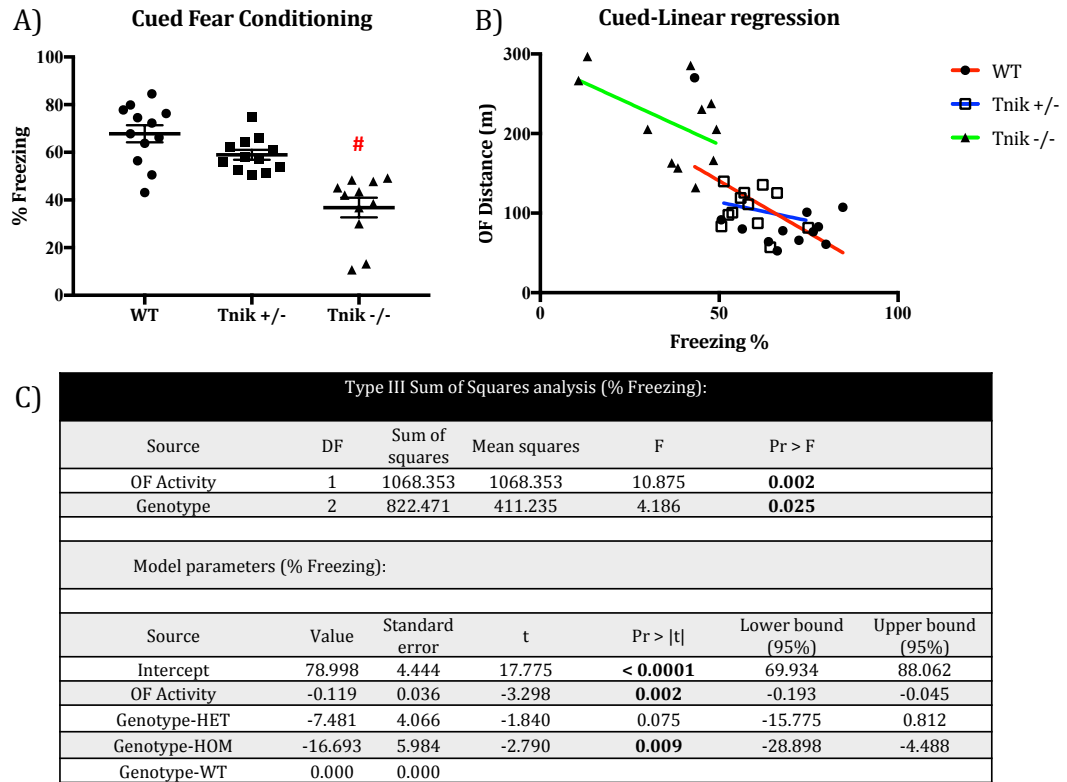


Figure 5- Cued Fear Test Shows Fear Memory Deficit in *Tnik* -/- Mice.

Tnik -/- mice display less freezing on cued fear testing (A, C)[F(2,32)=4.186, adjusted $p=0.025$, one way ANCOVA with type III sum of squares). There was also a significant effect of OF distance on cued fear test freezing levels (B, C)[F(2,32)=10.875, adjusted $p=0.002$, one way ANCOVA with type III sum of squares) revealing baseline activity to be a covariate which also partially explains the effect of genotype on cued fear test freezing. Mice per group were as follows: WT=12, *Tnik* +/- =12 and *Tnik* -/- =11. All graphs display values $\pm$ SEM. (#= significant at $p<0.05$)

***Tnik* ^{-/-} Mice Show a Spatial Memory Deficit in Y-Maze Testing**

The Y-maze, novel arm test, was performed as a measure of spatial memory in which recognition of the novel arm as a new environment is measured by time spent in and entries made into the novel arm. *Tnik* ^{-/-} mice spent a lower percentage of time and made a lower percentage of total entries in the novel arm than WT mice (Figure 6B and D). These measures indicate that *Tnik* ^{-/-} mice do not recognize the novel arm as a novel environment, which is evidence of a generalized spatial memory deficit. *Tnik* ^{-/-} mice traveled farther and made more total arm entries during the Y-maze test than *Tnik* ^{+/-} and WT mice, which are both additional measures indicating hyperactivity in *Tnik* ^{-/-} mice (Figure 6A and C).

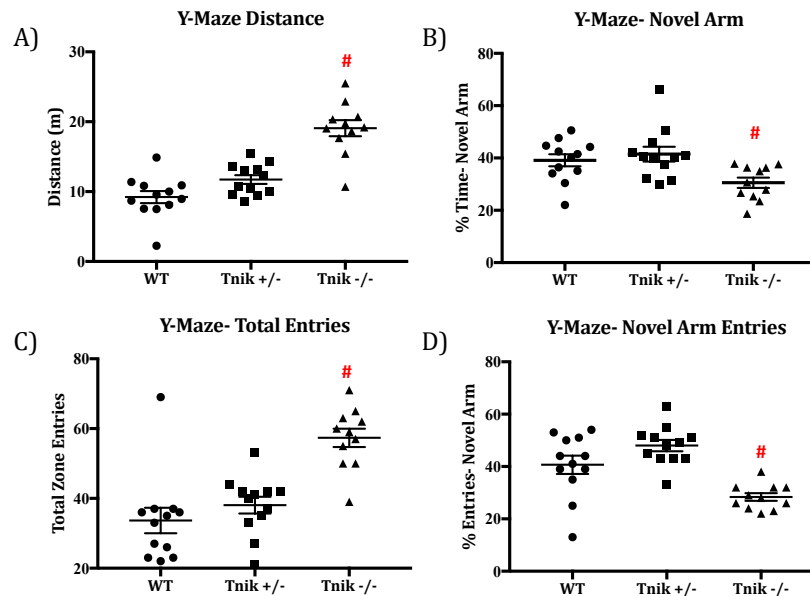


Figure 6- Y-Maze Reveals Spatial Memory Deficit in *Tnik* ^{-/-} Mice.

Y-maze testing revealed a spatial memory deficit evidenced by *Tnik* ^{-/-} mice spending less time (B)[F(2,32)=5.545, p=0.0086, one-way ANOVA with Dunnett's multiple comparisons test, WT vs. *Tnik* ^{+/-}, p=0.7626, WT vs *Tnik* ^{-/-}, p<0.0088] and making fewer entries into the novel arm (D)[F(2,32)=14.7, p<0.0001, one-way ANOVA with Dunnett's multiple comparisons test, WT vs. *Tnik* ^{+/-}, p=0.1156, WT vs *Tnik* ^{-/-}, p<0.0001]. *Tnik* ^{-/-} mice also traveled farther (A)[F(2,32)=32.15, p<0.0001, one-way ANOVA with Dunnett's multiple comparisons test, WT vs. *Tnik* ^{+/-}, p=0.1251, WT vs *Tnik* ^{-/-}, p<0.0001] and made more total entries to arms (C)[F(2,32)=17.58, p<0.0001, WT vs. *Tnik* ^{+/-}, p=0.5397, WT vs *Tnik* ^{-/-}, p<0.0001] which are evidence of hyperactivity. Mice per group were as follows: WT=12, *Tnik* ^{+/-} =12 and *Tnik* ^{-/-} =11. All graphs display values $\pm$ SEM. (#= significant at p<0.05)

EZM Reveals Decreased Anxiety-Like Behavior in *Tnik* $-/-$ Mice

Tnik $-/-$ mice traveled significantly farther than both WT and *Tnik* $+/-$ mice on the EZM, which is yet another measure of hyperactivity in *Tnik* $-/-$ mice (Figure 7A). Results revealed that *Tnik* $-/-$ mice spent a greater percentage of total time in the open arms of the maze than both the WT and *Tnik* $+/-$ mice, even when including total distance traveled as a covariate (Figure 7B). Open arm percentage was significantly affected by total distance traveled during the EZM though this did not diminish the effect of genotype on these results. Increased time in the open arms is indicative of lessened fear of the open area that is normally seen as less desirable to rodents. This shows a lower level of anxiety-like behavior in *Tnik* $-/-$ mice relative to both WT and *Tnik* $+/-$ mice.

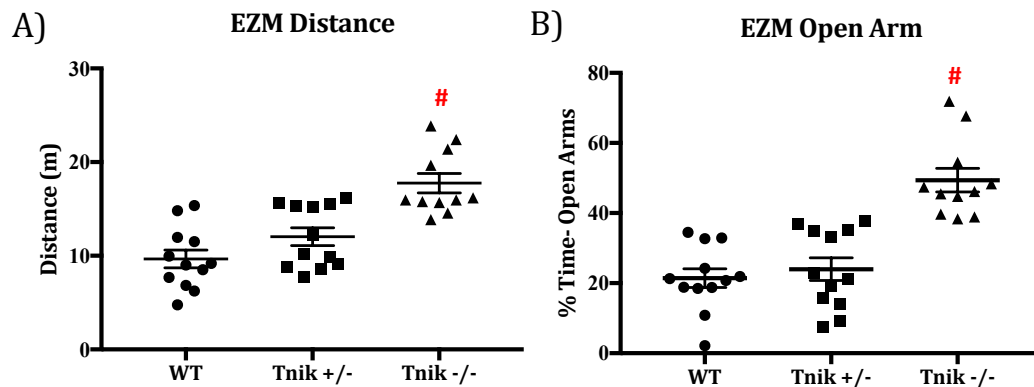


Figure 7- Elevated Zero Maze Shows Decreased Anxiety-Like Behavior in *Tnik* $-/-$ Mice.

Tnik $-/-$ mice display hyperactivity on the EZM evidenced by greater total distance traveled (A)[$F(2,32)=17.77$, $p<0.0001$, one-way ANCOVA with Dunnett's multiple comparisons test]. *Tnik* $-/-$ mice spent significantly more time in the open arms of the EZM (B)[$F(2,32)=5.787$, adjusted $p=0.007$, one way ANCOVA with type III sum of squares] which displays a reduction in anxiety-like behavior in *Tnik* $-/-$ mice relative to WT ($t=2.681$, $p=0.012$). Mice per group were as follows: WT=12, *Tnik* $+/-$ =12 and *Tnik* $-/-$ =11. All graphs display values $\pm$ SEM. (#= significant at $p<0.05$)

Hot Plate Test

Hot plate was performed as a measure of normal nociception in *Tnik* ^{-/-} and *Tnik* ^{+/-} as pain sensitivity differences can alter responsiveness to footshocks and fear memory testing results. There were no significant main effects on the hot plate test (Figure 8). This shows *Tnik* ^{-/-} mice have normal nociception, which is important as abnormal nociception can confound fear-conditioning results.

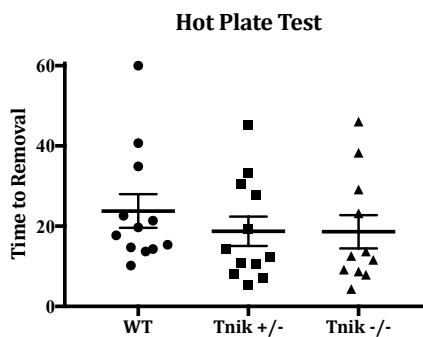


Figure 8- Hot Plate Reveals no Nociceptive Differences.

Mice did not display any significant differences on the hot plate test [$F(2,32)=0.55$, $p=0.58$]. Mice per group were as follows: WT=12, *Tnik* ^{+/-} =12 and *Tnik* ^{-/-} =11. All graphs display values $\pm$ SEM. (#= significant at $p<0.05$)

Behavioral Summary

Behavioral Test	<i>Tnik</i> ^{+/-}	<i>Tnik</i> ^{-/-}
OF Activity	↑	↑↑*
Y-Maze Novel Arm Entries	=	↓*
EZM Open Arm %	=	↑↑*
Cued FC Freezing %	↓	↓*#
Context FC Freezing %	↓	↓↓*
Hot Plate	=	=

Table 2. Summary of *Tnik* ^{+/-} and *Tnik* ^{-/-} Mouse Behavior. *Tnik* ^{-/-} mice display, relative to WT littermates, hyperactive locomotor behavior on the OF test of baseline activity, a spatial memory deficit on the Y-maze, fear memory deficit on contextual and cued fear memory tests and an anxiety-like behavioral decrease on the EZM. They display normal nociception on the hot plate. *Tnik* ^{+/-} mice do not display any significant differences from WT mice on any task. They do however display a slight intermediate phenotype according to baseline activity and fear memory performance.

Discussion

The present study sought to investigate the behavioral consequences of *Tnik* loss of function by studying *Tnik* $-/-$ mice. *Tnik* was identified as a gene of interest in the observed phenotypic differences between FS and FR mice, which were bred to study the intrusive thoughts and memories observed in PTSD patients. Since FS and FR mice display differences in fear memory on cued and contextual fear tests as well as anxiety like differences we studied both these behaviors in *Tnik* $-/-$ mice. We also examined spatial memory, baseline activity and nociception in *Tnik* $-/-$ mice. These behavioral phenotypes were investigated to determine if *Tnik* is a candidate gene for PTSD. *Tnik* $+/-$ mice were also tested on all of the same behavioral tests but no significant differences were found between the WT mice and the *Tnik* $+/-$ mice.

Spatial and Cued, Contextual Fear Memory Impaired in *Tnik* $-/-$ Mice

Contextual fear memory involves multiple brain regions, including the hippocampus, so it was not surprising to see a deficit in contextual fear memory considering the deficit also seen on the Y-maze. It appears as though the *Tnik* $-/-$ mice do indeed display hippocampal dysfunction according to these 2 results combined. Interestingly, the cued fear test also revealed a significant deficit in learning for the *Tnik* $-/-$ mice, even after correcting for hyperactivity. This may be due to the observed anxiety-like behavior differences or *Tnik* expression in brain regions other than the hippocampus such as the amygdala.

Interpretation of the Y-maze data shows a generalized spatial memory deficit in the *Tnik* $-/-$ mice (Figure 6B and D). *Tnik* $-/-$ animals spent approximately one

third of their time and made the decision one third of the time to enter the novel arm showing their performance did not differ from random chance. The latter measure, percentage of entries into the novel arm is definitively independent of hyperactivity, also observed on this task, as it measures the choice each mouse makes when it enters the center area. A spatial memory deficit is occasionally observed in some patients suffering from PTSD (153, 158). When the Y-maze and fear memory results are taken together we see a cognitive deficit that is tied to the hippocampus. These data support the hypothesis that *Tnik* is important for normal hippocampal function and loss of *Tnik* signaling leads to a deficit in hippocampal dependent memory.

Anxiety also affects freezing and since *Tnik* *-/-* mice display decreased anxiety on the EZM this influence may be causing the difference between *Tnik* *-/-* and WT mice on cued fear testing. The spatial memory deficit is consistent with what was observed on the Y-maze and has implications for spatial memory deficits seen in PTSD, schizophrenia and AD (55, 68, 158). The difference in cued fear memory, if indeed a reliable aspect of the *Tnik* *-/-* mouse phenotype, would indicate that *Tnik* may in fact play a role in generalized fear responses in those suffering from PTSD and not only on spatial memory. This would be a very interesting finding and may make *Tnik* a multifaceted disease risk factor for PTSD.

Altered Anxiety-like Behavior in *Tnik* *-/-* Mice

The elevated zero maze is a test of anxiety-like behavior in which rodents usually show a preference for the closed arms of the maze while displaying reluctance to enter the exposed arms (18). *Tnik* *-/-* mice show decreased anxiety-

like behavior by spending significantly more time in the open arms of the maze than both WT and *Tnik* +/- mice (Figure 7B). Altered anxiety in *Tnik* -/- mice strengthens its association to PTSD, a disorder that is strongly associated with heightened levels of anxiety (4). Anxiety differences in *Tnik* -/- mice show that *Tnik* may be involved in more than fearful memory formation and possible cognitive deficits associated with *Tnik*, but also a mediator of one of the other major symptoms of PTSD. Differences in anxiety-like behavior were a potential finding in *Tnik* -/- mice since FS and FR mice show differences in anxiety-like behavior (106). Anxiety-like behavior differences can have an effect on both the cued and contextual fear responses described previously (125). A generalization of decreased fear responses to both context and cue would implicate *Tnik* in more than the spatial working memory deficit and increase the importance of its potential relationship to PTSD (4). *Tnik* -/- mice did not spend more time in the center of the OF apparatus during the test but we believe this is an artifact of the hyperactivity the mice showed on this test. *Tnik* -/- mice traveled in circles in the periphery of the maze and did not show the usual exploratory behavior making these results difficult to interpret.

Hyperactive Locomotor Behavior in *Tnik* -/- Mice

Tnik -/- mice also display hyper locomotor activity, with the largest effect size of all behavioral differences between *Tnik* -/- and WT mice. This activity difference was observed across all tasks where distance traveled was measured (OF, EZM and Y-maze). This is a potential confounding variable for fear conditioning, considering freezing is the opposite of continuous movement, but it is also interesting for known *Tnik* function. In relation to PTSD, patients display

hypervigilance, which includes an increase in behaviors used to scan the environment (4). Hypervigilance may be similar to hyperactivity in rodents though this association has not been established.

***Tnik* ^{-/-} Mice as an Animal Model of PTSD Symptoms**

We initially identified *Tnik* in the hippocampus of FS mice, which were selectively bred according to their fear memory performance and is a proposed experimental model of the intrusive memories and heightened anxiety seen in PTSD (106, 125). To relate our findings back to FS mice and to PTSD, we focused on major aspects of PTSD like behavior observed in *Tnik* ^{-/-} mice including: decreased anxiety-like behavior in *Tnik* ^{-/-} mice and spatial memory deficit with related hippocampal structural disorganization and hyperactivity in *Tnik* ^{-/-} mice (17, 125). In the following sections, these behavioral phenotypes, their potential neural underpinnings and related transcripts were compared back to FS mice, as well as the symptoms and cognitive deficits observed in PTSD.

Phenotype	<i>Tnik</i> ^{-/-} Mice	FS Mice	PTSD
<i>Tnik</i> Expression	↓	↑	↑?
Fear Memory	↓	↑	↑
Anxiety	↓	↑	↑
Spatial Memory	↓	Normal	↓

Table 3- Phenotypes in *Tnik* ^{-/-} Mice, FS Mice and PTSD.

Summary table of phenotypes across *Tnik* ^{-/-} mice, FS mice and PTSD displaying consistencies and discrepancies across all 3 based on known and anticipated TNIK expression across all 3. Shows that *Tnik* ^{-/-} mice do indeed model some behaviors of both FS mice and PTSD (fear memory and anxiety) while differences in spatial memory do not occur as expected (?=hypothesized).

Chapter 4: The Role of *Tnik* in the Schaffer Collateral Pathway and Cell Survival

Introduction

Tnik and Hippocampal Neuronal Morphology

Previous work, including our own confirms that *Tnik* $-/-$ mice display deficits in behavior related to the normal function of the hippocampus (3, 28, 161). In fact, neurons in the dentate gyrus and the pyramidal cell layer of the hippocampus express *Tnik* in the post-synaptic density in wild type mice (16). However, the exact role *Tnik* is playing in these neurons that are crucial to the creation and recall of memories are not fully understood.

Tnik function has mostly been studied using knockdown methods *in vitro* (28, 73). In primary hippocampal cells, using *Tnik* siRNA to reduce *Tnik* expression, neuronal process growth was impaired, evidenced by both overall process length and decreased arborization (73). The mechanisms by which *Tnik* alters neuronal process extension are poorly understood, as they have not been directly studied, but *Tnik* is involved in a major neurodevelopmental pathway, which may explain some of the observed differences (103).

Tnik has been identified as an important mediator of the non-canonical Wnt signaling pathway in a number of previous studies in cancers of the colon/rectum where aberrant Wnt signaling is a major driving force of cancer growth (103, 123, 149). More recently, as interest in *Tnik* as a psychiatric disease risk factor began, *Tnik* is a key player in non-canonical Wnt signaling in the brain (28, 63). *Tnik* is

involved in Wnt signaling as a regulator of the microtubule associated kinase GSK3 β (28). *Tnik* directly phosphorylates and inactivate GSK3 β and loss of *Tnik* function in *Tnik* $-/-$ mice leads to an increase in GSK3 β activity (28). Interestingly, the hyperactivity observed in *Tnik* $-/-$ mice is reversed by pretreatment with a GSK3 β inhibitor showing that the effect *Tnik* has on behavior may also be mediated by Wnt signaling (28).

Tnik is specifically expressed in neurons in the granule cell layer of the dentate gyrus and the pyramidal cell layer in regions, CA1 through CA3 (16). The neurons of the granule cell layer mostly synapse onto neurons in the pyramidal cell layer of CA3, which in turn synapse onto neurons in the pyramidal cell layer of CA1. The connections between CA3 and CA1 are called the Schaffer collateral pathway which is one of the most widely studied neural circuits in the brain (10, 31, 157, 181). Disruption of function in this pathway affects the consolidation and retrieval of explicit memories (10, 157, 181). Considering the expression pattern and functions of *Tnik* along with the memory deficits we observed the effect *Tnik* loss of function has on this circuit is a topic that needs to be investigated.

The Role of *Tnik* in Neurogenesis and Cell Survival

Previous work in *Tnik* $-/-$ mice discovered a decrease in adult hippocampal neurogenesis in *Tnik* $-/-$ mice and a memory deficit corresponding to this neurogenesis decrease (28). Wnt signaling has been tied to hippocampal neurogenesis so this is likely to be the mechanism by which *Tnik* loss of function alters neurogenesis in the hippocampus (93, 165). Further to this point, GSK3 β transgenic mice also show a smaller dentate gyrus although this is not confirmed to

be due to a decrease in neurogenesis (35, 122). The similarities between the dentate gyrus morphology of *Tnik* $-/-$ mice and GSK3 β transgenic mice further confirms that it is likely Wnt signaling is affected leading to hippocampal changes in these mice and that *Tnik* is an important mediator in non-canonical Wnt signaling.

In addition to being linked to the transition of neural progenitors into mature neurons, *Tnik* also appears to be linked to cellular proliferation. Specifically, aberrant *Tnik* signaling has been linked to the progression of colorectal cancer and novel *Tnik* kinase inhibitors are being developed to specifically target these cancers (15, 70, 105, 149). Knockdown of *Tnik* in cultured colorectal cancer cells inhibits growth and this effect is specifically tied to the kinase activity of *Tnik* (149).

The role *Tnik* plays in cell proliferation in neurons has not been explored however *Tnik* has been associated with GSK3 β which has known effects on neural proliferation and apoptosis (58, 148, 172). Treatment with a GSK3 β inhibitor decreased neural cell death in CA1 in the hippocampus in a mouse model of AD (148). Loss of *Tnik* function could therefore, theoretically lead to neuronal cell death in the brain through the loss of GSK3 β inhibition.

Summary and Hypothesis

With the multiple roles *Tnik* may be playing in the hippocampus we sought to investigate what changes occur due to *Tnik* loss of function. *Tnik* is involved in neuronal process extension, adult neurogenesis and indirectly to cell survival via GSK3 β . We therefore hypothesize that loss of *Tnik* function will lead to altered neuronal process extension in the Schaffer collaterals and have effects on cell survival or neurogenesis leading to atrophy in the dentate gyrus.

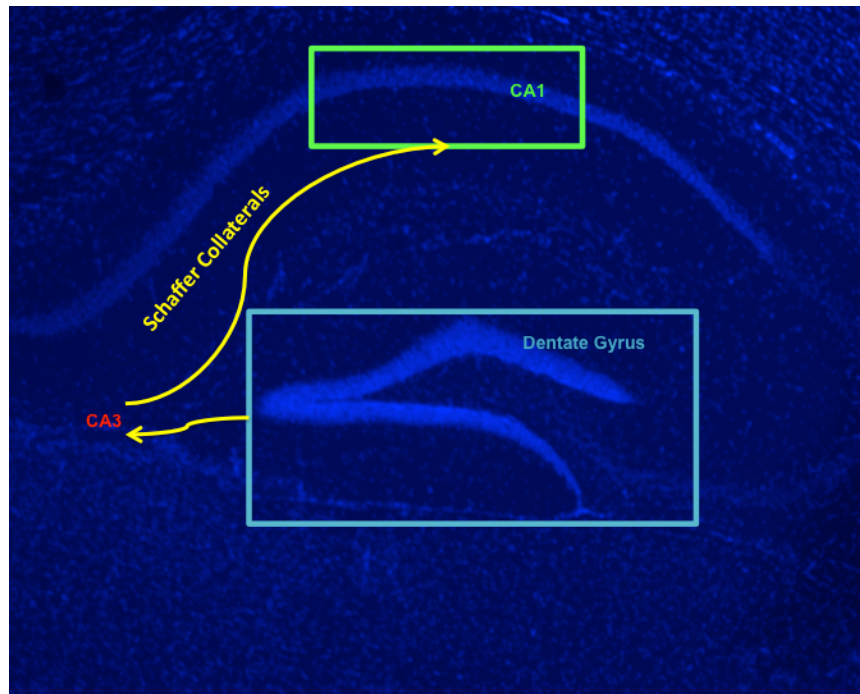


Figure 9- Schaffer Collateral Pathway.

Simplified summary of synaptic connections and flow of activity in the hippocampus. The blue rectangle outlines the dentate gyrus, which we examined with Cresyl Violet staining. The green rectangle outlines CA1, which we examined with MAP2 staining and fluorescent microscopy to study synaptic connections.

Results

Disruption of the Schaffer Collateral Pathway in *Tnik* $-/-$ Mice

To assess the condition of the Schaffer collateral pathway, which is critical to memory formation and retrieval, we focused on the projections entering the CA1 region of the hippocampus in WT and *Tnik* $-/-$ mice. We did not include *Tnik* $+/-$ mice in this analysis, as they did not display significant differences from WT in either the behavioral or RNAseq experiments. Looking at these projections we noticed disorganization in the projections in the *Tnik* $-/-$ animals relative to WT. WT mice showed unidirectional projections, which appeared to successfully synapse in CA1 (Figure 10A). *Tnik* $-/-$ mice showed shorter projections that appeared to terminate early, and oftentimes were perpendicular to the anticipated direction from observations in WT mice (Figure 10B).

To quantify this difference we performed analysis using the Directionality plugin in FIJI (ImageJ). Using this tool we determined the preferred orientation of the neuronal processes in each image. FIJI generated histograms with all orientations calculated on a 360° axis, superimposed on the image by the program. From those histograms, the plugin also calculated the standard deviation of the Gaussian curve formed, which is called the “Dispersion” for each image. Higher dispersion values mean there are fewer structures with the same preferred orientation. Dispersion values were averaged across condition and a two-tailed t-test was performed on the averages (WT vs. *Tnik* $-/-$). The t-test revealed a significant difference between WT and *Tnik* $-/-$ mice [$t(13)=3.7$, $p=0.0026$],

demonstrating that projections entering CA1 in the *Tnik* ^{-/-} mice were less organized than those in the WT mice (Figure 10). This lack of organization could lead to synaptic dysfunction and may be the underlying cause of the hippocampal dependent memory differences we observed during our behavioral experiments (119, 161).

The same analysis was performed on images from the cortex of the WT and *Tnik* ^{-/-} mice as a comparison structure to the hippocampus. No significant differences were found between groups according to a two-tailed t-test [$t(13)=0.2$, $p=0.8585$](Figure 10). This leads us to theorize that the effects of *Tnik* loss of function are not generalized to the entire brain; however, other structures not looked at in this study may be affected.

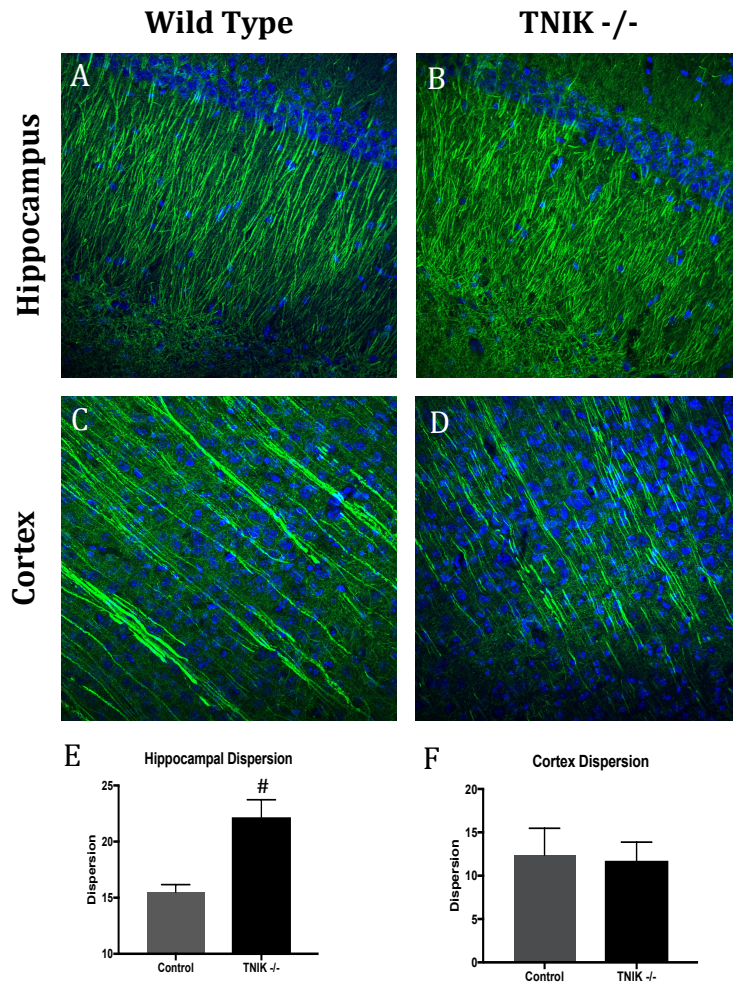


Figure 10- MAP2 Staining of Hippocampus and Cortex.

Confocal microscopic images of MAP2 stained brains from *Tnik* ^{-/-} and WT mice from the cortex and hippocampus (A-D). Unpaired two-tailed t-test revealed a significant difference in dispersion values between *Tnik* ^{-/-} and WT mice in the hippocampus [$t(13)=3.7$, $p=0.0026$]. There was no significant difference between dispersion values in the cortex [$t(13)=0.2$, $p=0.8585$]. Two images per brain region per animal were analyzed with 4 mice in each group.

Decrease in Dentate Gyrus Size in *Tnik* ^{-/-} Mice

It has been previously reported that the dentate gyrus shows atrophy in *Tnik* ^{-/-} mice (28). We also observed a smaller dentate gyrus when studying the hippocampus of *Tnik* ^{-/-} mice relative to their WT littermates (Figure 11). A decrease in the size of the DG can lead to impairment on hippocampal dependent memory tasks and may partly explain the observed hippocampal memory deficit (28).

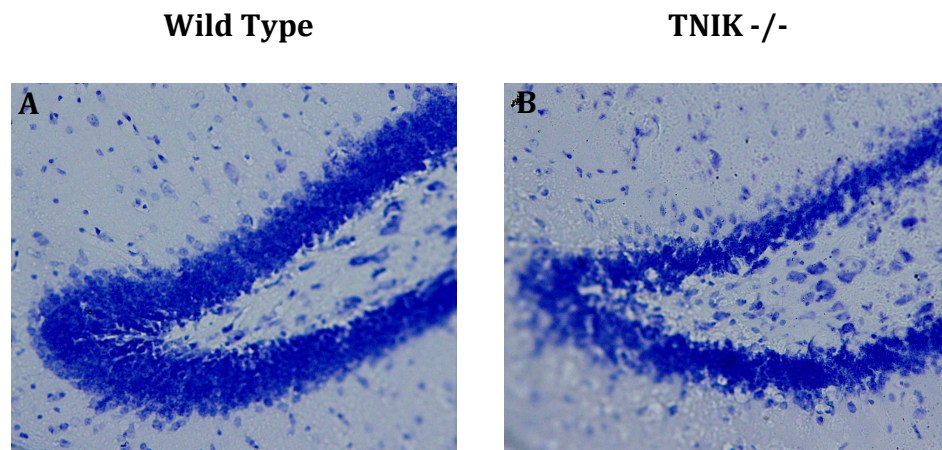


Figure 11- Dentate Gyrus of *Tnik* ^{-/-} and WT Mice.

Cresyl violet stained sections of the dentate gyrus of *Tnik* ^{-/-} (B) and WT (A) mice. Sections show the decreased volume of the dentate gyrus in *Tnik* ^{-/-} mice relative to their WT littermates.

Loss of Cell Viability in *Tnik* CRISPR/Cas9 Transfected Cells

In an attempt to create cell lines with a *Tnik* deletion for experiments investigating the effect of *Tnik* loss of function we discovered cells were not viable following transfection with CRISPR/Cas9 plasmid with *Tnik* gRNAs spliced into it. This effect was true for both P19 mouse embryonic carcinoma cells and Neuro2a mouse neuroblastoma cell lines. Cells transfected with the CRISPR/Cas9 *Tnik* gRNA plasmid had significantly lower cell counts than control cells 48 hours after transfection (Figure 12). Interestingly, pretreating cells with the GSK3 β inhibitor

CHIR99021 returned the cell counts to similar levels as control cells (Figure 12).

This reversal implies that the effect on cell viability is reliant on the Wnt signaling pathway in which *Tnik* and GSK3 β are both known mediators, with TNIK normally phosphorylating and inactivating GSK3 β (28). Previous studies utilizing Neuro2a cells and other immortalized cell lines have confirmed cell loss following disruptions of Wnt signaling (37, 75, 149). This is not surprising considering that TNIK has been investigated as a drug target for cancer treatment and Wnt signaling is found to be increased in numerous cancers (15, 70, 149).

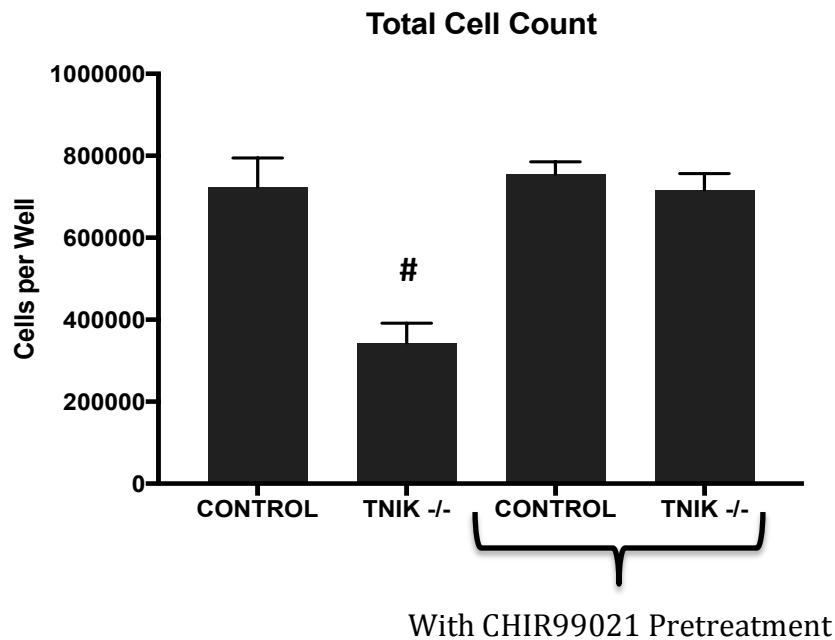


Figure 12- Cell Counts from *Tnik* -/- CRISPR/Cas9 Transfected Cells.

Cell counts from P19 cells transfected with a CRISPR/Cas9 plasmid with gRNAs targeted to *Tnik* with or without treatment with the GSK inhibitor CHIR99021. Cell counts decreased significantly following plasmid transfection but returned to normal when cells were pretreated with CHIR99021.

Discussion

Loss of *Tnik* Function Leads to Hippocampal Specific Alterations

Comparing brain slices from WT and *Tnik* $-/-$ mice stained for neuronal processes revealed an interesting phenotypic abnormality in the hippocampus of the *Tnik* $-/-$ mice (Figure 10). We were particularly interested in the Schaffer collateral pathway which is involved in the encoding and retrieval of spatial memory, which is impaired in *Tnik* $-/-$ mice (28)(Figure 6B and D, Figure 4A). Looking at the cortex in WT and *Tnik* $-/-$ mice did not show any significant differences based on genotype.

The Schaffer collateral pathway is one of the most heavily studied functional circuits in the brain. Many of the early studies in electrophysiology focused on this circuit, stimulating CA3 and recording action potentials in CA1 where most projections from CA3 terminate (31). A population of cells within the pyramidal cell layer of CA1 have been termed place cells by researchers (110, 117, 139). Particular environments specifically activate these cells, which shows how important these cells are to spatial memory. In fact, specific cells activated during fear conditioning can be optogenetically stimulated in a novel environment and elicit freezing behavior (128). These false memories only occur when cells in CA1 are stimulated, which again shows the importance of these pyramidal cells to spatial memory and in this case fear memory specifically (128).

Disruption of the connections between CA3 and CA1, the Schaffer collateral pathway, would lead to a spatial memory deficit like the one we observed in *Tnik* $-/-$ mice. The importance of this pathway in spatial memory function has been well established and the *Tnik* loss of function in our mice appears to disrupt this the organization of extensions in this pathway (Figure 10). While *Tnik* also has an active role in the adult CNS, evidenced by the efficacy of a GSK3 β inhibitor in correcting hyperactivity in *Tnik* $-/-$ mice, there is no evidence that the memory deficit is reversible (28). This leaves open the possibility that the effect of *Tnik* loss of function is during development, as the morphological changes we observed appear to be. This evidence appears to show that TNK may be a disease risk factor not only because of its role in the adult CNS but also due to a role during CNS development.

In addition to the changes to the Schaffer collateral pathway we also observed changes to the dentate gyrus, which had previously been observed in another *Tnik* transgenic mouse (28)(Figure 10). The dentate gyrus is one of the areas that receives new neurons throughout adulthood via adult hippocampal neurogenesis in the subgranular zone in the dentate gyrus (27). Adult neurogenesis in the dentate gyrus is required for spatial memory acquisition and has also been tied to *Tnik* function and Wnt signaling and interaction with GSK3 β (27, 28, 93). *Tnik* loss of function leads to a decrease in hippocampal neurogenesis and spatial memory deficits (28). Likewise, an increase in Wnt signaling and a decrease in GSK3 β activity leads to an increase hippocampal neurogenesis and rescues memory deficits in a mouse model designed to mimic the pathology of AD (37, 148). The role of GSK3 β in neuronal polarization and process elongation and branching is very

complex (145). In the hippocampus, inhibition of GSK3 β is required for the initiation of neuronal process elongation (111). However, GSK3 β expression is required for branching of neuronal processes, which is an important process in the normal development of the CNS and in establishing appropriate synaptic connections (172, 179). The complex role GSK3 β appears to play in neuronal development, specifically in the process of process elongation and branching explains why *Tnik* loss of function would have such a detrimental effect on the development of processes in the hippocampus. It appears as though strict regulation of GSK3 β activity to the appropriate time and place is required for the normal development of neuronal processes. Loss of *Tnik* function removes a kinase that regulates GSK3 β activity, and would therefore lead to a disruption of normal neuronal development wherever GSK3 β is crucial to neuronal development. This all further highlights the importance of the non-canonical Wnt signaling pathway and specifically *Tnik* and its downstream effectors in the structure and function of the hippocampus.

***Tnik* Loss of Function Leads to Loss of Cell Viability via GSK3 β**

The original goal of transfecting Neuro2a and P19 cell lines with a CRISPR/Cas9 plasmid with gRNA targeted to *Tnik* was to establish *Tnik* loss of function cell lines for studying neuronal differentiation. After creating the plasmid and transfecting the cells it became apparent that the cell lines could not continue to grow in the absence of *Tnik* signaling (Figure 12). Since GSK3 β is a known downstream effector of *Tnik* it was a possibility that GSK3 β may have been playing a role in the loss of viability that we observed. Treating with a GSK3 β inhibitor we discovered that the cell loss could be rescued back to control levels (Figure 12). *Tnik*

has previously been linked to cancer growth and kinase inhibitors are being developed as therapeutic agents (15, 70). Treatment with *Tnik* shRNA leads to cell death in other immortalized cell lines as well so *Tnik* function appears to be crucial to cell survival (63).

This finding has many implications for the role *Tnik* may be playing in the brain during disorders previously associated with Wnt signaling and *Tnik* (63, 148). TNIK is linked to a number of disorders in which there is cell death in the hippocampus leading to hippocampal atrophy including AD, schizophrenia and recently through our work to PTSD (81, 90, 148, 151, 167). In all of these disorders there is cell death in the hippocampus leading to deficits in spatial memory (37, 55, 153). In a mouse injected in the dorsal hippocampus with preformed A β fibrils which are one of the major pathological changes in AD, activating Wnt signaling rescues neurodegeneration and the behavioral deficits (spatial memory impairment) caused by the A β fibrils (37). Likewise, treatment with a GSK3 β inhibitor prevents cell death and reverses learning and memory deficits in another animal model of AD, mice overexpressing amyloid precursor protein and tau (148). These results show the importance of a pathway in which TNIK is confirmed to be a mediator in the pathology of major a psychiatric disorder. Linking TNIK to cell survival in the brain, specifically the hippocampus could also help explain how TNIK contributes to pathology observed in other TNIK linked psychiatric disorders.

Chapter 5: Comparison of *Tnik* ^{-/-} and WT Hippocampal Transcript Expression

Introduction

Important Roles of *Tnik* in Neurological Structure and Function

Tnik and Hyperactive Locomotor Behavior

Tnik and the pathway in which *Tnik* is a mediator, Wnt signaling, have been implicated in locomotor activity by utilizing transgenic mouse lines (28, 35). Knockout of *Tnik* leads to an increase in baseline activity in 2 different transgenic mouse lines (28). These 2 mouse lines display hyperactivity across a number of different behavioral tasks making it the most consistent and robust aspect of the *Tnik* ^{-/-} behavioral phenotype (28)(Figure 3A-B, Figure 6A, Figure 7A). Interestingly this hyperactive behavior was reversed by an inhibitor of a previously established downstream effector of *Tnik*, GSK3 β (28). A transgenic mouse, overexpressing GSK3 β in neurons in the hippocampus and cortex, also displays hyperactivity, further establishing GSK3 β as a downstream effector of *Tnik* (35, 68, 122). While both *Tnik* and GSK3 β are known mediators in Wnt signaling it is unknown what other genes, Wnt regulated or not, are involved in the hyperactive motor behavior observed in *Tnik* ^{-/-} mice (103, 149).

Tnik in Hippocampal Structure, Function and Spatial Memory

Hyperactivity is not the only behavioral phenotype of *Tnik* ^{-/-} mice. *Tnik* ^{-/-} mice also display hippocampal dysfunction evidenced by a hippocampal dependent

memory deficit (28)(Figure 6B and D, Figure 4A). There are 2 different ways *Tnik* loss of function appears to alter the hippocampus that may lead to the observed deficits in memory. *Tnik* ^{-/-} mice display decreased hippocampal neurogenesis, leading to dentate gyrus atrophy in the granule cell layer which leads to memory deficits (27, 28, 91).

In addition to the atrophy in the dentate gyrus, caused by a decrease in adult hippocampal neurogenesis, we also observed changes to the Schaffer collateral pathway (161, 174)(Figure 10 A-B). In *Tnik* ^{-/-} mice the projections from CA3 to CA1, which are crucial to spatial memory acquisition and retrieval, are disrupted and lack organization (Figure 10A-B and E). This same disorganization is seen in mouse models of neurodegenerative disease where Wnt signaling is negatively impacted causing neurodegeneration (36, 134). *Tnik* is also implicated in neurodegenerative diseases and the hippocampal disorganization seen in *Tnik* ^{-/-} mice may be part of how *Tnik* contributes to the pathology of those diseases. While *Tnik* is involved in the structure and function of the hippocampus the mechanism by which *Tnik* mediates these effects are unknown. Investigating the changes in transcript expression caused by *Tnik* loss of function and how they may lead to these structural changes will help explain how *Tnik* is a risk factor for disease.

Tnik in Cell Proliferation and Survival

Tnik has long been implicated as a risk factor for colorectal cancer in which Wnt signaling has been observed to be overactive (123, 149). Correspondingly, *Tnik* catalytic inhibitors have been developed as therapeutic agents to target colorectal cancer and inhibit cell proliferation during *in vitro* studies (15, 63, 70, 123). These

Tnik inhibitors decrease cell proliferation and tumor growth through activation of the Wnt signaling pathway and interaction with Wnt related transcription factors (149).

In *Tnik* ^{-/-} mice it has previously been observed that there is decreased adult hippocampal neurogenesis in the granule cell layer of the dentate gyrus of the hippocampus (28). Our previous results confirm that an immortalized cell line, which shares pluripotent traits with neural precursors, show decrease cell numbers following ablation of *Tnik* via the CRISPR/Cas9 gene editing system (Figure 12). It is not known whether *Tnik* is important for maintenance of the pool of precursor cells or whether *Tnik* is involved in differentiation from those precursors (73, 149, 170). Considering these results, and the previously established role *Tnik* plays in cell proliferation, it is one hypothesis that *Tnik* loss of function may be affecting pathways related to cell proliferation and survival.

Summary

Tnik is a mediator in Wnt signaling which is a major pathway that alters gene expression in all cell types including neurons (36, 75, 93, 103, 165). Both via Wnt signaling and via direct phosphorylation, *Tnik* is implicated in a number of functions including cell proliferation, cell survival, neurogenesis, learning and memory and neuronal process extension that are relevant to our other findings. Which of these pathways, and what genes within those pathways, are contributing to the phenotypes we observed in the *Tnik* ^{-/-} mice are unknown. It is for this reason that we performed whole exome sequencing from hippocampal tissue of WT and *Tnik* ^{-/-} mice for comparison. We focused on the hippocampus due to the behavioral and

morphological results we previously obtained to further understand the possible molecular mechanisms underlying the differences we observed between *Tnik* $-/-$ and WT animals.

Results

Transcript Identification

Non-biased hierarchical clustering was performed to determine what samples show similar expression patterns across all of the transcripts that were accurately detected during sequencing (Figure 13). This was done as a measure of validity, confirming expression changes were consistent within genotype, and different between genotypes before interpreting results from differential expression analysis. Data were modeled several ways to determine optimal cutoffs of both p-value and fold change for trimming down transcripts to those of interest. A scale of adjusted p-values (Q-values) was made to determine an optimal significance threshold of 0.05 (Figure 15). A volcano plot, taking into account both adjusted p-value and log2 fold change, was used to identify transcripts with a significant difference between groups and a high fold change that are of particular interest and have a low false discovery rate (Figure 15). Many of the data points in green on the volcano plot are presented in the Heatmap below, broken down by individual mouse (Figure 16). Many of those transcripts, also with relevant known functions, are presented in the literature review below.

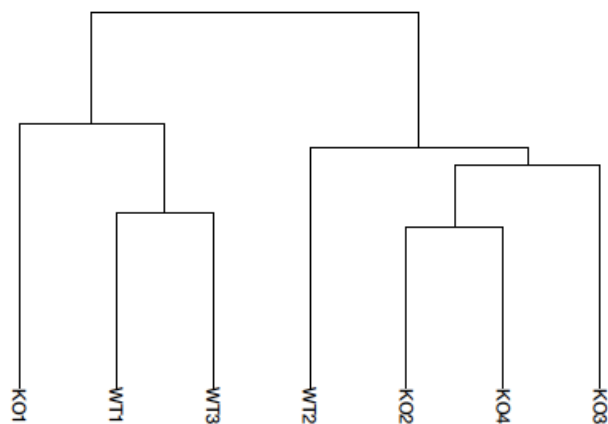


Figure 13- Hierarchical Clustering.

Unbiased hierarchical clustering of samples based on all 23,514 accurately detected transcripts. Clustering shows consistent grouping of samples, with 3 of 4 *Tnik* $-/-$ mice closely clustered and 2 of 3 WT mice likewise clustered together. Hierarchical clustering performed in GenePattern (132).

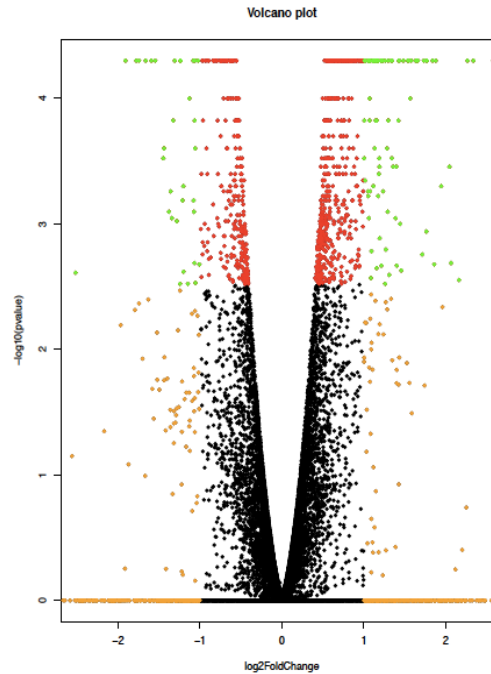


Figure 14-Volcano Plot of Transcripts.

Volcano plot of all transcripts in the comparison of WT and TNIK $-/-$ mice. Log2(fold change) on the x-axis reveals transcripts of high fold change to the far left and right of the plot. Adjusted p-values (Q-values) on the y-axis show significant Q-values in green dots, farthest from the x-axis.

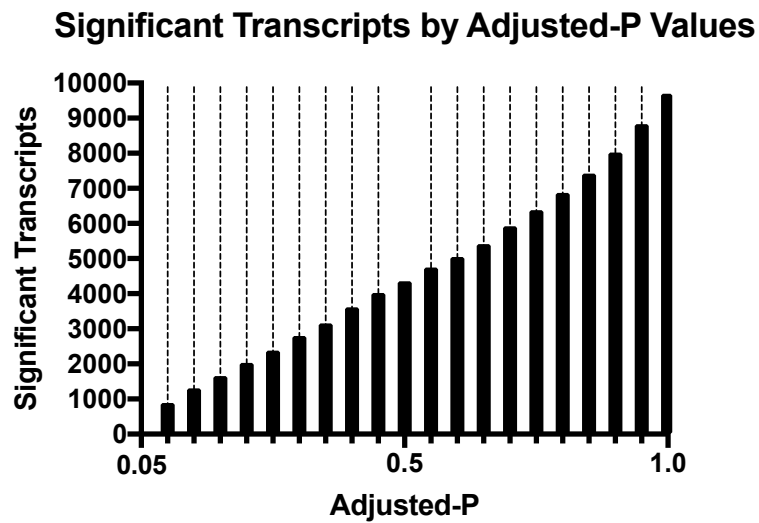


Figure 15-Fragments Significant by Adjusted P-Values.

Fragments significantly differentially expressed across varying adjusted p-values.

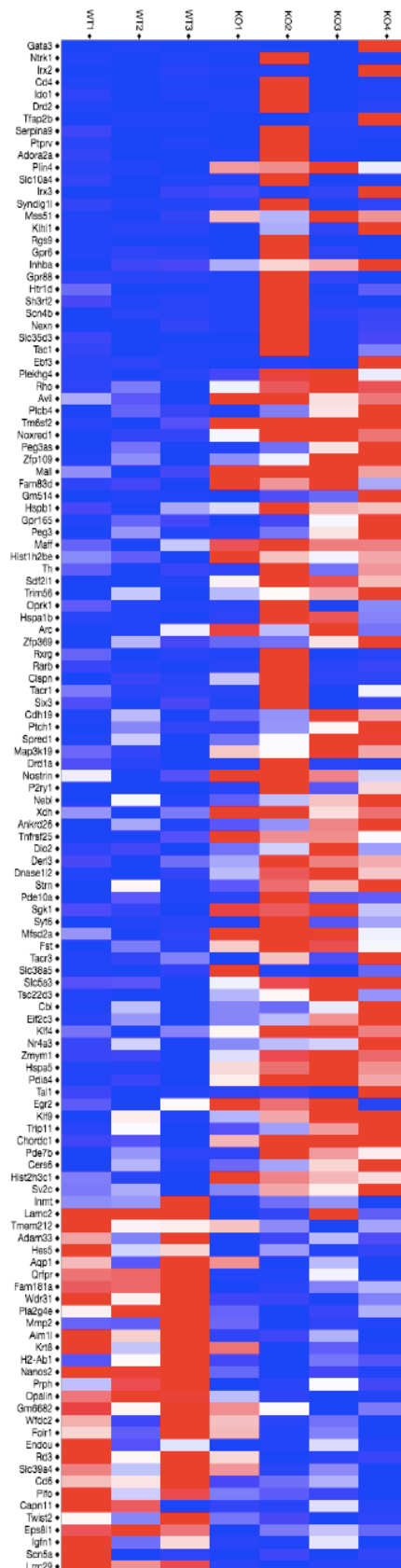


Figure 16-Heatmap of Top Transcripts.

Heatmap of the top 128 fold change genes with adjusted p-value less than 0.05 visually displaying each subject's breakdown of transcript expression by individual transcript. Red values indicate high expression and blue values indicate low expression. Heatmap created using Genepattern (132).

GO Biological Process Analysis

Introduction

From whole transcriptome profiling we identified 891 total transcripts that displayed Q-values (p values corrected for multiple comparisons) less than 0.05, which were deemed to be significantly different between genotypes. From these transcripts we narrowed our criteria, separating out only genes that showed a greater than 2-fold change between genotypes, which further reduced our transcript of interest list to 135 genes (102 higher expression in *Tnik* ^{-/-}, 33 higher expression in WT)(Figure 16).

GO biological pathway analysis utilizing PANTHER tools was performed on the 135 transcripts that differed significantly by genotype, searching for genes with particular physiological relevance (Table 5). There were a number of pathways with significance to our previous findings including: associative learning, locomotor behavior, negative regulation of cell survival, regulation of apoptosis and neurogenesis (Table 5). Performing a literature review of the genes that cross over multiple of these pathways revealed a number of genes of interest with possible ties to the results we obtained in behavioral phenotyping the *Tnik* ^{-/-} mice and analyzing the Schaffer collateral pathway in the hippocampus of *Tnik* ^{-/-} mice.

Hippocampal Structure, Function and Spatial Memory

Special focus was given to transcripts with known functions within the hippocampus since that was the source of the tissue and that is where we observed changes to neural structure. *Tnik* ^{-/-} mice also display altered behavior on tasks that

rely on normal hippocampal function (Figure 6B and D, Figure 4A, Figure 10 A-F, Figure 10).

There are a number of genes with known hippocampal function that showed differential transcript expression including: Adenosine A2a Receptor (*Adora2a*), Dopamine Receptor 1a (*Drd1a*), Dopamine receptor 2 (*Drd2*), Folate Receptor 1 (*Folr*), G-protein receptor 88 (*Gpr88*), Heat shock protein 5 (*Hspa5*), Indolamine 2,3-Dioxygenase 1 (*Ido1*), Matrix metalloprotease 2 (*Mmp2*) and Serum and glucocorticoid inducible kinase 1 (*Sgk1*).

Adora2a has previously been linked to both neurogenesis in the hippocampus and resulting alterations in hippocampal volume (72). *Tnik* *-/-* have impaired neurogenesis within the hippocampus as well (28). Blockade of *Adora2a* leads to an increase in hippocampal neurogenesis and improvements in working memory (92, 173, 182). The directionality is consistent, as *Tnik* *-/-* mice display a higher transcript expression of *Adora2a*, an impairment in spatial memory and decreased adult hippocampal neurogenesis (28).

Another gene we identified that affects the dentate gyrus is *Mmp2* which is an enzyme involved in reorganization of the extracellular matrix (94). *Mmp2* is activated in the dentate gyrus by activity and is involved in dendritic remodeling during synaptic plasticity (156). This area of remodeling is crucial to hippocampal function and memory formation and is in the same region where *Tnik* expression is particularly high (16, 128).

Both *Gpr88*, an orphan G-protein coupled receptor expressed in the CNS, and *Rarb*, a nuclear transcriptional regulator which affects *Gpr88* expression, emerged

from our analysis of differentially expressed transcripts. Rarb has been studied in the hippocampus and Rarb deficiency eliminates both LTP and LTD in the hippocampus, displaying that Rarb signaling is crucial to synaptic plasticity (25). In mice with disrupted Rarb signaling, induced via benzo(a)pyrene treatment, there is a marked deficit in spatial learning and memory (22). Interestingly, Gpr88 displays a different directional relationship in our data than has been previously established. Gpr88 increases along with Rarb in the *Tnik* *-/-* group which is the opposite effect than was seen in Rarb *-/-* mice when analyzing striatal tissue (112). In conjunction with Gpr88 displaying the opposite effect previously observed it also has an inverse effect on spatial working memory relative to Rarb (107). Mice with loss of function of Gpr88 present with improved spatial learning on numerous tests of spatial learning and memory (107). Relative to the known functions of *Tnik* only Gpr88 matches up directionally with our previous results. Gpr88 is at a higher transcript expression level in *Tnik* *-/-* mice and has been established to interfere with spatial memory which makes it a potential downstream effector of *Tnik* (107). The current work does not establish whether Rarb is driving Gpr88 expression and it is unclear why these genes display opposite effects on spatial memory while showing the same directionality in expression in *Tnik* *-/-* mice (22, 107, 112).

Sgk1 is another gene related to spatial learning and memory that shows a significant alteration in transcript expression. Increased activity of Sgk1 enhances contextual fear memory while inhibition of Sgk1 by multiple mechanisms impairs spatial memory (21, 88, 89, 163). *Tnik* phosphorylates and deactivates GSK3 β , which is more active in hippocampal neurons of *Tnik* *-/-* mice (28). Also interacting

with GSK3 β is the gene *Klhl1*, which interacts with GSK3 β to promote neuronal process extension and is more highly expressed in *Tnik* $-/-$ mice (77, 146). *Klhl1* is upregulated by increased GSK3 β activity so it is not surprising that it is upregulated in *Tnik* $-/-$ animals which have heightened GSK3 β activity (28, 146).

Dopamine Signaling Related Transcripts

Two different dopamine receptors, *Drd1a* and *Drd2* were discovered in our analyses. Both of these receptors are expressed in the hippocampus with known functions (53, 64, 119, 175). *Drd1a* has an effect on spatial memory as well as fear memory affects synaptic plasticity within the Schaffer collateral pathway from CA3 to CA1 (119). *Drd1* $-/-$ mice display both a spatial working memory deficit as well as decreased contextual fear conditioning as well as an impairment of synaptic plasticity in the Schaffer collateral pathway (119). *Drd2* also has several known actions within the hippocampus. Notably, loss of function of *Drd2* also leads to decreases in working memory performance on tests of spatial memory (175, 180). In addition, *Drd2* receptors mediate hyperactivity in a mouse model of ADHD and direct activation of *Drd2* also leads to hyperactivity (44, 48). *Drd2* also mediates a pathway involving GSK3 β , which leads to activation of apoptosis in hippocampal neurons (59). The functions of these 2 dopamine receptors and *Tnik* overlap significantly. *Tnik* is involved in spatial memory performance, hyperactivity and apoptosis, all of which are also mediated by these receptors showing functional overlap indicating these receptors may mediate some of the observed differences between *Tnik* $-/-$ mice and their WT littermates.

Strn or striatin, is another dopamine related transcript within our data set. Striatin is in fact regulated by signaling via dopamine acting at Drd1a which is also upregulated in *Tnik* $-/-$ mice (9, 19). Striatin is primarily expressed in dendrites, particularly dendritic spines in a similar manner to *Tnik* (16, 19). Targeting of striatin by missense mutation leads to decreased locomotor activity, which is consistent in directionality with what we observe in *Tnik* $-/-$ mice which display hyperactivity and an increase in striatin transcript expression (9).

Another gene involved in dopaminergic signaling that emerged from our analysis of the transcript list is Ido1. Ido1 is the rate limiting enzyme in the kynurenine pathway of tryptophan catabolism and is primarily expressed in astrocytes and microglia (60). Kynurenic acid, one of the products of the kynurenic acid pathway, increases the amount of extracellular dopamine (118). Kynurenic acid is also a direct antagonist of NMDA type glutamate receptors and inhibits LTP (118). Elevated levels of kynurenic acid impair hippocampal function and lead to spatial working memory and contextual fear memory deficits while having no effect on cued fear memory (23, 24). Increased transcript expression of Ido1 in *Tnik* $-/-$ mice presents yet another potential mechanism by which *Tnik* loss of function mediates a spatial memory deficit.

Cell Proliferation and Survival

There was a significant enrichment of genes involved in cell proliferation, survival and apoptosis in the list of significant differential transcripts. Many of the genes are linked to cancer and are crucial to proliferation and survival of cancer cells (Hspa5, Inhba, Ptprv, Gata3, H2-Ab1, Ido1, Inhba, Klf4, Ntrk1, Ptch1, Ptprv,

Rarb, Strn, Tfap2b, Twist2, Xdh). The relationship between these transcripts and cancer mirrors the main area of disease relevance previously established for *Tnik* which is in the growth and metastasis of colorectal cancer (149).

Many of the transcripts on our list are specifically tied to cell survival, proliferation, including adult hippocampal neurogenesis, and apoptosis within the hippocampus (53, 59, 72, 87, 96, 178). Adora2a is a mediator of neuroinflammation which leads to cell death within the hippocampus leading to decreased hippocampal volume (72).

Drd2 is involved in the Akt/GSK3 β signaling pathway, deactivating the pathway and leading to apoptosis in the hippocampus when activated (59). Also within the Akt/GSK3 β pathway is Twist2, which is low in expression in *Tnik* $-/-$ mice (104). Twist2 is a pro-survival gene, promoting activity of the Akt/GSK3 β pro-survival pathway (49, 95, 104). *Tnik* is known to phosphorylate and inactivate GSK3 β , making it interesting that several transcripts altered in *Tnik* $-/-$ mice also interact with GSK3 β and appear to deactivate a pro-survival pathway which can lead to apoptosis (28).

Klf4 is also linked to apoptosis and acts as a pro-apoptotic factor that is activated by glutamate stimulation of neurons via the p53 pathway (183). Klf4 is upregulated in *Tnik* $-/-$ mice, which may lead to higher rates of apoptosis in those mice. Like Klf4, Ptprv is upregulated in *Tnik* $-/-$ mice and is also involved in the p53 pathway (40). Interestingly, Ptprv is a direct target of p53 but does not induce apoptosis (39, 40). Instead Ptprv acts to promote cell cycle exit when activated by p53 which could lead to a decreased pool of neural progenitors in *Tnik* $-/-$ mice (40).

Like *Ptprv*, *Folr1* displays numerous actions on neuronal survival and proliferation within the hippocampus, including altering cell cycle progression (71, 87, 178). Decreased signaling via *Folr1*, by its ligand folate, leads to impaired adult hippocampal neurogenesis via cell cycle arrest at G0/G1 (178). Folate deficiency has leads to apoptosis in hippocampal neurons (178). *Folr1* is lower in expression in *Tnik* *-/-* mice which may in part lead to the decreased adult hippocampal neurogenesis and atrophied dentate gyrus in *Tnik* *-/-* mice that has been both previously observed and confirmed in the current study (28)(Figure 10). *Flr1* also leads to axonal regeneration following injury, as does *Gata3* which is conversely upregulated in *Tnik* *-/-* mice (76, 84).

Disease Risk Factors

There were many transcripts identified that were related to psychiatric disorders in the comparison between the *Tnik* *-/-* mice and their WT littermates. The most notable disease state implicated by the genes affected by *Tnik* loss of function are not surprisingly related to schizophrenia, as is *Tnik* via its association with DISC1, the first genetic risk factor identified for schizophrenia (170).

Th, *Drd1a*, *Drd2*, *Folr1*, *Ido1*, and *Strn* are important in dopamine signaling and include both the rate limiting enzyme in dopamine generation (*Th*) as well as the 2 primary receptors for dopamine (*Drd1a*, *Drd2*). The dopaminergic hypothesis of schizophrenia posits that dopamine signaling is altered in brain regions such as the PFC, striatum and hippocampus which leads to disease symptoms (143). It is obvious how *Th*, *Drd1a* and *Drd2* can alter dopaminergic signaling but the other transcripts related to dopamine signaling are more complex in the way they

influence dopamine synaptic transmission. Ido1 expression has been found to be increased in the frontal cortex of patients with schizophrenia (46). Kynurenic acid acts as a direct agonist of NMDA receptors which may be how it leads to an increase in extracellular dopamine in the frontal cortex of schizophrenics (23, 24, 46, 118).

In addition to schizophrenia there are a number of genes related to Alzheimer's disease, which are also present in our dataset. This is not a surprising finding since *Tnik* is important for learning and memory and regulates GSK3 β which is one of the kinases confirmed to hyper-phosphorylate Tau and lead to its aggregation in the brain of AD patients (35, 68, 148). *Adora2a* has been confirmed as a risk factor for both Alzheimer's and mild cognitive impairment and its expression is negatively correlated with hippocampal volume in those disease states (72). *Hspa5a* is an anti-apoptotic protein that is activated during disease progression of Alzheimer's disease as well as Huntington's disease (169). Seeing this transcript increases in *Tnik* $-/-$ mice may indicate that there is a similar pathological process occurring in the *Tnik* $-/-$ mice as is seen in neurodegenerative disease. There is another gene related to Alzheimer's that is also expressed significantly differently between *Tnik* $-/-$ and WT. *Mmp2* is a protease that is released from neurons into the extracellular space where it cleaves A β (7). Serum levels of *Mmp2* are lower in people with Alzheimer's relative to control subjects and may be partially responsible for disease progression (7, 67, 94, 126).

Associative Learning	Fold Change	Q-Value	Negative Regulation of Cell Survival	Fold Change	Q-Value
<i>Th*</i>	1.27	0.035	<i>Inhba*</i>	1.76	0.028
<i>Sgk1*</i>	1.11	0.003	<i>Ntrk1*</i>	4.02	0.005
<i>Tac1</i>	1.55	0.003	<i>Gata3*</i>	4.44	0.047
<i>Drd1a*</i>	1.16	0.003	<i>Klf9</i>	1.02	0.003
<i>Drd2*</i>	2.74	0.003	<i>Ptprv</i>	2.34	0.003
<i>Tacr1</i>	1.19	0.013	<i>Adora2a*</i>	2.26	0.003
<i>Gpr88*</i>	1.73	0.003	<i>Twist2*</i>	-1.59	0.003
<i>Oprk1</i>	1.23	0.018	<i>Strn*</i>	1.12	0.003
Locomotor Behavior			<i>Drd2*</i>	2.74	0.003
<i>Th*</i>	1.27	0.035	<i>Klf4*</i>	1.07	0.003
<i>Tal1*</i>	1.05	0.019	<i>H2-Ab1</i>	-1.2	0.017
<i>Adora2a*</i>	2.26	0.003	<i>Xdh*</i>	1.15	0.003
<i>Drd1a*</i>	1.16	0.003	<i>Ptch1*</i>	1.18	0.003
<i>Drd2*</i>	2.74	0.003	<i>Ido1*</i>	2.96	0.003
<i>Strn*</i>	1.12	0.003	<i>Tfap2b*</i>	2.70	0.003
<i>Klhl1*</i>	1.84	0.003	<i>Rarb*</i>	1.21	0.003
<i>Ido1*</i>	2.96	0.003	Generation of Neurons		
<i>Gpr88*</i>	1.73	0.003	<i>Mmp2*</i>	-1.13	0.005
Regulation of Apoptosis			<i>Th*</i>	1.27	0.035
<i>Arc</i>	1.23	0.003	<i>Inhba*</i>	1.76	0.028
<i>Mmp2*</i>	-1.13	0.005	<i>Sgk1*</i>	1.11	0.003
<i>Inhba*</i>	1.76	0.028	<i>Irx3</i>	1.94	0.016
<i>Sgk1*</i>	1.11	0.003	<i>Drd1a*</i>	1.16	0.003
<i>Hspa1b</i>	1.23	0.003	<i>Ntrk1*</i>	4.02	0.005
<i>Aqp1</i>	-1.07	0.007	<i>Tal1*</i>	1.05	0.019
<i>Ntrk1*</i>	4.02	0.005	<i>Gata3*</i>	4.44	0.047
<i>Gata3*</i>	4.44	0.047	<i>Adora2a*</i>	2.26	0.003
<i>Adora2a*</i>	2.26	0.003	<i>Egr2</i>	1.05	0.18
<i>Twist2*</i>	-1.59	0.003	<i>Strn*</i>	1.12	0.003
<i>Nr4a3*</i>	1.07	0.025	<i>Nr4a3*</i>	1.07	0.025
<i>Klf4*</i>	1.07	0.003	<i>Drd2*</i>	2.74	0.003
<i>Map3k19</i>	1.17	0.018	<i>Klf4*</i>	1.07	0.003
<i>Xdh*</i>	1.15	0.003	<i>Klhl1*</i>	1.84	0.003
<i>Hspb1*</i>	1.33	0.003	<i>Hes5</i>	-1.05	0.018
<i>Sdf2l1</i>	1.26	0.003	<i>Ptch1*</i>	1.18	0.003
<i>Ido1*</i>	2.96	0.003	<i>Folr1</i>	-1.31	0.003
<i>Tnfrsf25</i>	1.14	0.003	<i>Avil</i>	1.44	0.026
<i>Tsc22d3</i>	1.07	0.003	<i>Hspb1*</i>	1.33	0.003
<i>Hspa5*</i>	1.06	0.003	<i>Six3</i>	1.18	0.012
<i>Tfap2b*</i>	2.70	0.003	<i>Hspa5*</i>	1.06	0.003
<i>Cbl</i>	1.07	0.005	<i>Trip11</i>	1.02	0.015
<i>Rarb*</i>	1.21	0.003	<i>Rarb*</i>	1.21	0.003

Table 6. GO annotation pathway analysis of differentially expressed transcripts (Fold change= log(2) fold change). Negative fold change transcripts are upregulated in WT while normal fold change transcripts are upregulated in TNIK -/- mice (*= gene occurs in multiple pathways, bold transcripts are in WT or TNIK -/- top 25).

<i>WT</i>	<i>Fold Change</i>	<i>Q-Value</i>	<i>KO</i>	<i>Fold Change</i>	<i>Q-Value</i>
Rpl34-ps1	2.51	0.045	Gata3	4.44	0.047
Lrrc29	1.9	0.003	Ntrk1	4.02	0.005
Scn5a	1.78	0.003	Irx2	3.56	0.034
Igfn1	1.74	0.003	Cd4	3.54	0.003
Eps8l1	1.66	0.003	Ido1	2.96	0.003
Twist2	1.59	0.003	Drd2	2.74	0.003
Capn11	1.54	0.003	Tfap2b	2.70	0.003
Pifo	1.44	0.012	Serpina9	2.58	0.003
Cd6	1.44	0.022	Ptpv	2.34	0.003
Slc39a4	1.38	0.018	Adora2a	2.26	0.003
Rd3	1.36	0.025	Plin4	2.07	0.040
Endou	1.33	0.007	Slc10a4	2.05	0.013
Folr1	1.31	0.003	Irx3	1.94	0.016
Wfdc2	1.28	0.025	Syndig1l	1.88	0.003
Gm6682	1.24	0.049	Mss51	1.86	0.041
Opalin	1.24	0.003	Klhl1	1.84	0.003
Prph	1.24	0.003	Rgs9	1.77	0.003
Nanos2	1.21	0.02	Gpr6	1.77	0.003
H2-Ab1	1.2	0.017	Inhba	1.76	0.028
Krt8	1.2	0.045	Gpr88	1.73	0.003
Aim1l	1.15	0.049	Htr1d	1.71	0.037
Mmp2	1.13	0.005	Sh3rf2	1.66	0.003
Pla2g4e	1.1	0.003	Scn4b	1.63	0.003
Wdr31	1.1	0.019	Nexn	1.58	0.003
Fam181a	1.1	0.023	Slc35d3	1.57	0.005

Table 7. Top 25 fold change (fold change= log(2) fold change)transcripts in TNIK -/- and WT mice presented with Q-value (adjusted p-value) and fold change relative to opposite condition.

Discussion

Dopamine Hypothesis of Schizophrenia and TNIK

TNIK was first identified as a psychiatric disease risk factor by its association with DISC1 which was the first risk factor gene identified for schizophrenia (16, 170). There was a noticeable enrichment of genes associated with dopamine signaling in the differential transcript expression analysis (*Drd1*, *Drd2*, *Strn*, *Ido1*, *Th*) and hyperactive dopamine signaling in frontal cortex regions is one of the primary drivers of positive symptoms of schizophrenia (99). Interestingly, frontal cortex dendritic spines, in addition to DG and CA1-3 in the hippocampus, is one of the regions in which TNIK is expressed (16). If TNIK drives similar RNA expression profile alteration in the PFC cortex this may explain how TNIK is driving hyperactive dopaminergic signaling in the PFC, either by increases in DA receptors D1 and D2 or by increased DA generation by increases in tyrosine hydroxylase which is an enzyme in the pathway of DA synthesis (53).

There is another more direct way TNIK could alter DA signaling in the PFC during schizophrenia pathology that directly involves the role of TNIK in the development and function of the hippocampus. Hyperactivity in the Schaffer collateral pathway in the hippocampus, which *Tnik* structurally and functionally alters (Figure 10A-B and E, Figure 10)(28), has been directly tied to increased DA release in the PFC (98). Aberrant CA1 activity is observed in mice treated with mitotoxin methylazoxymethanol acetate (MAM) and subsequently display schizophrenia like symptoms (98). Injection of tetrodotoxin into the ventral hippocampus attenuates the DA release in the PFC observed in schizophrenia (61,

98). Since *Tnik* affects the structure and function of the same region of the hippocampus it may underlie aberrant signaling in the hippocampus that leads to schizophrenia symptoms (28).

Aside from *Tnik* altering DA associated transcripts and the hippocampus altering DA release in regions associated with schizophrenia symptoms, DA also directly affects hippocampal function and dependent memory (61, 66, 98, 175). Both D1 and D2 receptors are expressed on neurons throughout the hippocampus (53). Dopamine release, via projections from the locus coeruleus, into the hippocampus activates D1 receptors and increase spatial learning and memory (83). Likewise, activation of D2 receptors in the hippocampus causes an enhancement of spatial memory, while antagonism leads to a spatial memory deficit (175). Activation of D1 receptors is necessary for long-term potentiation at CA1 pyramidal neurons and also required for consolidation and retrieval of spatial memory (64, 65, 120, 150). The interaction of *Tnik*, dopamine and working memory is of interest to schizophrenia, and PTSD, because spatial working memory deficits are one of the most frequently reported cognitive deficits in both disorders (55, 90, 151). This spatial memory deficit is so pervasive that it is observed in adolescents and siblings at risk of developing schizophrenia (81, 152).

Tnik and the resulting differentially expressed transcripts resulting from *Tnik* loss of function appear to have numerous ties to schizophrenia and its related disease processes. DA signaling in the frontal cortex is aberrant in schizophrenics and is correlated with positive symptoms of schizophrenia (1, 100). Activity in the Schaffer collateral pathway, notably hyperactivity in CA1, is behind this release in

DA (98). *Tnik* loss of function alters this same pathway, both structurally as in our current study revealing a disorganization of projections into CA1 as well as functionally with modified quantal glutamate release at baseline in *Tnik* $-/-$ mice (28). DA also to directly modulates hippocampal activity and lead to differences in spatial working memory which is a common cognitive deficit seen in schizophrenics (90). These mechanisms all represent way that *Tnik* may affect disease progression and symptom presentation in schizophrenics and may represent a future therapeutic target for treating positive symptoms of schizophrenia (98).

TNIK and Alzheimer's Disease

In addition to schizophrenia, there are several transcripts linked to AD and its progression in our dataset. *Tnik* negatively regulates GSK3 β via phosphorylation, and GSK3 β is known to hyper phosphorylate Tau when constitutively active which leads to aggregations of Tau, one of the hallmark pathophysiological markers of AD (28, 35, 68). Decreases in hippocampal neurogenesis, which is caused by *Tnik* loss of function, play a role in progression of neurodegenerative disease (Figure 11)(28). Considering the potential link between *Tnik* and AD we were interested to see if there were any genes associated with AD in our dataset.

Adora2a, which encodes A2A receptors, has been specifically linked, to hippocampal volume in AD and mild cognitive impairment (72). SNPs within or upstream of A2A are correlated with larger hippocampal volume and an improvement in memory scores (72). The higher level of Adora2a transcript expression in *Tnik* $-/-$ mice may be involved in the decreased neurogenesis observed in *Tnik* $-/-$ mice as well as the memory impairment observed in these mice (28).

This adenosine receptor has been studied in animal models of AD including mice with over accumulation of A β as well as mice treated with injections of streptozotocin (STZ). Both caffeine, which is a non-specific antagonist of A2A receptors, and specific antagonists of A2A receptors improve memory performance and decrease neurodegeneration in the hippocampus in mouse models of AD (34, 47, 92, 142). This neuroprotective effect of A2A receptor antagonism is not limited to AD and is also observed in animal models of Parkinson's disease (80, 142). A2A and D2 receptors also interact in Parkinson's disease pathology, leading to a number of alterations in neuronal function including excitability and protein phosphorylation. (50, 52).

Mmp2 is differentially expressed between *Tnik* $-/-$ and WT mice, but is one of the transcripts showing higher expression levels in WT mice (Figure 13). Mmp2 is lowered in blood of Alzheimer's patients and has known functions in altering disease progression (94). Mmps are released from cells into the extracellular space and certain Mmp proteins, not yet Mmp2, break down A β and slow disease progression thereby improving cognitive performance and elevation during AD (67, 101, 156).

Implications for PTSD

We discovered *Tnik* as a potential disease risk factor following its identification in an RNA sequencing experiment from hippocampal tissue of FS and FR mice. These mice were selectively bred based on fear memory performance, with high freezing animals being bred to create FS mice and low freezing mice bred together to create FR mice (106). From these mice we sequenced RNA from the

hippocampus and identified *Tnik* as one of the highest fold change expression transcripts with higher expression in the FS mice. Fear conditioning is used as an animal model to study aspects of PTSD, which make these mice an animal correlate of humans with risk or resilience of developing PTSD following trauma (5, 45, 108).

Considering that we identified TNIK as a possible gene of interest in PTSD risk and resilience we are interested in transcripts discovered that may also play a role in PTSD. Interestingly, PTSD has significant comorbidity with schizophrenia and stress has also been linked to AD, which were previously reviewed above (62, 147). Specifically, with regards to schizophrenia, comorbidity with PTSD is associated with an increase with positive symptoms (147). Previously, a number of the transcripts differentially expressed between *Tnik* *-/-* and WT mice are also implicated in the Schaffer collateral pathway. CA1 hyper-stimulation leads to increased activity in the PFC dopaminergic system, which may indicate a common neural pathway between schizophrenia and PTSD (61, 97). In addition atrophy in the hippocampus is seen in patients with PTSD as well as schizophrenia and Alzheimer's disease, potentially implicating both adult neurogenesis and hippocampal neurodegeneration as common features of a number of psychiatric disorders (8, 82, 168, 176).

Chapter 6: General Discussion and Conclusions

Studying *Tnik* $-/-$ as an Animal Model of PTSD Symptoms

We originally identified *Tnik* in the hippocampus of FS mice, which were selectively bred according to their fear memory performance and is a proposed experimental model of the intrusive memories and heightened anxiety seen in PTSD (106, 125). To characterize the functional significance of *Tnik* we utilized *Tnik* $-/-$ mice and investigated PTSD related behavior including spatial memory and anxiety-like behavior and related hippocampal structural disorganization and transcriptome profiling in *Tnik* $-/-$ mice (17, 125). In the following sections, these behavioral phenotypes, their potential neural underpinnings and related transcripts were compared back to FS and FR mice, as well as the symptoms and cognitive deficits observed in PTSD.

Anxiety-Like Behavior in *Tnik* $-/-$ Mice: Implications for PTSD

Tnik loss of function leads to a decrease in anxiety-like behavior according to the results of the elevated zero maze (Figure 7). *Tnik* $-/-$ mice did not display any anxiety-like behavior, spending as much time in the open areas of the maze as the closed ones (Figure 7B). Conversely, FS mice show higher *Tnik* expression in the hippocampus and increased anxiety-like behavior (33). Taken together these results indicate a potential relationship between *Tnik* expression and anxiety-like behavior. Increased anxiety-like behavior is often seen to coincide with an increase in freezing to both cue and context, which was also observed in FS mice relative to their FR counterparts (106). It would appear that *Tnik* is an underlying genetic risk factor

common to both anxiety and fear memory. Heightened anxiety is one of the hallmark symptoms of PTSD, and if *Tnik* is a risk factor for PTSD higher expression levels of *Tnik* would be observed in patients with PTSD with corresponding heightened anxiety and a potentiated fear response, which are in fact observed (17, 45, 135). Together these results indicate *Tnik* is a potential risk factor for PTSD as it modulates a major behavioral aspect of the disorder.

Tnik is also related to PTSD and anxiety through its relationship with GSK3 β , which is a known downstream effector of *Tnik* (28). In a mouse model of juvenile stress mice are exposed to early life stress and show increased anxiety in adulthood as a result (14). This animal model is similar to PTSD in humans, where trauma leads to a long lasting increase in anxiety, and childhood trauma predisposes individuals to anxiety disorders such as PTSD (162). In this mouse model, GSK3 β levels are decreased in the hippocampus and anxiety is increased. This effect is directionally consistent with what we observed in *Tnik* $-/-$ mice, where GSK3 β is elevated in the hippocampus and anxiety-like behavior is decreased (28). Also observed in this juvenile stress mouse model was heightened levels of DISC1, a protein known to bind TNK1 in the PSD, and a disease risk factor psychiatric illnesses including MDD, AD and potentially PTSD (16, 170). DISC1 is known to bind both TNK1 and GSK3 β , with mutations in both of these regions being risk factors for psychiatric illnesses (154). While DISC1 interacts with both TNK1 and GSK3 β , it is unknown whether DISC1 is involved in facilitating the interaction between TNK1 and GSK3 β (154). If this were the case, it would explain how these three genes have all emerged as risk factors for the same psychiatric diseases, and understanding the

mechanistic interactions of these molecules would be an important topic of research in understanding illnesses where this pathway is implicated.

Although *Tnik* appears to alter anxiety-like behavior, we did not investigate the structure of the amygdala, which is one of the major structures involved in anxiety and anxiety-like behavior. Recently, it was discovered that in the lateral amygdala of FS mice there was an increase in the branching of projections distal to the cell body (20). Changes in the processes of elongation and branching are consistent with what we observed in the hippocampus of *Tnik* $-/-$ mice and with what has been seen in GSK3 β overexpressing mice (134, 145, 148). Examining the amygdala of *Tnik* $-/-$ mice, and comparing results with FS and FR mice, is one future direction in which research into *Tnik* and its role in the CNS should be directed, as anxiety is one of the main symptoms of PTSD and how *Tnik* affects brain regions linked to anxiety would be of great interest.

Fear and Spatial Memory Deficits in *Tnik* $-/-$ Mice: Implications for PTSD

Investigating fear memory revealed that loss of *Tnik* function leads to a deficit in both cued and contextual fear behavior, which matches directionally with what we observed in FS and FR mice. FS mice showed higher levels of freezing than FR mice on cued and contextual fear memory tests and likewise showed higher transcript expression of *Tnik* in the hippocampus (106). This suggests that *Tnik* is a potentially contributes to fear memory performance, which is designed to model aspects of PTSD, because its expression levels correlate with fear memory performance in *Tnik* $-/-$ and FS and FR mice (17).

Loss of *Tnik* function also appears to lead to a generalized spatial memory deficit as evidenced by a deficit on the Y-maze novel arm test of spatial memory. *Tnik* ^{-/-} mice displayed a decreased interest in the novel arm on the second phase of the task displaying that they did not recognize it as a novel environment (27). FS and FR mice did not show a difference in generalized spatial memory (33). This memory deficit has implications for PTSD since patients with PTSD have also reported to have deficits in spatial memory (153, 158). Spatial memory deficits are a common aspect of many mental disorders, including PTSD, schizophrenia and AD (55, 68, 81). This result is also supported by the significant difference in freezing observed between *Tnik* ^{-/-} and WT mice on the contextual fear conditioning experiment.

There is a disruption in the Schaffer collateral pathway of the hippocampus in *Tnik* ^{-/-} mice, which is crucial to spatial learning and memory (10, 157, 181). The disorganization we observed in the Schaffer collateral pathway of *Tnik* ^{-/-} mice is in line with the effects of *Tnik* knockdown observed *in vitro* where *Tnik* is required for neuronal process extension (28, 73). Disruption or inhibition of neurons in this pathway inhibits spatial learning and memory (21, 119, 161). Also, known functions of *Tnik*, such as AMPA receptor trafficking, are known to be crucial for LTP which is also required for spatial learning and memory (28). We also observed atrophy in the dentate gyrus which has been linked to impairment in hippocampal function and spatial learning and memory deficits, including experiments previously performed in *Tnik* ^{-/-} mice (21, 27, 28). The effect *Tnik* has on the hippocampus provides multiple ways *Tnik* may be required for proper hippocampal function as well as

learning and memory. These results in *Tnik* ^{-/-} mice relate to PTSD as well.

Decreased volume in the hippocampus has been observed in PTSD, which may in part be driven by a decrease in neurogenesis, although this is unknown and atrophy could also be due to other factors (42, 136).

Associative learning is one of the top pathways identified when significantly differentially expressed transcripts between *Tnik* ^{-/-} and WT mice were investigated. Among those transcripts, there are a number of genes with known hippocampal function that showed differential transcript expression including *Adora2a*, *Drd1a*, *Drd2*, *Folr*, *Gpr88*, *Hspa5*, *Ido1*, *Mmp2* and *Sgk1*. Many of these genes are for receptors, including 2 dopamine receptors, highlighting how *Tnik* may have a major impact on the responsiveness of neurons to external stimuli (53, 60, 107, 182). This would have major implications for PTSD, as alterations in dopamine signaling pathways have been reported in patients with PTSD across several populations and types of trauma (32, 41, 43, 144). If *Tnik* affects dopamine signaling this would have implications for its role in the pathogenesis of PTSD.

Across all of our experiments we observed a consistent effect of *Tnik* loss of function on learning and memory. Taken together, our results confirm *Tnik* plays an important role in learning and memory and in normal hippocampal development. In addition, transcriptome profiling revealed many ways *Tnik* might be affecting the formation and retrieval of memories. These experiments reveal a number of paths for future studies into *Tnik* function, including dopamine-signaling alterations and functional studies in the brain of *Tnik* ^{-/-} mice. These are just a few of the ways *Tnik*

may be affecting learning and memory and studies in this area would have implications for PTSD and other mental disorders.

Hyperactivity in *Tnik* $-/-$ Mice: Implications for PTSD

Tnik $-/-$ mice display increased locomotor activity across all tasks where distance traveled was measured (OF, EZM, and Y-maze). This is an interesting finding, as one of the disorders most commonly diagnosed during adolescence in soldiers who develop PTSD is ADHD, in which adolescents show heightened activity levels (2, 56). This association is surprising however, as *Tnik* $-/-$ mice may display increased activity levels, while this is the opposite of the expected effect we would expect if *Tnik* is more highly expressed in the brains of those with PTSD. Alterations of hippocampal activity generally do not impact activity, and this behavior is most likely associated with brain regions outside of those studied in this project.

In a previous study of *Tnik* $-/-$ mice, pretreatment with an inhibitor for the microtubule associated kinase GSK3 β inhibited hyperlocomotor activity (28). GSK3 β is a substrate of *Tnik* and it is deactivated by *Tnik* mediated phosphorylation (28). In a GSK3 β transgenic mouse model, overexpressing GSK3 β in neurons in the hippocampus and cortex, there is also an increase in locomotor activity, further confirming GSK3 β as a downstream effector of *Tnik* (35). This link between *Tnik* and GSK3 β is important in the association between *Tnik* and PTSD as well, through the relationship between GSK3 β and anxiety in a mouse model of juvenile stress and adult anxiety previously mentioned (14). These behavioral phenotypes, heightened anxiety and hyperactivity, appear to have common underlying mechanisms related to changes in Wnt signaling (28, 148).

***Tnik* -/- Mice, FS Mice and PTSD**

Several aspects of the phenotype of the *Tnik* -/- mice compare as expected with the FS mouse phenotype and symptoms of PTSD (106). Anxiety-like behavior and differences in cued and contextual fear memory performance are consistent with what would be expected if *Tnik* was underlying the same behaviors in FS mice and PTSD. These 2 behaviors have strong ties to PTSD, with PTSD being characterized by increased anxiety, particularly in response to circumstances reminding the person of the initial trauma (4). FS mice display heightened anxiety and an exaggerated fearful response to traumatic situations, which mimics PTSD accurately (106, 125). FS mice have a higher expression level of *Tnik* in the hippocampus according to our pilot work. *Tnik* -/- mice, which have a loss of *Tnik* function, present with lower anxiety-like behavior and a deficit in cued and contextual fear memory, as one would expect if *Tnik* contributes to the behavioral differences between FS and FR mice. The decrease in anxiety-like behavior, and the deficit in cued and contextual fear memory are both directionally consistent with PTSD symptoms, leading to the possibility of *Tnik* as a factor for risk and resilience.

Considering the effect *Tnik* has on spatial memory performance we do not see the expected directionality between *Tnik* -/- mice, FS mice and PTSD (Table 7). We would expect patients with PTSD, if they have higher expression levels of *Tnik*, to display an improvement in spatial memory, not a deficit similar to *Tnik* -/- mice (Table 7) (153, 158). Similarly, *Tnik* -/- mice and PTSD both present with pathological changes to the hippocampus, decreased dentate gyrus volume in *Tnik* -/- mice and overall decreased hippocampal volume in PTSD (13, 28, 82, 176). These

changes are not identical, but still contradict the predicted directionality of TNIK expression and hippocampal changes in PTSD (13). One explanation for this difference is the susceptibility to damage of the hippocampus in a number of pathological conditions including traumatic brain injury (TBI), AD, seizure disorders and encephalitis (8, 78, 116, 121, 177). With how easily injured the hippocampus can be, especially in states of heightened activity such as seizures, prolonged activity during PTSD could in theory lead to cell death which would lead to decreased hippocampal volume and result in dysfunction (78).

Phenotype	<i>Tnik</i> ^{-/-} Mice	FS Mice	PTSD
<i>Tnik</i> Expression	↓	↑	↑?
Fear Memory	↓	↑	↑
Anxiety	↓	↑	↑
Spatial Memory	↓	Normal	↓

Table 8- Phenotypes in *Tnik* ^{-/-} Mice, FS Mice and PTSD.

Summary table of phenotypes across *Tnik* ^{-/-} mice, FS mice and PTSD displaying consistencies and discrepancies across all 3 based on known and anticipated TNIK expression across all 3. Shows that *Tnik* ^{-/-} mice do indeed model some behaviors of both FS mice and PTSD (fear memory and anxiety) while differences in spatial memory do not occur as expected (?=hypothesized).

PTSD and Suicide Risk

One of the major emerging threats to the health and lives of American service members is suicide. Historically, rates of suicide in the military have been lower than those of the civilian population but this trend was reversed in 2007 when suicide rates in the military exceeded those in the civilian population for the first time (11). During this period, suicide became one of the major causes of death in the US Army, rising from 9 per 100,000 in 2001 to more than double that rate at 22 per 100,000 soldiers by 2009 (11).

This alarming rise in suicide in the US Army led to the creation of the army Study to Assess Risk and Resilience in Service members (STARRS) and a continuing longitudinal study that seeks to identify procedures that can be followed to prevent suicide, suicidal ideation and affect other mental issues facing US service members (164). Suicide and suicidal ideation are very complex behaviors and have been tied to a number of external factors including military rank at time of deployment (lower rank led to higher risk) gender (women showed higher rates than men) and a protective effect of marriage on suicide rate during deployment (54, 141).

One of the greatest risk factors discovered during STARRS in the military, and numerous previous studies in civilians, is comorbidity with a wide range of mental/behavioral disorders (113, 114, 129). Both MDD and PTSD have been linked to suicide in the military, with soldiers with comorbid MDD and PTSD showing more than 3 times the likelihood of attempting suicide (129). Interestingly, MDD has been significantly linked to suicidal ideation but not with an increased likelihood of creating a suicide plan or attempting suicide when comorbidity is controlled for

during analysis (113). However, suicidal planning and attempting is predicted by mental disorders where patients display heightened anxiety and agitation, such as PTSD (113, 115). The rate of both PTSD and MDD in the US Army also rose significantly since 2001, during the same time period as the increase in suicides (129). The predictive accuracy of PTSD regarding attempted suicide highlights the importance of preventing and treating PTSD in the military and the civilian population, where PTSD is predictive of suicide (114, 115). Addressing the spike in suicide seen since the start of the wars in Iraq and Afghanistan is one of the most pressing health and safety concerns facing the US military. Greater understanding of comorbid disorders such as MDD and PTSD and how they affect the structure and function of the brain are crucial aspects of addressing the overall public health crisis currently facing the military and civilian populations.

To identify factors that confer risk and resilience to developing PTSD would be a major step in treating and preventing the disorder. To this end, we identified and investigated a gene, *TNIK*, in an animal model designed to study specific aspects of PTSD. We observed that *Tnik* $-/-$ mice exhibit behavioral phenotypes associated PTSD related behaviors including anxiety and fear memory. *TNIK* is an important mediator in the Wnt signaling pathway as well as regulator of GSK3 β , which is overactive in a number of psychiatric diseases that display some similar symptomology to aspects of PTSD (102, 124). Our current study links this pathway to PTSD via the FS and FR mouse model and implicates Wnt signaling, and *TNIK*, as factors potentially conferring risk and resilience to developing PTSD. Further translational research in humans investigating the Wnt signaling pathway and *TNIK*

should be pursued to validate our current results. The outcome of those studies would lead to both a greater understanding of the underlying etiology of PTSD as well as help identify therapeutic approaches to treat this detrimental disorder.

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