A Potential Role For Sap97 In Psychiatric Disorders

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A Potential Role For Sap97 In Psychiatric Disorders

Abstract
The goal of this dissertation is to further understand the genetic architecture of neuropsychiatric disorders, such as autism spectrum disorder (ASD) and schizophrenia (SCZ). We attempt to understand the functional significance of the gene synapse associated protein of 97KDa (SAP97) and identify a novel role for SAP97 in the etiology of neuropsychiatric disorders.

SAP97 belongs to a family of scaffolding proteins, the membrane-associated guanylate kinases (MAGUKs), that are highly enriched in the postsynaptic density of synapses and play an important role in organizing protein complexes necessary for synaptic development and plasticity. Large-scale genetic studies have implicated MAGUKs in neuropsychiatric disorders such as intellectual disability, ASD, and SCZ, but knock-out mice have been impossible to study because the Sap97 null mice die soon after birth due to a craniofacial defect. In Chapter 2, we studied the transcriptomic and behavioral consequences of a viable, brain-specific conditional knockout of Sap97 (SAP97-cKO). RNA sequencing (RNAseq) from hippocampi from control and SAP97-cKO male animals identified 67 differentially expressed transcripts, which were specifically enriched for SCZ-related genes. Subjecting SAP97-cKO mice to a battery of behavioral tests revealed a subtle anxiety-like phenotype present in both male and female SAP97-cKO animals, as well as a mild male-specific cognitive deficit and female-specific motor learning deficit. Collectively, this work suggests that loss of Sap97 alters behavior, and may contribute to some of the endophenotypes present in SCZ. In Chapter 3, we discuss how the SAP97-cKO mouse may serve as a novel model system for interrogating aspects of the cellular and molecular defects underlying SCZ and other related neuropsychiatric disorders.

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A POTENTIAL ROLE FOR SAP97 IN PSYCHIATRIC DISORDERS

Preetika Gupta

A DISSERTATION

in

Neuroscience

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DEDICATION

This thesis is dedicated to my family, Sunil and Seema Gupta, Utsav Gupta, Rebecca Neff, Ethan Neff (dog), and Rajni Gupta (cat). Thank you for always helping me become a better version of myself, while standing by my side throughout the entire process.

“most importantly love
like it’s the only thing you know how
at the end of the day all this
means nothing
this page
where you’re sitting
your degree
your job
the money
nothing even matters
except love and human connection
who you loved
and how deeply you loved them
how you touched the people around you
and how much you gave them”

--Rupi Kaur, from milk and honey
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ABSTRACT

A POTENTIAL ROLE FOR SAP97 IN PSYCHIATRIC DISORDERS

Preetika Gupta
Robert G. Kalb

The goal of this dissertation is to further understand the genetic architecture of neuropsychiatric disorders, such as autism spectrum disorder (ASD) and schizophrenia (SCZ). We attempt to understand the functional significance of the gene synapse associated protein of 97KDa (SAP97) and identify a novel role for SAP97 in the etiology of neuropsychiatric disorders.

SAP97 belongs to a family of scaffolding proteins, the membrane-associated guanylate kinases (MAGUKs), that are highly enriched in the postsynaptic density of synapses and play an important role in organizing protein complexes necessary for synaptic development and plasticity. Large-scale genetic studies have implicated MAGUKs in neuropsychiatric disorders such as intellectual disability, ASD, and SCZ, but knock-out mice have been impossible to study because the Sap97 null mice die soon after birth due to a craniofacial defect. In Chapter 2, we studied the transcriptomic and behavioral consequences of a viable, brain-specific conditional knockout of Sap97 (SAP97-cKO). RNA sequencing (RNAseq) from hippocampi from control and SAP97-cKO male animals identified 67 differentially expressed transcripts, which were specifically enriched for SCZ-related genes. Subjecting SAP97-cKO mice to a battery of behavioral tests revealed a subtle anxiety-like phenotype present in both male and female SAP97-cKO animals, as well as a mild male-specific cognitive deficit and female-specific motor
learning deficit. Collectively, this work suggests that loss of Sap97 alters behavior, and may contribute to some of the endophenotypes present in SCZ. In Chapter 3, we discuss how the SAP97-cKO mouse may serve as a novel model system for interrogating aspects of the cellular and molecular defects underlying SCZ and other related neuropsychiatric disorders.
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CHAPTER 1: INTRODUCTION

This thesis attempts to elucidate the potential contribution of *SAP97* to neuropsychiatric disorders, such as autism spectrum disorder and schizophrenia. In particular, we examine the effects of loss of *Sap97* to mouse behavior and alterations in the transcriptome. Here, I will outline the critical information needed to understand the progress made in this subject.

SYNAPTOPATHIES: DISORDERS OF THE SYNAPSE

The genomic revolution has transformed the field of neuroscience by providing a platform to decipher the brain and its disorders. Advancements in whole exome and deep sequencing technologies allow for examination of the genetic architecture of psychiatric disorders using large patient cohorts. These large-scale genetic studies have implicated key overlapping molecular and cellular pathways that are impacted across mental disorders, such as chromatin regulation and the post-synaptic density, with dysfunction of the synapse being a convergence point (Grant, 2012; De Rubeis et al., 2014; McCarthy et al., 2014; Ardiles et al., 2017; Luo et al., 2017). This observation coined the term “synaptopathies,” or diseases of the synapse, to collectively classify these disorders.

Human mutations in genes encoding synaptic proteins are increasingly identified in neurodevelopmental disorders such as epilepsy, intellectual disability, autism spectrum disorder, and schizophrenia. While these disorders each present with unique symptoms, there is increasing recognition of the overlapping comorbidities between these disorders, in addition to the large genetic heterogeneity within these disorders
As a result, addressing the common underlying genetic mechanisms may be key for developing therapies across a broad range of behavioral symptoms.

In this thesis, we choose to focus on the potential contribution of SAP97 to autism spectrum disorder and schizophrenia, two neuropsychiatric conditions that are prevalent in today’s society and share a subset of risk-associated genes. Below, we provide a background for each disorder, and discuss both the importance and complications associated with each current genetic model.

AUTISM SPECTRUM DISORDERS

Epidemiology and clinical presentation

Autism spectrum disorder (ASD) is diagnosed in approximately 1 out of 110 children in the United States and is 4-5 times more common in male children than in females (The Autism Genome Project Consortium, 2007; Sungur et al., 2017). Newer prospective studies of younger siblings of children with ASD who are at elevated risk provide evidence that the age of onset for the majority of cases of ASD is the second year of life (Zwaigenbaum et al., 2006; Martínez-Pedraza and Carter, 2009). Several studies document that the mean age at which parents first report concerns to a medical professional is between 18 and 24 months of age (Sullivan et al., 2007; Martínez-Pedraza and Carter, 2009). Although ASD behaviors lie on a continuum in the general population, individuals with ASD are characterized by severe and pervasive impairments in reciprocal social interaction and communication and exhibit stereotyped behaviors, as well as restricted interests and activities (Martínez-Pedraza and Carter, 2009). Most parents first report concerns in the area of speech and language in addition to extreme sensory over- or underreactivity and disturbances in the acquisition of social
communication, play, and motor development (Young et al., 2003). Parents also report concerns regarding sleep and eating, which may be associated with sensory sensitivities or with the insistence on sameness (Goodlin-Jones et al., 2008; Sharp et al., 2013).

**Neuropathologic profile of ASD**

Neuropathologic explorations in human postmortem tissue allow for investigation at the cellular and cytoarchitectural levels from individuals with ASD. Global brain development abnormalities are seen in the archicortex, cerebellum, brainstem, and other subcortical structures, with region-specific severity of neuropathology in young children with ASD (Wegiel et al., 2014; 2015). Brain size as well as head circumference of a subset of subjects with ASD is increased compared to normal age-based values (Sacco et al., 2015). An assessment of the microarchitecture of cortical areas commonly implicated in ASD between subjects with ASD and controls reveals disorganization of gray and white matter, and disorganized cortical structure and nodules of misplaced neurons (Casanova et al., 2013; Stoner et al., 2014; Wegiel et al., 2015). These defects reflect alterations in neuronal maturation and migration processes in subjects with ASD. A study from one subject with ASD described finding pencil fibers consisting of oligodendrocytes, astrocytes, and glia that disrupted cortical lamination in the prefrontal cortex (Hashemi et al., 2016).

Young children with ASD also have significantly reduced neuronal and cytoplasmic volumes in the majority of examined areas compared to age-matched controls (Wegiel et al., 2014; 2015). The distribution of neuronal sizes becomes more comparable between control and ASD individuals in adulthood, which is likely a result of opposing developmental trajectories (Wegiel et al., 2014; 2015). Subjects with ASD also have a significant increase in neuropil, comprising the dendrites, non-myelinated axons,
synapses, vasculature, and glial cell processes present in between cell bodies (Varghese et al., 2017). Neurons show reduced dendritic branching in the hippocampus of ASD subjects as compared to controls (Raymond et al., 1996). Studies measuring spine density on apical dendrites in the cortex of ASD subjects report slower pruning of spines in the temporal lobe (Hutsler and Zhang, 2010; Tang et al., 2014). This results in the difference in spine densities between ASD and controls being greater in adolescence than in early childhood (Tang et al., 2014). These changes appear to be related to alterations that occur during early pregnancy, such as reduced programmed cell death and/or increased cell proliferation, altered cell migration, abnormal cell differentiation, abnormal neurite sprouting, and pruning that cause atypical wiring of the brain (Lacivita et al., 2017).

The biggest motivation for studying ASD is that the disorder has no known cure. Psychotropic medications currently available alleviate psychiatric and behavior problems, such as aggression, self-injury, hyperactivity, anxiety, and mood symptoms, but they do not have an effect on the core symptoms of ASD (Young and Findling, 2015). To date, the only approved drugs to treat symptoms of ASD are risperidone and aripiprazole, both used to treat aggression, self-injury, and severe tantrums (Lacivita et al., 2017). The lack of treatment is due in part to the multifactorial nature of the disorder.

Another motivation for studying ASD is that prevalence rates have dramatically increased in the past decade (Christensen et al., 2016). There are various reasons for this increase, including broadening of the spectrum to include milder forms of the disorder, improved clinical detection, and higher public awareness (Levy et al., 2009). As a result, ASD has recently emerged as a major public health issue worldwide. Due to the lack of promising treatment, there is an urgent need for ASD research. One of the obstacles in studying the disorder is that hundreds of risk genes have been identified,
with not one major causative gene. Rare variants have been identified that are highly penetrant, and common variants can contribute to small effect sizes (Lacivita et al., 2017). Below, we discuss what is currently known regarding the genetic underpinnings of ASD.

**Genetic susceptibility to ASD**

Interestingly, family genetic data supports a first-degree relative recurrence risk of approximately 5-10%, which points to disruption of genetic architecture as being a leading cause of the disease (Ritvo et al., 1989; Sumi et al., 2006). It is believed that the neurocognitive phenotype of ASD is the result of a complex and highly heterogeneous set of genetic and environmental causes (Lacivita et al., 2017). In some patients, the cause of the disorder is purely genetic (due to known chromosomal mutations), while in other patients, the disorder is more likely related to environmental causes such as prenatal exposure to chemical pollutants, toxins, viruses, or drugs (Persico and Merelli, 2014; Lacivita et al., 2017). For this thesis work, we have chosen to focus primarily on the genetic contribution to ASD and other related psychiatric disorders. The genetic abnormalities associated with ASD and other related psychiatric disorders may be grouped into three classes: 1) at least 5% are caused by single gene mutations, 2) approximately 10% are copy number variations including duplications, large deletions, inversions, and translocations of chromosomes, and 3) many are polygenic risk factors due to accumulation of common variants, each contributing to a portion of the risk (Varghese et al., 2017). Most research done in the laboratory to model and study the unique contribution of each risk-associated gene utilizes rodent models.
GENETIC MOUSE MODELS OF ASD

There are two types of animal models for ASD: environmentally induced (by exposure of the pregnant animals to certain toxins or infection/inflammation) and those that are induced by genetic manipulations. In this introduction, we have chosen to focus on genetic mouse models of ASD. More than a hundred de novo single gene mutations and copy-number variants have been implicated in ASD, each occurring in a small subset of cases (Kazdoba et al., 2015). Mutant mouse models with syntenic mutations offer investigators a tool for understanding the role of each gene in modulating biological and behavioral phenotypes relevant to ASD (Kazdoba et al., 2015). Investigations of ASD, schizophrenia, and other related psychiatric disorders indicate a highly polygenic architecture with small effect sizes of each implicated risk variant (Ebert and Greenberg, 2013; Fromer et al., 2014; Kato, 2014; Smoller et al., 2018). As a result, mouse modeling of these disorders by targeting one such risk variant typically demonstrates a moderate, or incomplete manifestation of the human disorder. Below, we have highlighted the most prominent genetic mouse models of ASD. Extensive characterization of these models demonstrates that while ASD is genetically complex, these studies are useful in describing the direct contribution of each gene.

As mentioned previously, a remarkable number of risk genes for ASD code for synaptic proteins, including cell adhesion proteins, neuroligins and neurexins, and postsynaptic scaffolding proteins such as the PROSAP/SHANK family. Mice with targeted mutations in many of these genes have been generated and characterized, as described below.

CNTNAP2

The contactin associated protein-like 2 (CNTNAP2) gene, a cell adhesion
molecule located on chromosome 7, encodes contactin-associated protein-like 2 (CASPR2), a member of the neurexin superfamily (Rodenas-Cuadrado et al., 2013). Several mutations in the CNTNAP2 locus, including rare, common and deletion variants, have been associated with ASD (Alarcón et al., 2008; Arking et al., 2008; Rossi et al., 2008; Poot et al., 2009). A recessive nonsense mutation in CNTNAP2 was shown to cause a syndromic form of ASD, cortical dysplasia, and focal epilepsy syndrome (Alarcón et al., 2008; Arking et al., 2008). The CNTNAP2 variant that increases risk for the language endophenotype in ASD was shown to lead to abnormal functional brain connectivity in human subjects (Weinstein-Fudim and Ornoy, 2016). Knockout mice for the mutation show migration abnormalities, reduced number of interneurons, and abnormal neuronal network activity (Scott-Van Zeeland et al., 2010). Mice lacking Cntnap2 also exhibit behavioral abnormalities such as reduced juvenile ultrasonic vocalizations, reduced social interaction time, and increased repetitive behaviors (Peñagarikano et al., 2011).

**Neuroligins and Neurexins**

Neuroligins are cell adhesion molecules located at the postsynaptic side of the synapse and interact with neurexins, their presynaptic partner protein (Bang and Owczarek, 2013). Neuroligins contribute to synaptic neurotransmission through their influence on synaptic formation (Hu et al., 2015). Neuroligin (NLGN) proteins encoded by X-linked genes, such as NLGN3 and NLGN4, have been associated with ASD in large genome-wide studies (Auranen et al., 2002; Glessner et al., 2009). Using amino acid sequencing in linkage and proband case studies, deletions and frameshifts in NLGN3 and NLGN4 sequences have been identified in individuals with ASD (Laumonier et al., 2004; Lawson-Yuen et al., 2008). Knockout mouse models have been created for
four neuroligin isoforms—*Nlgn1*, *Nlgn2*, *Nlgn3*, and *Nlgn4*. *Nlgn1* KO mice show minimal social deficits, but have increased grooming and spatial learning impairments along with impaired hippocampal long-term potentiation (Blundell et al., 2010). *Nlgn2* KO mice show no social deficits, but display increased anxiety-like behavior, decreased pain sensitivity, and poor motor coordination (Blundell et al., 2010; Wöhr et al., 2013). In addition, *Nlgn2* KO mice had decreased inhibitory neurotransmission, as well as decreased immunostaining of inhibitory synapse markers (Blundell et al., 2010). *Nlgn3* knock-in (R451C) mice, with a ASD-related point mutation, did not display robust ASD-like behaviors, but rather had mild developmental differences, enhanced spatial learning, and reduced acoustic startle (Tabuchi et al., 2007; Chadman et al., 2008; Etherton et al., 2011). These results would suggest that this ASD-related point mutation delayed development, altered learning, and reduced sensitivity to stimuli. *Nlgn* knock-in mice also exhibited increased inhibitory neurotransmission in the barrel cortex, increased excitatory neurotransmission and enhanced long-term potentiation in the hippocampus, and increased dendritic branching in the hippocampus (Tabuchi et al., 2007; Etherton et al., 2011). *Nlgn3* KO mice show no social deficits, but are impaired in fear conditioning and olfaction, and are hyperactive. *Nlgn3* KO mice also show decreased total brain volume (Radyushkin et al., 2009). And lastly, *Nlgn4* KO mice show reduced sociability and ultrasonic vocalizations, as well as a reduction in total brain volume (Jamain et al., 2008; El-Kordi et al., 2013). Genetically modified mice have also been made for neurexins (NRXN), the neuroligin partner protein. Numerous association studies have identified mutations in the *NRXN1* gene, located on chromosome 2, in intellectual disabilities and ASD (Feng et al., 2006; Szatmari et al., 2007; Zahir et al., 2007; Glessner et al., 2009). *Nrnx1* KO mice display increased grooming, reduced locomotor activity, reduced sensorimotor gating, and increased aggression (Etherton et al., 2009;
Grayton et al., 2013). Together, these studies demonstrate that the \textit{Nlgn} and \textit{Nrxn} genes may not play a prominent role in social behavior, but may instead regulate anxiety and cognition.

**SHANK/ProSAP2 Family**

The \textit{SHANK} family of genes, located on chromosome 22q, encodes scaffolding proteins that assist in the synaptic organization of excitatory glutamatergic neurons by binding to postsynaptic density proteins, signaling molecules, postsynaptic receptors, and cytoskeletal proteins (Grabrucker et al., 2014). Genetic studies have identified \textit{de novo} and inherited mutations in \textit{SHANK1}, \textit{SHANK2}, and \textit{SHANK3} (Berkel et al., 2010; Boccuto et al., 2012; Sato et al., 2012). 22q13 deletion syndrome, also known as Phelan-McDermid syndrome, is caused by a deletion on the distal part of the long arm of chromosome 22 and is associated with ASD-like behaviors (Phelan and McDermid, 2011; Kolevzon et al., 2015). \textit{SHANK3} is one of the most commonly mutated genes within the Phelan-McDermid critical region (Phelan and McDermid, 2011). Genetically-modified mouse models have been generated and characterized for the three \textit{Shank} isoforms. \textit{Shank1} KO mice do not display robust social deficits, but emit fewer ultrasonic vocalizations and have motor impairments (Silverman et al., 2011; Wöhr et al., 2011). \textit{Shank1} KO mice also display dendritic spine abnormalities, including weaker basal synaptic neurotransmission (Hung et al., 2008). \textit{Shank2} KO mice, however, show reduced sociability in addition to abnormal ultrasonic vocalizations (Schmeisser et al., 2012). \textit{Shank2} KO mice also had reduced number of hippocampal dendritic spines and reduced glutamatergic neurotransmission in the hippocampus (Schmeisser et al., 2012). Multiple transgenic mouse models of \textit{Shank3}, with deletions in various domains of the gene, have also been generated and characterized. Reduced sociability, reduced
ultrasonic vocalizations, and high levels of repetitive self-grooming were dependent upon which isoform of Shank3 was deleted (Peça et al., 2011; Wang et al., 2011b; Kouser et al., 2013). Reduced basal neurotransmission, as well as abnormalities in neuronal morphology (neuronal hypertrophy, dendritic spine deficits) have been identified in most of these models (Peça et al., 2011; Wang et al., 2011b; Kouser et al., 2013). Overall, these studies highlight that the Shank gene family may be responsible for normal social behavior, maintaining normal synaptic function and neuronal structure, and that complete or partial loss of Shank may also induce repetitive behaviors.

**SCHIZOPHRENIA**

**Epidemiology and clinical presentation**

In addition to ASD, we also chose to examine the potential contribution of SAP97 to schizophrenia (SCZ). SCZ affects approximately 5 out of every 1000 individuals (Wu et al., 2006). The age of onset varies between men and women, where men tend to have a younger onset, with the peak incidence for men and women lies between 15-24 years of age (Wu et al., 2006). Men have about a 30-40% higher lifetime risk of developing SCZ. Like many other psychiatric disorders, SCZ is diagnosed by its symptoms, which fall into three main categories: positive, negative, and cognitive. Positive symptoms include hallucinations, delusions, and disorganized thinking (Lehman et al., 2006; Tandon et al., 2009). Negative symptoms include social withdrawal, blunted affect, and a decreased in incentive motivation (Lehman et al., 2006; Tandon et al., 2009). Cognitive symptoms encompass deficits in processing speed, working memory, attentional set-shifting, and verbal memory (Lehman et al., 2006; Tandon et al., 2009). Typically, the negative and cognitive symptoms are more predictive for the long-term prognosis of the disorder (Green et al., 2000). Similar to ASD, there is evidence
suggesting a strong genetic component of SCZ. Classical twin studies demonstrated a 50% concordance rate for SCZ among monozygotic twins and a reduced rate of 15% if the twins are dizygotic (Canetta and Kellendonk, 2018).

**Neuropathologic profile of SCZ**

Abnormalities in neurodevelopment might be responsible for the cognitive deficits in SCZ (Tripathi et al., 2018). In SCZ, abnormal brain development begins as early as prenatal life, which intensifies during childhood and continues until adulthood (Tripathi et al., 2018). Many brain areas are altered in SCZ, such as the third and lateral ventricles, prefrontal cortex, amygdala, medial temporal lobe, basal ganglia, thalamus, corpus collosum, and cerebellum (Tripathi et al., 2018). Abnormalities in neurotransmission, including the neurotransmitters dopamine, serotonin, and glutamate, have also provided the basis for theories on the pathophysiology of SCZ (Lavretsky et al., 2008). Other theories implicate aspartate, glycine, and GABA as part of the neurochemical imbalance of SCZ (Lavretsky et al., 2008). The core symptoms of SCZ, such as negative symptoms and executive dysfunction, are thought to result directly from altered neuroplasticity (Voineskos et al., 2013). SCZ alters brain derived neurotrophic factor (BDNF), which is associated with hippocampal neuroplasticity, attributing to the cognitive deficits present in the disorder (Nieto, 2013). Abnormal activity at dopamine receptor sites is also thought to be associated with many symptoms of SCZ. Low dopamine levels within the nigrostriatal pathway are thought to affect the extrapyramidal system, leading to motor symptoms (Lavretsky et al., 2008; Patel et al., 2014). The mesolimbic pathway may play a role in the positive symptoms of SCZ in the presence of excess dopamine (Lavretsky et al., 2008; Patel et al., 2014). Negative symptoms and cognitive deficits may also be due to low mesocortical dopamine levels (Lavretsky et al., 2008).
One of the leading motivations for studying SCZ is that it has no known cure. Current pharmacological agents, such as second-generation antipsychotics, are used to treat the symptoms of the disorder rather than the underlying cause (Lewis and Lieberman, 2018). However, similar to ASD and many other neuropsychiatric disorders, one obstacle in studying the genetic cause of SCZ is that whole genome studies have identified over 100 gene variants that are associated with the disorder (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). While few studies of common variants have produced important insight into possible biological mechanisms of SCZ, most common variants only minimally increase the risk for the disorder. In order to elucidate the contributions of each genetic variant to the etiology of SCZ, various genetic mouse models have been generated and characterized.

GENETIC MOUSE MODELS OF SCHIZOPHRENIA

SCZ has both genetic and environmental components, and an attempt has been made to model both aspects of the disorder. In this thesis introduction, we focus on describing genetic models of SCZ. Several mouse models have been useful for studying the behavioral consequences of specific synaptic gene alterations and the mechanisms potentially underlying the pathogenesis of SCZ.

Neuregulin

Neuregulin 1 (NRG1) is part of a family of growth and differentiation factors, and was first suggested as a potential candidate gene for SCZ in a study of the Icelandic population (Stefansson et al., 2002). This association between NRG1 and SCZ was later confirmed in Scottish and Irish populations (Stefansson et al., 2002; Corvin et al., 2004). NRG1 is essential for neurodevelopment, with key roles in synapse formation,
neuronal migration, synaptic plasticity, and regulation of neurotransmitter systems (Falls, 2003). While homozygous null mice for Nrg1 die midgestation, heterozygous mutant mice are viable (Gerlai et al., 2000). Nrg1 hypomorph epidermal growth factor-like domain models result in hyperactivity with impaired PPI (Gerlai et al., 2000; Duffy et al., 2008). Additionally, most NRG1 proteins are synthesized with a transmembrane (TM) domain, and Nrg1 hypomorph TM models also result in hyperactivity and exhibit impaired PPI, altered habituation, increased aggression, and decreased functional NMDA receptors (Stefansson et al., 2002; Karl et al., 2007; O’Tuathaigh et al., 2008). Nrg1 immunoglobulin-like domain mutant mice, while not hyperactive, are impaired in the latent inhibition task (Rimer et al., 2005). A more recent model focusing on the deletion of a specific Nrg1 isoform (Type III) produces mice with a more pronounced PPI deficit, impaired performance on delayed alteration memory tasks, enlarged lateral ventricles, and decreased spine density (Chen et al., 2008). Collectively, these studies indicate that Nrg1 may play a more prominent role in the sensorimotor gating phenotype of SCZ, while its effects on activity and memory remain unclear.

**DISC1**

Disrupted-in-schizophrenia-1 (DISC1) was identified as a candidate gene when it was found to be disrupted by a balanced translocation that cosegregates with SCZ and related psychopathologies (Millar et al., 2000; 2001). DISC1 plays an important role in neurite outgrowth, cell migration, and cell signaling (Mackie et al., 2007). A mutant mouse model of Disc1 carrying a deletion variant displayed impairments in working memory, deceased mPFC volume, altered synaptic transmission in the hippocampus, and reduced dendritic growth in the dentate gyrus (Koike et al., 2006; Kvajo et al., 2008). An inducible Disc1 C-terminal fragment transgenic model exhibited abnormal spatial
working memory, deficits in social interaction, and decreased hippocampal dendritic complexity (Li et al., 2007). A model expressing the dominant negative C-terminal truncated Disc1 exhibited hyperactivity, disrupted PPI, depressive-like symptoms, and enlarged lateral ventricles (Hikida et al., 2007). Inducible expression of mutant human DISC1 also produced mice with enlarged lateral ventricles, deficits in spatial working memory, impaired social interaction, and hyperactivity (Pletnikov et al., 2007). And lastly, truncated Disc1 transgenic mice exhibit enlarged lateral ventricles, decreased cortical neurogenesis, increased immobility and reduced vocalization in depression-related tests, as well as impairment in latent inhibition (Shen et al., 2008). These studies demonstrate that while Disc1 clearly contributes to the pathophysiology of SCZ, the nature of the mutation has profound effects on the range of observed behavioral phenotypes.

**Dysbindin**

Several studies have implicated dysbindin (DTNBP1) as a SCZ candidate gene (Straub et al., 2002; Tang et al., 2003; Kirov et al., 2004). DTNBP1 binds to dystrobrevins, components of the dystrophin-associated glycol-protein complex (DGC), and is thought to play a fundamental role in regulating synaptic structure and signaling (Benson et al., 2001). Dtnbp1 deletion mice on a DBA/2J background strain were found to have increased anxiety and impaired social interaction, as well as deficits in working and recognition memory (Hattori et al., 2008; Takao et al., 2008). These mice also displayed increased freezing response to a conditioned stimulus, suggesting deficits in emotional and motivated learning and memory (Bhardwaj et al., 2009). These mice also had decreased dopamine levels, reduced steady state levels of snapin (synaptic priming regulator), and deficiencies in neurosecretion (Murotani et al., 2007; Feng et al., 2008;
However, a study of \textit{Dtnb1} KO mice on a C57Bl/6 strain showed no evidence of increased anxiety, although replicated the spatial learning and memory deficit (Cox et al., 2009). While \textit{Dtnb1} partially contributes to a SCZ phenotype, the extent remains unclear due to complications with the background strain.

\subsection*{22q11.2 deletion}

The 22q11.2 deletion is a rare chromosomal mutation spanning ~3 Mb that has also been associated with SCZ (Drew et al., 2011). 22q11.2 deletion syndrome (22q11DS) is characterized by a 25-fold increased risk for developing SCZ as well as cardiac and facial anomalies (Karayiorgou et al., 2010). This deletion syndrome also increases the risk of other psychiatric disorders, such as attention-deficit hyperactivity disorder, bipolar disorder, anxiety, and affective disorders (Murphy et al., 1999; Niklasson et al., 2001). 22q11.2 microdeletion carriers also show language delay, decreased full scale IQ, learning disabilities and mental retardation, and deficits in attention and working memory (Niklasson, 2001; Karayiorgou et al., 2010). Although this rare microdeletion is present in only 1-2\% of patients with SCZ, its high penetrance for the disease makes 22q11DS genetic models an excellent opportunity to investigate the pathogenesis underlying certain behavioral abnormalities present in SCZ and other related disorders (Karayiorgou et al., 2010).

A region of mouse chromosome 16 is homologous to the 22q11.2 region in humans, containing murine versions to all genes except \textit{CLTCL1}, with minimal reorganization of gene order (Paylor and Lindsay, 2006). Several mouse models have been generated and characterized with deletions that fall within or encompass the microdeletion (Drew et al., 2011). For example, the Df(16)A+/- mice show impairments in the acquisition of a delayed non-match to sample T-maze task that relies on spatial
working memory (Stark et al., 2008). These mice also display reduced synchronous activity between the dorsal hippocampus and medial prefrontal cortex during task acquisition, suggesting that deficits in communication between the hippocampus and prefrontal cortex may underlie working memory impairments (Sigurdsson et al., 2010). Another 22q11DS mouse model, Df(16)1+/-, showed weakened auditory thalamocortical connections in post-adolescent, but not pre-adolescent animals, mirroring the developmental timeline of behavioral impairments in PPI in these mice (Chun et al., 2014). This reduction in thalamocortical strength was specific to the auditory cortex, and was due to an unexpected increase in dopamine D2 receptors in the medial geniculate nucleus of the thalamus (Chun et al., 2014). Thalamocortical strength and PPI were normalized following acute administration of haloperidol, suggesting that the therapeutic effects of antipsychotic medications in SCZ could be due to targeting thalamic D2 receptors (Chun et al., 2014). Overall, the 22q11DS mouse models may highlight the SCZ-related behaviors, along with the associated mechanisms, linked to this chromosomal region.

**SIGNIFICANCE OF CURRENT RODENT MODELS**

The above discussion of current and popular rodent models for ASD and/or SCZ highlight the polygenic origin of these disorders. The literature suggests that, at least in the mouse, disruption of a single gene is typically not the cause of psychiatric disorder. However, study of these risk variants in genetically modified mice allow us the means to elucidate which behavioral domains are regulated by each candidate gene. Furthermore, we can investigate the specific molecular and cellular pathways regulated by each candidate gene that underlie the behavioral domain(s) in question. In this thesis work, we make the first attempt to understand the contribution of candidate gene **SAP97**...
to psychiatric disorders such as ASD and SCZ. Below, we discuss the background of
SAP97’s gene family, the membrane associated guanylate kinases. We discuss their
significance in synapse biology and the current evidence suggesting their role in
psychiatric disorders.

MEMBRANE ASSOCIATED GUANYLATE KINASES

In this section of the introduction, we discuss the membrane associated
guanylate kinases (MAGUKs), an integral group of synaptic genes known to play a
prominent role in a broad range of psychiatric disorders, including ASD and SCZ. We
choose to focus in depth on the MAGUK family as it is extensively expressed in the brain
and well conserved throughout evolution. Below, we provide a general background and
rationale for focusing on the contribution of MAGUKs to ASD, SCZ, and other related
psychiatric disorders.

MAGUK Subfamily Classification

The MAGUK protein family is classified phylogenetically in 10 subfamilies by
comparison of the genomic sequences of the core PDZ-SH3-GUK region and by the
supplemental domains they possess (Oliva et al., 2011). Of the ten MAGUK
subfamilies, members of the subfamilies DLG, CASK, MPP, CACNB, and MAGI are
expressed in the central nervous system (CNS) where they play various roles in the
formation and function of synapses (Laura et al., 2002; Jing-Ping et al., 2005; Deng et
al., 2006). Members of the ZO family are not expressed in neurons, but are present in
the brain where they play an import role in the formation and maintenance of the blood-
brain barrier (Wolburg and Lippoldt, 2002). The DLG and CASK subfamilies are the
most well-studied MAGUKs due to their clear role in synapse formation and function
(Oliva et al., 2011). While both subfamilies are also expressed in epithelial tissues and the peripheral nervous system, previous groups have focused on their function in the CNS (Oliva et al., 2011). For this body of work, we have chosen to focus on the DLG subfamily of MAGUKs.

**Expression Pattern of the DLG MAGUK Subfamily**

The DLG subfamily of MAGUKs (Dlg-MAGUK) is expressed in the CNS at all stages of development (Kim and Sheng, 2004; Funke et al., 2005). All members can be found presynaptically and postynaptically, however some are mainly found in the postsynaptic compartment of excitatory synapses and restricted to the postsynaptic density (Kim and Sheng, 2004; Funke et al., 2005). The Dlg-MAGUK family also differs in their temporal and spatial expression. PSD-95 is expressed at low levels during embryonic and early postnatal development, but is enhanced during postnatal development, and reaches maximum expression at adulthood (Hsueh and Sheng, 1998; Al-Hallaq et al., 2001). PSD-93 shows a similar expression profile to PSD-95 in the hippocampus (Sans et al., 2000). SAP102, however, is highly expressed in the hippocampus during the first postnatal week, remains stable by postnatal day P35, and decreases into adulthood (Muller et al., 1996; Sans et al., 2000). SAP97 displays an expression pattern opposite to PSD-95 and PSD-93 in the hippocampus and other brain tissues, where expression levels decrease from embryo to adult stages (Cai et al., 2008). This observation suggests that SAP97 participates in developmental processes of the nervous system (Cai et al., 2008).
The role of Dlg-MAGUKs in Synapse Formation and Function

The expression pattern of Dlg-MAGUKs during development suggests that they are involved in the regulation of synaptogenesis, a highly regulated process of building neural circuits. Studies done in mammals as well as Drosophila neuromuscular junction suggest that Dlg-MAGUKs are necessary for the clustering and stabilization of glutamate receptors once the pre- and postsynaptic sites have been contacted and stabilized by adhesion proteins (Chen and Featherstone, 2005; Waites et al., 2009). The participation of Dlg-MAGUKs in the maturation of mammalian synapses has been shown via overexpression experiments. Overexpression of PSD-95 and SAP97 increases the size of spines and the formation of multi-innervated spines in hippocampal neurons (Nikonenko et al., 2008; Poglia et al., 2010). Additionally, overexpression of SAP97 promotes dendritic growth and requires the binding to the AMPA receptor subunit (AMPAR), GLUA1 (Zhou et al., 2008). SAP97, PSD-95, and SAP102 overexpression also enhance the expression of presynaptic proteins such as synatophisin, synapsin, and bassoon (Regalado, 2006). The overexpression of several PSD-95 interacting proteins also has an effect in spine morphogenesis (Lee et al., 2008).

While Dlg-MAGUK overexpression studies have been informative, loss of function experiments have not produced consistent results. Knockout mice for Psd-95, Psd-93, or Sap102 do not have defects in synapse development (Miguad et al., 1998; McGee et al., 2001; Cuthbert et al., 2007). Psd-95 mice carrying a targeted mutation that introduces a stop codon in the third PDZ domain show only altered dendritic spine density in the hippocampus (Vickers et al., 2006). Moreover, acute knockdown of Psd-95 using shRNA does not produce defects in dendritic spine density in primary hippocampal cultures (Elias et al., 2006). Sap97 knockout animals have not been possibly to study, as the mutant mice display a cleft palate and die prematurely
(Caruana and Bernstein, 2001). However, studies conducted on neuronal cultures from Sap97 knockout animals do not show any defect in glutamate receptor distribution or AMPAR mediated currents (Howard et al., 2010). As it has been demonstrated that PSD-95, PSD-93, and SAP102 can compensate for each other, the lack of defects observed in the knockout mice can be explained by functional redundancy (Elias et al., 2006; Howard et al., 2010).

The Dlg-MAGUK family also plays an integral role in glutamate receptor clustering and trafficking, both of which are essential processes for the efficiency and plasticity of glutamatergic synapses. PSD-95 is the most well-studied Dlg-MAGUK for its role in clustering and trafficking glutamate receptors, especially NMDARs (Elias and Nicoll, 2007). PSD-95 is also implicated in the trafficking of AMPARs, although indirectly via transmembrane AMPAR regulatory proteins (TARPs) (Chen et al., 2000).

SAP97 has been implicated in receptor trafficking by various studies. Sap97 occurs as two splice variants (α and β). SAP97α is mostly found at the postsynaptic density, while SAP97β is found in the perisynaptic region (Oliva et al., 2011). Both splice variants are able to bind the GLUA1 AMPAR (Oliva et al., 2011). Current evidence suggests that the ratio between these two isoforms can regulate the distribution of GLUA1, and as a result, synaptic strength (Waites et al., 2009). Acute overexpression of SAP97β promotes trafficking of AMPARs and NMDARs to the synapse in immature pyramidal neurons but not in mature neurons (Howard et al., 2010). However, chronic overexpression in vivo during development enhances synaptic transmission in mature neurons (Howard et al., 2010). These findings suggest that SAP97β plays a role in receptor trafficking during development rather than in adult plasticity.
THE CONTRIBUTION OF MAGUKs TO PSYCHIATRIC DISORDERS

The prominent role of Dlg-MAGUKs at glutamatergic synapses and in synaptic plasticity suggests that mutations in MAGUK genes would be involved in synaptic-related disorders, notably ASD and SCZ. Below, we outline the evidence implicating the Dlg-MAGUKs in ASD, SCZ, and related neuropsychiatric disorders, and discuss the current genetic models available.

Sequencing techniques and analytics have been used to identify PSD-95 mutations in ASD and SCZ patients. Whole-exome sequencing studies of SCZ and ASD patients show disrupted mutations of proteins located in excitatory synapses of the PSD, such as NMDAR and PSD-95 (Fromer et al., 2014; Purcell et al., 2014). Studies from postmortem SCZ patients reveal a significant decrease in PSD-95 mRNA and protein expression levels in the dorsolateral and dorsomedial prefrontal cortex, suggesting an association between PSD-95 dysfunction and SCZ (Ohnuma et al., 2000; Catts et al., 2016). PSD-95 is also involved in a network of interactions with high-risk ASD genes that include SHANK, HOMER, NLGN, and FMR1 (Gilman et al., 2011; Tsai et al., 2012; De Rubeis et al., 2014). Furthermore, PSD-95 is a candidate gene disrupted in intellectual disability, a cognitive disorder characterized by a reduction of dendritic spines (Lelieveld et al., 2016). PSD-95 has direct interactions with intellectual disability-related proteins within the excitatory PSD that include ARC and IL1RAPL1, which are responsible for regulating spine density and function (Pavlowsky et al., 2010; Valnegri et al., 2011; Fernández et al., 2017). To model with in mice, null animals have been made and characterized. Feyder et al. characterized the Psd-95 knockout mice and the mice exhibit increased repetitive behaviors, abnormal communication, hyper-social behavior, impaired motor coordination, and increased stress-reactivity and anxiety-related...
responses (Feyder et al., 2010). The extent to which the *Psd-95* null mice faithfully report on the contribution of *Psd-95* to psychiatric disease is an open question.

Mutations in the gene *SAP102* are found in patients with X-linked mental retardation (Tarpey et al., 2004). The mutations identified introduce premature stop codons within or before the third PDZ domain, and it is likely that this impairs the ability of SAP102 to interact with NMDAR and other proteins involved downstream of NMDAR signaling pathways (Tarpey et al., 2004; Zanni et al., 2009). The disruption of the ability to bind NMDARs may lead to altered synaptic plasticity and explain the intellectual impairment observed in individuals with *SAP102* mutations (Tarpey et al., 2004; Zanni et al., 2009). Cuthbert et al. report the first characterization of *Sap102* KO mice, and find that *Sap102* mutant mice display cognitive deficits with a specific spatial learning deficit (Cuthbert et al., 2007).

A variety of evidence implicates *SAP97* in the etiology of ASD and SCZ. Single nucleotide polymorphisms in *SAP97* have been linked to an increased risk of SCZ in males, which supports the possible involvement of *SAP97* gene variation in the susceptibility to SCZ and in the genetic basis for sex differences in the disorder (Uezato et al., 2012). The human *SAP97* gene resides in the chromosomal region 3q29, where multiple genome-wide analyses on copy number variations found an excess of microdeletions in SCZ (Kirov et al., 2011; Levinson et al., 2011; Kushima et al., 2016; Marshall et al., 2016). A meta-analysis demonstrated the 3q29 deletion confers a 40-fold increased risk for SCZ (Mulle, 2015). Additionally, individuals with 3q29 microdeletions spanning the *SAP97* locus display autism and intellectual disability (Quintero-Rivera et al., 2010). In another study of the expression levels of multiple postsynaptic density proteins, including PSD-95, PSD-93, and SAP102, the authors found a specific decrement in the level of *SAP97* in post mortem frontal lobe from
schizophrenic patients (Toyooka et al., 2002). SAP97 levels were decreased to less than half that of control levels, and concordantly, its binding partner GLUA1 was similarly decreased in the same brain region (Toyooka et al., 2002). SAP97 is also the only member of the Dlg-MAGUK family that directly binds to the extreme C-terminus of the GLUA1 AMPAR, a subunit that promotes dendritic growth and patterned synaptic innervation (Zhou et al., 2008; Zhang et al., 2017). Thus, it is plausible that defects in these SAP97-dependent mechanisms contribute to an ASD and SCZ phenotype. However, unlike the other members of the Dlg-MAGUK family, the issue has been difficult to study because Sap97 knockout mice die a few days after birth from a craniofacial defect (Caruana and Bernstein, 2001).

STATEMENT OF MOTIVATION AND HYPOTHESIS

The goal of this thesis is to understand the direct contribution of SAP97 to neuropsychiatric disorders such as ASD and SCZ. While mouse models for Psd-95, Psd-93, and Sap102 have been previously generated and characterized behaviorally, Sap97 null animals have been impossible study. In Chapter 2, we determine whether Sap97 directly contributes to the pathophysiology of ASD and SCZ, and in what capacity, by generating mice that have a conditional knockout of Sap97 targeted to neurons using the Cre-loxP system. Given the substantial evidence supporting the involvement of the Dlg-MAGUK family in neuropsychiatric disorders, we hypothesized that loss of Sap97 would contribute partially to the endophenotypes of ASD and/or SCZ-like phenotype. In order to test this hypothesis, we subjected the Sap97 conditional knockout mice to a battery of behavioral tests and biochemical studies to screen for an ASD or SCZ-like phenotype. We report that loss of Sap97 results in subtle sex-specific behavioral abnormalities and alters the expression of SCZ risk-associated gene.
transcripts in the hippocampus. This thesis works provides the first broad behavioral and transcriptomic characterization of Sap97 deficient animals, and provides a stepping-stone for understanding the molecular mechanism by which SAP97 contributes for neuropsychiatric disorders.
CHAPTER 2: SAP97 REGULATES BEHAVIOR AND EXPRESSION OF SCHIZOPHRENIA RISK ENRICHED GENE SETS IN MOUSE HIPPOCAMPUS

SUMMARY

Synapse associated protein of 97KDa (SAP97) belongs to a family of scaffolding proteins, the membrane-associated guanylate kinases (MAGUKs), that are highly enriched in the postsynaptic density of synapses and play an important role in organizing protein complexes necessary for synaptic development and plasticity (Cai, 2006; Elias and Nicoll, 2007; Zhou et al., 2008; Chen et al., 2015; Zeng et al., 2016). Large-scale genetic studies have implicated MAGUKs in neuropsychiatric disorders such as intellectual disability, autism spectrum disorders (ASD), and schizophrenia (SCZ), but knock-out mice have been impossible to study because the Sap97 null mice die soon after birth due to a craniofacial defect. We studied the transcriptomic and behavioral consequences of a brain-specific conditional knockout of Sap97 (SAP97-cKO). RNA sequencing (RNAseq) from hippocampi from control and SAP97-cKO male animals identified 67 differentially expressed transcripts, which were specifically enriched for SCZ-related genes. Subjecting SAP97-cKO mice to a battery of behavioral tests revealed an anxiety-like phenotype present in both male and female SAP97-cKO animals, as well as a male-specific cognitive deficit and female-specific motor learning deficit. These data suggest that loss of SAP97 regulates behavior, and may contribute to some of the endophenotypes present in SCZ. The SAP97-cKO mouse serves as a novel model system for interrogating aspects of the cellular and molecular defects underlying SCZ and other related neuropsychiatric disorders.
INTRODUCTION

Intellectual disabilities and neuropsychiatric behavioral disorders affect about 17.9% of individuals over their lifetime and interfere with the ability of people to experience a fulfilling and productive life (nimh.nih.gov). Some of these disorders are clearly developmental. For example, autism spectrum disorders (ASD) are characterized by impairments in social interaction and communication, and by restricted, repetitive behaviors and about 1% of children show signs and symptoms that lead to the diagnosis of ASD (Ebert and Greenberg, 2013; Uchino and Waga, 2013). Schizophrenia (SCZ) is another mental disorder that is characterized by disordered thought processes and disturbed emotional responsiveness (Grabrucker et al., 2014). The symptoms of SCZ usually appear during young adulthood, with an overall prevalence of about 0.7% (Fromer et al., 2014; Grabrucker et al., 2014). Technological advances have brought unprecedented insights into the genetic architecture of these and many other neuropsychiatric disorders (De Rubeis et al., 2014; Fromer et al., 2014; Zhao et al., 2014; Xing et al., 2016).

Exome-sequencing technology has allowed us to systematically scan genes for de novo mutations at the single-base resolution, potentially offering insights into risk-determining genes (Ghosh et al., 2013; Fromer et al., 2014). Whole-exome sequencing results from patients with ASD or SCZ reveal significantly enriched copy number variant (CNV) mutations in the synaptic gene set (Fromer et al., 2014). Among the most prevalent synaptic genes that have been uncovered in large-scale genomic studies have been alterations in the neurexins/neurolignins along with the PROSAP/SHANK family. Various genetically manipulated mice of these gene families recapitulate some of the behavioral features of ASD, SCZ, and intellectual disability (Peça et al., 2011; Wang et al., 2011b; Kouser et al., 2013; Han et al., 2014). However, none of these models
completely phenocopy disease in humans, consistent with the polygenic origin of these disorders.

Another important group of synaptic genes that has been implicated to be involved in ASD or SCZ is the Discs-large (Dlg) family of membrane associated guanylate kinases (MAGUKs) (Kristiansen et al., 2006; Funk et al., 2009; Feyder et al., 2010; Xing et al., 2016; Winkler et al., 2017). The Dlg family is the most comprehensively studied family of MAGUKs, and is comprised of PSD-95, PSD-93, SAP102, and SAP97. They share a common domain structure comprised of three PDZ domains, along with an SH3 and GUK domain. The Dlg-MAGUK family directly binds to many proteins in the postsynaptic density (i.e. glutamate receptor subunits, TARPS, and neurexin/neuroligin clusters), and regulates synaptic nanoscale structure and synaptic transmission (Bats et al., 2007; Mondin et al., 2011; Giannone et al., 2013). Mice with a targeted deletion of Psd-95, Psd-93, and Sap102 show a range of phenotypes also displayed by individuals with psychiatric disorders (Cuthbert et al., 2007; Feyder et al., 2010; Winkler et al., 2017).

A variety of evidence implicates SAP97 in the etiology of ASD and SCZ: 1) single nucleotide polymorphisms in SAP97 have been linked to an increased risk of schizophrenia in males (Uezato et al., 2012), 2) individuals with microdeletions spanning the SAP97 locus display autism and intellectual disability (Quintero-Rivera et al., 2010), and 3) a study of expression levels of multiple postsynaptic density proteins found a specific decrement in the level of SAP97 in post mortem frontal lobe from schizophrenic patients (Toyooka et al., 2002). SAP97 is also the only member of the Dlg-MAGUK family that directly binds to the extreme C-terminus of the GLUA1 AMPA receptor (AMPAR), a subunit that promotes dendritic growth and patterned synaptic innervation (Zhou et al., 2008; Zhang et al., 2017). Thus, it is plausible that defects in these SAP97-
dependent mechanisms contribute to a ASD and SCZ phenotype. While these findings advocate for the participation of SAP97 in the etiology of neuropsychiatric disorders, the issue has been difficult to study because Sap97 knockout mice die a few days after birth from a craniofacial defect (Caruana and Bernstein, 2001).

In order to determine whether SAP97 directly contributes to the pathophysiology of ASD and SCZ, we generated mice that have a conditional knockout of Sap97 targeted to neurons using the Cre-loxP system. We then subjected these mice to a battery of behavioral tests and biochemical studies to screen for an ASD or SCZ-like phenotype. Overall, our results suggest that loss of Sap97 results in sex-specific behavioral abnormalities as well as regulates transcripts of SCZ risk-related genes.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee. The Cre-loxP system was used to generate a Sap97 conditional knockout (cKO) mouse. SAP97fl/- mice were generated as previously described (RRID: IMSR_JAX:013097). Nestin-cre+/- mice on a C57Bl/6 background were purchased from Jackson Labs (stock number 003771, RRID: IMSR_JAX:003771). Nestin-cre+/-; SAP97fl/- mice were generated by crossing male Nestin-cre+/- with female SAP97fl/- mice. Nestin-cre+/-; SAP97fl/- male mice were then crossed with female SAP97fl/- mice to generate Nestin-cre+/-; SAP97fl/fl (SAP97-cKO) and littermate control animals. Littermate control animals (genotype: Nes-cre+/-, SAP97fl/fl, and wild-type) were averaged and compared to cKO animals. Genomic DNA was extracted from tail snips using the Phenolcholoroform acetate method to confirm genotypes. The primers used for genotyping were as follows: Sap97 flox fwd-
Behavioral Experiments

Open Field

Assessment of general exploratory behavior and anxiety were evaluated using the open field paradigm. Mice were placed in a white, opaque plexiglass box (40cm x 40cm) and were given 15 minutes to explore the apparatus. Exploratory locomotor activity (total distance traveled, average speed, and mean distance from border) was scored using the Any-MAZE tracking software (San Diego Instruments, San Diego, CA, RRID:SCR_014289).

Elevated Plus Maze

Assessment of anxiety-like behaviors was evaluated using an elevated plus maze (Coulbourn Instruments, Whitehall, PA). The mouse was initially placed in the center “free zone”, and was allowed to freely explore the apparatus for the 5-minute trial time. Time in the open arms versus the closed arms, as well as number of entries to these arms, was measured using the Any-MAZE tracking software.

Accelerating Rotarod

Assessment of motor learning and motor coordination was evaluated using the accelerating rotarod (Ugo Basile, Varese, Italy). The starting acceleration was 4 rpm, and accelerated to 40 rpm over a 5-minute trial time. Mice underwent 3 trials per day for 4 consecutive days, for a total of 12 trials. Latency to fall from the rod was manually measured and compared across the 4 days.
**Novel Object Recognition (NOR)**

Assessment of cognition was evaluated using the NOR paradigm. The testing apparatus was a white, opaque plexiglass box (40cm x 40cm). On day 1, mice were habituated to the testing apparatus for 15 minutes. On day 2, the mice were reintroduced to the testing apparatus and allowed to explore two identical objects equally spaced from the walls of the apparatus (objects A and A’) for 5 minutes and the animal was then removed. Any-MAZE tracking software was used to measure the time spent investigating each object, and a preference index (PI) was calculated by dividing time spent investigating A’ by time spent investigating A (A’/A). One hour later after identical object exploration, the mouse was placed back in the testing apparatus where one of the identical objects had been replaced with a novel object that differed in shape, color, and texture (object B). Again, the mouse was given 5 minutes to explore the two objects, and preference for the novel object was calculated by dividing time spent investigating B by time spent investigating A (B/A). Significant preference for the novel object was assessed by comparing the PI from the training phase to the PI from the testing phase.

**Three Chambered Social Choice**

Assessment of sociability was evaluated using the standard three-chambered social choice paradigm. A white, opaque plexiglass rectangular box was used, with three partitions (each 20cm x 40cm). The mouse was first given 5 minutes to habituate to the empty apparatus. After habituation, into the left and right compartments was placed either with an inanimate object (non-social zone) or an age and sex-matched C57Bl/6 mouse (social zone). The object and mouse were placed under clear, plexiglass cylinders with perforations to allow odor detection. During the testing phase, the test mouse was allowed five minutes to explore either zone. Time in each zone was
measured using the Any-MAZE tracking software and sniffing time of either the inanimate or social target was manually scored. Social zone preference was calculated by dividing social zone time by total zone exploration time, and social sniffing preference was calculated by dividing time spent sniffing the social target by total sniffing time.

**Cued Fear Conditioning**

Assessment of amygdala dependent fear learning was evaluated using the cued fear conditioning paradigm. Fear conditioning paradigms pair an emotionally neutral stimulus, such as light/tone (conditioned stimulus or CS) with an aversive stimulus, such as footshock (unconditioned stimulus or US), leading to the expression of a threat response (freezing) to presentation of the neutral CS alone. For these experiments, the context was altered between training (context A) and testing (context B) to isolate the light/tone (CS) cued response from the hippocampal dependent contextual response.

The cued fear conditioning paradigm used in this study was modified from experiments described in (Newton et al., 2004; Wolff et al., 2014). The CS consisted of simultaneous auditory (75dB, white noise, 20s) and light stimuli (yellow light pulses, 20s, flickering at 4 Hz) generated by built in audio and light stimuli generators (Med Associates, Fairfax, VT). The US consisted of a footshock (1.05mA, 1.5s) delivered through the metal grid floor. During CS-US pairings, the US was delivered immediately following the cessation of the CS. On day 1 of this paradigm, animals underwent fear conditioning training in context A, a rectangular conditioning chamber (21.6 cm × 17.8 cm × 12.7 cm) with Plexiglas and metal walls, and a metal grid floor (Med Associates, Fairfax, VT). Animals were allowed to freely explore the chamber for 1 min before experiencing 3 CS-US pairings (60s interstimulus interval).
One min after the final CS-US pairing (5 min total), mice were removed from context A and placed back in their home cage. Twenty-four hours later (day 2) animals underwent behavioral testing to measure freezing in context B, a custom made triangular conditioning chamber with black striped Plexiglas walls and a smooth, opaque black plastic floor, scented with organic vanilla extract. Mice were allowed to freely explore the chamber for 1 min before experiencing 3 presentations of the CS alone (60s, interstimulus interval). Again, animals were removed from context B after a total of 5min. During testing, freezing behavior was scan sampled every 5th second from the onset of the first CS presentation to the end of the trial (4 min total). Freezing was defined as a total lack of movement aside from respiration at the instant of every 5th second. The total number of freezing spells was then divided by total observations to generate a freezing percentage per animal.

**Biochemistry**

Mice were anesthetized with a pentobarbital solution and decapitated. The brain was removed, and each hemisphere of the cerebellum, cerebral cortex, and hippocampus was dissected. One hemisphere was rapidly transferred to a mortar and pestle prechilled on dry ice, and ground into a fine powder to be processed for RNA extraction by the RNeasy mini kit (Qiagen, Catalogue #74134) according to the manufacturer’s instructions. Once RNA extraction was complete, conversion to cDNA was done using the iScript Supermix (Bio-Rad, Catalogue #1708841, Hercules, CA). The other hemisphere was transferred to a dounce prechilled on ice, and lysed in 1% Triton-X lysis buffer with protease and phosphatase inhibitors for generation of protein lysates.
**Antibodies**

The following antibodies were used in this study as follows: immunoblotting of SAP97 (Thermo Fisher Scientific, catalogue # PA1-741, RRID:AB_2092020);
immunoblotting of PSD95 (NeuroMab, catalogue # 75-348, RRID:AB_2315909);
immunoblotting of PSD93 (NeuroMab, catalogue # 75-284, RRID:AB_11001825);
immunoblotting of SAP102 (NeuroMab, catalogue # 75-058, RRID:AB_2261666);
immunoblotting beta-actin (Cell Signaling Technology, catalogue # 3700 (mouse), RRID:AB_2242334 or Sigma-Aldrich, catalogue # A2066 (rabbit), RRID:AB_476693).
Secondary antibodies for immunoblots (IRDye) were purchased from Li-COR (Catalogue # 925-32210, RRID: AB_2687825 and Catalogue # 925-68021, RRID: AB_2713919).

**Western Blot**

Western blot was performed according to standard procedures (David and Kalb, 2005; Kim et al., 2005; Mojsilovic-Petrovic, 2006).

**Quantitative PCR**

Quantitative real-time PCR (qPCR) was carried out as previously described using the delta delta Ct method to calculate relative gene expression levels (Livak and Schmittgen, 2001). Ribosomal S17 and S18 (RS17, RS18) were used as reference genes. Each reaction consisted of cDNA, primers, and Power SYBR Green PCR Master Mix (Applied Biosystems, Catalogue # 4367659, Waltham, MA) with a total 25uL reaction volume. Melting curve analysis of the target sequences showed that all primers used in this study generated amplification of a single peak, without primer-dimer artifacts. Primer and cDNA concentrations were optimized prior to use in qPCR experiments. Each qPCR experiment consisted of 4-6 biological replicates, as well as
three technical replicates per sample. The primers used for qPCR included: Glua1 fwd-CCCTGAGAGGTCCCGTAAAC rev- GTCAGAGGCACTGGTCTTGT; Glua3 fwd-CCATGCTCTTGTGAGCTTCG rev- AGTCCACCTATGCTGATGGT; Glua4 fwd-TGAATGAAACAGCCTCTGTGG rev- AGGCTTGCCATTGCTTTCTT; Nrcam fwd-AAGACCGGCTGGACTTGGGG rev- GGCTTGCCATTGCTTTCTT; Huwe1 fwd-GTTGGGGATTTCACCAGAG rev- CAGTCTGCAGGACTTCTGATT; Pten fwd-CCTGAGAAAGACTTGAAGGG rev- CTGTGCAACTCTGAGTTAAA; Adam10 fwd-GGTGGGGAGGTCTGGAACG rev- CTGTGGAAGCCTGACTGCTCT; Was fwd-TCAGCTGAACAGACCCCTG rev- CATGCATCAGCCGACCTACT; Erbb4 fwd-ACCCAGGAGGGTAACG rev- TGGTAAAGTAGAATGGGCC; Sema4C fwd-GGTGGCCGAGTCAACCG rev- TTCAGTCAGCAGCCTCTT; Kcna3 fwd-TCCGAAAAGCCCGAGTAAC rev- CTGTGAGGGTCCCTGTCTTG; Kcna4 fwd-CACCGCTGGGAAATGGAAGGT rev- GGAAGGTGGTGACGCAGT; Kcna5 fwd-TAGGACACTGGCTGGGAATGG rev- ACGCACAAGGCAGCTCAA; Gng13 fwd-TTGCTGTCTTGGACACCCCTG rev- TTCCTCTTTGAGCCACTT; Fzd7 fwd-AGAACCCTCGGCTACAACGTG rev- ACCGAACAAAGGAAAGAATGC; Dlgap4 fwd-TTGGCTTGTCTCAGCAGCTCC rev- TGATGAACATGCACTGAG; Ctnn1 fwd-CAGTTGGCTGAGAATGAC rev- ACCTGTGAAACAGAGGCTCC; Calm3 fwd-GAGTAACCTCGAGCAGGTG rev- GAAGGCTTCTTGAACTTG; Kcnc1 fwd-CTACGCGCGGTATGTTGG rev- TCGGTCTTGGTCAGATGGG; Axin2 fwd-CAGGCGCAAGGAAAT rev- AGGCTCTTCTCCTTACAGCA; Lef1 fwd-GTCGACTTCAGGGTGAAGAAGAAG rev- TGCTGTCAAGGTCTGTG; β-catenin fwd-GTCAGTGCAGGAGGCC rev- CAGGTCAGGCTTCATAG; Runx2 fwd-GCCCTGAAGTTGTAGCC rev- GTTCTCATCATCCGGCC; Kalirin fwd-GAGTTCAGGGGTGGGAT rev- CCATCATCCGAAAGATCCTC; Nudel1 fwd-
RNA Sequencing

RNA was isolated from four control and four SAP97-cKO male hippocampi, quality evaluated by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and sequenced with an Illumina HiSeq 4000 High-Throughput Sequencing System. The RNA-seq reads were aligned to the mouse genome mm10.GRCm38.p5 using STAR version 2.5.3a (Dobin et al., 2012). Next, normalization and quantification were performed with the PORT version 0.8.2a-beta pipeline (http://github.com/itmat/Normalization) which first removes reads that map to ribosomal RNA sequences or mitochondrial DNA and then uses a read re-sampling strategy for normalization to account for batch effects and differences in sequencing depth among the samples. After the normalization procedure, the gene level quantification was done by PORT with respect to the Ensemblv90 annotation. The normalized count of reads mapping to exon 10 of Sap97 showed almost 30-fold reduction from an average of 242 in the control samples to an average of 8 in the SAP97-cKO samples affirming the efficacy of the knockdown procedure. The differential expression analysis was performed using the R Bioconductor package limma-voom (Law et al., 2014; Ritchie et
al., 2015). The top 566 genes with FDR < 0.5 and fold-change of greater than 1.6 were used for general pathway enrichment analyses, which were performed using Ingenuity IPA.

**Analysis of overlap between differentially expressed genes and risk-associated disease genes**

The significance of overlap between the set of differentially expressed genes (DEGs) and risk-associated ASD, SCZ, and other neuronal disorder genes was analyzed using non-parametric analysis. The ASD gene list was chosen from research by Silvia De Rubeis et al. (De Rubeis, et al., 2014), while the SCZ and ataxia gene lists were chosen from online resources (szdb.org, genedx.com). Details of the lists chosen and overlap analysis are discussed in results section. The mean and variance of the corresponding hypergeometric distribution were calculated. The p value of the significance of the overlap was estimated using the hypergeometric probability test.

**Statistics**

Data were analyzed using Prism (GraphPad Software, La Jolla, CA, RRID:SCR_015807). Significant differences within groups were determined using either Student’s t-test, one-way ANOVA followed by Tukey’s test for multiple comparisons, or repeated-measures two-way ANOVA followed by Tukey’s test for multiple comparisons. For all tests except for RNAseq, the significance threshold was set to p<0.05. The significance threshold for DEGs in the RNAseq experiment was set to FDR <0.25.

**RESULTS**

*Targeted deletion of SAP97 to neurons*
Global Sap97 knockout mice have been generated, but die soon after birth due to a craniofacial defect. In order to study the effect of loss of Sap97 on neuronal development and behavior, we conditionally knocked out Sap97 by crossing Nestin-cre mice with Sap97 floxed mice (see Methods). SAP97-cKO mice were born at Mendelian ratios and were grossly normal. At two months of age, we harvested tissue from the cerebellum, hippocampus, and cortex from control and SAP97-cKO animals of both sexes and prepared protein lysates for western blot analysis. In all three brain regions, the abundance of SAP97 was significantly reduced (Cerebellum: Ctrl 3.998 ± 0.9833, n=4; SAP97-cKO 1.373 ± 0.348, n=5, p=0.0279; Hippocampus: Ctrl 4.252 ± 0.6751, n=5; SAP97-cKO 1.215 ± 0.4314, n=5, p=0.0053; Cortex: Ctrl 0.287 ± 0.08557, n=5; SAP97-cKO 0.06179 ± 0.01072, n=5, p=0.0311), indicating that we successfully generated cKO animals (Figure 1A-D). Additionally, in order to ensure male and female animals had comparable expression of SAP97, we harvested tissue from the cerebellum, hippocampus, and cortex from male and female C57Bl/6 animals and prepared protein lysates for western blot analysis. In all three brain regions, the abundance of SAP97 was not significantly different between male and female animals (Cerebellum: Male 0.3029 ± 0.03097, n=4; Female 0.3162 ± 0.03791, n=4; Hippocampus: Male 0.3296 ± 0.03764, n=4; Female 0.3135 ± 0.03483, n=4; Cortex: Male 0.3272 ± 0.0134, n=4; Female 0.257 ± 0.04237, n=4) (Figure 1E-F).

No apparent compensation by other DLG-MAGUK family members

Previous work shows that the Dlg-MAGUKs (PSD-95, PSD-93, SAP102, and SAP97) can have redundant functions in electrophysiological assays. In order to examine whether loss of SAP97 led to compensatory changes in the abundance of the other Dlg-MAGUK family members, we measured total protein levels in the cerebellum,
hippocampus, and cortex. There was no significant difference in the abundance of any other Dlg-MAGUK at the protein level in control versus SAP97-cKO male or female animals (Figure 2). These data suggest that if members of the Dlg-MAGUK family compensate for the lack of SAP97, they do so without a change in overall abundance.

**No changes in gene expression level of known SAP97 binding partners or interactors**

SAP97 is a scaffolding protein that allows for a large number of protein-protein interactions. Thus, the absence of SAP97 could potentially affect the expression level of numerous proteins. To determine whether loss of Sap97 contributes to changes in expression levels of other identified members of the postsynaptic density, we conducted a directed qPCR screen. We measured mRNA levels firstly of all AMPAR subunits, as SAP97 is known to be the only Dlg-MAGUK to directly bind GLUA1. mRNA levels of Gluα1, Gluα3, and Gluα4 remained unchanged in the three brain regions that were probed (cerebellum, hippocampus, and cortex) (Figure 3A). Results from Gluα2 were highly variable and thus removed from the study.

We next sought to determine whether the levels of other genes known to interact with Sap97 were affected by loss of Sap97. We measured mRNA levels of 16 genes in the hippocampus. From our selection of 16 genes, we observed no differences in the mRNA expression level between control and SAP97-cKO animals (Figure 3B). We also measured mRNA levels of 4 of these 16 genes in the cerebellum and cortex and observed no differences in the mRNA expression level between control and SAP97-cKO animals (Figure 3B). These results would suggest that the abundance of genes from our selection is not significantly regulated by Sap97 expression.
SAP97 also shares direct and indirect binding partners with DISC1, a gene with strong association with neuropsychiatric disorders. Our lab has previously shown that SAP97 and DISC1 contribute to maintaining Wnt/β-catenin signaling within a homeostatic range. In order to address whether loss of Sap97 has an effect on the Wnt/β-catenin pathway in the SAP97-cKO mice, we measured mRNA levels of 4 pathway-related genes in the hippocampus, and 2 of these genes in the cortex. In both brain regions, we observed no differences in the mRNA expression level between control and SAP97-cKO animals (Figure 3C). We also measured mRNA levels of 7 Disc1 pathway-related genes in the cortex and observed no differences in expression between control and SAP97-cKO animals (Figure 3D). These results would suggest that the abundance of genes from our selection is not significantly regulated by Sap97 expression.

Identification of SAP97-regulated transcripts in the hippocampus

Given that we observed no group differences in our directed qPCR screen, we sought a broader, unbiased approach by performing RNAseq analysis on hippocampi from SAP97-cKO and control mice (n = 4 per group). For each animal, we verified the presence or absence of SAP97 by western blot on brain tissue before submitting hippocampal samples for sequencing.

A total of 66 genes were found to be significantly downregulated in the SAP97-cKO animals as compared to control hippocampi (FDR < 0.25) (Figure 4B, Table 1). In contrast, only one gene was upregulated in the hippocampi of SAP97-cKO animals as compared to control hippocampi. Gene ontology analysis of the DEGs revealed enrichment for numerous cellular and molecular functional categories, including those related to “Cell Morphology,” “Cellular Development,” and “Cell-To-Cell Signaling and
Interaction” (Table 2A-B). Additionally, the top enriched ID Associated Network Functions included “Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function,” and “Developmental Disorder, Embryonic Development, Organ Development” (Table 2C). Gene ontology terms to describe gene products known to be associated with ASD or the neurexin-neuroligin-SHANK complex in mice frequently include “Cell Communication” and “Nervous System Development”, which overlaps with the findings in our RNAseq study (Patel et al., 2015). Previous studies that have conducted RNAseq on SCZ patients and performed gene ontology analysis on the resulting DEGs have identified regulation of the actin cytoskeleton as a key pathway (Zhao et al., 2014). While the actin cytoskeleton was not directly implicated by our RNAseq study, it is essential for many of the gene ontology analysis terms listed in our data set. As proper arrangement of the actin cytoskeleton is essential for neuronal cell maturation and migration, neurite outgrowth, and maintenance of synaptic density and plasticity, dysregulation of these pathways in the nervous system could have severe consequences in psychiatric disorders such as SCZ.

Schizophrenia risk enrichment in DEG set

In order to determine whether the DEG set had a significant overlap with genes implicated in psychiatric disorders such as ASD and SCZ, we compared our DEG set with disease-related gene databases. For determining overlap with ASD-related genes, we used the gene set previously generated from the transmission and de novo association test (TADA), which consists of 107 genes. When we matched our DEG list to the TADA ASD gene list, we did not find the match percentage to be significant based on the hypergeometric distribution (Distribution mean = 0.30, standard deviation = 0.30; SAP97-cKO DEG 0.0) (Figure 4C). We next chose to compare our DEG list to SCZ risk-
related genes found from SZDB: A Database for Schizophrenia Genetic Research (szdb.org). The distilled list of genes from this database gives a score for each gene based on criteria such as convergent functional genomics, copy number variation, differential expression, genome wide association study, and linkage and association studies. The more categories a certain gene is implicated in, the higher the score for that gene. Based on this model, we chose the top 1,000 genes from this database to match to our SAP97-cKO DEG list. Interestingly, we found the SAP97-cKO DEG list to have a significant amount of overlap to the SZDB list based on the hypergeometric test (Distribution mean = 2.79, standard deviation = 1.63; SAP97-cKO DEG 13.43, \( p=0.0018 \)) (Figure 4C). Finally, we matched our SAP97-cKO DEG list to ataxia risk-related genes as a negative control, as ataxia is not classified as a neuropsychiatric disorder and SAP97 has not previously been implicated in ataxia. We used a list of ataxia risk-related genes compiled from GeneDx, whose clinical team compiled using multiple sources, including Online Mendelian Inheritance in Man (OMIM), Human Gene Mutation Database (HGMD), and Human Phenotype Ontology (HPO) terms. The total number of genes in this list was 993, which would also allow us to control for the size of the SCZ gene list used. When we compared our SAP97-cKO DEG list to the GeneDx ataxia gene set, we found no significant match percentage (Distribution mean = 2.77, standard deviation = 2.65; SAP97-cKO DEG 4.48) (Figure 4C). Together, these results suggest that SAP97-cKO DEGs are specifically enriched for SCZ risk-related genes.

**Behavioral analysis of SAP97-cKO mice**

Next, we performed a battery of behavioral tests to screen for behavioral deficits in the SAP97-cKO mice.
Anxiety-like phenotype in SAP97-cKO mice

We first performed the open field test to examine general ambulation and center exploration behavior. In the males, we observed no change in the total distance traveled (Ctrl 64.8 ± 2.102, n = 29; SAP97-cKO 61.76 ± 3.763, n = 19) (Figure 5A) or the speed of the animals (Ctrl 0.07214 ± 0.002259, n = 29; SAP97-cKO 0.06847 ± 0.004183, n = 19) (Figure 5B), but saw a reduction in the distance from the border (Ctrl 0.06531 ± 0.001558, n = 29; SAP97-cKO 0.05939 ± 0.00237, n = 19, p=0.0346) (Figure 5C). This indicates that the male SAP97-cKO mice stay closer to the perimeter of the apparatus as compared to littermate control, implying that while SAP97-cKO animals do not have a basic impairment in movement, they may have an anxiety-like phenotype. Female SAP97-cKO animals exhibited decreases in total distance traveled (Ctrl 64.39 ± 3.495, n = 24; SAP97-cKO 52.1 ± 4.519, n = 12, p=0.0446) (Figure 5A) and speed (Ctrl 0.0717 ± 0.004071, n = 24; SAP97-cKO 0.058 ± 0.005053, n = 12, p=0.0497) (Figure 5B) in addition to a reduction in distance from the border (Ctrl 0.06063 ± 0.001486, n = 24; SAP97-cKO 0.05369 ± 0.001995, n = 12, p=0.0088) (Figure 5C). Overall, these observations indicate an anxiety-like phenotype present in both male and female SAP97-cKO animals.

Anxiety is a common comorbidity associated with various psychiatric disorders. In order to further gauge whether SAP97-cKO mice had alterations in anxiety-like behavior, we performed the standard elevated plus maze. When comparing time spent in open arms versus the closed arms, we saw no significant differences between genotypes in both males (open arms: Ctrl 69.75 ± 4.098, n = 33; SAP97-cKO 62.47 ± 9.331, n = 15; closed arms: Ctrl 160.8 ± 5.339, n = 33; SAP97-cKO 179.3 ± 8.893, n = 15) and females (open arms: Ctrl 68.17 ± 6.734, n = 20; SAP97-cKO 64.85 ± 7.72, n = 13; closed arms: Ctrl 156.6 ± 8.515, n = 20; SAP97-cKO 172.5 ± 8.559, n = 13) (Figure
Likewise, the number of entries into the open versus closed arms was similar between genotypes of both sexes (Figure 6B). Total distance traveled in the maze was also measured and compared between Ctrl and SAP97-cKO animals to ensure no significant differences in overall exploration of the maze (Figure 6C). Together with the open field results, these observations indicate an anxiety-like phenotype in both male and female SAP97-cKO animals that is particular to specific behavioral tasks.

No changes in cued fear conditioning behavior in male SAP97-cKO mice

Amygdala circuitry is key for regulating anxiety-like responses in mice, and amygdala neuronal activity has been shown to be increased in the open field paradigm (Wang et al., 2011a). Given the observed increase in anxiety-like behavior in the SAP97-cKO mice and the wide expression pattern of Sap97, we were interested to know whether dysfunction in the amygdala might contribute to these observations. We decided to test the mice in the standard cued fear-conditioning paradigm (see Methods), which is thought to be an amygdala specific behavior. Both control and SAP97-cKO male and female mice exhibited normal freezing behavior (Male: Ctrl 46.96 ± 3.652, n = 15; SAP97-cKO 58.53 ± 6.029, n = 10; Female: Ctrl 35.89 ± 5.249, n=3; SAP97-cKO 40.1 ± 3.932, n=4) (Figure 7). These results suggest that the SAP97-cKO mice have no deficit in cued fear conditioning behavior and the described anxiety phenotype may be independent of amygdala circuitry.

Male-specific cognitive deficit in SAP97-cKO mice

Cognitive deficits are another endophenotype observed in various psychiatric conditions. Given that cognitive deficits are also present in several mouse models of human psychiatric disorders, we examined SAP97-cKO mice for this behavior. The
novel object recognition task is a standard test for cognition that measures ability to recall an object previously observed, as indicated by preference for a novel object (see Methods). During the training phase of this task, we observed no significant differences between the ratio of time spent investigating the two identical objects A and A’ for both males and females (Male: Ctrl 1.364 ± 0.1438, n = 29; SAP97-cKO 1.441 ± 0.1578, n = 15; Female: Ctrl 1.3 ± 0.2506, n = 22; SAP97-cKO 1.301 ± 0.2775, n = 10) indicating that the animals had no prior bias. During the testing phase, control male mice displayed a marked increase in the preference index for the novel object, while SAP97-cKO male mice showed no significant increase in novel object preference index (Ctrl A-A 1.364, Ctrl A-B 2.457; SAP97-cKO A-A 1.441, SAP97-cKO A-B 1.941, $F_{(3, 79)} = 5.311$, p=0.0022) (Figure 8). When we examined this behavior in the females, we observed a trending, but not significant, increase in preference index for the novel object in both control and SAP97-cKO animals (Ctrl A-A 1.3, Ctrl A-B 2.255; SAP97-cKO A-A 1.301, SAP97-cKO A-B 2.575, $F_{(3, 57)} = 2.59$, p=0.0616) (Figure 8). These findings suggest a male-specific cognitive deficit in the SAP97-cKO animals.

**Female-specific motor learning deficit in SAP97-cKO mice**

Alterations in motor learning and motor coordination have also been observed in mouse models of ASD. In order to determine whether this behavioral change is present in the SAP97-cKO mice, we performed the standard rotarod task (see Methods). Analysis of both sexes showed significant time effects (Male: $F_{(3, 6)} = 12.31$, p=0.0057; Female: $F_{(3, 6)} = 9.126$, p=0.0118), while only female animals showed a trend for genotype effects and significant time and genotype interaction effects (genotype effect: $F_{(1, 2)} = 11.53$, p=0.0769; time x genotype effect: $F_{(3, 6)} = 5.099$, p=0.0434) (Figure 9A). Control animals of both sexes showed a significant increase in latency to fall from the
rod from day 1 to day 4 (Male: Day 1 157.5 ± 19.12, Day 4 211.1 ± 2.074, n = 30, p=0.0010; Female: Day 1 171.6 ± 16.53, Day 4 237.3 ± 3.439, n = 21, p=0.0021), indicating learning of the task (Figure 9A-B). However, while male SAP97-cKO mice showed no learning impairment (Day 1 154.4 ± 16.64, Day 4 229.4 ± 0.8372, n = 18, p=0.0002), female SAP97-cKO mice showed no significant learning over the timecourse of this task (Day 1 186 ± 7.927, Day 4 213.3 ± 9.988, n = 16) (Figure 9A-B). In order to determine whether this female motor learning impairment was dependent on age, we tested a subset of aged animals (8-9 months) on the rotarod task. While both aged control and SAP97-cKO female animals did not display significant learning over the 4-day task (Control: Day 1 146.6 ± 15.06, Day 4 185.3 ± 3.153, n = 7; SAP97-cKO: Day 1 95.07 ± 8.834, Day 4 129.2 ± 7.136, n = 5), female SAP97-cKO animals performed worse overall as compared to littermate controls. These results suggest that there is a female-specific motor learning deficit present in the SAP97-cKO mice that persists with age.

**No social deficits present in SAP97-cKO mice**

Problems with socialization are often seen in patients with ASD, and many genetic mouse models of ASD have been able to mimic this behavioral deficit. We looked for this endophenotype in the SAP97-cKO mice using the three-chambered social choice paradigm (see Methods). During the testing phase of this paradigm, we measured preference for the social target zone versus the nonsocial target zone. Control and SAP97-cKO animals of both sexes exhibited a strong preference for spending time in the social target zone (Male: Ctrl-Nonsocial 0.334, Ctrl-Social 0.666, n = 15; SAP97-cKO-Nonsocial 0.4164, SAP97-cKO-Social 0.5836, n = 11, $F_{(3, 48)} = 24.67$, p<0.0001; Female: Ctrl-Nonsocial 0.3641, Ctrl-Social 0.6359, n = 8; SAP97-cKO-Nonsocial 0.4011, SAP97-cKO-Social 0.5989, n = 7, $F_{(3, 46)} = 22.67$, p<0.0001).
Nonsocial 0.3045, SAP97-cKO-Social 0.6955, n = 8, $F_{(3, 20)} = 14.53$, p<0.0001) (Figure 10A). Manual scoring of sniffing preference for the social target versus the nonsocial target was also measured for the male animals. Control and SAP97-cKO male animals also exhibited a strong preference for sniffing/investigating the social target (Ctrl-Nonsocial 0.2663, Ctrl-Social 0.7337, n = 8; SAP97-cKO-Nonsocial 0.3513, SAP97-cKO-Social 0.6487, n = 8; $F_{(3, 28)} = 54.84$, p<0.0001). These results suggest no social deficit in the SAP97-cKO mice.

**DISCUSSION**

*SAP97* is a member of the Dlg-MAGUK family that has repeatedly been implicated in neuropsychiatric disorders (Quintero-Rivera et al., 2010; Uezato et al., 2012; 2015; Xing et al., 2016), although its direct role in contributing to pathology has been unexplored. We generated and studied mice that were null for *Sap97* in the nervous system and make three principal observations. First, there are no compensatory changes in expression levels of other Dlg-MAGUKS or AMPARs in the SAP97-cKO versus controls. Second, loss of *Sap97* is associated with changes in gene transcripts related to SCZ. And third, SAP97-cKO animals of both sexes display an anxiety-like phenotype, as well as a male-specific cognitive deficit and female-specific motor learning deficit. Our results argue that *Sap97* is required for normal brain function and its absence leads to specific behavioral deficits and transcriptomic changes associated with SCZ.

**ASD and SCZ as polygenic disorders**

Investigations of ASD, SCZ, and other related psychiatric disorders indicate a highly polygenic architecture with small effect sizes of each implicated risk variant.
Mouse modeling of these disorders by targeting one such risk variant typically demonstrates a moderate, or incomplete manifestation of the human disorder. This is well illustrated by human and mouse studies of the PROSAP/SHANK family member SHANK3. Human genetic studies link mutations in SHANK3 to a broad range of neuropsychiatric disorders. For example, deletions of exons 1-9 or exons 1-17 of SHANK3 have been found in patients exhibiting severe language delay and significant intellectual disability. Mice generated to mimic these deletions were generated by Peca et al. and the main behavioral effects were repetitive grooming and deficits in social interaction (Peça et al., 2011). Jiang et al. used a different targeting strategy to mimic the human deletions and the mice displayed repetitive behaviors, deficits in social interaction, abnormal ultrasonic communication patterns and learning and memory deficits (Jiang and Ehlers, 2013). In a second well-studied family, affected individuals displayed ASD-features and this was linked to a deletion of SHANK3 exon 21 (an exon that included the Homer binding domain). Mice generated to mimic this genetic lesion were created by Kouser and Speed et al. and ~2.5 month old animals exhibit defects in spatial learning and memory, motor-coordination deficits, hypersensitivity to heat, novelty avoidance, but minimal social abnormalities and no repetitive grooming behavior (Kouser et al., 2013). Together, this work demonstrates that creating a mouse with a genetic lesion that closely mimics, or is identical, to the gene defect in humans with neuropsychiatric disease only partially recapitulates the human behavioral phenotypes.

This disparity between genetic lesions associated with psychiatric phenotypes and mice created to mimic the human condition also extends to the Dlg-MAGUK family. Nonsynonymous missense mutations in the Dlg-MAGUK family members have been found in ASD and SCZ patients, and decreased protein expression of PSD-95, PSD-93, and SAP97 has been observed in the cortex of postmortem SCZ patients. To model
with in mice, null alleles of *Psd-95, Psd-93,* and *Sap102* have been created—*Psd-95* and *Sap102* knockout animals share spatial learning memory deficits (Migaud et al., 1998; Cuthbert et al., 2007), while animals null for *Psd-95* or *Psd-93* share a hyper-social phenotype (Winkler et al., 2017). *Psd-95* and *Sap102* knockout animals display a mild, and *Psd-93* knockout animals display a severe, motor function defect (Cuthbert et al., 2007; Winkler et al., 2017). The *Psd-95* null mouse has been the most extensively investigated animal. Feyder et al. characterized the *Psd-95* knockout mice and the mice exhibit increased repetitive behaviors, abnormal communication, hyper-social behavior, impaired motor coordination, and increased stress-reactivity and anxiety-related responses (Feyder et al., 2010). The extent to which the *Psd-95* null mice faithfully report on the contribution of *PSD-95* to psychiatric disease is an open question.

*SAP97 splice variants and their differing roles in the nervous system*

*SAP97* has wide molecular diversity, which is created by extensive alternative splicing. The two most well-studied *Sap97* splice variants are *Sap97α* and *Sap97β*. In *Sap97α*, the prototypic N-terminal L27 domain is replaced with a putative palmitoylation motif. Overexpression of *SAP97α* (but not *SAP97β*) was shown to enhance the synaptic levels of AMPARs and to compensate for the shRNA-mediated loss of PSD-95 in organotypic slices (Waites et al., 2009). *SAP97* isoform-specific biology may also extend into human SCZ data. Uezato and colleagues identified a new *SAP97* splicing variant that is transcribed from a previously unreported 95-base-pair exon (exon 3b). In post-mortem prefrontal cortices of patients with SCZ, mRNA expression of exon 3b was significantly reduced, specifically in patients with early-onset SCZ (Uezato et al., 2015). How reduced levels of the *SAP97* 3b transcript may be involved in the susceptibility and pathophysiology of early-onset SCZ is unknown. While our study provides a broad, all-
around characterization of the effect of Sap97 on brain function, it will be necessary to conduct future studies aimed at addressing the individual roles of prominent splice variants.

**The role of the Serpin family as a molecular module in SCZ**

The RNAseq study we conducted on the hippocampi of SAP97-cKO animals indicated 67 DEGs, which were specifically enriched for SCZ-related risk genes. The specific SCZ-related risk genes we identify in our data are *Serping1*, *Runx3*, *Clec7A*, *Serpinh1*, *Cd1*, *Ap1S2*, *Xbp1*, *Serpind1*, and *C4b*. These observations lead us to hypothesize that Sap97 is a component of a “molecular module” of gene products that together subserve aspects of normal behavior. Further, we hypothesize that abnormalities in the operation of this molecular module give rise to select behavioral alterations. Defects in many molecular modules in aggregate manifest as the complex psychiatric disorder we recognize as SCZ. The components of this module may interact physically, functionally, developmentally, or in terms of localization. Future work will be required to elucidate: 1) how the components of this hypothesized molecular module mechanistically interact, and 2) how this impacts brain function and behavior.

Our attention is drawn to three genes that were differentially expressed in the hippocampus of SAP97-cKO mice versus controls—serine peptidase inhibitors (serpins), as this group of genes has previously been reported in the literature to be associated with SCZ (Madani et al., 2003; Hoogendoorn et al., 2004; Saetre et al., 2007; Allswede et al., 2017; Chang et al., 2017; Reumann et al., 2017). *SERPING1* was found to be upregulated in postmortem brain tissue from SCZ patients (Saetre et al., 2007; Chang et al., 2017). Additionally, a study of adult Swedish twins enriched for SCZ showed an association between gene expression level of *SERPING1* and thickness across the
cortex, a characteristic that is potentially involved in the pathogenesis of SCZ (Allswede et al., 2017). Polymorphisms in the promoter regions of genes on 22q11, a chromosomal region that has been associated with various psychiatric illnesses including SCZ, resulted in activity differences in the gene SERPIND1 (Hoogendoorn et al., 2004). Another well-studied member of the serpin family previously implicated in SCZ, but not directly by our RNAseq data, is neuroserpin (SERPINI1). SERPINI1 is restricted to regions in the brain where synaptic changes are associated with learning and memory (cortex, hippocampus, amygdala, and olfactory bulb) (Reumann et al., 2017). SERPINI1 has also been implicated in dendrite growth, as overexpression studies in primary neurons leads to increased dendritic arborization and altered dendritic spine shape (Borges et al., 2010). Additionally, mice with dysregulated expression of Serpin1 show selective reduction of locomotor activity in novel environments, anxiety-like responses, and neophobic response to novel objects (Madani et al., 2003). These behavioral phenotypes in the Serpin1 deficient mice are reminiscent of the defects we see in the SAP97-cKO animals. Serpin1 is also a known inhibitor of the extracellular protease tissue-type plasminogen activator (tPA). Conditions that affect the activity of tPA have consistently been described in drug-naïve cases of SCZ (Halacheva et al., 2009; Delluc et al., 2013; Song et al., 2014; Gris et al., 2015). Interestingly, psychotic patients on chronic warfarin therapy for deep-vein thrombosis showed remission of psychotic symptoms, indicating that defective modulation of the coagulation pathway might contribute to the pathogenesis of SCZ (Hoirisch-Clapauch et al., 2015). C4B, or complement component b, is another gene directly listed from our RNAseq study that has known roles in the coagulation pathway and is an important cofactor to the serine protease family. The strongest genetic association of SCZ at a population level involves variation in the Major Histocompatibility Complex (MHC) locus, where the association of
SCZ with the MHC locus arises substantially from many diverse alleles of the \textit{C4} genes (Rezende, 2003; Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016; Allswede et al., 2017). These prior observations along with our findings from the SAP97-cKO RNAseq study may highlight the mechanism by which \textit{SAP97} contributes to the etiology of SCZ.

\textbf{Sex-specific differences in psychiatric disease}

Psychiatric disorders are characterized by substantial sex-differences in their prevalence, symptomology, and treatment response (Kokras et al., 2014). Women are more likely than men to develop dementia, panic disorder, post-traumatic stress disorder, and major depression (Kessler et al., 2008; Wittchen et al., 2011). Conversely, the incidence of neurodevelopmental disorders such as ASD and SCZ is higher in males (Fombonne, 2003; Häfner, 2003). In our study, we conducted RNAseq on male hippocampal tissue from SAP97-cKO tissue and found the resulting DEGs to be specifically enriched for SCZ risk-related gene sets. However, our behavioral screen was undertaken on both male and female SAP97-cKO animals and identified interesting sex-specific differences. This raises the possibility that the RNAseq profile of female SAP97-cKO mice may be at least partially distinct from the male SAP97-cKO dataset.

One potential limitation of our study of female behavior is the lack of assessment of the estrous cycle. Female mice in distinct stages of the estrous cycle have been previously shown to perform differently in behavioral tasks related to anxiety and cognition. Furthermore, it is thought that oestrogens play a protective role against SCZ (Kulkarni et al., 2013). It will be vital to perform behavioral testing at different stages of the female estrous cycle, as well as corroborate behavioral findings with RNAseq data in order to have a complete understanding of the role of \textit{SAP97} in the female brain.
Conclusion

Our study provides the first broad behavioral and transcriptomic characterization of Sap97 in the mouse nervous system. Despite study limitations, we show that loss of Sap97 contributes to enrichment of SCZ related genes, as well as behavioral abnormalities in both male and female animals. Our findings are congruous with previous literature of monogenic mouse models of psychiatric disorders reporting a partial manifestation of the disease phenotype and thus are a first step to understanding the molecular mechanism by which SAP97 contributes to neuropsychiatric disorders.
Figure 2.1. **SAP97 protein is sufficiently knocked down in SAP97-cKO animals.** (A) Western blots showing reduced SAP97 band intensity in male SAP97-cKO cerebellum, hippocampus, and cortex. (B) Quantification of male western blot analysis. (C) Western blots showing reduced SAP97 band intensity in female SAP97-cKO hippocampus and cortex. (D) Quantification of female western blot analysis. (E) Western blots showing no significant changes in SAP97 band intensity between male and female C57Bl/6 animals. (F) Quantification of male versus female C57B/6 western blot analysis. *P<.05, **P<.01 (two-tailed Student’s t test). Data are presented as mean ± SEM.

Figure 2.2 **No compensation by Dlg-MAGUK family abundance in SAP97-cKO animals.** (A) Western blots and quantification showing no significant change in abundance of PSD-95 in cerebellum, hippocampus, and cortex of either male or female SAP97-cKO animals. (B) Western blots and quantification showing no significant change in abundance of PSD-93 in cerebellum, hippocampus, and cortex of either male or female SAP97-cKO animals. (C) Western blots and quantification showing no significant change in abundance of SAP102 in cerebellum, hippocampus, and cortex of either male or female SAP97-cKO animals. n.s., no significance (two-tailed Student’s t test). Data are presented as mean ± SEM.

Figure 2.3. **No change in mRNA expression level of AMPAR subunits, selected SAP97 interactor genes, selected Wnt/β-catenin pathway targets, and selected DISC1 pathway targets in SAP97-cKO animals.** (A) qPCR results showing no significant change
in abundance of \textit{Glua1}, \textit{Glua3}, or \textit{Glua4} mRNA transcripts in selected brain regions. (B) qPCR results showing no significant change in abundance of mRNA levels of selected Sap97 interactor genes in selected brain regions. (C) qPCR results showing no significant change in abundance of Wnt/β-catenin pathway targets in selected brain regions. (D) qPCR results showing no significant change in abundance of \textit{DISC1} pathway targets in cortex. n.s., no significance (two-tailed Student’s \(t\) test). Data are presented as mean ± SEM.

Figure 2.4. Loss of SAP97 leads to downregulation of DEGs and enrichment of SCZ risk-related genes. (A) qPCR verification of top DEG (\textit{Sgk1}) from RNAseq study. (B) Heat map representation of downregulation of DEGs in SAP97-cKO hippocampus. (C) DEGs are specifically enriched for SCZ risk-related genes. *\(P<.05\) (two-tailed Student’s \(t\) test), **\(P<.01\) (hypergeometric probability test).

Figure 2.5. Comparison of open field behavior indicates anxiety-like phenotype in both male and female SAP97-cKO animals. (A) No group differences seen in average distance traveled in male animals. Female SAP97-cKO animals display significantly less distance traveled. (B) No group differences seen in average speed in male animals, while female SAP97-cKO show decreased speed. (C) Both male and female SAP97-cKO animals show decreased average distance from border of apparatus. n.s., no significance, *\(P<.05\), **\(P<.01\) (two-tailed Student’s \(t\) test). Data are presented as mean ± SEM.

Figure 2.6. Comparison of elevated plus maze behavior between control and SAP97-cKO animals. (A) No group differences seen in average total time spent in open arms vs closed arms of maze. (B) No group differences seen in total open arm entries or closed arm entries. (C) No group differences seen in average distance traveled in elevated plus maze
apparatus. n.s., no significance (two-tailed Student’s t test). Data are presented as mean ± SEM.

**Figure 2.7. Comparison of cued fear conditioning behavior between control and SAP97-cKO animals.** (A) Freezing behavior during habituation phase. Both control and SAP97-cKO male and female animals exhibit low levels of freezing with no significant differences between groups during habituation. (B) Freezing behavior during testing phase. Both male and female SAP97-cKO animals show no differences in freezing behavior compared to littermate controls. n.s., no significance (two-tailed Student’s t test). Data are presented as mean ± SEM.

**Figure 2.8. Comparison of novel object recognition behavior indicates male-specific cognitive deficit.** Control male animals exhibit preference for novel object (Ctrl A-A vs Ctrl A-B), while SAP97-cKO male animals do not show preference. Both control and SAP97-cKO female animals show trend for preference of novel object, but did not reach significance. n.s., no significance, **P<.01 (ordinary one-way ANOVA with Tukey’s test for multiple comparisons). Data are presented as mean ± SEM.

**Figure 2.9. Comparison of rotarod behavior indicates female-specific motor learning deficit.** (A) Both control and SAP97-cKO male animals show learning over the 4-day course of rotarod paradigm. Control female animals show increased motor learning over course of 4 days, while female SAP97-cKO show no significant increase in motor learning. (B) Plots showing comparison of Day 1 versus Day 4 rotarod data for control and SAP97-cKO animals indicates female-specific motor learning deficit. (C) Aged SAP97-cKO males show learning impairment over the 4-day course of rotarod. Both aged control and SAP97-cKO
cKO female animals show no significant increase in motor learning, however, aged female
SAP97-cKO animals have significantly worse performance on the task as compared to aged
littermate controls. (D) Plots showing comparison of Day 1 versus Day 4 rotarod data for
aged control and SAP97-cKO animals. n.s., no significance, **P<.01, ***P<.001 (repeated-
measures two-way ANOVA with Tukey’s test for multiple comparisons). Data are presented
as mean ± SEM.

**Figure 2.10. Comparison of social choice behavior between control and SAP97-cKO
animals.** (A) No significant differences observed between control and SAP97-cKO male or
female animals in preference for social target. (B) No significant differences observed
between control and SAP97-cKO male animals in preference for sniffing/investigating social
target. **P<.01, ***P<.001, ****P<.0001 (ordinary one-way ANOVA with Tukey’s test for
multiple comparisons). Data are presented as mean ± SEM.
Table 2.1. List of genes with significant expression differences between control and SAP97-cKO mice.

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Table 2.2A. List of top diseases identified through IPA that were affected in hippocampus of SAP97-cKO animals.

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Table 2.2B. List of top molecular and cellular functions identified through IPA that were affected in hippocampus of SAP97-cKO animals.

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Table 2.2C. List of top networks identified through IPA that were affected in hippocampus of SAP97-cKO animals

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Figure 2.1

A

B

C

D

E

F

Cerebellum
SAP97 level (normalized to actin)

Control
Ctrl
cKO
CB
HP
CORT

Hippocampus
SAP97 level (normalized to actin)

Female Cerebellum
SAP97 level (normalized to actin)

M
F
CB
HP
CORT

Female Hippocampus
SAP97 level (normalized to actin)

Female Cortex
SAP97 level (normalized to actin)

C57Bl/6 Cerebellum
SAP97 level (normalized to actin)

C57Bl/6 Hippocampus
SAP97 level (normalized to actin)

C57Bl/6 Cortex
SAP97 level (normalized to actin)

Actin

SAP97

n.s.

p = .06

n.s.
Figure 2.2

A

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</table>
Figure 2.3

A. AMPAR Subunits Cerebellar mRNA

B. SAP97 Interactors Cerebellar mRNA

C. Wnt/B-catenin Targets Hippocampal mRNA

D. DISC1 Targets Cortical mRNA

Fold Change

Control

cKO

n.s.
Figure 2.4

A. Hippocampal SGK1 mRNA

B. Disease-Related Gene Set Overlap

C. Match %

** Figure 2.4 **

A

C

B
Figure 2.5

A

Distance Traveled

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Distance Traveled (m)
n.s.

B

Average Velocity

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Velocity (m/sec)
n.s.

C

Average Distance From Border

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Distance (m)

**
Figure 2.6

A

Time Spent in Open Arm

Time (sec)

Control
cKO
0
50
100
150
200
n.s.
33 15

Time Spent in Closed Arm

Time (sec)

Control
cKO
0
50
100
150
200
n.s.
33 15

B

Open Arm Entries

# Entries

Control
cKO
0
5
10
15
20
n.s.
33 15

Closed Arm Entries

# Entries

Control
cKO
0
5
10
15
20
n.s.
33 15

C

Distance Traveled

Distance (m)

Control
cKO
0
5
10
15
20
n.s.
20 13

Dark Arm Entries

# Entries

Control
cKO
0
5
10
15
20
n.s.
20 13
Figure 2.7

**A**

%Freezing During Habituation

<table>
<thead>
<tr>
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<td>10</td>
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<tr>
<td>Female</td>
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**B**

%Freezing

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Figure 2.8

**Novel Object Recognition**

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<td>15</td>
</tr>
<tr>
<td>ckO A-B</td>
<td>15</td>
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</tbody>
</table>

For males:

- Control A-A: 29
- Control A-B: 29
- ckO A-A: 15
- ckO A-B: 15

For females:

- Control A-A: 22
- Control A-B: 22
- ckO A-A: 10
- ckO A-B: 10

n.s. indicates non-significant difference.
Figure 2.9

A

Rotorod

Latency to Fall (sec)

male control n = 30
male cKO n = 18

B

Rotorod Day 1 vs Day 4

Latency to Fall (sec)

male control n = 8
male cKO n = 8

C

Rotorod (Aged Males)

Latency to Fall (sec)

male control n = 7
female cKO n = 5

D

Rotorod Day 1 vs Day 4 (Aged Males)

Latency to Fall (sec)

male control n = 21
female cKO n = 16

Latency to Fall (sec)

male control n = 8
male cKO n = 8

Latency to Fall (sec)

male control n = 7
female cKO n = 5

Latency to Fall (sec)

male control n = 21
female cKO n = 16
Figure 2.10

A

Social Choice

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B

Social Choice (sniff time)

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CHAPTER 3: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis work, I have attempted to further understand the direct role of SAP97 in psychiatric disorders, such as ASD and SCZ. In Chapter 2, I characterize mice conditionally lacking Sap97 in the nervous system at the behavioral and transcriptomic level. I find that while SAP97-cKO mice have relatively subtle behavioral deficits, loss of Sap97 results in an enrichment of SCZ risk-related DEGs. Below, I will describe the implications for this work and discuss remaining questions for future work.

THE MOLECULAR MODULE HYPOTHESIS

Investigations of ASD, SCZ, and other related psychiatric disorders indicate a highly polygenic architecture with small effect sizes of each implicated risk variant. Mouse modeling of these disorders by targeting one such risk variant typically demonstrates a moderate, or incomplete manifestation of the human disorder, as discussed in the Introduction of this thesis. Results from this thesis work coincide with previous results indicating psychiatric disorders to be polygenic in nature. While SAP97-cKO mice did not show robust changes across the behavioral domains related to ASD and/or SCZ, we observed subtle, sex-specific abnormalities. Likewise, loss of Sap97 resulted in mild changes at the transcriptomic level as well, with 67 DEGs. However, this DEG set was enriched for SCZ risk-related genes, where 4 of 9 of these SCZ risk genes play a role in common pathways. These genes are Serping1, Serpinh1, Serpind1, and C4b.

This first three of these genes (Serping1, Serpinh1, Serpind1) are known as serine protease inhibitors, or serpins. Protease inhibition by serpins controls an array of biological processes, including coagulation and inflammation, and this family of genes
has previously been reported in the literature to be associated with SCZ (Madani et al., 2003; Saetre et al., 2007; Borges et al., 2010; Chang et al., 2017; Reumann et al., 2017; Weickert et al., 2018). *C4B*, or complement component b, has known roles in the coagulation pathway and is an important cofactor to the serine protease family. The strongest genetic association of SCZ at a population level involves variation in the Major Histocompatibility Complex (MHC) locus, where the association of SCZ with the MHC locus arises substantially from many diverse alleles of the *C4* genes (Rezende, 2003; Hoirisch-Clapauch et al., 2015; Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016).

These findings suggest that *Sap97* is a member of a tight cluster of genes, or “molecular module” that interacts and subserves aspects of normal behavior. Defects in many molecular modules in aggregate may result in the complete manifestation of the disorder. The components of this module may interact physically, functionally, developmentally, or in terms of localization. In order to elucidate how the components of this hypothesized molecular module interact, and how this impacts organismal behavior, a number of experiments can be done. Below, I outline a few potential future experiments to address these questions.

**Interaction within the SAP97 molecular module**

One question to be addressed is whether *Sap97, Serping1, Serpinh1, Serpind1,* and *C4b* physically interact. One method to address this question would be by coimmunoprecipitation (CoIP) experiments. We can begin by overexpressing *Sap97* and one or a combination of SCZ risk genes in HEK cells, and subsequently perform CoIP. We may additionally examine whether this molecular module interacts
endogenously by performing CoIP experiments on brain tissue lysate generated from control and SAP97-cKO animals.

It is also plausible that the SAP97 molecular module interacts in the region-specific manner. For example, members of the serpin family, such as SERPINI1, are restricted to regions in the brain where synaptic changes are associated with learning and memory (i.e. cortex, hippocampus, amygdala). One could argue that as a scaffolding protein with multiple protein-protein binding domains, SAP97 may aid in tethering members of the molecular module to synaptic regions, where they act together to ensure proper synapse formation, function, and behavioral output. To address this question, we can prepare synaptosomes from control and SAP97-cKO animals and examine physical interaction within this proposed molecular module.

Previous evidence from the literature also suggests that members of the SAP97 molecular module may interact functionally as well. For example, the serpin family of genes, namely Serpini1, has also been implicated in dendrite growth (Borges et al., 2010; Reumann et al., 2017). And similar to SAP97, overexpression studies in primary neurons leads to increased dendritic arborization (Borges et al., 2010). Additionally, characterization of mice lacking Schnurri-2, or MHC-binding protein 2, show immature dendritic spine morphology characterized by increases in spine length and decreases in spine diameter (Nakao et al., 2017). Schnurri-2 knockout mice also exhibited increases in C4b gene, which is thought to mediate synapse elimination during postnatal development, and show SCZ-like behaviors (Takao et al., 2013). Other observed changes in these mice included a significant reduction in GLUA1 and a trend for decreased expression of PSD-95, both of which have strong association with SAP97 (Nakao et al., 2017). Overall, these results suggest that the SAP97 molecular module we have identified may possibly function at the synapse and play a role in dendrite and
spine morphology, and ultimately, proper behavioral output. We can attempt to address this question in the future by conducting co-knockdown experiments (i.e. knockdown of serpin family/C4B in addition to SAP97) and using dendritic growth as a readout. We can also employ the use of targeted viral vectors to further the knockdown level of other module members within the SAP97-cKO mice. It is plausible that a more severe knockdown effect of this module will result in a larger behavioral defect compared to what we observed in our studies.

**Neuroinflammation and psychiatric disorders**

Results from previous studies also indicate that our identified SAP97 molecular module may also serve as a potential interface between inflammation and synaptic dysfunctions. Inflammation has been posited as a potential mechanism underlying the development and progression of SCZ and other related neuropsychiatric disorders, and meta-analyses have demonstrated that patients with SCZ reliably exhibit increased markers of inflammation (Potvin et al., 2008; Miller et al., 2011; Goldsmith et al., 2016; Miller and Goldsmith, 2016; Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016). Furthermore, there is growing literature showing that increased inflammatory cytokines may be linked to negative symptoms in patients with SCZ (Garcia-Rizo et al., 2012; Liu et al., 2012; Asevedo et al., 2014; Kissi et al., 2018). In particular, TNFα and interleukin-6 were found to be associated with deficit syndrome, a distinct subtype of SCZ characterized by primary and enduring negative symptoms (Goldsmith et al., 2018). A previous study identified TNFα as a critical molecule involved in the synaptic alterations seen in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of brain inflammation (Centonze et al., 2009). TNFα has the potential to promote dendritic spine loss in EAE brains through an excitotoxic
mechanism (Centonze et al., 2009). SAP97 also binds to TNFα converting enzyme (TACE) via the SAP97 PDZ3 domain (Peiretti, 2003). Interestingly, overexpression of SAP97 reduced the release of three different TACE-processed substrates, including TNFα (Peiretti, 2003). This suggests that SAP97 participates in regulating the inflammatory response. Given that the serpin family of genes and C4B are also known to play important roles in the inhibition of the inflammatory response, an interesting potential future experiment would be to examine inflammation in the SAP97-cKO animals by measuring levels of cytokines such as TNFα and interleukin-6 in plasma collected from control and SAP97-cKO animals. As the SAP97 molecular module is downregulated in the SAP97-cKO animals, we would expect these animals to have a heightened immune response. This increased immune response could be a potential mechanism underlying the specific behavioral deficits observed in the SAP97-cKO mice, and may serve as a target for therapeutic intervention.

ENVIRONMENTAL MODELS OF PSYCHIATRIC DISORDERS IMPLICATE IMMUNE RESPONSE

In this thesis work, we have chosen to focus predominately on examining and modeling the genetic etiology of psychiatric disorders. However, genetics do not account for all patient cases, and environmental factors are thought to contribute significantly to disease risk. Multiple environmental rodent models of psychiatric disorders implicate a heightened immune response.

A commonly-used environmentally induced model of ASD is the propionic acid (PPA) model. PPA is a short chain fatty acid, a metabolic end-product of enteric bacteria in the gut, and a common food preservative. Various studies have indicated that PPA causes ASD-like behaviors and neuroinflammatory response in rats (MacFabe
et al., 2007; Shultz et al., 2008; MacFabe et al., 2011). For example, Shultz et al.
reported that exposure to PPA resulted in impaired social behavior measured as
distance apart, proximity, and play behavior in rats (Shultz et al., 2008). MacFabe et al.
demonstrated that rats treated with PPA showed restricted behavioral interest to a
specific object among a group of objects, impaired social behavior, and impaired
reversal in a T-maze task compared to controls, in addition to reactive astrogliosis and
activated microglia in the brain (MacFabe et al., 2011).

As SCZ is considered a neurodevelopmental disorder, early environmental
factors potentially play a role in the etiology of the disease. One early life factor
associated with SCZ is maternal infection during pregnancy (Brown and Derkits, 2010).
Early epidemiological studies found an increased rate of SCZ among offspring who were
in utero during major influenza epidemics as compared to non-epidemic periods (Brown
and Derkits, 2010). This association was replicated in several geographic populations
(Brown and Derkits, 2010). Additional studies found an increased risk of SCZ among
offspring of mothers who received diagnosis of influenza, toxoplasmosis, rubella, or
bacterial infection during pregnancy (Brown et al., 2000; Brown et al., 2004; Brown et al.,
2005; Sorensen et al., 2009). High levels of pro-inflammatory cytokines in the maternal
serum during pregnancy were also found to increase risk of SCZ in in utero offspring
(Canetta et al., 2014). Maternal immune activation (MIA), is not specific to
schizophrenia, but may also increase the risk for ASD, bipolar disorder, and depression
(Canetta et al., 2014).

Several groups have studied prenatal infection in rodent models. Both direct viral
infection of the fetus, as well as abnormal activation of the maternal immune system,
resulted in behavioral impairments relevant to SCZ (Shi et al., 2002; Shi et al., 2005;
Meyer and Feldon, 2012). The maternal immune system can be activated with a
synthetic double-stranded RNA, polyinosinic-polycytidylic acid (PolyIC). The PolyIC model of SCZ has gained wide recognition in the scientific community as it successfully accounts for several aspects of SCZ: epidemiology, pathophysiology, symptomology, and treatment (Meyer and Feldon, 2012).

PolyIC is a commercially available synthetic analog of double-stranded RNA. Double-stranded RNA is generated during viral infection as a replication intermediate for single-stranded RNA or as a byproduct of symmetrical transcription in DNA viruses (Takeuchi and Akira, 2007). It is recognized as a foreign by the mammalian immune system through the transmembrane protein toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001). Upon binding to TLRs, double stranded RNA, or the synthetic analog PolyIC, stimulates production and release of many pro-inflammatory cytokines, including interleukin-1B, interleukin-6, and TNFα (Fortier et al., 2004; Cunningham et al., 2007). PolyIC is also a potent inducer of the type 1 interferons INF-a and INF-b (Kimura et al., 1994; Traynor et al., 2004). Administering PolyIC can therefore mimic the acute phase response to viral infection. Maternal exposure to PolyIC is capable of altering pro- and anti-inflammatory cytokine levels in the three relevant compartments of the maternal-fetal interface of rodents, namely the placenta, amniotic fluid, and the fetus (Meyer et al., 2006). This allows the model to include aspects of maternal/fetal inflammation, taking into account one of the most relevant immunological mechanisms suggested to be crucial for mediating the long-term effects of prenatal infection on brain and behavioral development (Patterson, 2009; Meyer et al., 2016). The PolyIC mouse model of SCZ recapitulates behavioral phenotypes such as sensorimotor gating deficits, impaired working memory, and reduced social behavior (Ibi et al., 2009).

Increased susceptibility to environmental stress remains an open question in the SAP97-cKO animals. As discussed earlier, the literature supports the idea that inhibition
of the SAP97 molecular module may induce a heightened immune response in the SAP97-cKO animals. However, as the behavioral deficits observed were moderate, it is plausible to assume that the potential immune response in these animals was not sufficient to induce a large-scale behavioral effect. A complementary experiment to using targeted viral vectors to further the knockdown level of other SAP97 molecular module members would be to expose SAP97-cKO animals to environmental stress (i.e. maternal immune activation), and measure whether we obtain a more complete manifestation of SCZ as compared to the environmental stress or SAP97-cKO model alone. Results from these proposed experiments would elucidate the mechanism by which loss of SAP97 contributes to the etiology of SCZ.

LIMITATIONS OF RODENT MODELS

How useful are rodent models, in general, for understanding human psychiatric disorders? Animal models aim to recapitulate behavioral symptoms, but this approach has limitations as some of the behavioral symptoms are distinctive for humans and are not measurable in animals (i.e. delusions or hallucinations) (Canetta and Kellendonk, 2018). Additionally, compensatory mechanisms present in mice may not have the same effects in humans (Deconinck et al., 1997).

One classical example is the mdx mouse model for Duchenne muscular dystrophy (DMD). DMD in humans is caused by lack of dystrophin, a large membrane-associated protein expressed in muscle and the brain (Tinsley et al., 1994). The mdx mouse model lacks dystrophin due to a mutation that results in a premature stop codon, but presents with a much milder form of the disease than in humans (Bulfield et al., 1984). Compensation for lack of dystrophin by structurally related proteins such as utrophin may also be more successful in the mouse, leading to a milder phenotype than
in humans (Deconinck et al., 1997). Dystrophin/utrophin double knockout mice have been generated and present with many more clinical signs of DMD than *mdx* mice (Deconinck et al., 1997). Nevertheless, the *mdx* mouse model is a popular model for studying DMD and has proven useful for examining potential therapeutics and molecular mechanisms underlying the disorder.

The story of the *mdx* mouse model may also be true for the SAP97-cKO model. SAP97 has wide molecular diversity, which is created by extensive alternative splicing. Uezato and colleagues identified a new SAP97 splicing variant that is transcribed from a previously unreported 95-base-pair exon (exon 3b) (Uezato et al., 2015). In post-mortem prefrontal cortices of patients with SCZ, mRNA expression of exon 3b was significantly reduced, specifically in patients with early-onset SCZ (Uezato et al., 2015). However, this exon is primate-specific (Uezato et al., 2017). It is plausible that this primate-specific exon of SAP97 is responsible for contributing to a more severe manifestation of SCZ in humans, while loss of mouse SAP97 is more easily compensated for by other genes. In this thesis work, we have shown that loss of SAP97 is not compensated for by change in overall abundance of the other Dlg-MAGUK family members. This does not rule out compensation in terms of localization (i.e. at the synapse versus whole tissue) or by activity. These open questions should be addressed in the future to further understand SAP97’s role in disease in humans versus the mouse.

**Difficulty in using DSM criteria for rodent models of psychiatric disorders**

An additional complication with using rodents to model psychiatric disorders is determining how symptoms in an animal model add up to a recognized human disorder. The Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IVTR) contains scant knowledge of the pathophysiology underlying these disorders (Nestler
and Hyman, 2010). Diagnoses are based solely on phenomenology, such as symptoms, signs, and course of illness. As a result, the boundaries between DSM-IVTR disorders, and the boundaries between disorder and normal variation, are unclear (Hyman, 2010). For example, two patients with major depression may exhibit no overlap of the 9 symptoms listed in the DSM criteria for Major Depressive Episode. The multiple symptom combination, in addition to the inability to assess certain symptoms in mice, means that different mouse models of depression would have little in common (Nestler and Hyman, 2010). This issue is extended to a variety of psychiatric disorders, including ASD and SCZ.

Additionally, DSM-IVTR diagnoses do not currently map onto abnormalities of molecules, synapses, cells, or neural circuits for psychiatric disorders. There are no molecular or cellular abnormalities in the human disease which could validate potential phenomenology in an animal (Nestler and Hyman, 2010). Individual symptoms observed in animal models may not have a simple, straightforward correspondence to human symptoms. As a result, animal models are unlikely to mirror the full extent of a given psychiatric disorder. While animal models of disease are useful, it is necessary to keep in mind the imperfections of rodent models when interpreting results.

CONCLUSIONS

This thesis work provides the first broad behavioral and transcriptomic characterization of $SAP97$ in the mouse nervous system. Despite study limitations, we show that loss of $SAP97$ contributes to enrichment of SCZ related genes, as well as moderate sex-specific behavioral abnormalities. We have potentially identified a module of genes where $SAP97$ and the serpin/$C4B$ family are participants, whose role is to regulate inflammatory response in the nervous system. While we have taken the first
steps to elucidate the contribution of *SAP97* to psychiatric disorders, further investigation is needed to validate the "molecular module" hypothesis and fully understand the downstream pathways and behaviors affected by this module. Further understanding of the role of *SAP97* in regulating inflammatory response and behavior may identify new targets for therapeutic intervention.


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