Exploring the Role of *Rapgef6* in Neuropsychiatric Disorders

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ABSTRACT

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Schizophrenia is highly heritable yet there are few confirmed, causal mutations. In human genetic studies, we discovered CNVs impacting *RAPGEF6* and *RAPGEF2*. Behavioral analysis of a mouse modeling *Rapgef6* deletion determined that amygdala function was the most impaired behavioral domain as measured by reduced fear conditioning and anxiolysis. More disseminated behavioral functions such as startle and prepulse inhibition were also reduced, while locomotion was increased. Hippocampal-dependent spatial memory was intact, as was prefrontal cortex function on a working memory task. Neural activation as measured by cFOS levels demonstrated a reduction in hippocampal and amygdala activation after fear conditioning. In vivo neural morphology assessment found CA3 spine density and primary dendrite number were reduced in knock out animals but additional hippocampal measurements were unaffected. Furthermore, amygdala spine density and prefrontal cortex dendrites were not changed. Considering all levels of analysis, the Rapgef6 mouse was most impaired in hippocampal and amygdala function, brain regions implicated in schizophrenia pathophysiology at a variety of levels. The exact cause of Rapgef6 pathology has not yet been determined, but the dysfunction appears to be due to subtle spine density changes as well as synaptic hypoactivity. Continued investigation may yield a deeper understanding of amygdala and hippocampal pathophysiology, particularly contributing to negative symptoms, as well as novel therapeutic targets in schizophrenia.

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I have been fortunate to have incredibly inspiring MD/PhD mentors throughout my career. Matt State and Joel Hirschhorn pushed me to think analytically and carefully about what I was reading and experiments I was performing. They also showed me that science can be exciting and rewarding and clinically relevant.

Dedication

> I am among those who think that science has great beauty. A scientist in his laboratory is... also a child placed before natural phenomena that impress him like a fairy tale... We should not allow it to be believed that all scientific discovery can be reduced to mechanisms, machines, [or] gearings.
>
> ~ Marie Curie

This work is dedicated to my family and friends who brought me to this point in my career and who encouraged and helped me in a myriad of ways. To my Bubby who passed away during my thesis, Grandma and Papa, my parents Sue and Dan, siblings Sam and Emily, and my entire extended family. To my husband Bryan who listened to all my experiments, and my wonderful in-laws Chris and Jeff. To my friends who edited, cheered, wined and dined, taught, commiserated, and laughed with me. Thank you all very much for your patience and love.

Chapter 1: Introduction

1.1 Clinical introduction to schizophrenia

Around the turn of the last century, neuropsychiatrists such as Eugen Bleuler and Emil Kraepelin attempted to refine both the nosology and diagnosis of mental disorders. In 1911, Bleuler first used schizophrenia as a diagnostic term when he described a group of psychotic disorders that shared the four A's: loosened association, incongruous affect, ambivalence, and autism (McGlashan 2011). His concern was accurate diagnosis on the basis of clinical symptoms. Bleuler believed the course and outcome for these patients varied, which was an ideological departure from Kraepelin's theory of psychosis. Kraepelin distinguished *folie circulaire* (repetitive madness, now bipolar disorder) from *dementia praecox* (early dementia, today diagnosed as schizophrenia) because he felt those with schizophrenia had an early adult onset with gradual and eventual demise into premature dementia (Tandon et al. 2009). He emphasized the cognitive decline and poor functional outcome as the hallmarks of disease diagnosis and prognosis, instead of clinical symptoms early in the disease process.

Whereas this divide of symptomatology as opposed to outcomes would separate American and European diagnostic approaches for decades, today both doctors' opinions are represented in the diagnosis of schizophrenia. A patient must present with at least two of the following symptoms for at least one month: delusions, hallucinations, disorganized speech, disorganized or catatonic behavior, or negative symptoms (such as affective flattening, alogia, or avolition) (Association 2000). These symptoms must not be dominated by depressive or manic features, nor due to any substance or medical condition. In research, the official diagnostic criteria are often regrouped into the positive, negative, and cognitive symptoms of schizophrenia (Figure 1.1). Positive symptoms include hallucinations or false sensory experiences, especially of

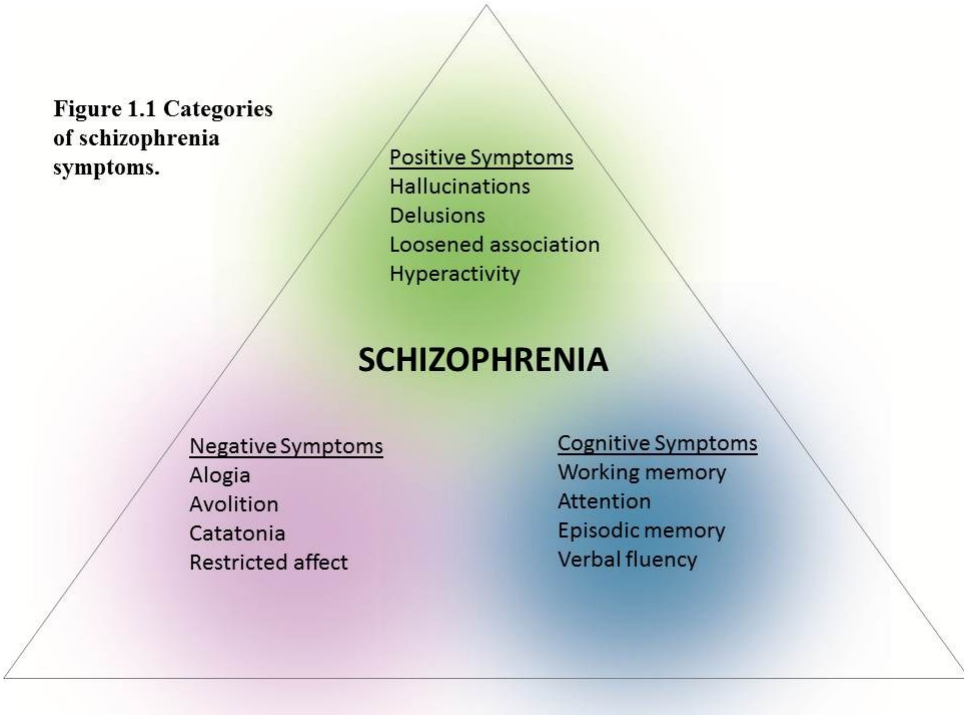


Figure 1.1 Categories of schizophrenia symptoms.

auditory phenomena such as voices, and delusions or bizarre thoughts and beliefs. The negative symptoms listed above indicate social withdrawal from emotion, language, and motivation. Finally the cognitive symptoms, so key to Kraepelin's formulation of the disease, are garnering more research interest since they include deficits in verbal fluency and episodic and working memory.

Due to the multifaceted diagnostic criteria, patients with the same diagnosis can be highly heterogeneous in their symptoms, presenting a challenge for research. Moreover, there are no biomarkers or physical signs that are diagnostic or prognostic with a high degree of certainty. Fortunately, diagnostic criteria in the United States and Europe yield over 70% concordance and high diagnostic agreement so clinical assessment is usually correct (Jakobsen et al. 2006). Thus clinical research in schizophrenia is complicated because diagnosis can never be biologically validated and the diagnostic criteria encompass a spectrum of symptoms.

There are two additional diagnoses within the schizophrenia spectrum. First, when mood

symptoms such as depression or mania are a consistent component along with positive, negative, and cognitive symptoms, the diagnosis of schizoaffective disorder applies. This diagnosis represents some of the clinical and genetic overlap between schizophrenia and affective disorders discussed below. Second, schizotypal personality disorder shares some clinical aspects with schizophrenia, such as strange thoughts and behavior and a lack of social interaction; however these symptoms are never as severe as the positive or negative symptoms in schizophrenia.

Disease incidence is 15.2 cases per 100,000 individuals per year, which yields a lifetime risk of 0.7% (McGrath et al. 2004; Tandon et al. 2008). While incidence is equivalent across dozens of countries and is not affected by national affluence as measured by gross national product, there are slightly higher rates of schizophrenia diagnosis in urban populations and in people who have migrated (McGrath et al. 2004; Saha et al. 2006). Men have an onset about 5 years before women (Hafner et al. 1998). Moreover, the ratio of male to female patients is 1.42:1 on meta-analysis of epidemiologic data, further indicating the possibility of estrogen or lifestyle influence (Aleman et al. 2003).

Disease onset is typically in young adults in their 20s and 30s, however many prospective and retrospective studies have identified a prodromal or premorbid period marked by milder symptoms in the years before the first psychotic episode. Analysis of 13 studies following prodromal patients found that prodromal diagnosis has on average 81-97% sensitivity (correct predictions of prodromal conversion to schizophrenia) and 59-67% specificity (accurate predictions of no prodrome remaining psychosis-free), depending on the type of diagnostic criteria (Chuma and Mahadun 2011). These results demonstrate that individuals at risk can be identified with accuracy; whether intervention with psychological or pharmacological treatment is beneficial given potential side effects remains a hotly debated ethical and scientific question.

The discovery of antipsychotic drugs in the 1960s put an end to earlier "treatments" including lifetime institutionalization, insulin coma, and prefrontal leucotomy. Antipsychotics are typically classed into first and second generation drugs, with first generation drugs sharing dopamine receptor-specific antagonism and second generation medications having more widespread neurochemical effects. Yet drug efficacy is proportional to dopamine D2 receptor antagonism among both first and second generation antipsychotic medications, and all antipsychotic drugs are more effective than placebo in treating positive symptoms of schizophrenia (Tandon et al. 2010). Multiple studies have demonstrated that newer drugs are no more efficacious for cognitive outcomes, quality of life, or first psychotic breaks than older, less expensive drugs (Davidson et al. 2009; Keefe et al. 2007; Swartz et al. 2007). While second generation drugs carry lower risk of motor side effects such as extrapyramidal symptoms and tardive dyskinesia, they carry metabolic side effects. Since negative and cognitive symptoms are poorly treated with available medications, it is critical to come to an understanding of the underlying biology of these symptoms so that targeted treatments can be developed with the potential to greatly reduce the burden of illness. Potential neurobiological targets for treating cognitive and negative symptoms include other dopamine, serotonin, glutamate, and acetylcholine receptors.

Thanks to modern treatment, the dire downward spiral described by Kraepelin is no longer the usual course of schizophrenia, however it is still a devastating lifetime disease. Approximately one quarter of patients have full remission and around 50% have partial recovery of quality of life (Harrison et al. 2001). Unfortunately, individuals with schizophrenia have a 2.5-fold mortality hazard compared with unaffected people (Saha et al. 2007). Part of the excess mortality is due to an elevated suicide rate of 5% (Palmer et al. 2005), and the rest is attributed to

increased medical and psychiatric comorbidities. Homelessness, incarceration, and reduced productivity or unemployment are all more common among patients with schizophrenia (Tandon et al. 2009). Having a family member with schizophrenia was rated as more challenging than any other medical diagnosis queried (Magliano et al. 2005). When patient care, medical and legal fees, and lost productivity are taken into account, schizophrenia was estimated to cost $62.7 billion in 2002 in the United States (McEvoy 2007). The financial and emotional costs of schizophrenia are staggering. While there is reason for optimism, there is still great concern for the welfare of patients and their families that urgently drives research.

1.2 Schizophrenia etiology

The etiology of schizophrenia remains unknown. This hampers the development of novel diagnostic tests, therapies, and prevention since neurobiological targets and risk factors are as yet unidentified. Analysis of epidemiological, morphological, and behavioral data from individuals with schizophrenia as well as animal models have yielded many theories concerning schizophrenia etiology, several of which are discussed here.

1.2.1 Neurotransmitter theories of schizophrenia

Changes in neurotransmission are a major area of schizophrenia research due to the known drug mechanisms on neurotransmitters. Dopamine is an excitatory neurotransmitter released from the ventral tegmental area in several pathways throughout the brain, notably into the prefrontal cortex in the mesocortical pathway and via the mesolimbic pathway into the nucleus accumbens and amygdala. Since all antipsychotic drugs are dopamine receptor antagonists, dopamine dysregulation is clearly important. Moreover, in vivo imaging showed that amphetamine given to patients with schizophrenia caused increased psychotic symptoms and dopamine release, measured as less D2 receptor availability (Laruelle et al. 1996). Measurement

of D1 receptor occupancy, however, found less dopamine binding in the dorsolateral prefrontal cortex that correlated with working memory impairments in patients (Abi-Dargham et al. 2002). This pattern of altered dopamine function is supported by animal models such as overexpression of the D2 receptor in the striatum that caused increased cortical D1 receptor activation, leading to behavioral deficits in prefrontal tasks (Kellendonk et al. 2006). Based on these findings, the modern dopamine theory of schizophrenia posits that there is an increase in mesolimbic dopamine release onto D2 receptors leading to positive symptoms and a decrease in mesocortical dopamine onto D1 receptors causing negative and cognitive symptoms (Simpson et al. 2010).

Glutamate is the major excitatory neurotransmitter of the central nervous system and its release is widespread, unlike dopamine. Glutamate NMDA receptor antagonists such as phencyclidine and ketamine can induce psychotic states in individuals with and without schizophrenia (Javitt and Zukin 1991; Lahti et al. 2001). Ketamine induced effects on episodic memory and psychosis may be localized to the cingulate cortex (Northoff et al. 2005). These results suggest both that these drugs can be used to model psychosis and that NMDA receptors are potential novel therapeutic targets. A mouse model with 90% reduced NMDA receptor subunit 1 expression demonstrated reduced sensorimotor gating, anxiety, and social interaction, suggestive of negative symptoms and diminished neural inhibition (Halene et al. 2009). One hypothesized relationship between glutamate and dopamine is that reduced glutamate in the corticostriatal system causes increased thalamic output, leading to elevated dopamine and sensory signal transmission that may fuel positive symptoms (Lang et al. 2007).

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the brain secreted by inhibitory interneurons. Several studies suggest GABA function is diminished in schizophrenia. The GABA producing enzyme GAD67 was reduced in postmortem prefrontal,

anterior cingulate, and superior temporal cortex, as well as striatum and thalamus (Thompson et al. 2009). Careful analysis of GABAergic interneurons in postmortem tissue across multiple studies found that interneurons are reduced in more superficial layers and increased in deeper cortical layers, implicating impaired interneuron migration in prenatal development (Benes 2012). In a GABA theory of schizophrenia, a loss of cortical inhibition could lead to positive symptoms mediated by altered dopamine and glutamate neurotransmission.

While almost every other neurotransmitter or neuropeptide has been implicated in at least one study of schizophrenia, the most significant and validated findings converge on dysregulation of dopamine, glutamate, and GABA. Despite much effort, there is still no theory to link these neurochemical findings together with environmental, genetic, and animal model results to yield one unifying explanation of the etiology of schizophrenia. In light of the heterogeneity found in genetic studies, it is unlikely that a single neurotransmitter underlies schizophrenia. Instead, it is more likely that a myriad of disruptions, from genetic and environmental sources, affect particular circuits, leading to pathophysiology.

1.2.2 Environmental factors in schizophrenia

While schizophrenia is highly heritable (Sullivan et al. 2003), indicating that genetic factors (described below) play a significant role in disease etiology, there is substantial risk attributable to environmental exposure. These exposures are typically divided into prenatal and childhood risk factors.

Prenatal risk factors associated with increased schizophrenia diagnosis include maternal infection, most notably with influenza, malnutrition in general and specifically deficiency of micronutrients such as vitamin D, advanced paternal age, and obstetric complications especially those that cause hypoxia and maternal stress (Brown 2011). Animal models of maternal infection

reliably generate neurobiological changes and behavioral deficits considered endophenotypes of schizophrenia (Brown 2011). These factors seem to converge onto oxidative stress, apoptosis, and inflammation pathways that could endanger fetal neurodevelopment. This negative impact on neurodevelopment may lead to the changes in brain volume found in adult patients, discussed further in Chapter 4 (Figure 1.2). Prenatal rodent models demonstrate that early life insults can cause adult behavioral and anatomical deficits, therefore it is possible that a similar timeline of pathophysiology may also occur in humans with regard to schizophrenia pathophysiology.

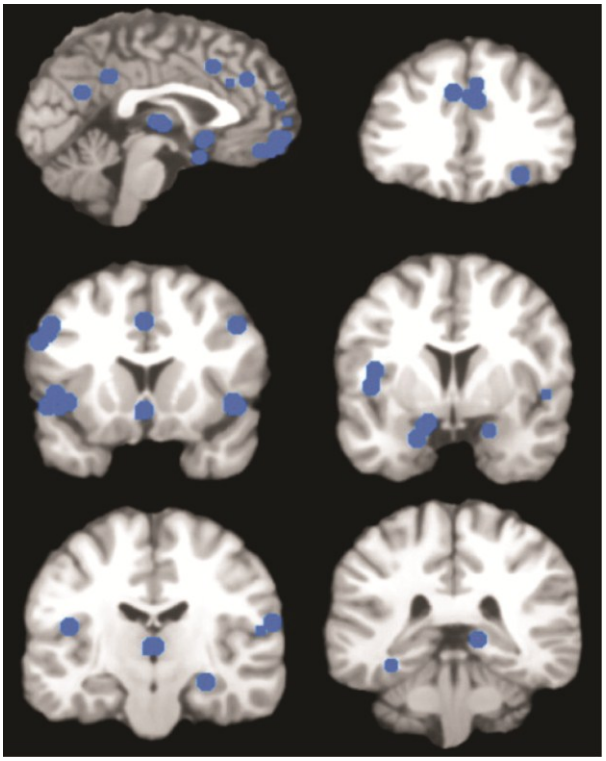


Figure 1.2 Brain regions affected in schizophrenia. A meta-analysis of brain regions determined to be reduced in gray matter density (volume) via structural imaging in individuals with chronic schizophrenia compared to controls. Many regions are affected including the frontal and temporal cortex, hippocampus, and thalamus. From (Shepherd et al. 2012).

As mentioned above, both urban living and migration appear to be childhood risk factors for developing schizophrenia (McGrath et al. 2004). Both of these may cause socioeconomic challenges or hypothalamus-pituitary-adrenal stress axis overactivity that could negatively impact development and puberty. Another risk exposure is cannabis, the use of which correlated with increased subsequent diagnosis in a dose-dependent fashion even after correction for

prodromal symptoms to rule out self-medication (Brown 2011). Cannabinoid receptors might alter dopamine release from the ventral tegmental area to the striatum, thereby worsening neurotransmitter imbalances (Dean et al. 2001).

Environmental factors are an important consideration because they may represent possible inroads to disease prevention that are safe and uncontroversial, as opposed to premorbid treatment. For example, maternal immunization against influenza and other viruses and prenatal education may represent feasible, safe, effective ways to limit schizophrenia risk slightly. Furthermore, environmental exposures may interact with genetic factors in determining risk.

1.3 Genetics of schizophrenia

Twin studies yielded an estimate of 81% heritability for schizophrenia based on meta-analysis of the difference between dizygotic twin diagnostic concordance (10-15%) and monozygotic twin concordance (40-55%) (Sullivan et al. 2003). This means that a major fraction of the predisposition to schizophrenia is attributable to genetic factors as opposed to environmental exposure; yet in most families schizophrenia is not a Mendelian disease with recessive or dominant inheritance patterns. To add to the complexity, only about one third of individuals with schizophrenia have family members with schizophrenia, meaning up to two thirds of cases are sporadic, implying the genetic contribution is of low penetrance or due to novel mutations (Tandon et al. 2008). Therefore the genetics of schizophrenia are quite complex and until recent advances in technology permitted assessment of newly discovered forms of genetic variation such as single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) there was little agreement on the genetic factors contributing to schizophrenia.

1.3.1 Common vs. rare variants (adapted from (Levy et al. 2012))

Most psychiatric disorders are multifactorial in nature with complex genetic etiologies.

Both risk allele frequency and penetrance contribute to disease susceptibility. The common disease-common variant (CDCV) hypothesis emphasizes the importance of relatively common or high frequency alleles, each of small effect, acting together to increase disease risk. The common disease-rare variant (CDRV) hypothesis, conversely, stresses the impact of individually rare yet highly penetrant alleles (Figure 1.3). Schizophrenia is undoubtedly caused by both common and rare alleles, yet the relative contribution of each hypothesis remains debatable.

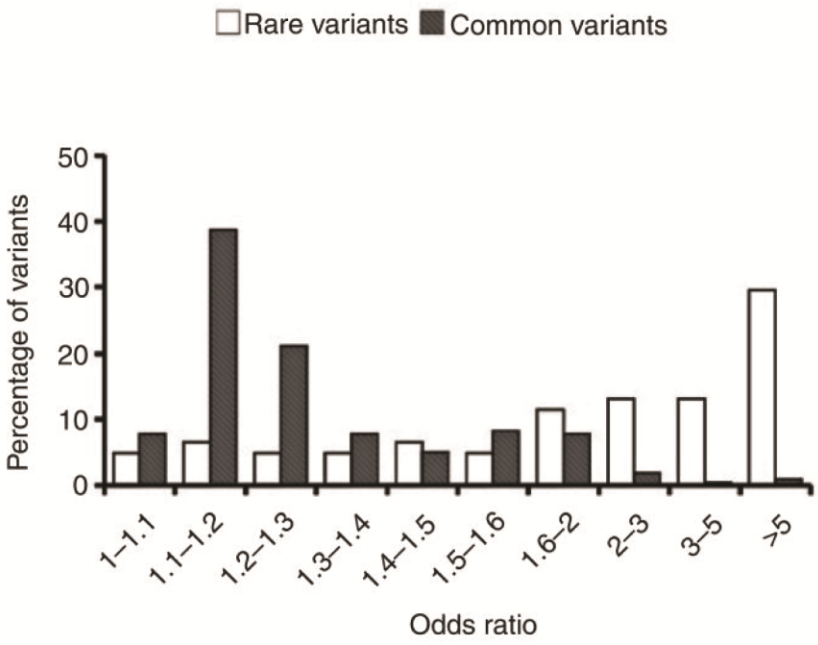


Figure 1.3 Comparison of rare and common variants. Common variants from the literature of all genetic diseases are more likely to have lower odds ratios than rare variants, in line with the effects of natural selection. From (Bodmer and Bonilla 2008).

Association studies to examine the CDCV hypothesis can either investigate candidate genes based on *a priori* functional or positional evidence or identify candidates using unbiased genome wide association studies (GWAS) (Altshuler et al. 2008). Some of the more recent GWAS identified additional putative loci for schizophrenia, as well as bipolar disorder and autism (Ferreira et al. 2008; O'Donovan et al. 2008; Wang et al. 2009). It is interesting to note that the implicated loci do not include any of the previous top candidate genes. After nearly 1,500 association studies on almost 800 candidate genes for schizophrenia (www.szgene.org), no genes have shown unequivocal and replicable evidence of association with schizophrenia (Allen

et al. 2008; Need and Goldstein 2009; Sanders et al. 2008). The most recent GWAS meta-analysis, with almost 10,000 cases and nearly 1 million SNPs, did not identify any individual risk loci, but did calculate that a minimum of 23% of schizophrenia risk is due to common variants (Lee et al. 2012). When the largest schizophrenia group to date (almost 18,000 cases) was analyzed by GWAS, seven loci reached genome-wide significance, but only two of those loci had been previously reported, indicating that greater power yields results but minimal replication (Schizophrenia Psychiatric Genome-Wide Association Study 2011). Thus common variant approaches have not been highly successful in defining specific predisposing genetic factors.

Because few families transmit psychiatric disorders in a Mendelian pattern and technology was inefficient to recognize rare alleles, until recently, the CDRV hypothesis had not received much attention. As technology to detect rare mutations improves, it is becoming increasingly clear that rare alleles may contribute substantially to both familial and sporadic cases of schizophrenia (Bodmer and Bonilla 2008; ISC 2008; Xu et al. 2008). Ironically, while the limitations of linkage studies spurred GWAS to elucidate common risk alleles, it was the unintended repurposing of GWAS data for copy number variation detection that provided strong supporting evidence for the rare variant hypothesis. With the discovery of copy number variants (CNVs) as a form of rare genetic variation, there has been a wave of research demonstrating that rare, occasionally recurrent, CNVs contribute to schizophrenia genetic heterogeneity and etiology. Plummeting sequencing costs and new arrays containing all known polymorphic exonic coding variants will result in many additional studies of rare mutations. This represents a conceptual shift in our understanding of the genetic architecture of schizophrenia.

Rare variants also help to explain how schizophrenia and other disorders can persist as genetic diseases. Schizophrenia dramatically reduces fertility rates, thereby limiting the

transmission of inherited causal variants (Laursen and Munk-Olsen 2010). This natural selection should reduce the incidence of schizophrenia, yet as discussed above the incidence remains stable. The evidence for rare mutations, especially de novo germline mutations, provides a constant source of new risk (Rees et al. 2012; Xu et al. 2012; Xu et al. 2008).

1.3.2 Copy number variant results in schizophrenia (adapted from (Levy et al. 2012))

Microdeletion of 22q11.2 was one of the first CNV described in schizophrenia (Karayiorgou et al. 1995). Since its initial discovery a strong and specific relationship has been established between 22q11.2 microdeletion and psychosis; as patients enter adulthood up to one-third of all individuals carrying this deletion developed either schizophrenia or schizoaffective disorder (Gothelf et al. 2007; Murphy et al. 1999; Pulver et al. 1994). A number of studies, including those using CNV arrays, indicated that 22q11.2 deletions account for as many as 1–2% of sporadic (non-familial) schizophrenia cases (ISC 2008; Karayiorgou et al. 1995; Stefansson et al. 2009; Xu et al. 2008). There were initial reports of an association of autism with both deletions and duplications at this locus, however a recent meta-analysis demonstrated that 22q11 duplication is significantly associated with autism and 22q11 deletion with schizophrenia, but there is no significant overlap between diagnostic categories and risk variants (Crespi and Crofts 2012). Thus 22q11 deletion represents the only confirmed, recurrent structural mutation responsible for introducing new, sporadic cases of schizophrenia specifically.

Following rapid developments in high-density microarray technologies designed to screen for structural variants throughout the whole genome and advancements in statistical analysis methods, several groups in rapid succession provided evidence that rare CNVs contribute to the genetic etiology of schizophrenia (Figure 1.4). A high resolution SNP array analysis performed on 159 schizophrenia and 200 control trios (affected individual and both

biological parents) determined that de novo or non-inherited CNVs are significantly more common in sporadic schizophrenia cases than controls, a 10% rate compared to 1.3%, respectively (Xu et al. 2008). In contrast with de novo CNVs, inherited rare CNVs were only 1.5 times more common in sporadic cases than controls, representing a smaller but significant increase from 20% to 30%. Analysis of the genes affected by the de novo CNVs demonstrated enrichment for pathways participating in neural development and RNA processing. Conversely, a parallel scan that focused on familial schizophrenia (cases with at least one first- or second-degree relative with schizophrenia) showed that rare inherited CNVs had a prominent role, being almost twice as common in familial cases of schizophrenia compared to sporadic cases or controls (Xu et al. 2009), while there was no enrichment of de novo CNVs. Nine of 12 families with inherited CNVs showed evidence of CNV and disease co-segregation, increasing the likelihood of pathogenicity.

The role of de novo as compared to inherited CNVs was investigated by several studies with similar family-based patient populations. Among 662 schizophrenia trios, the rate of de novo CNVs was 5.5% in sporadic cases as opposed to 5.1% in familial cases and 2.2% in controls (Kirov et al. 2012). In a smaller study of 177 schizophrenia trios, the de novo CNV rate was significantly increased over controls to an odds ratio of 5.0, although de novo CNVs were not more common in sporadic compared to familial cases (Malhotra et al. 2011). Alternatively, in a study of 631 families with multiple individuals with schizophrenia, the CNV rate was similar to nonfamily studies (Levinson et al. 2012). These studies present empirical evidence supporting the hypothesis that multiple rare CNVs contribute to the genetic risk of schizophrenia, including mutations unique to a family termed “private” CNVs that affect diverse genes. The results also suggest a divergence in the genetic architecture of familial and non-familial (i.e. sporadic)

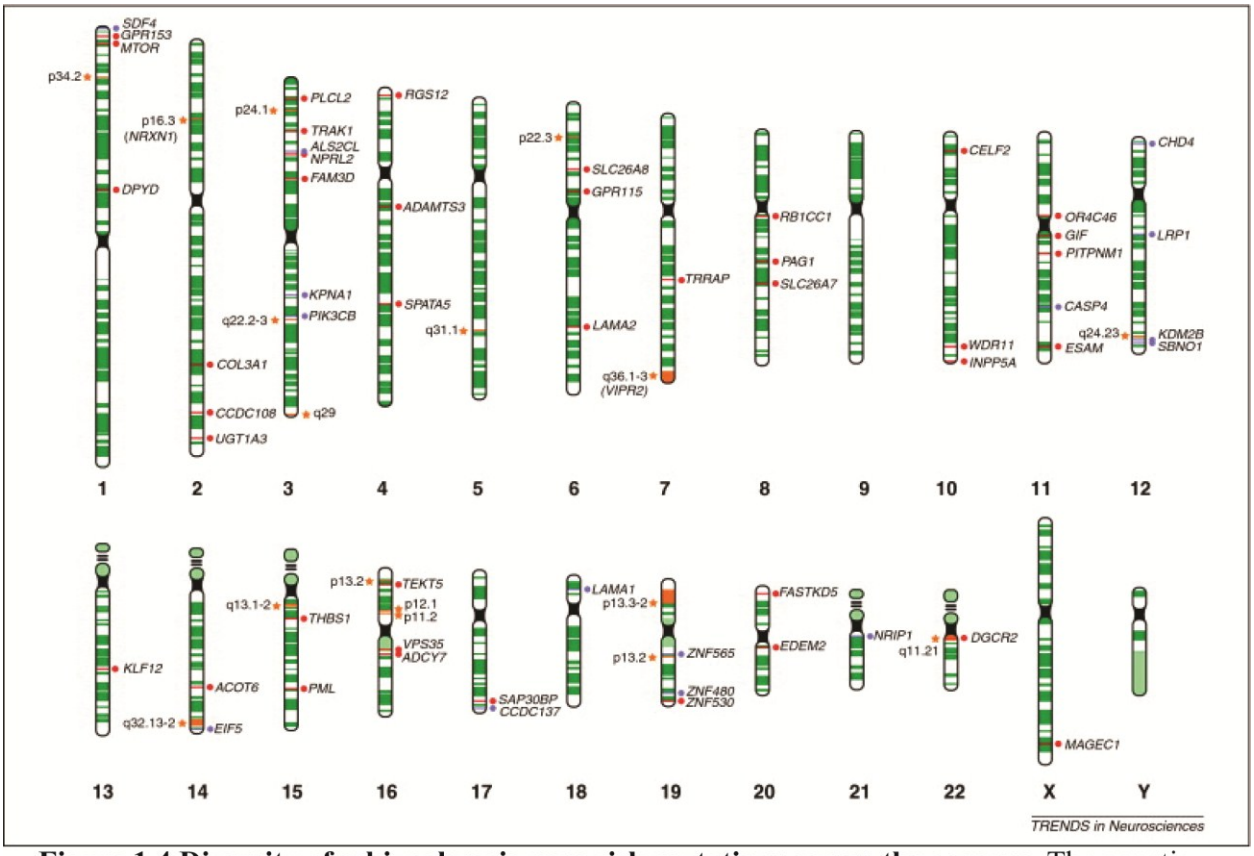


Figure 1.4 Diversity of schizophrenia rare risk mutations across the genome. The genetic etiology of schizophrenia is highly heterogeneous, as evidenced by recent studies of de novo mutations such as copy number variants (CNVs, stars) and point mutations (purple and red circles) identified in patients. As the pool of patients and controls increases, the list of potential rare disease-causing mutations is likely to grow as well. We only indicate de novo mutations for point of clarity and the fact they are more likely to be pathogenic. The de novo CNVs shown are those described in the literature as of July 2011. From (Arguello and Gogos 2012).

schizophrenia (Figure 1.5). While the overall frequency of rare structural variants is equivalent between the familial and sporadic cases (~40%), the heritable nature of variants is markedly different. Sporadic schizophrenia is characterized by a significant enrichment of rare de novo mutations and only a modest increase in the rate of rare inherited CNVs that do not appear to preferentially affect genes. By contrast, familial schizophrenia is characterized by enrichment in rare inherited genic CNVs that hypothetically have higher penetrance, while de novo mutations are found at lower frequency.

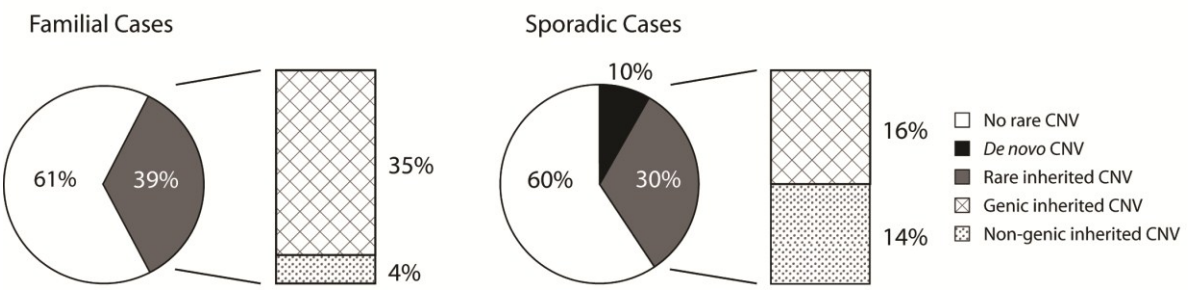


Figure 1.5 Divergence in the genetic architecture of familial and non-familial schizophrenia. Frequency distribution of rare CNVs identified in familial and sporadic cases of schizophrenia. De novo CNVs are higher in sporadic than familial cases or controls. There is a ~20% basal rate of inherited CNVs in controls, while the overall frequency of carriers of rare CNVs is the same between familial and sporadic cases (~40%). CNVs from familial cases are more likely to affect genes than those from sporadic cases, suggesting they inherit more pathogenic mutations. Adapted from (Xu et al. 2009).

Three initial case/control studies (ISC 2008; Stefansson et al. 2009; Walsh et al. 2008) also provided evidence for a collective enrichment of rare CNVs in schizophrenia, although they could not confirm the importance of CNV heritability or familial as opposed to sporadic schizophrenia on mutation rates. Initially, Walsh and colleagues used ROMA on 150 schizophrenia cases plus controls (Walsh et al. 2008). They reported a significant 3-fold increase in the frequency of rare genic CNVs among cases (15% compared to 5% in controls), and a 4-fold increase when only early-onset cases were considered (20% compared to 5% in controls). In an independent sample of 83 early-onset trios, they determined that rare genic CNVs were present in 28% of cases compared to 13% of non-transmitted parental chromosomes, a significant increase. One recurrent CNV was identified at 16p11.2. Genes affected in the cases were mostly involved in brain development and neural function and included ERBB4 and Neurexin1 (NRXN1).

The International Schizophrenia Consortium (ISC) analyzed 3,391 European schizophrenia cases and controls (ISC 2008). When rare CNVs with <1% overall frequency were

considered, cases had 1.15 times as many CNVs as controls. Furthermore, cases had 1.41 times as many genes affected by CNVs. This study confirmed that 22q11.2 deletions were significantly associated with schizophrenia and identified two other recurrent CNVs at 15q13.3 and 1q21.1 that had significantly increased frequency among cases.

Additionally, Stefansson et al. employed a three-step strategy that partially took advantage of family structure to provide additional evidence for the role of rare CNVs in schizophrenia (Stefansson et al. 2009). First, they identified 66 de novo CNVs in over 7,500 control families. Next, they tested these 66 CNVs for association with schizophrenia in 1,433 cases and over 33,000 controls. Three recurrent deletions at 1q21.1, 15q11.2, and 15q13.3 were nominally significant among cases with schizophrenia or psychosis, two of which were also reported in the ISC scan (ISC 2008). Finally, they performed association analysis in 3,285 additional cases and controls, and reported that all three CNVs, but not SNPs within these regions, were significantly associated with psychosis with high odds ratios. Moreover, the controls included individuals with other psychiatric disorders, thus possibly masking non-psychotic neuropsychiatric phenotypes associated with these CNVs.

Next, Need et al. performed both a GWAS and a CNV scan in 871 cases plus controls, followed by independent replication (Need et al. 2009). The GWAS yielded no significant results. CNV analysis, however, indicated an enrichment of large structural variants (>2 Mb) in the cases. CNVs within NRXN1, as well as at 1q21.1 and 22q11.2, replicated earlier findings.

Three smaller-scale studies also investigated the importance of rare CNVs in the etiology of schizophrenia. A scan of schizophrenia trios identified 13 rare CNVs in cases that were absent from controls or the CNV database (Kirov et al. 2008). The most interesting findings in light of the results discussed above were a deletion at 2p16.3 involving NRXN1 inherited by affected

siblings from their unaffected mother, a de novo duplication at 15q13.1, and a deletion at 16p12.2 inherited from a parent with a non-psychotic affective disorder. Another SNP array analysis of 54 Dutch cases discovered CNVs affecting NRXN1, as well as 3 other candidate genes (Vrijenhoek et al. 2008). A study in 471 UK schizophrenia cases investigated how CNV frequency, size, and copy number were associated with schizophrenia (Kirov et al. 2009). It was determined that CNVs with frequency <1% and length >1 Mb were 2.3 times more frequent in cases than controls. After combining their data with the Stefansson et al. (2008) and ISC (2008) studies in a preliminary meta-analysis, 1q21.1, 15q11.2, 15q13.3, and 17p12 demonstrated significant association with a broad schizophrenia diagnosis.

Subsequent to these initial reports, there have been dozens of additional studies and meta-analyses confirming the importance of rare CNVs in schizophrenia and other neuropsychiatric disorders, some of which are noted in Tables 1.1 and 1.2. Of note, several studies found an enrichment of synaptic genes affected by rare CNVs (Glessner et al. 2010; Kirov et al. 2012). While all the studies described here were based on blood samples, one study used postmortem brain tissue from a variety of neuropsychiatric disorders and, though underpowered to detect a change in CNVs in cases, replicated prior findings at 1q21.1 11q25, 15q11.2 and 22q11 in individuals with schizophrenia (Ye et al. 2012). Interestingly, a parallel expression microarray analysis found that CNVs have variable penetrance on gene expression.

There are parallel copy number findings in autism spectrum disorder. De novo CNVs are enriched in individuals with autism, especially in sporadic cases which had rates ten-fold higher than controls and three times higher than familial cases (Sebat et al. 2007). This finding of increased overall CNV burden especially de novo CNVs in sporadic cases has been repeated several times (Marshall et al. 2008; Sanders et al. 2011). Conversely, homozygosity mapping and

CNV analysis in consanguineous families identified fewer de novo and more inherited CNVs compared to other autism populations, confirming that de novo CNVs contribute less when there is an increased recessive risk burden (Morrow et al. 2008).

Overall, these studies suggest that rare structural rearrangements collectively contribute significantly to schizophrenia risk. De novo mutations appear to contribute more to sporadic than to familial schizophrenia. Several loci were discovered in multiple studies, indicating risk loci as well as specific genes (Tables 1.1 and 1.2). Thus, rare CNVs represent an important source of genetic heterogeneity in the etiology of complex psychiatric diseases such as schizophrenia.

Table 1.1: Loci Overlapping in CNV Reports

Locus of interest	***Study***	***Candidate genes***	***Odds Ratio (95%CI)****
1q21.1	(ISC 2008);(Stefansson et al. 2008);(Kirov et al. 2009);(Need et al. 2009)	*GJP8*	9.1 (4.2–19.4)
2p16.3	(Kirov et al. 2008);(Walsh et al. 2008);(ISC 2008);(Vrijenhoek et al. 2008);(Rujescu et al. 2009);(Need et al. 2009)	*NRXN1*	4.78 (2.44–9.37)
15q11.2	(Stefansson et al. 2008);(Kirov et al. 2009)	*CYFIP1*	2.8 (2.0–3.9)
15q13.1-q13.3	(ISC 2008);(Stefansson et al. 2008);(Kirov et al. 2008);(Kirov et al. 2009)	*CHRNA7, APBA2, NDNL2, TJP1*	11.4 (4.8–27)
16p11.2	(Walsh et al. 2008);(McCarthy et al. 2009)	*MAPK3, DOC2A, SEZ6L2*	8.4 (2.8–25.4)
16p12.2-p12.1	(Kirov et al. 2008);(ISC 2008)	*EEF2K, CDR2*	
16p12.4-p13.1	(Kirov et al. 2009);(Ingason et al. 2011)	*NDE1, NXPH2, NTAN1*	2.98 (non-significant)
22q11.2	(Xu et al. 2008);(ISC 2008);(Stefansson et al. 2008);(Need et al. 2009)	*DGCR8, ZDHHC8, PRODH, COMT, TBX1*	~30

*****Estimates from incomplete meta-analysis in (Kirov 2010).

Table 1.2: Candidate Genes from CNV Reports

Study	***Genes of Interest***	***Pathways of Interest***
(Walsh et al. 2008)	*ERBB4, NRXN1, SLC1A3, GRM7, PRKCD, SKP2, MAGI2, CAV1, PRKAG2, PTK2, DLG2, LAMA1, PTPRM*	Cell adhesion, glutamate receptors, cell cycle, cell growth and extension
(Kirov et al. 2008)	*NRXN1, APBA2, NDNL2, TJP1, EEF2K, CDR2*	Cell adhesion, amyloid processing, calmodulin signaling
(ISC 2008)	*CHRNA7, NRXN1, CNTNAP2, NOTCH1, PAK7, GJA8*	Cell adhesion, gap junctions, acetylcholine receptors
(Xu et al. 2008)	*RAPGEF6, EphB1, DICER1*	Cell signaling, Ephrin signaling, RNA processing
(Stefansson et al. 2008)	*GJA8, CYFIP1, CHRNA7*	Gap junctions, translation, acetylcholine receptors
(Vrijenhoek et al. 2008)	*NRXN1, MYT1L, ASTN2*	cell/synaptic adhesion
(Kirov et al. 2009)	*PMP22, NDE1, NXPH2, GJA8, CYFIP1, CHRNA7*	Myelin sheath, cell adhesion
(Xu et al. 2009)	*NRG3, RAPGEF2, PEX13, KIAA1841, AHSA2, USP34, C4orf45, PTPRN2, CSMD1, MACROD2, A26B3, LOC441956*	Neural differentiation, peroxisomal targeting, ubiquitination
(Ingason et al. 2011)	*NTAN1, NDE1*	Disc1 signaling, neuronal proliferation, memory regulation

1.3.3 Sequencing results in schizophrenia

As the costs of exon chip array and whole exome and genome sequencing are driven down by new technology, researchers are hopeful to discover some of the missing risk alleles for schizophrenia and other disorders. Sequencing the entire genome is more informative as it provides data on regulatory domains and as yet unidentified genes, however the cost and data informatics burden are currently prohibitive for large studies. To date there have been 3 whole exome studies of schizophrenia. Our laboratory reported the results of whole exome sequencing of 53 trios with a sporadic case of schizophrenia plus 22 control trios, then on a larger sample of 231 trios from South Africa (Afrikaner) and the United States with schizophrenia or

schizoaffective disorder plus 34 control trios (Xu et al. 2012; Xu et al. 2011). When the entire cohort of Afrikaner cases was considered, 73 out of 146 individuals had at least one de novo mutation predicted to be functionally disruptive. This mutation rate was not different from controls or statistical expectations, but the ratio of nonsynonymous to synonymous de novo mutations, 6.15:1, was significantly higher than the control ratio of 2.2:1 and the expected ratio of 2.23:1. This ratio was not affected among inherited mutations, suggesting a specific, deleterious accumulation of novel but not inherited mutations in sporadic cases, analogous to CNV findings (Xu et al. 2008). Moreover, four genes were affected by two de novo mutations each among cases, demonstrating that even rare mutations can cluster in novel candidate genes. Individuals with CNVs were excluded from the sequencing study. Thus in the Afrikaner schizophrenia population 9.9% of sporadic cases carry de novo CNVs while 17.6% harbor rare mutations, yielding an estimate that about a quarter of sporadic cases are due to de novo mutations.

In a smaller scale study of 14 trios with moderate exome coverage, there was an increase in the number of both de novo mutations and nonsense mutations in cases compared to controls (Girard et al. 2011). Although this sample was underpowered, the findings are consistent with our results.

Finally, a study of 166 individuals with schizophrenia or schizoaffective disorder who were treatment resistant or had a strong family history of schizophrenia found no significant enrichment of rare mutations (Need et al. 2012). They selected the 5155 variants that had a P value <0.05 and genotyped these in additional cases and controls, but no loci were significantly associated with schizophrenia after correction for multiple comparisons. The authors calculated that since they were adequately powered to detect rare (1-5% frequency) variants with relative

risk of 2-6 but failed to do so, the missing variation in schizophrenia must reside in very rare (<1%) mutations.

The coming months will surely bring additional sequencing data, especially since psychiatric genetics experts have recommended a focus on rare, penetrant mutations such as those that result from sequencing (Karayiorgou et al. 2012). It is probable that future sequencing results will confirm CNV findings in establishing the importance of rare mutations as causes of schizophrenia.

1.3.4 Interactions among rare and common variants

There are several pathways through which common and rare variants may interact in determining schizophrenia risk. First, common variants may identify loci affected by rare variants, or vice versa. Common variants discovered through SNP association studies are not usually deleterious, therefore they are presumed to be in linkage disequilibrium with rare variants that are causal (Bodmer and Bonilla 2008). Thus common variants might predict the location of rare variants for targeted sequencing of candidate regions. Alternatively, genes carrying rare variants may also carry common variants as well, as is the case with *DISC1*. While *DISC1* mutation was identified as a cause of neuropsychiatric disease including schizophrenia due to a truncating translocation in one family, there are many studies of common SNPs within the gene that are associated with schizophrenia as well (i.e. (Saetre et al. 2008)).

Next, common variants might modify rare variants within the same gene or in gene-gene interactions. In schizophrenia, such an interaction has been suggested between common CNVs affecting glutathione transferases *GSTM1* and *GSTT2* and rare CNVs elsewhere in the genome that also contribute to risk (Rodriguez-Santiago et al. 2010). In hereditary non-polyposis colon cancer Lynch I syndrome, which was thought to be a purely monogenic, rare disease, common

alleles affecting the same family of glutathione transferases may also modify the severity of disease symptoms and onset (Felix et al. 2006; Pande et al. 2008). Common variant risk modifiers have also been identified in β-thalassemia (Nuinoon et al. 2010).

The common variant hypothesis assumes that common alleles interact with each other, but there is also growing evidence for interactions among rare variants as well. For example, the rare CNV of 16p12.1 deletion was associated with developmental delay, but carrying additional CNVs in addition to 16p12.1 deletion predicted worse symptoms (Girirajan et al. 2010). This phenomenon has been termed the two hit hypothesis or oligogenic model of rare mutations interacting to affect disease phenotype (Coe et al. 2012; Girirajan et al. 2012). This suggests rare variants do not need to be 100% penetrant therefore explaining why identified CNVs occur at low levels in control populations. Such interactions may also help explain phenotypic variation, or the association of one primary CNV with multiple diagnoses. While this primary CNV increases neurodevelopmental disease sensitivity, differing secondary rare variants in each individual's genetic background may predispose to one particular disorder over another. These secondary variants could be CNVs or smaller mutations that presumably disable disorder-specific circuitry and molecular pathways that interact with genes altered by the primary CNV. As the spectrum of identified risk factors expands, understanding the relationships between variants will become an exciting new field.

1.3.5 Rare variants affect circuitry

Rare mutations that have been reported in schizophrenia do not frequently implicate the same genes, but they do converge on similar pathways. As mentioned, several CNV studies found that affected genes were significantly enriched for synaptic pathways (Glessner et al. 2010; Kirov et al. 2012; Walsh et al. 2008). Overall, there is a pattern among studies in

schizophrenia, bipolar disorder, and autism that genetic findings implicate synaptic and axonal pathways (Arguello and Gogos 2012). Moreover, sequence results from our laboratory determined that affected genes were more likely to be expressed during fetal development, indicating a temporal specificity for neurodevelopment (Xu et al. 2012). This convergence onto synaptic function and neurodevelopment could help explain how such a disparate mutational load can yield the same clinical disorder and informs our understanding of the neurobiological etiology. Given that several neurodevelopmental diseases are inter-related by the same pathways, however, this avenue of research does not help differentiate between pathophysiology mechanisms. It remains to be proven how these genes affect synaptic function, how disease variants compromise this function, and how changes at the cellular level can contribute to the complicated clinical spectrum of dysfunction.

In contrast to temporal and functional approaches, studying brain circuitry via animal models may be a faster and more specific way to investigate pathophysiology. Analysis of the 22q11 mouse model found altered hippocampal-prefrontal cortex connectivity during working memory performance (Sigurdsson et al. 2010). Careful assessment of animal models based on rare mutations with disease specificity will determine if there are other circuitry phenotypes that are unique to particular diagnoses. Future studies could assess circuitry via optogenetics approaches or in vivo recordings (Kvajo et al. 2012). Circuit analysis has the added benefit of directly explaining pathophysiology on a functional level.

1.4 Comorbidity and coheritability of schizophrenia with affective and anxiety disorders

There is much clinical overlap among patients with schizophrenia, mood, and anxiety disorders. The Diagnostic and Statistical Manual requires that for multiple diagnoses to be valid, the two or more sets of symptoms must be distinguishable in time. By one estimate, 30% of

patients with schizoaffective disorder and 17% of those with schizophrenia also have anxiety disorder diagnoses (Young et al. 2012). A meta-analysis of anxiety disorder comorbidity published prior to the release of that study found that among individuals with schizophrenia 12% were also diagnosed with obsessive compulsive disorder, 15% with social phobia, 11% with generalized anxiety disorder, 10% with panic disorder, and 12.4% with post-traumatic stress disorder (Achim et al. 2011). While some of this prevalence may be overlapping, a conservative estimate is that 30-40% of patients have a coexisting anxiety disorder. Among affective disorders, the comorbidity of depression is estimated at half of all patients with schizophrenia (Buckley et al. 2009). Patients with schizophrenia have 20 times higher risk of being diagnosed with bipolar disorder and those with schizoaffective disorder carry over 100 fold increased risk of bipolar comorbidity (Laursen et al. 2009). Having a comorbid diagnosis is associated with more severe symptoms and worse outcome.

This high degree of comorbidity may be due to a shared genetic etiology of neuropsychiatric disorders or a lack of diagnostic specificity. Genetic studies demonstrate overlap among risk loci for schizophrenia, bipolar, and autism spectrum disorders (Carroll and Owen 2009). Additionally, relatives of patients with either bipolar disorder or schizophrenia have an increased risk of having the other diagnosis (Van Snellenberg and de Candia 2009). While genetic overlap between schizophrenia and anxiety disorders is less prevalent, there are reports of variants in schizophrenia or anxiety candidate genes such as *DTNBP1*, *GRM1*, and the serotonin transporter being associated with anxiety diagnosis as well (Ayoub et al. 2012; Haddley et al. 2012; Voisey et al. 2010). Some researchers believe there may be a clinical and genetic spectrum connecting schizophrenia with affective disorders. This shared genetic risk may underlie a shared neuropathology of brain regions or neurotransmitter systems. Alternatively,

these diagnoses may predispose patients to other diseases, for example schizophrenia may increase the risk of traumatic events occurring and therefore the rate of post-traumatic stress disorder. The data suggest that any clinical or animal model should consider assessment of affective and anxiety symptoms as well as the core symptoms of schizophrenia.

1.5 Animal modeling of neuropsychiatric disease

1.5.1 Animal model validity

While epidemiology, in vivo imaging, and treatment outcome studies have greatly contributed to our understanding of schizophrenia, there are a multitude of questions remaining that can only be answered through experimentation. Animal models provide an ethical way to explore questions of disease etiology, pathophysiology, and treatment. In order to gauge whether an animal represents an accurate and useful model, there are three criteria to be met: face validity, construct validity, and predictive validity (Robbins and Sahakian 1979). Face validity requires that a model recapitulates disease symptoms especially behavioral symptoms and pathology. Construct or etiological validity means the animal model shares either the genetic or environmental etiology of the disease and therefore the ensuing neurobiological mechanisms are affected. Predictive validity is when animal models respond the same way humans do to treatments or interventions. The goal is to create models that are valid at as many levels as possible.

Schizophrenia is not an easy disease to model in rodents. Many of the clinical symptoms do not apply to rodent behavior making it challenging to prove face validity or to treat those symptoms with antipsychotic drugs to establish predictive validity. Moreover, as there are no genetic loci or environmental risks that have 100% penetrance for schizophrenia in humans, it is difficult to establish construct validity. Fortunately, there are some symptoms that translate and

some mutations that are amenable to modeling.

Several schizophrenia symptoms can be modeled and measured in rodents (Powell and Miyakawa 2006). Amphetamine induced hyperactivity in animals is similar to clinical findings of increased positive symptoms following the administration of amphetamines and other psychostimulant drugs. Social interaction deficits are analogous to the negative symptom of social withdrawal. Cognitive symptoms such as reduced working memory and attention are analogous to impairments on maze tasks with delays such as the T maze. Finally, sensorimotor gating deficits, a specific neurological phenomenon found in patients with schizophrenia, can be modeled in rodents as prepulse inhibition. A strong model should find converging evidence of deficits in several of these tests (Arguello and Gogos 2006). All of these behavioral domains will be discussed in detail and many of them experimentally tested in Chapter 3.

1.5.2 Rare mutation models of schizophrenia

Rare mutations are considered by some, including our laboratory, to be the most etiologically valid type of animal model (Arguello et al. 2010). Rare mutations are more likely to be causal because they are more likely to be deleterious; common variants are less damaging because they persist at high frequencies under natural selection. This is validated by the higher penetrance and risk of rare as opposed to common variants (Bodmer and Bonilla 2008). Due to high homology and synteny between the human and mouse genome, gene mutations or entire CNVs can be faithfully mimicked across species (Kas et al. 2009). Thus the ability to accurately model a pathologic mutation holds much promise for understanding the neurobiology and pathophysiology.

There are several well characterized models of rare mutations in schizophrenia including the 22q11 deletion syndrome, *Disc1*, and *Neurexin1a*. Four mouse models recapitulate the 22q11

human deletion to varying extends and many of the findings concur (Kvajo et al. 2012). Our laboratory developed the *Df(16)A*$^{+/-}$ mouse that faithfully models the 1.5Mb 22q11 deletion (Stark et al. 2008). Behaviorally, the heterozygous deletion mouse was hyperactive and anxious with impaired prepulse inhibition, fear conditioning, and working memory acquisition on the T maze but not the Morris water maze (Drew et al. 2011; Stark et al. 2008). In vivo and in vitro, hippocampal neurons had decreased dendritic arbors and reduced mushroom spine density and width (Mukai et al. 2008). On electrophysiology analysis, there was a reduction in inhibition in hippocampal area CA1, diminished long term potentiation, and decreased CA3 neural activation (Drew et al. 2011). Many of these findings were phenocopied by heterozygous deletion of *Dgcr8*, a microRNA processing gene within the deleted locus. *Dgcr8*$^{+/-}$ mice had deficits in prepulse inhibition and working memory, but locomotion and fear conditioning were normal (Stark et al. 2008). On analysis, these mice shared a reduction in the hippocampal CA1 dendritic arbor and mushroom spine width, although spine density was unaffected, while in the medial prefrontal cortex there was a reduction in neuron density and spine area (Fenelon et al. 2011; Stark et al. 2008). Multiple other genes within the homologous region on mouse chromosome 16 have also been explored via knock out models. The results indicate that hippocampal morphology and electrophysiology are affected by several genes, revealing a shared phenotype of pathology.

Truncation of *DISC1* in one family with high incidence of neuropsychiatric diagnoses drew attention to this gene as a rare cause of schizophrenia and other disorders (Millar et al. 2000). There are multiple models affecting this gene, varying from deletions to dominant negative knock in mice, but our laboratory generated a model that most accurately represents the effects of the human truncation (Koike et al. 2006). This mouse was specifically impaired on two

tests of working memory (Koike et al. 2006; Kvajo et al. 2008). Morphologically, Disc1 animals had reduced cortex volume and apical dendritic arbor length in the prefrontal cortex, as well as increased migration and diminished, misoriented dendritic arbors of adult born neurons in the dentate gyrus subregion of the hippocampus. When compared with the 22q11 and Dgcr8 models, both genetic manipulations converged on altered working memory and changes in the prefrontal cortex and hippocampus, the brain regions responsible for generating working memory. This highlights the importance of cognitive symptoms, especially working memory, in animal modeling.

As noted above, multiple CNV studies found deletions affecting *NRXN1* in schizophrenia as well as autism. When deleted in mice, the homologous gene *Neurexin1a* was proven to be important in hippocampal function. Deletion mice had decreased spontaneous excitatory post-synaptic current frequency and input/output ratios but inhibitory neurotransmission was unchanged, indicating a specific reduction in CA1 hippocampal excitatory synaptic strength (Etherton et al. 2009). On behavioral testing, these animals had reduced prepulse inhibition and nest building, increased grooming and motor learning on the rotarod, and no changes in social interaction, anxiety, or spatial memory. While the hippocampal deficits are in line with other rare schizophrenia models, the behavior phenotype is more suggestive of autism symptoms than of schizophrenia. This model highlights the issue of pleiotropy in genetics since rare mutations can be associated with multiple diagnoses. Although this mouse model is certainly valuable in terms of understanding similarities between schizophrenia and autism and the role of Neurexin1 in neural function, it does not advance our understanding of schizophrenia-specific pathophysiology. The same concern applies to animal models of *CNTNAP2* deletion and 16p11.2 duplication as these rare mutations are similarly shared between patients with schizophrenia and

autism (Horev et al. 2011; Penagarikano et al. 2011). In order to generate useful animal models of schizophrenia pathophysiology, we should focus on non-promiscuous, disease-specific mutations.

Although there are many animal models exploring genetic, pharmacologic, and environmental contributions to schizophrenia risk, there are very few animal models that target rare genetic mutations known to increase the risk of schizophrenia specifically.

1.6 Statement of hypothesis

We aim to understand the genetic etiology of schizophrenia by assessing rare mutations that are specific to schizophrenia and amenable to animal modeling. We identified *RAPGEF6* as a putative causal gene in a de novo case of schizophrenia; consequently we speculated that it contributes to neuropsychiatric development and function in both humans and rodents. We hypothesized that a mouse model knocking out *Rapgef6* will have neural and behavioral phenotypes associated with schizophrenia. We tested these hypotheses via the following experiments:

1) CNV and whole exome sequencing of schizophrenia cases as described in Chapter 2
2) Behavioral characterization of a mouse with *Rapgef6* deleted, including assessment of schizophrenia endophenotypes and amygdala and hippocampal activation, as described in Chapter 3
3) Morphological analysis of neurons in vivo and in vitro from the hippocampus, prefrontal cortex, and amygdala, regions associated with both the altered behavioral domains and clinical findings in schizophrenia, as described in Chapter 4

1.7 In the next chapter

Having covered recent genetic findings in schizophrenia, we will next probe deeper into

these results to explore the genetic convergence onto chromosome 5q and the *RAPGEF6* locus. A variety of genetic techniques discussed above identified *RAPGEF6* as a potential candidate gene for schizophrenia.

Chapter 2: *RAPGEF6* Genetic Findings

2.0 My role

I contributed to some of the probe design and execution of multiplex ligation-dependent probe amplification (MLPA) to validate inherited CNVs. I also designed primers and performed sequencing to confirm the mutation found via sequencing in *RAPGEF2*.

2.1 Introduction: 5q genetic results in schizophrenia

Prior genetic research in schizophrenia pointed to the *RAPGEF6* locus, including cytogenetics, linkage, and association studies (Figure 2.1). Early karyotype studies drew neuropsychiatric attention to chromosome 5q. An uncle and a nephew concordant for schizophrenia as well as facial abnormalities were found to carry, along with the proband's unaffected mother, a trisomy of chr5q11.2-q13.3 (Bassett et al. 1988). Another duplication of chr5q35.2-q35.3 was found in a child with microcephaly, mental and motor retardation, and facial abnormalities (Chen et al. 2006a). Neither of these duplications covered the *RAPGEF6* locus directly, but this gene was influenced by a 5q deletion (remapped in the current genome) in a woman with schizophrenia, mental retardation, and facial abnormalities (Bennett et al. 1997) and an insertion of 5q22.1-q31.1 in 10 family members leading to duplications and deletions of *RAPGEF6* and other genes, both of which led to developmental and anatomic abnormalities but not schizophrenia (Arens et al. 2004).

Linkage results for schizophrenia on chromosome 5 originated with associations at 5q11-q13 (Sherrington et al. 1988) and 5p14.1-p13 (Silverman et al. 1996). Assessment of specific European high risk families drew attention to the *RAPGEF6* locus on 5q31.1. Irish schizophrenia pedigrees showed linkage to 5q22-q31 with a maximum LOD >3 (Straub et al. 1997); German

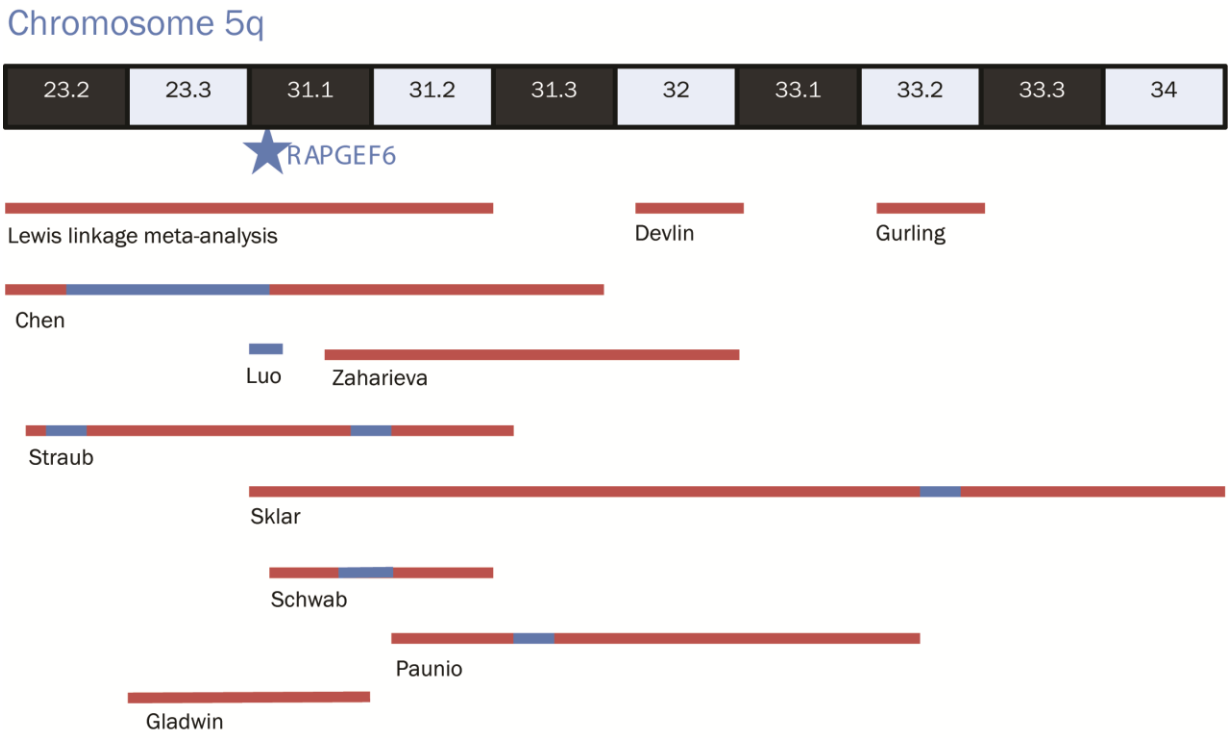


Figure 2.1 Genetic convergence onto chr 5q. Cytogenetic, linkage, and association data indicate that 5q is an inportant region in understanding schizophrenia risk. Red bars represent significant data, blue bars represent the most significan locus within a study. *RAPGEF6* is located within 5q31.1 as noted by the star.

and Israeli sibling pairs revealed linkage to 5q31.1-q31.2 with a combined LOD 1.27 (Schwab et al. 1997); Finnish families displayed linkage to 5q31.2-33.1 with LOD 3.16 (Paunio et al. 2001); and mixed families were found to be linked to 5q33.2 with LOD 3.6 (Gurling et al. 2001). Two studies of isolated cases of schizophrenia also showed linkage to 5q32 and 5q35 in Palau natives (Devlin et al. 2002) and 5q 166-176.6cM in European Costa Ricans (DeLisi et al. 2002). After these findings, a genome-wide meta-analysis of linkage results found that 5q21-q31 was the fourth most important linkage peak in schizophrenia although it did not reach genome-wide significance (Lewis et al. 2003). Subsequent to that meta-analysis, one final linkage study was published linking 5q31-q35 to schizophrenia, psychosis in schizophrenia, and bipolar disorder (Sklar et al. 2004).

With the advent of single nucleotide polymorphism (SNP) genotyping, researchers began to explore genome-wide association (GWA) with schizophrenia. Assessment of Irish pedigrees demonstrated association with 5q31.1 including *SPEC2*, *RAPGEF6*, and *ACSL6*; this finding was replicated in an independent sample, but the linkage disequilibrium between these genes was too strong to narrow the locus (Chen et al. 2006b). The region encompassing these three genes also was implicated in a Han Chinese population analyzing 8 SNPs representing a haplotype block across the locus (Luo et al. 2008). The researchers also determined that there was significant evidence for positive selection (reduced mutation) on *RAPGEF6* across animal species. A focused analysis of 5q31-q32 in family trios found a moderate association with Neuregulin2, protocadherins, and *IL9* (Zaharieva et al. 2008). Disequilibrium testing limited to 8 genes of interest within the Irish sample did not find a significant association for *RAPGEF6* (Edwards et al. 2008). Finally, a genome-wide study performed by grouping SNPs into regional loci using genotypes from 4 public data sets found association with schizophrenia at 5q23.3-q31.1 (Gladwin et al. 2012). Figure 2.1 clearly demonstrates that there was convergence of genetic data from a variety of studies onto the *RAPGEF6* locus prior to our copy number variation (CNV) and sequencing analysis.

2.2 Methods

2.2.1 Patient collection (adapted from (Xu et al. 2008))

Patient collection methods were described in (Abecasis et al. 2004). Both affected and control families were recruited from the Afrikaner population in South Africa. Afrikaner heritage was established by surname and by having Afrikaans-speaking grandparents. Diagnostic evaluations were done in person by trained clinicians using the Diagnostic Instrument for Genetic Studies (DIGS), which was translated and back-translated into Afrikaans. Upon

completion of the DIGS, the interviewers assigned DSM-IV diagnoses and completed a detailed narrative report. All narratives and DIGS were independently evaluated by trained clinicians in New York City. Family history was obtained by a nursing sister, who recorded detailed pedigree information, and by the clinical interviewer, who inquired in detail about family history during the clinical interview.

The cohort of 152 sporadic cases consisted of all probands collected from this population who satisfied two criteria: i) both biological parents were available for genotyping, and ii) there was no history of schizophrenia in first- or second-degree relatives. Similarly, our cohort of 48 familial cases consisted of all probands collected from this population who satisfied two criteria: i) both biological parents were available for genotyping, and ii) there was a positive history of schizophrenia in first or second-degree relatives. Finally, the control cohort consisted of 159 families (triads) recruited from the Afrikaner community. A subset of the control families completed a detailed self-report questionnaire that inquired about several mental conditions, including phobias, anxiety, depression and history of treatment for any of these conditions. Also, family history of any mental illness up to three generations was excluded. Families were excluded only if sample failures occurred at the quality control and CNV identification step. Even if one of the three DNA samples included in each triad failed, the entire triad was excluded from analysis, as the de novo nature of CNV could not be established.

2.2.2 Copy number variant scan (adapted from (Xu et al. 2008; Xu et al. 2009))

We genotyped subjects using the Human Genome-Wide SNP Array 5.0 (Affymetrix), which contains 500,568 SNPs, as well as 420,000 additional nonpolymorphic probes that can assess copy number (CN) variation across the human genome. All sample processing was completed at the Vanderbilt Microarray Shared Resource according to manufacturer's protocol.

Following hybridization, we used the GeneChip Command Console (Affymetrix) to acquire raw genotyping data. Raw data in the form of CEL files was imported into the Genotyping Console (Affymetrix) for quality control analysis and genotype calling using the BRLMM-P algorithm (Affymetrix). Average call rate on arrays used in this study was 99.43%.

We analyzed CEL files from all chips using two software packages, DNA-Chip Analyzer (dChip) and Partek Genomics Suite (Partek software, version 6.3 Beta, build #6.07.1127), which enable detection of CNVs by performing scaling, normalization, and feature extraction of hybridization signal intensities, as well as hidden Markov model (HMM) analysis for inferring locus specific CNV status. The entire procedure included five steps: (i) quality control; (ii) CNV identification; (iii) de novo CNV detection; (iv) de novo CNV verification by intensity and genotyping filters, and (v) de novo CNV confirmation by duplex Taqman quantitative real time PCR (qPCR) or inherited CNV confirmation by multiplex ligation-dependent probe amplification (MLPA).

We considered a CNV as inherited only if there was 50% or more overlap in size with a variant in its biological parental chromosomes. Inherited rare CNVs detected in subjects and their parents were considered only if they involved at least ten consecutive probe sets and did not show 50% overlap with a CNV detected in any parental chromosome other than those of the biological parents. An alternative criterion (CNVs not showing >50% overlap with the CNV collection downloaded from Database of Genomic Variants [hg18 version 3]) was also applied with almost identical results.

A number of rules were applied to determine whether a candidate CNV in a child is a true inherited CNV. 1) Average inferred CN of the normal parent should be close to two for an autosomal region. The average inferred CN of the child and the mutation-carrying parent should

be at least 20% greater (for duplication) or less (for deletion) than the normal parent; 2) if a stretch of homozygosity was observed within a candidate deletion CNV, the latter was considered to be a true positive call. Moreover, observation of the same homozygosity in a child and mutation-carrying parent, when the normal parent maintains proper heterozygosity, was considered a strong indication of an inherited CNV; and 3) presence of Mendelian inconsistencies between the genotype of a child and the normal parent was considered a good indicator of the existence of an inherited CNV in the child.

2.2.3 CNV validation by qPCR and MLPA (adapted from (Xu et al. 2008; Xu et al. 2009))

Real-time quantitative PCR validation was performed using the ABI Prism 7900HT System (Applied Biosystems) to confirm candidate de novo CNVs. The TaqMan method was used, according to the manufacturer's guidelines. All PCR primers and probes were custom designed (Sigma-Aldrich). All target CNV probes were 5' FAM and 3' BHQ™-1 Dual labeled. For normalization, a 5' JOE™ and 3' BHQ™-1 dual labeled TaqMan probe to the VEGFA locus (vascular endothelial growth factor) was used as control. For each candidate de novo CNV, genomic DNA samples of the subject and his/her parents were tested simultaneously within one run. An aliquot of the genomic DNA from each triad was pooled as the template to obtain a qPCR standard curve for each run. For each CNV a duplex PCR reaction was set up containing a target gene primer probeset and a VEGFA primer probeset. We conducted 5 replicate PCR reactions for each CNV and each triad. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min on a 7900HT (Applied Biosystems). The relative levels of gene dosage were determined according to both comparative C_T method and the standard curve methods described in the ABI company manual. All results that showed a fold-change <0.7 or >1.25 were considered to be true positive results.

For MLPA validation, two to three pairs of MLPA target-probes were designed based on unique sequences within each CNV region. Additionally, three pairs of MLPA control probes from the unique sequences of the VEGFA locus were included in each MLPA reaction. All probes were synthetic oligonucleotides. MLPA reagents were prepared according to the instructions (MRC-Holland). Final PCR products were analyzed on an ABI3730XL for peak identification and quantification. The peak profiles of all test samples were visualized and parameters (height and area) were extracted using Peak Scanner Software v1.0 (Applied Biosystems). For copy number quantification, peak area and height for each probe was normalized to the mean value for all control probes. The relative ratio of each peak was calculated by comparisons between the proband sample and the samples of his/her relatives. Deletion was identified as relative ratio <0.8 and duplication as relative ratio >1.2.

2.2.4 Whole exome sequencing (adapted from (Xu et al. 2012))

For exome capture and sequencing, genomic DNA (~3 μg) was sheared to 200–300 bp in size using a Covaris Acoustic Adaptor. Fragments were then end repaired, polyA tailed and ligated to sequencing adaptor oligonucleotides (New England BioLab). Libraries were barcoded using the Illumina index read strategy, which uses 6-base sequences within the adaptor that are sequenced separately from the genomic DNA insert. Ligated products were size selected during purification steps. The DNA library was subsequently enriched for sequences with 5′ and 3′ adaptors by PCR amplification using primers complementary to the adaptor sequences (ligation-mediated PCR, LM-PCR). Exonic DNA was captured using two hybridization systems: Agilent SureSelect v2 (n = 85 trios) and NimbleGen SeqCap EZ v2 (n = 180 trios). After capture, another round of LM-PCR was performed to generate the final libraries. Each library was quantified by fluorescent methods (PicoGreen, Invitrogen), and fragment size was measured with

the Agilent Bioanalyzer. Finally, the molar concentration of each library was measured using the size information from the Agilent Bioanalyzer and DNA quantitation information from an RT-PCR assay (Kapa Biosystems). Each library was normalized to a 10 nM concentration and sequenced using an Illumina HiSeq 2000.

In the exome data analysis pipeline, raw sequencing data were mapped to the human reference genome (hg19) using the Burrows-Wheeler Aligner (BWA v0.5.81536). The Genome Analysis Toolkit (GATK, version 5091) was used to remove duplicates, perform local realignment and map quality score recalibration to produce a cleaned BAM file and then make genotype calls for all trios jointly. The resulting variant call format (VCF, version 4.0) files were annotated using the GenomicAnnotator module in GATK to identify and label the called variants that were within the targeted coding regions and overlap with known and likely benign SNPs reported in dbSNP v132. The filtered genotype calls were further validated using the mpileup module in SAMtools. Indel calls were made by Dindel software using one cleaned BAM file per run. The resulting VCF files were further revalidated using the same SAMtools procedure as for the point mutations. To determine potential mutations at splice donor or acceptor sites, GATK variant calls were made in a batch fashion (90 samples per batch) that covered each target coding region and the 50-bp flanking segments on each side of it. The variants in the resulting VCF files were annotated according to refGene-big-table-hg19.txt. A variant was annotated as a 'canonical splice-site mutation' if it disrupted the largely invariable core canonical 2-bp acceptor (AG) or donor (GU) sites. De novo variants within 10 bp of the exon-intron boundary, included in the consensus sequence flanking core canonical splice sites and therefore likely to modulate splicing efficiency, were annotated as 'consensus splice-site mutations.' Candidate de novo variants were tested using standard Sanger sequencing on an ABI 3730xl DNA Analyzer (Applied Biosystems)

to validate the presence of each mutation in the proband and its absence in the parental genomes, using custom primers designed using Primer3 software. The total number of de novo single nucleotide variants (SNVs) found and validated in a given cohort was divided by the total number of bases analyzed to calculate the per-base rate of point mutation in the captured coding sequence.

The two-sided exact binomial test was conducted using R. Fisher's exact test or the χ-squared test with Yates' correction was used for the analysis of contingency tables, depending on the sample sizes, using R.

2.3 Results

2.3.1 Copy number variant scan

A whole genome CNV scan in the general population determined that even healthy individuals carry on average 11 large scale insertions or deletions (Sebat et al. 2004). We speculated that some of these CNVs, specifically de novo and rare inherited CNVs, contribute causally to schizophrenia and other psychiatric disorders. To assess the CNV burden in disease, we analyzed trios of an affected patient and his or her parents. The resulting CNV data were analyzed separately for familial (at least one first or second degree relative was also affected) and sporadic (no family history) schizophrenia to determine if they had differing large scale copy number burdens. The results and implications for understanding the genetic basis of schizophrenia etiology were discussed in Chapter 1.

Because both de novo and rare inherited mutations have low allele frequency in the population, most genetic analysis will be under-powered if a specific mutation target is under investigation. Alternatively, we wanted to test the hypothesis that mutations affecting a specific protein family instead of a single gene could contribute to the risk of schizophrenia.

A de novo deletion of *RAPGEF6* occurred in a sporadic case of schizophrenia (Figure 2.2A) (Xu et al. 2008). This deletion was 114.3kb long starting at chr5:130,863,839 (hg18) affecting exons 2 through 11 of the gene. This CNV was confirmed by qPCR (Figure 2.2B).

There was an inherited duplication of 4q32.1-q32.2 that included *RAPGEF2*, another member of the RAPGEF protein family, in two sisters concordant for schizophrenia (Figure 2.2C,D) (Xu et al. 2009). The 716.4kb duplication spanned chr4:160104841-160821269 (hg18) and was confirmed by MLPA to be inherited from their mother, who was asymptomatic (Figure 2.2C). The other duplicated gene, *C4orf45*, is a predicted open reading frame.

2.3.2 Clinical history of patients

The patient carrying a *de novo* deletion on chromosome 5q, encompassing the *RAPGEF6* gene, was male, born in 1962. He had dropped out of school after 8th grade due to intellectual difficulty and had no family history of schizophrenia. He first presented to medical services in his 30s with 8 months of psychotic symptoms including thought disturbances, flattened affect, suicidal ideation, and alcohol use, meeting full criteria for a diagnosis of schizophrenia, paranoid subtype. He had long periods of paranoid ideation and bouts of aggression, auditory hallucinations, as well as poverty of ideation and withdrawal. He was noncompliant with medication, experienced marked deterioration in his functioning and eventually committed suicide by overdose of medication and alcohol.

The duplication on chromosome 4q that disrupts *RAPGEF2* is shared by 2 sisters affected with schizophrenia and was inherited from their mother. The elder sister, ECL, was born in 1950. She started training as a nurse but could not cope and did not complete her training. At that time, she experienced confusion and exhibited obsessive-compulsive behavior; including asking the same questions over and over again and re-checking everything that she had to do. She then

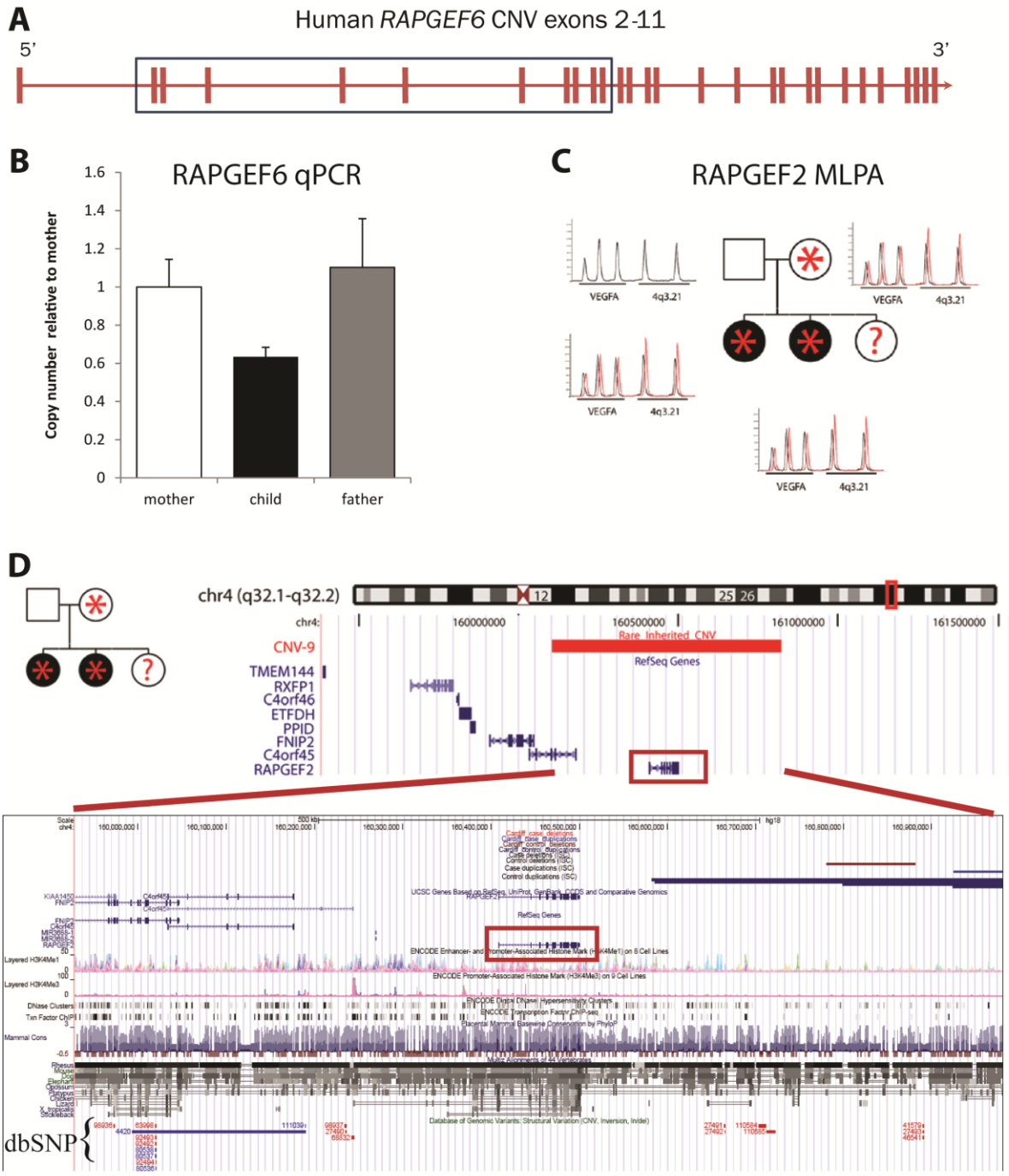


Figure 2.2 *RAPGEF6* and *RAPGEF2* copy number variation. A) Schema of deletion of exons 2-11 within *RAPGEF6*. **B**) qPCR validation of de novo *RAPGEF6* deletion in a sporadic case of schizophrenia. **C**) Inheritance of *RAPGEF2* dupliction (asterix) confirmed through MLPA via the asymptomatic mother in comparison to control *VEGFA*. **D**) Inherited duplication involving *RAPGEF2* (circled), shown as a genetic schema and with UCSC browser tracks of dbSNP CNVs in the region none of which affect *RAPGEF2*. Adapted from (Xu et al. 2009).

started experiencing persecutory delusions, auditory hallucinations, and decreased drive. In addition, several negative symptoms were present in the course of ECL's disease, including apathy, alogia, flat affect, inappropriate affect, depersonalization and derealization. The patient was anxious and socially withdrawn as a child. The younger sister, ML, now deceased, was born in 1952. Following her graduation from high school she completed a 3 year degree in primary school education. During her second year of teaching she became psychotic and was admitted to a psychiatric hospital for 3 months. At that time she was 25 years old. She experienced several delusions and hallucinations, including olfactory and visual hallucinations, which are overall rare. She had problems with socialization from a young age that worsened with time. Both sisters were unable to hold a job due to their principal illness and received disability grants. They are both unmarried and living with their parents. There was no history of learning difficulties, developmental delays, or mental retardation.

2.3.3 Whole exome sequencing

Our analysis demonstrated that de novo CNV rates were collectively increased in a schizophrenia cohort and can explain about 10% of sporadic case risk (Xu et al. 2008). Therefore, the purpose of whole exome sequencing was to assess whether our schizophrenia cohort had sources of genetic risk other than CNVs and if the pattern of risk found in CNVs, namely de novo mutations in sporadic cases and rare inherited mutations in familial cases, would also hold at the level of single base mutations. Individuals with CNVs as identified in prior studies were excluded from sequencing. Major findings concerning de novo mutations were discussed in Chapter 1.

We did not detect any de novo mutations in the whole exome sequencing data. However we discovered and validated two inherited mutations in *RAPGEF2*, chr4:160274698 S1223C and

chr4:160277184 V1450I (hg19), both localized to the C terminus of the protein. While the latter mutation of valine to isoleucine was predicted to be a tolerated mutation by SIFT software (sift.jcvi.org), the former mutation of serine to cysteine was predicted to be a damaging mutation. The serine site is conserved in Multiz Alignments of 46 vertebrates (UCSC genome browser) and the mutation had not been reported in the latest SNP database (dbSNP 135). Therefore, serine to cysteine conversion might have functional impact on the protein.

2.4 Summary of findings

Upon analysis of copy number variation in a genetically homogenous population, we discovered a de novo mutation in *RAPGEF6* in a sporadic case of schizophrenia and an inherited mutation in *RAPGEF2* in concordant familial cases, as well as two inherited exonic mutations in *RAPGEF2*. *RAPGEF6* lies within the region of high linkage and association in schizophrenia studies of chromosome 5q (Figure 2.1). These results represent a striking convergence of human genetics onto the *RAPGEF* family and *RAPGEF6* in particular. Moreover, the literature reviewed below demonstrates significant involvement of the Rapgef family in neural phenotypes in animal models, indicating that *Rapgef6* is a plausible functional candidate in schizophrenia.

2.5 Discussion

The human genetics results, from cytogenetics to copy number research, implicated *RAPGEF6* in the genetic liability to schizophrenia. In order to understand the functional implications of *RAPGEF6* deletion, we will review the relevant literature about this gene, its family, and its up- and downstream regulators, the Rap family.

2.5.1 Role of Rapgef6 in cell adhesion

To date, there have been no studies of RAPGEF6 function in the brain. One microarray

study found that Rapgef6 was upregulated in mouse lines with higher adult gliogenesis and downregulated in mice with higher neurogenesis (Kempermann et al. 2006). Several in vitro experiments using a variety of cell model systems demonstrated that, in both mouse and human cells, Rapgef6 has a definite role in stimulating adhesion to both other cells and to the extracellular matrix (Figure 2.3). *RAPGEF6* was initially cloned and characterized biochemically through its homology to other Rapgef members. In humans there are 3 major isoforms, the shortest of which lacks the PDZ domain and thus might affect localization (Kuiperij et al. 2003).

Expression and activity assays proved that RAPGEF6 activated Rap2 more than Rap1 by exchanging its GDP for GTP (Figure 2.3A), and that unlike other family members its activity was independent of GTP binding (Gao et al. 2001). The same group found that active MRas-GTP bound to the RA domain of RAPGEF6 and delivered it to the cell membrane to increase membrane concentrations of Rap1-GTP. MRas is therefore important for localization but not modulation of RAPGEF6 activity.

In lymphocyte precursor cells, MRas directed Rapgef6-dependent adhesion of integrin LFA1 to ICAM (Yoshikawa et al. 2007). This process of adhesion was triggered by TNFα administration. The mouse model of *Rapgef6* deletion, described further in Chapter 3, had no gross behavioral or anatomic abnormalities beyond splenomegaly (Yoshikawa et al. 2007). Knock out B cells taken from the spleen also demonstrated absent TNFα induced LFA1-ICAM adhesion, even though levels of LFA1, MRas, and TNFα receptor were unchanged and MRas and Rapgef6 levels were the same in B as well as unaffected T cells. Thus Rapgef6 is essential for specific forms of outside-in integrin signaling.

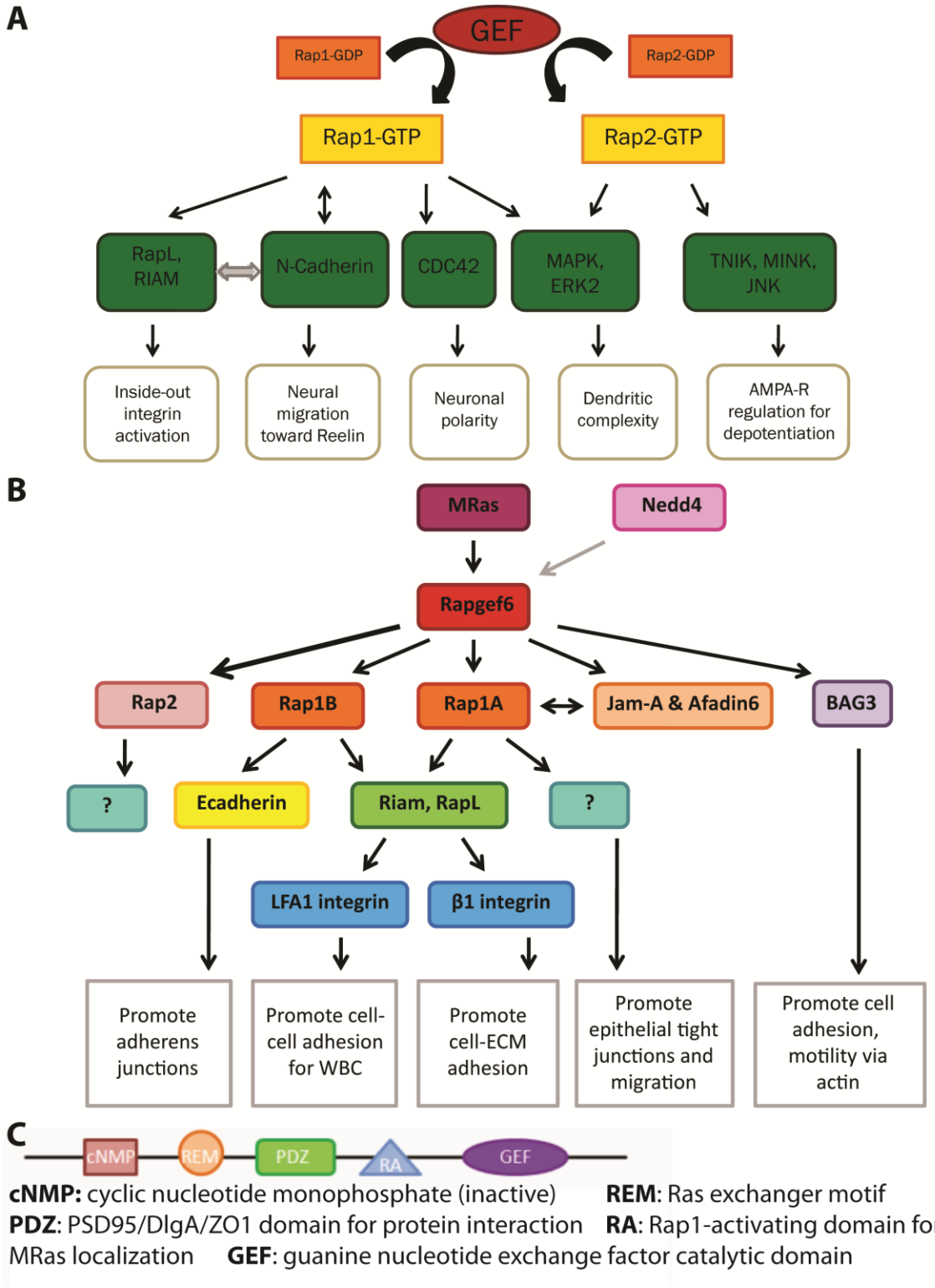


Figure 2.3 Rap and Rapgef6 pathways. A) GEFs activate Rap1 and Rap2, which have important neural effects. **B**) Rapgef6 influences integrin and cadherin adhesion. It is localized by MRas and may be ubiquitinated by Nedd4. **C**) Rapgef6 has several conserved domains.

Human epithelial cells also rely on RAPGEF6 for cell adhesion. Knocking down *RAPGEF6* or *RAP1A* led to immature Ecadherin containing adherens junctions between cells (Dube et al. 2008). Additionally, Ecadherin levels were reduced, suggesting RAPGEF6 affects its expression via RAP1, and F-actin was disorganized. The authors also repeated these findings in endothelial cells with VEcadherin junctions, which they successfully rescued by administering RAPGEF3/4 agonist 8CPT. Similar studies established that RAPGEF6 maintained β1-integrin tight junctions and cellular migration by regulating JAM-A and Afadin (AF6) as well as RAP1 activity and integrin expression (Severson et al. 2009). JAM-A was coimmunoprecipitated with the PDZ domain of RAPGEF6. Jam-A was identified as a marker of NG2 glia, including NG2 glia undergoing proliferation to generate oligodendrocytes (Stelzer et al. 2010). Its unpolarized expression did not suggest a role in cell fate determination. Afadin is important in remodeling of dentate gyrus to CA3 synapses in the hippocampus because its deletion resulted in decreased nectin, Ncadherin, and β-catenin-dependent puncta adherentia junctions at the mossy fiber to CA3 synapse in vivo (Majima et al. 2009) and also in hippocampal culture (Lim et al. 2008).

Expression of human *RAPGEF6* in Cos7 cells found a role for RAPGEF6 in BAG3-mediated cell migration (Iwasaki et al. 2010). BAG3 and RAPGEF6 associated on a yeast two hybrid screen and coimmunoprecipitated via the PPDY motif, which is a putative Nedd4 ubiquitination domain. Overexpressing *RAPGEF6* increased integrin-fibronectin adhesion and knock down studies demonstrated RAPGEF6 was necessary for the adhesion effects of BAG3. The Allen Brain Atlas predicts very low BAG3 brain expression.

miRNA binding prediction from TargetScan (release 6.2) identified only 4 potential binding sites with low preferential conservation. The predicted miRNA interactions are with miR135 at two potential loci, miR383, and miR217.

Finally, there are two protein structure reports of potential binding partners for RAPGEF6. The structure of tyrosine phosphatase PTP1E (also known as FAP-1) was solved by complexing its second PDZ domain with the C terminus of RAPGEF6, which proved to be an unusually complicated and high affinity PDZ binding arrangement (Kozlov et al. 2002; Milev et al. 2007). PTP1E also bound the Fas receptor, ephrins, and APC in a variety of cancer models (Kozlov et al. 2002). PTP1E was expressed in CA1 and dentate gyrus neurons where it blocked apoptosis (Savaskan et al. 2005). Protein interaction predictions as well as coimmunoprecipitation verified Interleukin-16 as another binding partner of RAPGEF6 (Chang 2010). In both human and mouse cell culture systems, Rapgef6 has a role in integrin and cadherin mediated cell adhesion. Although it is more effective at activating Rap2 in vitro, there are no published findings to date on cellular interactions between Rapgef6 and Rap2. Since integrin and cadherin signaling are crucial in neural migration and cell connectivity, these findings suggest Rapgef6 could have a role in neural development and function.

2.5.2 Neural role of Rapgef family members

While Rapgef6 has never been studied in the brain, many other family members have strong neural phenotypes (Table 2.1). The Rapgefs are a family of 6 proteins, of which Rapgef3 and Rapgef4 are highly homologous and cAMP-activated. Some family members, such as Rapgef1 and Rapgef2, are positively regulated by Rap1 activity.

In mice, deletion of *Rapgef1* is embryonic lethal because it is essential for vascular development (Ohba et al. 2001). Fibroblasts from early embryos were highly motile and poorly adherent. This finding was extended by demonstration that Rapgef1 is necessary for paxillin and β1-integrin mediated focal adhesion (Voss et al. 2003). A hypomorph mouse model was created that had cortical neuroepithelial overproliferation but diminished mature neurons due to elevated

Table 2.1 Neural roles of Rapgef family members

	Implicated in	Mouse KO Phenotype	Human Disease
RAPGEF1	**Neuroepithelial cell cycle via b-catenin, cortical migration via radial glia attachment and Reelin, neurite outgrowth**	**Hyperproliferation of neuroepithelium, reduction in neurons (vascular failure)**	
RAPGEF2	**Neurite outgrowth, neural migration, midline axon crossing**	**Cortical heterotopia, failed commissures (vascular failure)**	**Schizophrenia (CNV)**
RAPGEF3 RAPGEF4	**LTP/LTD, hippocampal function**	**Double KO reduces LTP, spatial memory, social interaction via miR124**	**Depression (assoc), autism (mutation)**
RAPGEF5	**Downregulated in neuronal differentiation**		
RAPGEF6	**WBC integrin-mediated cell adhesion; epithelial E-cadherin tight junction maintenance**	**Grossly normal, splenomegaly**	**Schizophrenia (CNV, assoc, karyotype, linkage)**

nuclear β-catenin preventing precursor cells from exiting symmetric mitosis (Voss et al. 2006). Beyond regulating neural proliferation, Rapgef1 also proved to be essential for radial glia orientation that directs cortical migration (Voss et al. 2008).

Rapgef2 is ubiquitinated for degradation by Nedd4, suggesting this is a potential mechanism for Rapgef6 regulation as well (Pham and Rotin 2001). It mediates NGF trophic signaling in the downstream cascade from TrkA receptors, promoting neurite outgrowth (Hisata et al. 2007). *Rapgef2* deletion limited to the forebrain resulted in grossly abnormal brain development with heterotopia and failure of white matter tracts (Bilasy et al. 2009). Upon more careful analysis, axons failed to decussate within the corpus callosum implying a role for

Rapgef2 in axon guidance (Bilasy et al. 2011).

RAPGEF3 and 4, also known as EPACs, have a variety of cellular roles and, due to their regulation by cAMP, share a specific activator 8-pCPT-2'-O-Me-cAMP (8CPT). Administration of 8CPT has a variety of effects, including increasing long term depression in CA1 of the hippocampus (Ster et al. 2009), stimulating integrin adhesion (Bos 2006), and increasing prepulse inhibition and improving fear conditioning memory in wild type mice (Kelly et al. 2009; Ma et al. 2009). Initial neural culture experiments localized Rapgef4 to both sides of the synapse, including colocalization with AMPA receptors (Woolfrey et al. 2009). 8CPT reduced spine area but not spine density, while dominant negative Rapgef4 increased spine area, implying that Rapgef4, unlike Rapgef1 and 2, depresses synapses. Individual knock out of either *Rapgef3* or *4* had no effect on behavior, but a double knock out led to reduced long term potentiation in the hippocampus as well as impairments in spatial memory and social interaction (Yang et al. 2012). Neither the single or double deletions caused a change in spine density in CA1, contradicting the knock down findings. Microarray and rescue experiments found that Rapgef3/4 activate Rap1 to inhibit miRNA124 expression, thereby derepressing Zif268. Sequence analysis of 9 autism candidate genes revealed 4 nonsynonymous cosegregating mutations in *RAPGEF4* (Bacchelli et al. 2003), while *RAPGEF3* SNPs were associated with anxiety and depression (Middeldorp et al. 2009).

Little is known about Rapgef5 except that it is inhibited by MRas (Rebhun et al. 2000) and downregulated in neural differentiation (Bithell et al. 2003).

Rapgefs are conserved back to fly and worm. In *Drosophila*, the Dizzy mutant is a deletion of the gene 50% homologous to Rapgef2 and Rapgef6 because it is a guanine nucleotide exchange factor for Rap1 (Lee et al. 2002). When it was deleted, the fly died without

photoreceptors in the eye, proper wing structure, or ovaries. Overexpression of Dizzy in fly macrophages caused normal migration but abnormally long cell protrusions and morphology, probably as a result of enhanced integrin adhesion (Huelsmann et al. 2006). Rapgef1 homolog C3G is necessary for muscle adhesion via integrins and its deletion was also lethal (Shirinian et al. 2010). In *C. elegans* the homolog of Rapgef2 and Rapgef6 is Pxf-1 which is essential for proper epithelial attachment and cuticle development and molting (Pellis-van Berkel et al. 2005).

Overall, the other Rapgefs have striking neural phenotypes when deleted and contribute to neural guidance, morphology, and electrophysiology. We hypothesized that Rapgef6 would have a moderate neural phenotype when deleted.

2.5.3 Neural role of Rap family members

Rapgefs activate Rap1 and Rap2 by exchanging GDP for GTP (Figure 2.3A). Raps mediate a number of signal cascades, several of which have neural significance such as cadherin and integrin signaling (Bos 2005), and have been associated with neuropsychiatric diseases (Stornetta and Zhu 2011). Rap1 was implicated early on in ERK activation for neuronal differentiation (York et al. 1998). Rap1 also directs neural polarity since its localization to a neurite tip correlated with that neurite developing into the axon (Schwamborn and Puschel 2004). When constitutively active in vitro, Rap1 recruited Afadin (a Rapgef6 binding partner) to spines resulting in thinner spines with fewer AMPA receptors (Xie et al. 2005). Dominant negative Rap1 reduced in vitro neural complexity whereas constitutively active Rap1 had the opposite effect, suggesting Rap1 supports neural growth (Chen et al. 2005), but another study found no effect of active Rap1 on neural morphology (Fu et al. 2007).

In vivo deletion of *Rap1* limited to the cortex resulted in reduced long-term potentiation and increased basal firing rates in the cortico-amygdala pathway but not the thalamo-amygdala

connections in the setting of normal presynaptic function (Pan et al. 2008). This correlated with impaired cued and conditional fear conditioning but normal spatial memory. Electroporation of dominant negative Rap1 into developing neurons in vivo did not affect neural migration or neurite outgrowth but led to a loss of neural orientation with the axon misguided (Jossin and Cooper 2011). Additional rescue experiments implicated Ncadherin, Rac1, Cdc42, and Ra1 as the downstream effectors of Rap1 in neural polarity. Both this and an additional study found that Reelin was the upstream activator of Rap1 in directing early neural migration and orientation (Franco et al. 2011).

In human postmortem studies, Rap1 was reduced in both expression and activation in the prefrontal cortex and hippocampus of suicidal, depressed individuals (Dwivedi et al. 2006). Moreover, protein analysis of frontal cortex for ERK pathway members determined that Rap1 levels were reduced in individuals with schizophrenia or depression but not bipolar disorder, increasing support for the idea of neuropsychiatric disorders as synaptic dysfunction disorders (Yuan et al. 2010).

Rap2 experiments show that it may counteract Rap1 in neural growth and synaptic transmission. Rap2 constitutive activation impaired the length and complexity of hippocampal neurons in culture by increasing retraction (Fu et al. 2007). This also caused reduced electrical activity due to diminished GluR2 subunits of the AMPA receptor, and while spine density was normal the total number of spines was reduced due to the shorter arbor. Other studies replicate the negative effect of Rap2 on glutamate transmission, finding that constitutively active Rap2 reduces AMPA but not NMDA current through TNIK activation, which blocks long-term potentiation (Zhu et al. 2005). In vivo, knocking in a constitutively active *Rap2* transcript to forebrain neurons led to fewer, shorter CA1 spines with increased long-term depression (Ryu et

al. 2008). Behaviorally this correlated with impaired spatial learning on a water maze and decreased contextual fear extinction plus hyperactivity, the first two of which implicate the hippocampus. Thus Rap2 may counteract Rap1 by inhibiting spines and increasing synaptic depression.

MRas is not as well studied as the Raps, but it was found to promote filopodia outgrowth and actin rearrangement, most likely via Rapgef6 binding partner Afadin (Matsumoto et al. 1997; Quilliam et al. 1999). In neural-like PC12 cells, NGF activated MRas leading to neural differentiation, and constitutively active MRas increased neurite outgrowth (Sun et al. 2006). Surprisingly, deletion of *MRas* did not cause any morphology changes in the hippocampus or cortex, nor any behavior issues on fear conditioning or the water maze (Nunez Rodriguez et al. 2006). The authors found MRas was expressed in astrocytes as well as neurons and that FGF and EGF signaling in astrocytes was disrupted.

The importance of Raps and MRas in neural development further confirms that Rapgef6 is both a plausible functional and genetic candidate for modeling schizophrenia.

2.5.4 Rare genetic models

The large and small scale mutations we defined in *RAPGEF6* and *RAPGEF2* were so rare they were private, affecting no control individuals nor aligning with any findings in dbSNP. Rare mutations such as these are considered important in developing animal models of disease. Not only are rare mutations an essential component of schizophrenia genetic risk, they are more likely to be deleterious and causal and therefore more etiologically valid for understanding the neurobiology affected in the disorder (Arguello et al. 2010). We use the rare mutation approach to dissect affected pathways in schizophrenia, in this case pathways affected by *Rapgef6* deletion that may lead to schizophrenia endophenotypes.

2.6 Future directions

2.6.1 Genetics

We plan to continue discovering the full spectrum of genetic risk associated with *RAPGEF6* by extending our exome sequencing to include additional cases. Unfortunately we did not sequence any individuals with rare CNVs, so it would be useful to sequence the three cases of *RAPGEF* CNVs to determine if there are any interacting mutations in related genes that might worsen the phenotype, per the two hit hypothesis. We are also monitoring the literature for evidence of mutation or CNV findings in this gene family from other populations.

Several of the genetic findings discussed above are SNP association studies pointing to *RAPGEF6,* but an association study of this population did not generate a genome-wide significant result at this locus (unpublished data). We could use imputed SNP genotype data from publically available data sets to query this gene family and associated genes. Such a focused analysis would greatly increase power by reducing the need to correct for multiple comparisons. Moreover, there are growing maps of expression quantitative trail loci (eQTLs), SNPs that correlate with gene expression (Cookson et al. 2009). eQTLs for Rapgef family members are already identified in public databases so we could run an association study to determine if individuals with schizophrenia are predicted to have altered expression of these genes.

2.6.2 In the next chapter

Next we will consider the generation of a *Rapgef6* deletion mouse and its behavioral phenotype. We performed a detailed behavioral assessment focused on endophenotypes of neuropsychiatric disorders.

Chapter 3: Animal Behavior

3.0 My Role

The Rapgef6 knock out mouse used in the experiments described in this thesis was provided by the Kataoka laboratory. I validated two additional knock out lines that were not included in this project. I controlled the breeding of the mouse line, executed all behavior paradigms contained in this chapter, and analyzed all the data, with the exception of the auditory evaluation performed by the Olson laboratory. While many of our behavior protocols were already established in the lab, I had to develop and assess new protocols for novel object recognition and T maze testing, as well as a novel immunohistochemistry protocol to study cFOS expression after our laboratory's standard fear conditioning protocol.

3.1 Introduction

3.1.1 Validity and utility of rodent modeling

Animal models, especially genetically altered mice, are used extensively in neuroscience research. Animal behaviorists agree upon three core features to validate a strong model: face, construct, and predictive validity (Robbins and Sahakian 1979). Face validity questions whether there is behavioral analogy between the animal and human phenotypes and whether the animals reproduces key disease symptoms. Construct validity ensures that there is a shared basis of etiology, be it a genetic or environmental insult, that leads to analogous neurobiological pathway abnormalities. Predictive validity is demonstrated when animals have similar behavioral responses to agonists, antagonists, and other pharmacology interventions as human patients.

In neuropsychiatric disorders, there is an incomplete understanding of the underlying neurobiology and a lack of adequate pharmacology, thus predictive validity can be challenging to

prove. As mentioned below, the clinical phenotypes available to generate face validity are similarly limited by the complexity of psychiatric symptoms (Nestler and Hyman 2010). Thus it is the opinion of our lab and others that construct validity is the most fruitful starting point to build a useful animal model (Arguello et al. 2010; Kas et al. 2009). By working from rare human genetic mutations that are more likely to be causal and highly penetrant, we intend to create models that more accurately reflect genetic dysfunction and thus lead toward better neurobiological understanding and pharmacological therapeutics that can be reapplied to patient care.

3.1.2 Endophenotypes of schizophrenia amenable to modeling

As described in Chapter 1, the core symptoms of schizophrenia are classified as positive (notably hallucinations and delusions), negative (such as flattened affect and catatonia), and cognitive deficits (attention and working memory impairments). On initial consideration, these symptoms appear to have no rodent correlate, nor would one expect a single mouse to recapitulate such a heterogeneous disorder. Fortunately, careful behavior assessments can be used to investigate some clinical phenotypes of schizophrenia.

Endophenotypes are heritable, quantitative traits that are associated with a disease and may be present in unaffected relatives, thus representing experimentally approachable subphenotypes (Amann et al. 2010). Thus endophenotypes are considered useful targets for rodent behavior assessment. What endophenotypes of schizophrenia can be accurately modeled for face validity? Cognitive working memory impairments, prepulse inhibition (PPI) deficits, hyperlocomotion, and altered social interaction are paradigms currently considered to have the most analogy between rodents and humans (Powell and Miyakawa 2006).

Since cognitive symptoms can present prior to psychosis, are refractory to current

treatment, and exist in unaffected relatives (Elvevag and Goldberg 2000; Green and Nuechterlein 2004), this is considered by some to be the most pressing area for schizophrenia research and novel therapeutics (Arguello and Gogos 2010). Patients have deficits in a variety of cognitive domains (Lee and Park 2005), but working memory and attention are most amenable to animal modeling as they can be tested through mazes and goal set-shifting tasks which rely upon executive control of information over temporal delays (Arguello and Gogos 2010).

PPI deficit is so established in the human literature that this paradigm is part of the cognitive neuroscience treatment research to improve cognition in schizophrenia (CNTRICS) focus for schizophrenia research (Green et al. 2009). PPI impairment is thought to be a failure of top-down cortical suppression of irrelevant information and thus may represent a cognitive symptom (Arguello and Gogos 2006). Also known as sensorimotor gating, PPI involves a reflexive reduction in startle when a sound or tactile stimulus is closely preceded by a milder stimulus. PPI is diminished in individuals with schizophrenia and their unaffected relatives, making this failure to suppress startle an endophenotype; yet is also decreased in other neuropsychiatric disorders (Powell et al. 2009). As it has strong relevance to neuropsychiatric disease and is easy to evoke in both humans and animals, PPI is a commonly investigated phenotype.

Positive symptoms have only been successfully modeled by hyperactivity, which is thought to represent psychomotor agitation. Quantified activity either by home cage observation or on open field exploration should be considered both at baseline and after challenge with dopaminergic and serotonergic psychostimulants, which may cause an inordinate increase in locomotion in schizophrenia models (Brookshire and Jones 2009).

Conversely, social interaction deficits may model negative symptoms such as social

withdrawal (Powell and Miyakawa 2006). In animals this can be tested in a number of protocols that bring together two animals for quantitative observation of social interest and establishment of dominance (Crawley 2008). This behavioral paradigm is more controversial since human and rodent social behaviors are not especially analogous.

Working memory, PPI, hyperlocomotion, and social interaction are 4 hypothetically important phenotypes in animal models of schizophrenia, but we have also tested additional domains. Clearly a hypothesis-driven approach to understanding mouse behavior phenotypes is the most focused approach, yet it is important in animal models to consider the divergent effects of pleiotropy (when one gene influences multiple phenotypes). Many behaviorists recommend systematic phenotyping of novel models since 96% of mutant models were found to have unexpected behavioral findings (Beckers et al. 2009).

3.1.3 Rodent behavior domains explored

In our experiments, we explored both schizophrenia-associated phenotypes as well as more general domains (Crawley 2008). In the open field test, mice are placed in a well-lit chamber and their movement is assessed by a laser beam grid. The resulting data assesses locomotion and anxiety-like behavior. Animals naturally tend to avoid the center of the chamber since it is less protected, so an increase in anxiety-like behavior is indicated by more time and distance in the chamber margins and anxiolysis by more transit in the center (Prut and Belzung 2003). Altered locomotion can complicate analysis of other behavior tests therefore it is an important component of basic behavioral screening.

During novel object recognition, animals are habituated to two identical objects then re-exposed to one familiar and one novel object at two later time points. The mice should indicate recognition of novel objects by exploring those items more (Lyon et al. 2012). Novel object

recognition is a type of spatial memory that can be altered by psychostimulants such as PCP and methamphetamine and rescued by antipsychotics, thus it is dopamine sensitive and has relevance to schizophrenia modeling (Amann et al. 2010). The other assessment of spatial memory we used was the Morris water maze, in which mice learn to swim to a visibly marked platform, then to a hidden platform, relying on extramaze cues (D'Hooge and De Deyn 2001). Water maze performance is affected after deletion of schizophrenia candidate genes dopamine D1 receptor, glutamate NMDA receptor 2B, and some Disc1 models therefore it is relevant to schizophrenia research (Arguello and Gogos 2010).

On the T maze, animals are trained to run to the end of one open maze arm then on the next trial in the maze when both arms are available they must choose the opposite arm for a reward. Increasing the delay time between the two trials increases the working memory load and this learning paradigm is dependent on the prefrontal cortex (Sanchez-Santed et al. 1997). The importance of the T maze as a measure of schizophrenia endophenotype working memory was discussed above.

Mice are exposed to auditory tones with or without a preceding quieter prepulse tone and their reactions are measured by a force sensitive restraining chamber during prepulse inhibition assessment. PPI assays measures auditory function, baseline startle, and top-down sensorimotor gating of the startle reflex (Powell et al. 2009). As discussed, sensorimotor gating is an endophenotype of schizophrenia.

Fear conditioning assesses both baseline fear and learned fear by quantifying freezing behavior at baseline and on re-exposure to the same chamber (contextual conditioning) and tone (cued conditioning) associated with the animal receiving foot shocks. Contextual conditioning requires intact hippocampal and amygdala circuitry and cued conditioning relies mostly on the

amygdala (Gerlai 2001; Maren 2008). Contextual fear is diminished in Gαs and Reelin deletion while Dntbp1 deletion causes cued fear sensitization thus mouse models of schizophrenia have varying fear impairments (Amann et al. 2010). There is growing interest in the amygdala in schizophrenia as the possible basis of emotional blunting since the NMDA receptor antagonist ketamine can block amygdala activation and fear conditioning and the atypical antipsychotic clozapine can reverse these effects (Pietersen et al. 2007).

3.1.4 Role of Rapgef and Rap proteins in behavior

While several genes in the Rapgef and Rap families have been knocked out or into mouse models, only some are nonlethal and thus amenable to behavior assessment. For instance, there are several interesting findings regarding *Rapgef3* and *Rapgef4*. In wild type animals, administration of Rapgef3/4 agonist 8CPT leads to an increase in PPI without a change in startle, as well as an enhancement in fear learning (Kelly et al. 2009). These findings are supported by results that 8CPT delivered to the hippocampus after fear conditioning increases contextual fear memory (Ma et al. 2009). Double knock out (KO) of both *Rapgef3/4* causes deficits in social interaction and water maze acquisition and reversal impairments in both constitutive and inducible knock out animals, while single gene deletion mice were not affected (Yang et al. 2012). This study also demonstrated that overexpression of miR124 or knock down of transcription factor *Zif268* could recapitulate the phenotype, and that the opposite changes in these genes could rescue the phenotype, identifying a pathway downstream of *Rapgef3/4*.

Cortex specific KO of *Rap1a* and *Rap1b* caused fear conditioning impairments while water maze performance was unaffected (Pan et al. 2008). Specifically there was a reduction in KO freezing in both short and long term testing of cued fear and long term contextual fear, as well as a short term reduction in fear generalization to a novel context. When the shock was

lengthened to strengthen learning, the deficits were reversed, suggesting a limited role of the cortex in fear conditioning. This fear dysfunction correlated with electrophysiology showing reduced plasticity from the cortex to the lateral amygdala but increased basal cortical glutamate release onto the lateral amygdala. The authors hypothesized that Rap1 suppresses cortico-amygdala synaptic transmission to permit amygdala plasticity underlying fear conditioning.

Constitutively active *Rap2* overexpressed in the cortex led to poor spatial performance on the water maze, normal fear conditioning but decreased fear extinction, and open field hyperactivity (Ryu et al. 2008). The authors also attempted to make a dominant negative *Rap2* knock in, but this mouse did not demonstrate altered Rap2 activity levels. They posit that Rap2 may inhibit the Ras-ERK pathway signaling, thereby inhibiting spine growth to increase synaptic depression.

Knock out of *MRas* led to no neural morphological or behavioral phenotype; animals had no change in water maze learning or fear conditioning (Nunez Rodriguez et al. 2006). MRas localizes Rapgef6 to the membrane but does not activate it in lymphocytes (Gao et al. 2001), however it is unknown if this is the major Rapgef6 regulator in the brain or if MRas can affect Raps through any other Rapgef protein.

Predictions based on these findings would be that since Rapgef6 positively regulates Rap1/2 activity as do Rapgef3/4, *Rapgef6* KO may lead to impaired social interaction, decreased spatial performance on the water maze, and a reduction in fear conditioning. If MRas is truly the only upstream regulator of Rapgef6, then we predict no difference on either the water maze or fear conditioning.

3.2 Methods

3.2.1 Animal model generation and housing

Rapgef6 animals were generated and shared by the Kataoka laboratory at Kobe University (Yoshikawa et al. 2007). Exon 21 (encoding the GEF enzymatic domain) was isolated from a BAC with restriction enzymes MfeI and BsgI, then ligated into the floxed position in a vector that also had a 3' floxed, inverted *TK-neo* cassette. Large regions of *Rapgef6* were added up and downstream of this cassette to direct homologous recombination. This vector was linearized and electroporated into 129/Ola embryonic stem cells. Positive *Rapgef6*^{flox} cells were confirmed via Southern blot, then injected into C57Bl/6 blastocysts to generate chimeras. Resulting *Rapgef6*$^{+/flox}$ mice after a generation of backcrossing to C57Bl/6 were bred with *CAG-Cre* mice to yield *Rapgef6*$^{+/-}$ animals lacking the GEF domain.

After shipment from RIKEN facility, *Rapgef6*$^{+/-}$ mice were housed on a 12 hour light cycle in the Kolb animal facility with water and food ad libitum except as noted. Heterozygous animals were mated to generate litters of wild type, heterozygous, and homozygous deletions. Up to 5 littermates of the same gender were co-housed. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees established by Columbia University under federal and state regulations.

Genotyping was performed using the following primers: GAGCCTTGAGATACAGAAACTTG located 5' to exon 21, CTTGACAACAGGGAAGAGTG within exon 21, and CTAGGGAGGTGTCAGCAAAG 3' of exon 21. PCR was performed with 2uL DNA extracted from tail clippings on PureTaq PCR beads (GE) with the protocol 94^{o}C 5min, 35 cycles of 94^{o}C 30s 63^{o}C 40s 72^{o}C 60s then 72^{o}C 10m. On an agarose gel, this yielded products of 792bp from a WT mouse, 316bp from a KO mouse, and both from a HET mouse.

At least one week prior to the initial behavior, animals were moved to the Rodent Neurobehavioral Analysis Core (RNAC) facility in NYSPI or a satellite testing facility in the Black Building (T maze and PPI cohort). Animals continued to be group housed, except the T maze cohort that was individually housed with plastic igloos and nesting squares for enrichment. There was at least 1 week in between behavioral assessments. The first cohort (14 WT, 12 HET, 7 HOM) underwent open field, novel object recognition, water maze, then fear conditioning. The second cohort (12 WT, 12 HET, 9 HOM) was tested on open field, novel object recognition (novel paradigm), and fear conditioning. The third cohort experienced T maze then PPI. The fourth cohort was used for fear conditioning followed immediately by sacrifice for cFOS staining.

In an attempt to generate a different knock out mouse, ES cells were purchased with a trap inserted into intron 1 (CF0294) or 8 (AY0426) (Wellcome Sanger Institute). Trap insertion was validated by PCR and sequencing. The ES cells trapped in intron 1 were sent for 129 blastocyst injections (MMRRC). The resulting chimera was shipped to Columbia University, where it was bred and the offspring backcrossed to C57Bl/6 for 5 generations using microsatellite-assisted speed congenics (Taconic) to select the most backcrossed mice from each generation to continue breeding. These HET mice were then mated together, but as mentioned above, no HOM offspring were ever recovered.

3.2.2 Open field

Animals were identified and habituated individually in cages for 30-60 minutes, then placed into transparent open field chambers (25cm square) with infrared motion detection beams (Coulbourn) for 1 hour on day 1 and 30 minutes on day 2, exactly 24 hours later. Locomotion in the horizontal and vertical planes was tracked via laser beam breaks and the data reported in

TruScan software as distance traveled or time in the margins (<2.5 beams from wall) and center (rest of chamber) as well as rearing. The time spent and distance traveled in center vs. margin zones was extrapolated using separate formulae from individual animal coordinates taken at 1s intervals. Due to reduced sampling frequency, this extrapolation formula yielded a discrepancy between zone distances and total distance, but no such discrepancy for time measurements.

3.2.3 Novel object recognition

The initial cohort of animals was tested using a protocol that failed to demonstrate accurate spatial memory for identical object placement. A new protocol was then executed with the second cohort of animals. On the first two days, animals were habituated to a 9x18in empty cage for 10 minutes. On the third day, they were returned to that cage with two identical green plastic toys affixed at either end and videotaped for 5 minutes. One hour later, they were returned to the cage except one green toy was replaced with a yellow plastic toy, balanced across genotypes for left and right sides, and videotaped for 5 minutes. Three hours later, the novel toy was replaced again with a red plastic toy. All toys were distinct in texture, shape, and color. Videotape was hand-scored by a blind observer for the time spent in direct contact sniffing each item over every 5 minute period. Percent time per novel object was calculated as (total time investigating novel object)/(total time investigating both objects).

3.2.4 Morris water maze

In the initial platform training, mice were placed in a 3 gallon bucket of room temperature water level with a plastic platform and time until they climbed onto the platform was noted. This training was repeated twice a day 1 hour apart for 3 days using opaque water due to nontoxic white tempera paint on the second and third days. After each trial, animals were warmed on a thermoregulated heating pad. Animals were consistently tested either in the

morning or afternoon throughout training, with both groups balanced with respect to genotypes.

Once animals were trained to find a platform, 2 days of visual platform training began. The platform was connected to a brightly colored pole and flag then submerged in an 8 foot diameter pool of room temperature opaque water. The walls of the room containing the pool were uniquely decorated to provide color, pattern, and textural extra-maze cues, and the tester's location was concealed with curtains. Mice were placed into the pool at one of 8 randomly chosen entrance points using 3 pathways to the pool to prevent association with any one location or the location of the tester, however the platform was never moved. Three times a day for two days, mice were manually timed and automatically tracked with AnyMaze Software (Stoelting) and a camera (Logitech) until they located the visible platform and remained on it for 30 seconds.

Next in the hidden platform training, the platform marker was removed and animals were timed and tracked until they located the unmarked platform and remained for 30 seconds. After 3 trials a day for 5 days of hidden platform testing, the two probe trials occurred. One hour after the last hidden trial, the platform was removed and mice were automatically tracked while swimming for 1 minute. This probe was repeated 24 hours later.

3.2.5 T maze

In order to increase the reward of food, animals were weighed for 3 days then food restricted over the course of a week to achieve 80-90% of their starting weight. Animals were weighed daily and fed accordingly to maintain this weight. As noted above, mice were individually housed with igloo and nesting enrichment for the duration of this test.

The T maze apparatus has one start arm 38cm long by 10cm wide with two choice arms 26cm long holding blue 3cm dishes affixed to the floor at the end of each arm plus walls 12cm

high. Sliding doors separated the beginning of the start arm, as well as each choice arm. Extra food pellets (BioServ) were placed beyond each choice arm in order to mask any olfactory cues.

Mice were allowed to explore the entire maze for 10 minutes on the first day of habituation. On the second and third days of habituation, food pellets were placed into each dish and were replenished once the mouse ate all 3 pellets.

On two days of forced alternation training, mice were released from the start zone and allowed to explore the only open arm which did not have food (pseudorandomized left and right across 10 trials), then prodded back to the start zone. During a 5 second intratrial delay this arm was closed off and the opposite, goal arm opened with a food pellet in the dish at the end of the goal arm. Mice were allowed to explore the opposite, goal arm and eat the food, then returned to the start zone. This was repeated 10 times with an intertrial delay of 40 seconds for 2 days. On these and all other sessions, the animals were videotaped and manually timed and scored.

Next animals advanced into choice training. The goal arm of the maze was closed off with a food pellet in the dish, but no food pellet in the open arm. Each mouse was timed until it reached the empty food dish of the open arm, then returned to the start zone for 5 seconds. The center area of the maze was wiped with 70% ethanol to remove olfactory cues and both arms were opened. The mouse was then released and the choice of arms (goal or non-goal) and time to the food dish were recorded. Once the animal's body entered one arm, the door to the other arm was closed. There was a 40 second intertrial delay. This was repeated 10 times daily, pseudorandomized for the goal arm. If mice developed a strong turn bias, goal arms were biased toward the avoided side and 5 extra trials were added daily until the bias was corrected.

Training continued until each mouse reached criterion of 7 out of 10 correct choices on 3 consecutive days. On the day after the criterion was met, animals began 3 days of working

memory testing. The intratrial delay was increased to 10, 20, or 30 seconds with 4 trials of each delay time pseudorandomized across the session.

3.2.6 Prepulse inhibition

On the first day, mice were weighed and then placed into the restraining startle chamber of the PPI box (Hamilton Kinder). The Startle Monitor software was programmed to deliver a 5 minute habituation period, then 6 blocks of pseudorandomized trials with 10-20 second randomized intertrial delays. Each block was comprised of the following 7 trials: no sound, 40ms 120dB burst (the startle stimulus), or a 20ms prepulse of 74, 78, 82, 86, or 90dB 100ms prior to the 120dB startle stimulus. Background noise of approximately 70dB was consistent throughout the experiment. After each stimulus, the equipment recorded force transduced over 65ms and the maximum force (startle) was reported. Percent PPI of a startle response was calculated as: 100 – [(startle response on acoustic prepulse + startle stimulus trials / startle response alone trials) × 100].

To determine startle threshold, 24 hours following PPI animals were weighed and returned to the startle chamber. After a 5 minute habituation, 13 auditory stimuli were presented 5 times each in pseudorandomized order with 10-20s intertrial delays. Tested stimuli were 70, 74, 78, 82, 86, 90, 94, 98, 102, 106, 110, 114 and 118dB over a background noise of 70dB. Maximum startle was sensed and reported as above.

3.2.7 Auditory testing

Two pairs of WT and HOM 5-7 week old female animals were brought to Elizabeth Olson's laboratory at Columbia University Medical Center for auditory testing. Mice were anesthetized with ketamine (13 mg/kg) and urethane (1.5 mg/g). The analgesic buprenorphine (0.1 mg/kg) was also administered for maintenance of anesthesia. At the end of the experiment,

the animal was sacrificed with an overdose of urethane or sodium pentobarbital. During the experiment, animal body temperature was maintained at ~ 37° C using a thermostatically controlled heating blanket. A tracheotomy was performed to maintain a patent airway. The left pinna was removed. To measure the compound action potential (CAP) thresholds, the bulla was opened with great care.

Acoustic stimuli were generated and collected digitally using Tucker Davis Technologies (TDT) System III. Stimulus and acquisition programs were written in Matlab and TDT Visual Design Studio. The sampling frequency of the TDT system was 200 kHz. Data were stored following removal of the first 4096 points of the response waveform to avoid the transient, and time-averaging the remaining waveform, typically in 50 time-locked segments. Responses were later analyzed by Fourier transform in Matlab.

The ear was acoustically stimulated via a 40 – 1377 tweeter (Radio Shack). The tweeter and a probe-tube microphone (Bruel and Kjaer model 4134) were coupled together via a T tube and coupled to the ear canal. The probe-tube microphone served as the ear canal pressure monitor. The transfer function of the probe-tube microphone was accounted for when setting the sound pressure level (SPL, decibels relative to 20 Pa peak) and analyzing the data. With a 1 second data acquisition time, the microphone noise level (with probe-tube) was ~ -10 to 25 dB SPL up to 50 kHz. The noise level was determined by the average FFT value of the six adjunct points at frequencies below and above the stimulus. The level of distortion products produced by the system (mainly the driver) has been discussed previously (Dong and Olson 2006; Dong and Olson 2008). With current settings, system distortion was ~ 60 dB smaller than the 80 dB SPL primaries. Therefore, system distortion was not a concern in the results. System distortion was also checked with postmortem responses at the end of each experiment.

Sound-evoked CAP recordings (.5 to 80 kHz) were made from a silver wire electrode firmly connected to the cochlear bony shell at the round window. The reference electrode was connected to the neck muscle and the animal was grounded. To get the CAP waveform, anti-phase single tone (1 second duration) stimulus were used and averaged 30 times to remove the cochlear microphonic responses. 5 uV peak-to-peak criteria was used to determine the threshold sound pressure level to each stimulation.

Two equal-intensity tones (1-2s duration) with fixed $f_2/f_1 = 1.05$ or 1.25 were used in DPOAE measurements. The primary frequencies were swept from 1 to 60 kHz in 500 Hz steps.

3.2.8 Fear conditioning

On the first day of fear conditioning, animals were individually habituated in rectangular cages with wire rack tops for 5-10 minutes. They were then placed into the sound-attenuated fear conditioning chambers (Med Associates) and videotaped and monitored via FreezeFrame software (Coulbourn). Paper towel scented with lemon extract was placed inside each chamber to create an odor. After 3 minutes of habituation, a 30 second tone of 85 dB and 2kHz was delivered then a 0.7mA shock was delivered for 2 seconds immediately after the tone ceased. This pairing of tone and shock was repeated 1 minute later.

Twenty-four hours after conditioning, animals were re-exposed to the same context. They were habituated in the same rectangular cages, placed into the same chambers with lemon scent, and tracked for 6 minutes. Two hours later, animals were tested for cued conditioning. In order to create a novel context, they were habituated in triangular cages with plastic lids, then placed into the chambers but with plastic, colored floor and wall inserts and a vanilla scent. After 3 minutes in this “novel” chamber, the tone played for 3 minutes.

Percent time spent freezing was automatically scored by FreezeFrame after the

freezing/motion threshold was manually set for each animal on each trial. Animals were balanced with respect to genotype for time of day (morning vs. afternoon) and 4 available testing chambers.

3.2.9 cFOS activation after fear conditioning

Fear conditioning was executed as described above on 2-4 animals per session. Following cued conditioning assessment, animals were left in the chambers for 90-120 minutes. Each animal was then taken directly to anesthesia and perfusion with PBS and 4% PFA. After overnight post-fixation, brains were sliced 60um thick on a vibratome and every other section was stained with rabbit anti-cFOS 1:5000 (Calbiochem), mouse anti-NeuN 1:300 (Millipore), and TOPRO 1:2500.

These slides were then viewed at 20x on a confocal microscope to permit manual counting of cFOS+ cells in the dentate gyrus, CA3, and CA1 subregions of the hippocampus as well as the lateral, basolateral, and central nuclei of the amygdala. Regions of interest were defined using a mouse atlas to set anatomic boundaries and analyzed from Bregma -1.3 to -1.9 (Paxinos and Franklin 2004). The dentate gyrus is a clearly demarcated structure. Dorsal CA1 hippocampus was defined from the end of the blades of the dentate gyrus to the end of the mossy fiber pathway. Dorsal CA3 began at the end of the mossy fiber pathway and terminated at the midline. The basolateral nucleus of the amygdala was defined as the lower half of the region within the forking of the external capsule while the lateral nucleus was the upper half. The central nucleus was medial to the lateral/basolateral nucleus.

3.2.10 Data analysis

All data was analyzed and graphed using Prism 5 (GraphPad). For all paradigms one way ANOVA was used to assess genotype effects, or two way repeated measures ANOVA to test

genotype and time effects. Bonferroni correction post-hoc testing was then used to compare genotypes for significance. On novel object recognition and T maze paradigms, one sample t tests were used to compare mean percentages against 50% (chance) performance. Chi^2 tests were used to assess expected vs. observed numbers of mice on startle and T maze tests.

3.3 Results

3.3.1 Generation of mouse model

Rapgef6 animals were generated and shared by the Kataoka lab (Yoshikawa et al. 2007). Briefly, exon 21 that encodes the GEF enzymatic domain was deleted via homologous recombination of a floxed allele that was excised via crossing with a *Cre* expressing mouse line (see Methods for details) (Figure 3.1A). The Kataoka laboratory published proof of trap insertion and *Cre*-assisted deletion (Figure 3.1B-D). We confirmed by replication the absence of the expected protein bands on Western blot from homozygous knock out (HOM) animal brain samples (Figure 3.1E).

In an effort to more closely emulate the deletion in the patient with schizophrenia, we also attempted to generate knock out lines with a knock out cassette trap inserted into intron 1 or 8 of *Rapgef6*. Although the location of the traps could be validated via PCR and sequencing on both ES cells and mouse tissue, Northern blot results were inconclusive on *Rapgef6* expression due to either inadequate probe specificity or low RNA concentration (data not shown). Heterozygous knock out (HET) mice were born from the intron 1 ES cell injections, but HET x HET matings never yielded HOM pups as validated by qPCR, PCR, and Western blot results (data not shown). Timed matings were established to check if HOM embryos were dying prior to birth, but no HOM animals were detected from E15.5 onward. Since sequencing results demonstrated the initial 700 bases of the trap were deleted, we assumed there was an issue with

the trap integration leading to either failure of the trap to block expression or a duplication of the gene locus that prevented complete knock out.

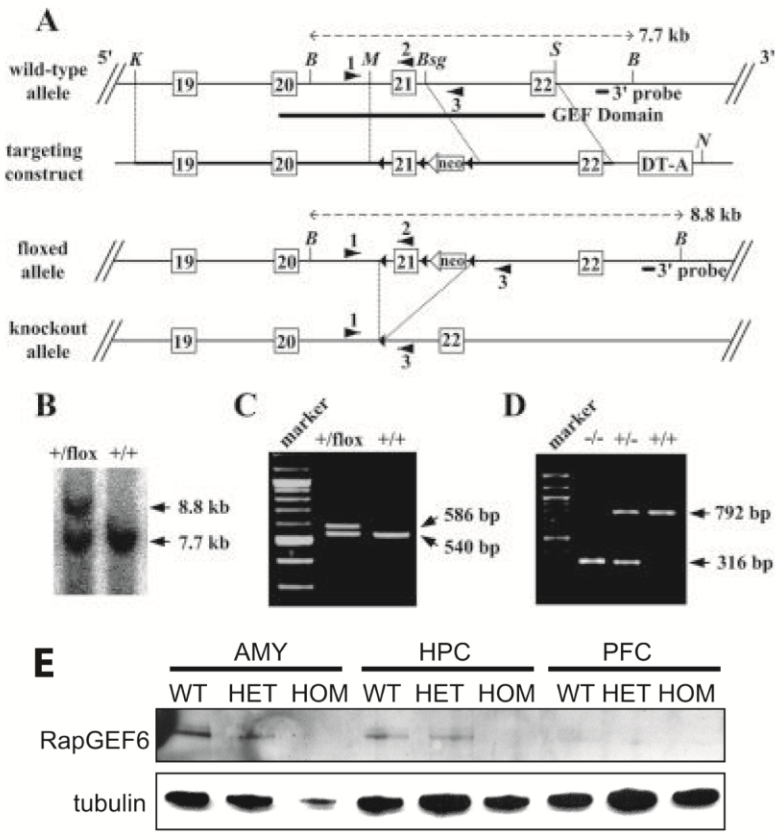


Figure 3.1 *Rapgef6* knock out mouse model. **A**) Schema of generation of the knock out via floxing the GEF domain exon 21. **B**) Southern blot from the probes indicated in the schema to demonstrate trap incorporation. **C**) PCR of the upstream flox locus from primers indicated in the schema. **D**) PCR of the mouse after crossing to Cre, from the indicated primer locations, demonstrating successful deletion of exon 21 (Yoshikawa et al. 2007). **E**) Successful elimination of Rapgef6 on Western blot of amygdala, hippocampus, and prefrontal cortex from our lab.

3.3.2 Open field

Two cohorts of littermate males were allowed to explore an open field chamber for 60 minutes on the first day and 30 minutes on the second. Since the two cohorts did not significantly differ in total distance traveled (P=0.3 on day 1, P=0.1 on day 2), the data sets were combined. Data was analyzed in 1 minute bins for effects of genotype and test time, or averaged across each test day for effect of genotype. On day 1 of testing, there was no significant effect of genotype on total distance (P=0.15), margin distance (P=0.15), or center distance (P=0.63) although there was a significant effect of test time (P<0.0001 for all 3 comparisons) (Figure 3.2A-C). While there

was no significant effect of genotype on time spent in the margin or center zone (P=0.89) there was a significant effect of test time (P<0.0001) as well as a significant interaction of genotype and test time, indicating the genotypes varied in their response over time (P=0.005) (Figure 3.2D,E). Entries into the center zone were not significantly affected by genotype (P=0.36) but were influenced by test time (P<0.0001) (Figure 3.3A). Rearing, an indicator of locomotion and exploration, was significantly different by genotype (P=0.018) and test time (P<0.0001) as well as an interaction between time and genotype (P=0.001) (Figure 3.3B). Post-hoc testing demonstrated significantly increased (P<0.05) rearing in HET animals over WT at 57 minutes and in HOM over WT at 27 and 55 minutes.

When measurements were averaged over the hour of testing on day 1, there was no significant difference in total distance (P=0.17), center distance (P=0.73), margin or center time (P=0.64) (Figure 3.2A-E), or center entries (P=0.47) (Figure 3.3A). There was a significant effect of genotype on margin distance (P=0.014) with HET animals traveling more distance than WT (Figure 3.2B). Additionally, rearing rates were significantly affected (P=0.008) with HOM and HET animals increased over WT (Figure 3.3B).

On day 2 of testing, there was a significant effect of both test time (P<0.0001) and genotype (P=0.029) on total distance with HOM more active than WT at 30 minutes (Figure 3.4A). There was only a significant effect of test time (P<0.0001) but not genotype (P=0.13) on margin distance, while both test time and genotype were significant for center distance (P<0.0001, P=0.017 respectively) (Figure 3.4B,C). Margin and center times were significant for neither test time (P=0.15) nor genotype (P=0.24) (Figure 3.4D,E). Center entries were affected by test time (P<0.0001) and genotype (P=0.049) with HOM entries greater than WT at 29 minutes (P<0.01) (Figure 3.5A). Rearing, which was significantly different by genotype over test

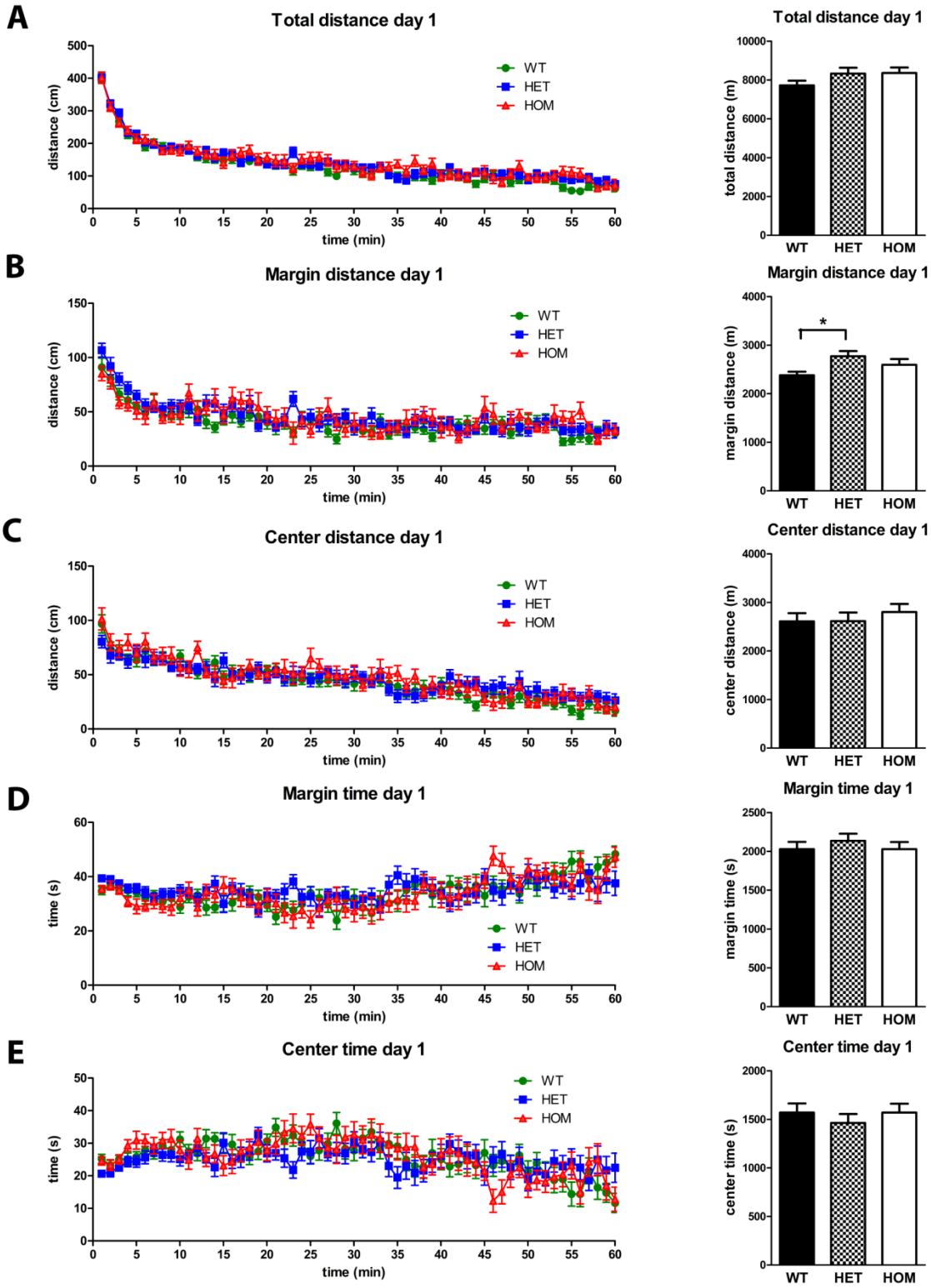


Figure 3.2 Open field results day 1. No significant effect of genotype on **A**) total, **B**,**D**) margin, or **C**,**E**) center distance or time across test time, but **B**) a significant increase in HET margin distance overall. N = 26 WT, 24 HET, 16 HOM Error bars +/- SEM for this and all figures.

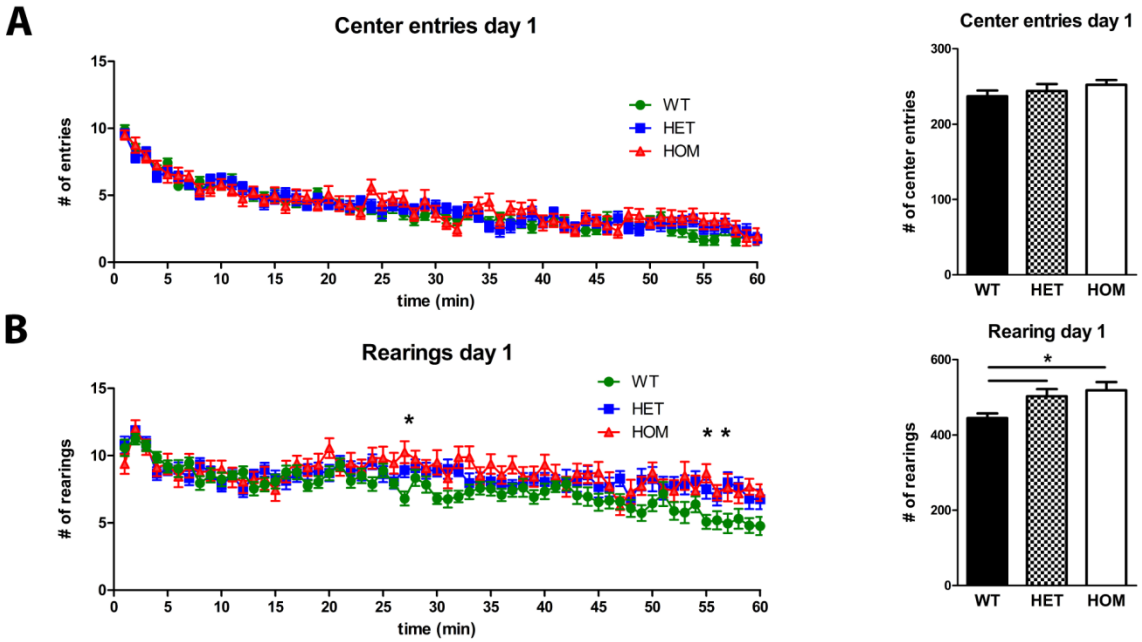


Figure 3.3 Open field results day 1. No significant effect of genotype on **A**) center entries, but a significant effect of genotype on **B**) rearing across time and overall with HET and HOM increased over WT.

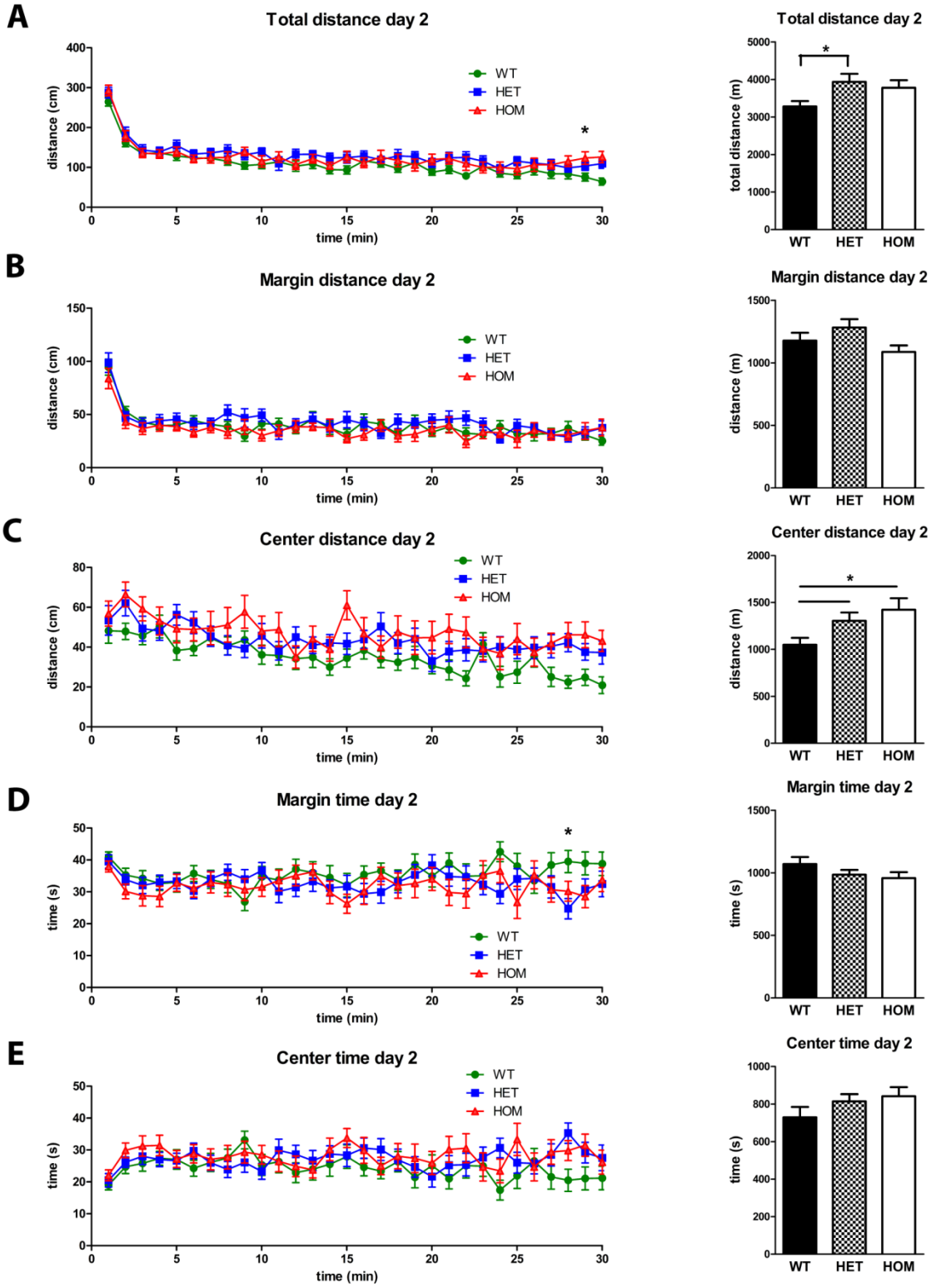


Figure 3.4 Open field results day 2. No significant effect of genotype on **A-C**) distance or **D**,**E**) time measures across test time except **D**) an increase in WT margin time. Overall, an increase in **A**) HET total distance and **C**) HET and HOM center distance.

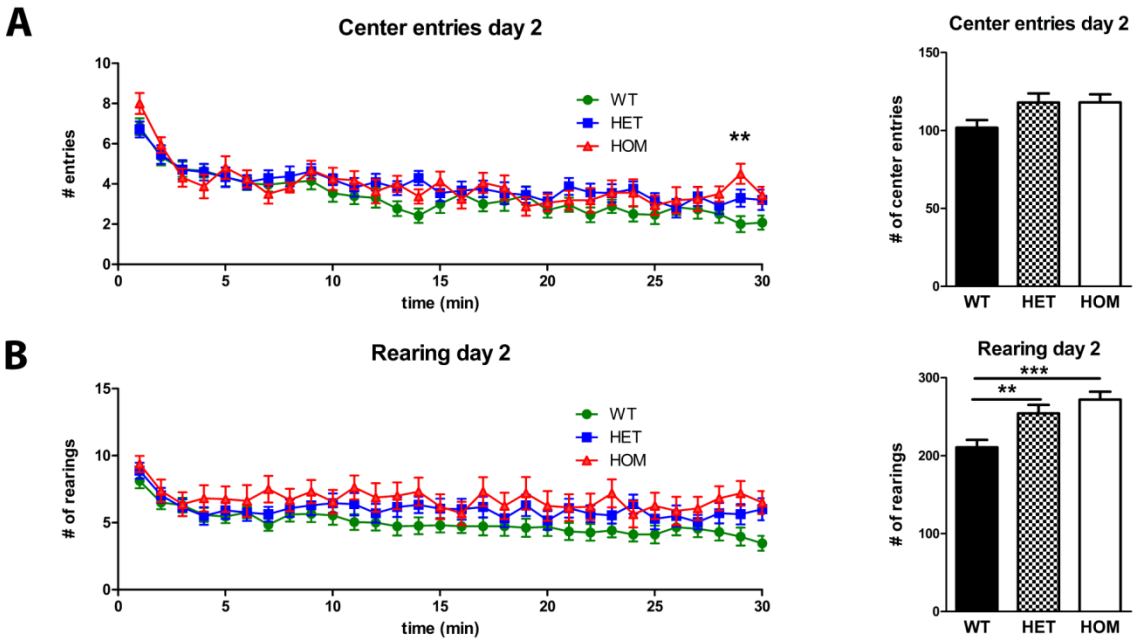


Figure 3.5 Open field results day 2. **A**) A significant increase in HOM animals on center entries, and **B**) HET and HOM animals on rearing.

time, was only affected by test time (P<0.0001) but not genotype (P=0.09) (Figure 3.5B).

Upon averaging measurements across day 2, there was no significant effect of genotype on margin distance (P=0.13), or center or margin time (P=0.24) (Figure 3.4B,D,E). Total distance was dependent on genotype (P=0.029) with HET mice traveling more than WT, as was center distance (P=0.017) for which HOM and HET animals traveled more in the center than WT (Figure 3.4A,C). Center entries was affected by genotype (P=0.049) with no post-hoc comparisons significant though HOM and HET were elevated over WT (Figure 3.5A). Finally, rearing was significant (P=0.0003) and increased in HOM and HET over WT (P<0.001 and P<0.01, respectively) (Figure 3.5B).

3.3.3 Novel object recognition

When mice were exposed to two identical objects at opposite ends of an empty cage, there was no significant effect of genotype on either the time (P=0.82) or percent time (P=0.7)

spent investigating either object out of the total time of investigation (Figure 3.6A). These mean percent time values were not significantly different from 50% chance (P=0.999, P=0.45, P=0.81 for WT, HET, HOM respectively). One hour later, there was no effect of genotype on the time or percent time (P=0.91, P=0.31 respectively) spent investigating the novel object (Figure 3.6B). There was a significant increase in time spent with the novel object for all genotypes (P=0.002, P=0.015, P=0.0008 for WT, HET, HOM) (Figure 3.6D). Finally, three hours later, there was no genotype difference in time (P=0.4) or percent time (P=0.57) with the second novel object, yet all genotypes persisted in investigating the second object more than chance (P=0.0005, P=0.03, P=0.01 for WT, HET, HOM respectively) (Figure 3.6C,E).

3.3.4 Morris water maze

During two days of three trials per day of visible platform training, mice learned to swim to a submerged platform with a large, brightly colored flag marking its location. There was no significant effect of genotype on the latency to reach the platform (P=0.46) or the distance swum prior to reaching the platform (P=0.58), although all genotypes significantly improved over trials (P<0.0001 for both measures) (Figure 3.7A,B).

The flag was then removed and animals trained to reach the hidden platform for 2 or 3 trials per day for 5 days. Again there was no significant effect of genotype on the latency to reach the platform (P=0.87) or the distance covered prior to reaching the platform (P=0.98), with all genotypes showing improvement across trials (P<0.0001 for both measures) (Figure 3.7C,D).

One hour after the last hidden trial, the platform was removed and the mice were tracked for 60 seconds. Analysis demonstrated a significant preference (P<0.0001) with an increase in all genotypes to spend more time in the NW quadrant that previously contained the platform (P<0.001 for all post-hoc comparisons of NW to other quadrants), but no difference between

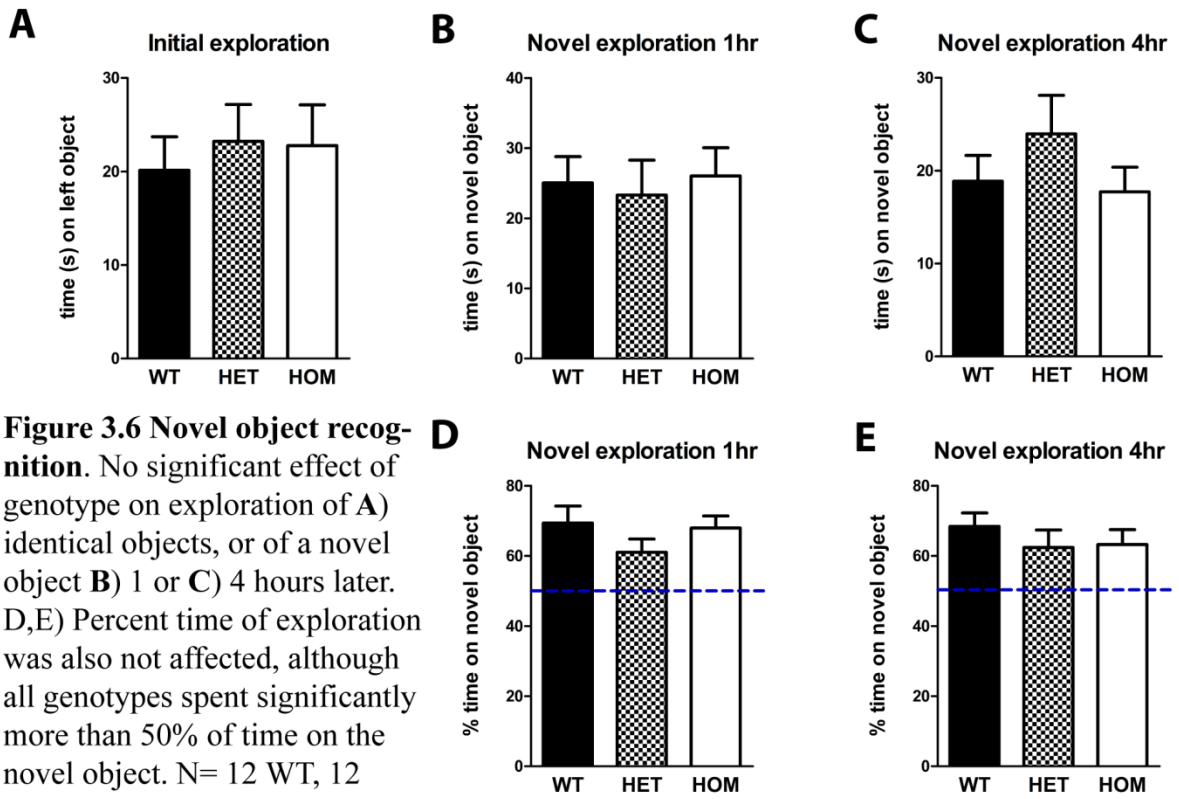


Figure 3.6 Novel object recognition. No significant effect of genotype on exploration of **A**) identical objects, or of a novel object **B**) 1 or **C**) 4 hours later. D,E) Percent time of exploration was also not affected, although all genotypes spent significantly more than 50% of time on the novel object. N= 12 WT, 12 HET, 9 HOM

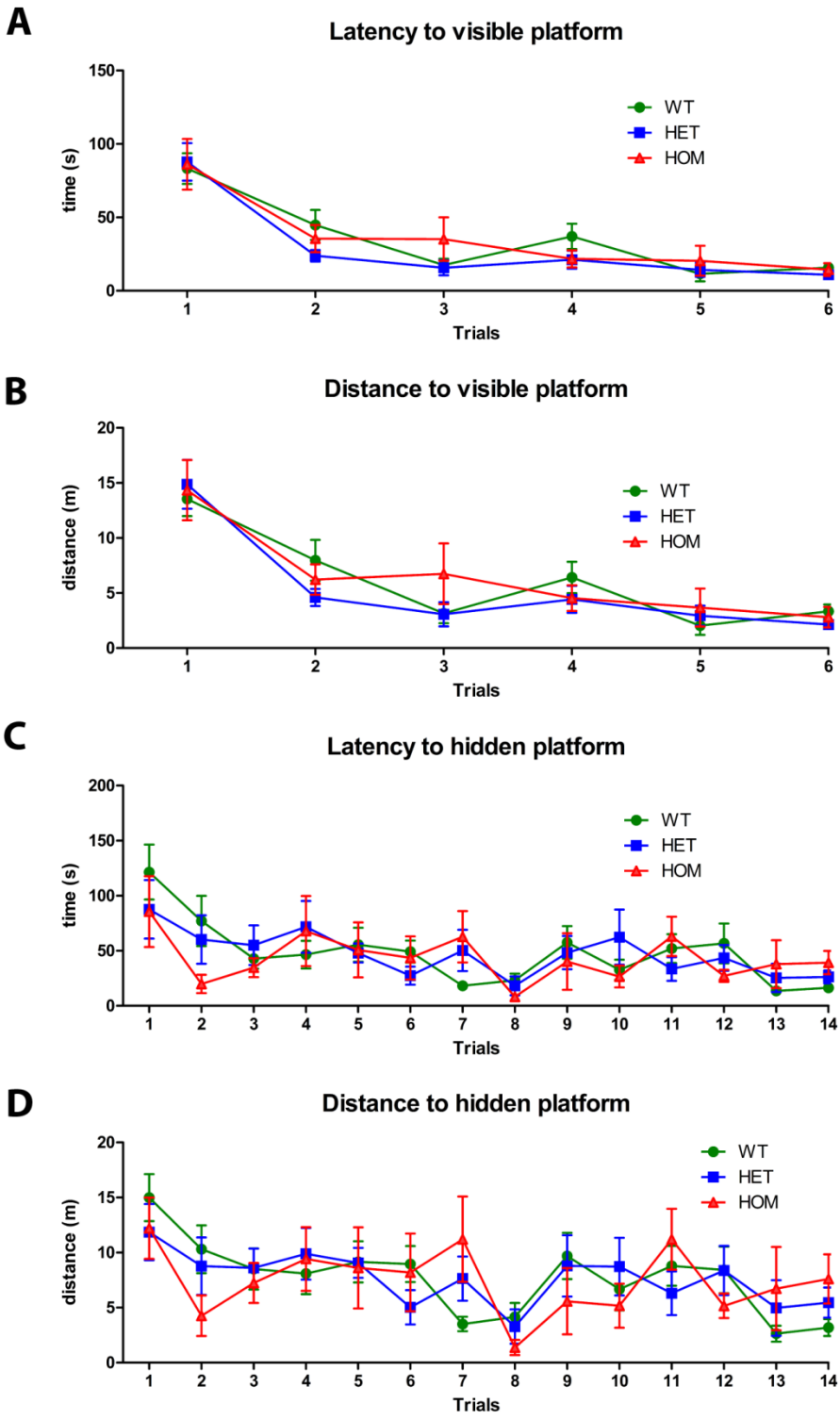


Figure 3.7 Water maze learning. No significant effect of genotype on **A**) latency or **B**) distance covered to reach the visible platform, nor on **C**) latency or **D**) distance to reach the hidden platform during learning of the water maze. N= 14 WT, 12 HET, 7 HOM

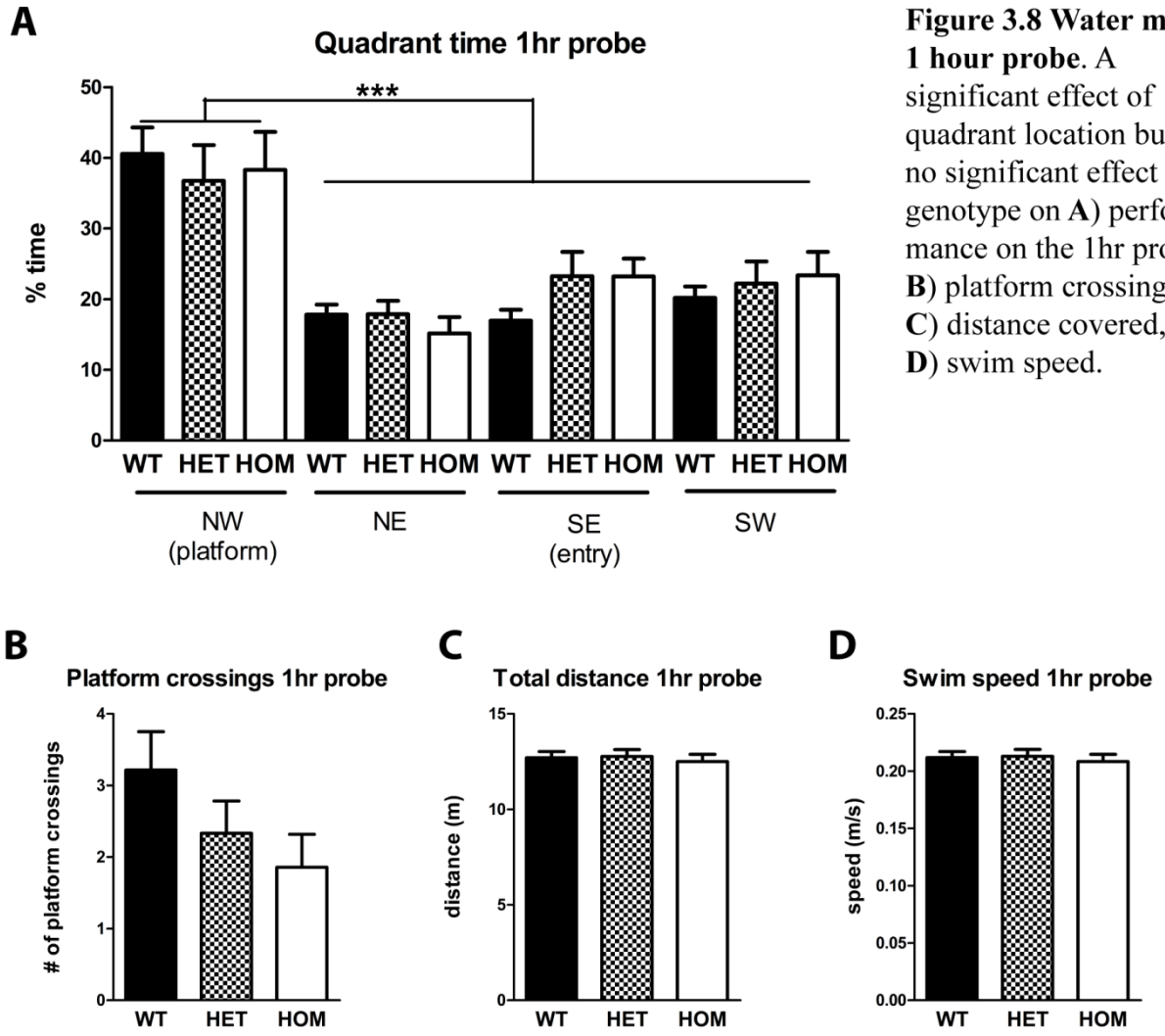


Figure 3.8 Water maze 1 hour probe. A significant effect of quadrant location but no significant effect of genotype on **A**) performance on the 1hr probe, **B**) platform crossings, **C**) distance covered, or **D**) swim speed.

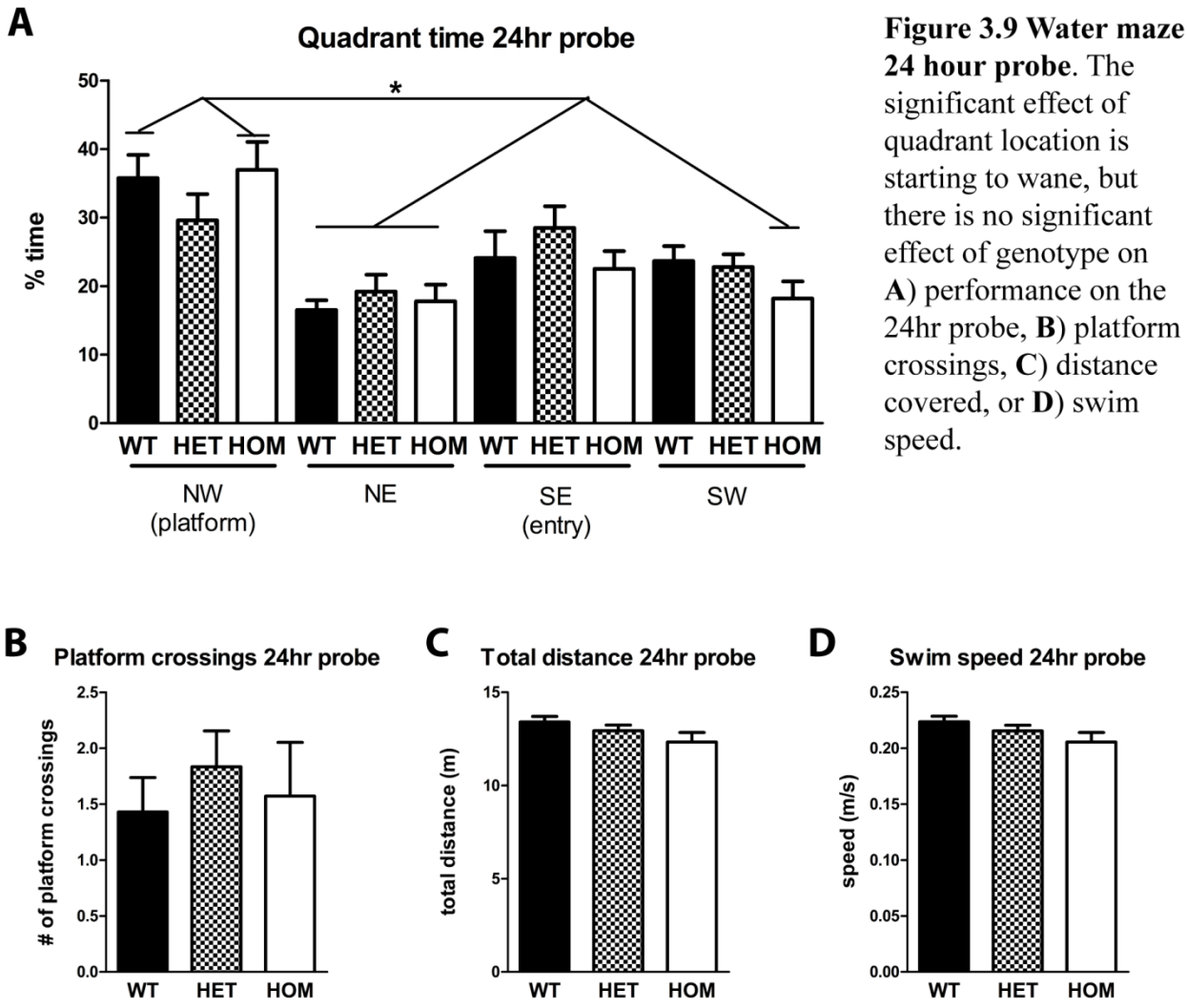


Figure 3.9 Water maze 24 hour probe. The significant effect of quadrant location is starting to wane, but there is no significant effect of genotype on **A**) performance on the 24hr probe, **B**) platform crossings, **C**) distance covered, or **D**) swim speed.

genotypes (Figure 3.8A). When the specific location of the platform was considered, there was no significant effect of genotype on platform location crossings (P=0.20) (Figure 3.8B). In view of the hyperactivity noted in HOM and HET animals on open field, the total distance covered and swim speed were calculated, but there was no significant effect of genotype (P=0.89 distance, P=0.88 swim speed) (Figure 3.8C,D).

Twenty-four hours later, the same test was re-administered and there was a significant increase in time spent in the NW quadrant (P<0.0001) (Figure 3.9A). On post-hoc comparison, WT and HOM animals still spent significantly more time in the NW quadrant than in the NE or

SW ($P<0.05$), but HET animals did not. Platform location crossings were not significantly different ($P=0.68$), nor were distance swum ($P=0.15$) nor speed ($P=0.14$) (Figure 3.9B-D).

3.3.5 T maze

Mice were considered to have learned the T maze task to criterion when they could correctly choose the goal arm 7/10 times for 3 consecutive days. The number of days of training required to reach this criterion was not affected by genotype ($P=0.55$) (Figure 3.10A). While more HOM animals failed to reach criterion by 12 days of training, this was not significant via ANOVA ($P=0.69$) or Chi^2 analysis ($P=0.68$). Upon reaching criterion, all genotypes were equally successful at the task ($P=0.19$) (Figure 3.10B).

During the 3 days of working memory testing with increasing intratrial delays, there was no effect of genotype on performance ($P=0.78$), although there was an expected effect of intratrial delay time ($P=0.007$) with performance decreasing significantly as delays increased (Figure 3.10D). At each delay value, all genotypes performed above chance (10s delay $P<0.0001$ for all genotypes; 20s delay $P<0.0001$ WT, HET $P=0.0002$ HOM; 30s delay WT $P=0.0001$ HET $P=0.025$ HOM $P<0.0001$) (Figure 3.10E-G).

Some animals developed a turn bias during the testing phase. This perseverance was evaluated by calculating the percent of left turns for each set of trials, then computing the absolute percent difference from 50% which represents both chance and the correct number of left turns. There was no significant effect of genotype on turn bias ($P=0.92$) (Figure 3.10C).

Given the open field hyperlocomotion phenotype in HET and HOM animals, time to reach the forced run and choice arms were compared during the three testing days, separated by intratrial delay time to avoid confounding of working memory effects on speed. No genotype effects were found at 10s delay for the forced arm time ($P=0.08$), but time navigating the choice

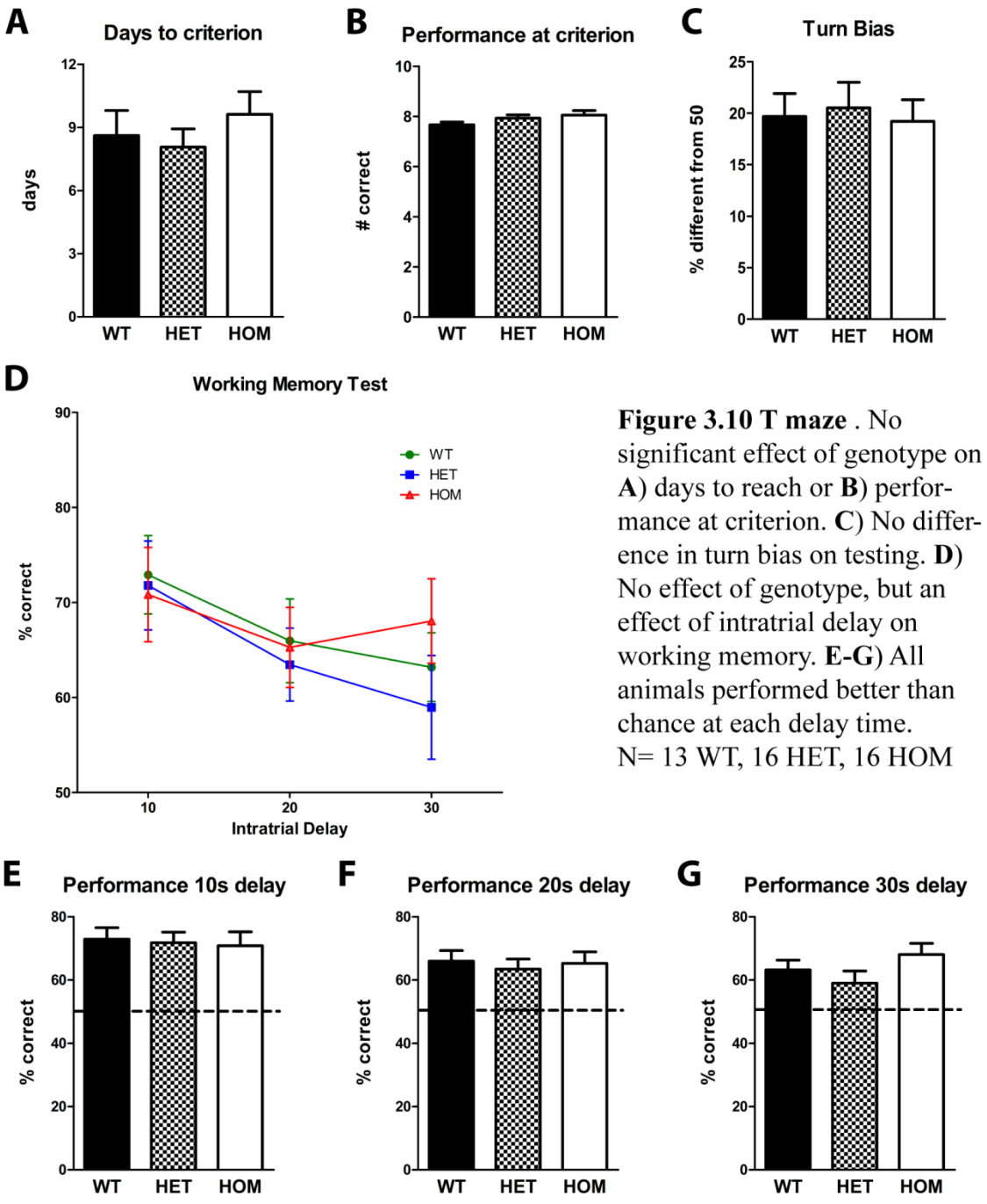


Figure 3.10 T maze . No significant effect of genotype on **A**) days to reach or **B**) performance at criterion. **C**) No difference in turn bias on testing. **D**) No effect of genotype, but an effect of intratrial delay on working memory. **E-G**) All animals performed better than chance at each delay time. N= 13 WT, 16 HET, 16 HOM

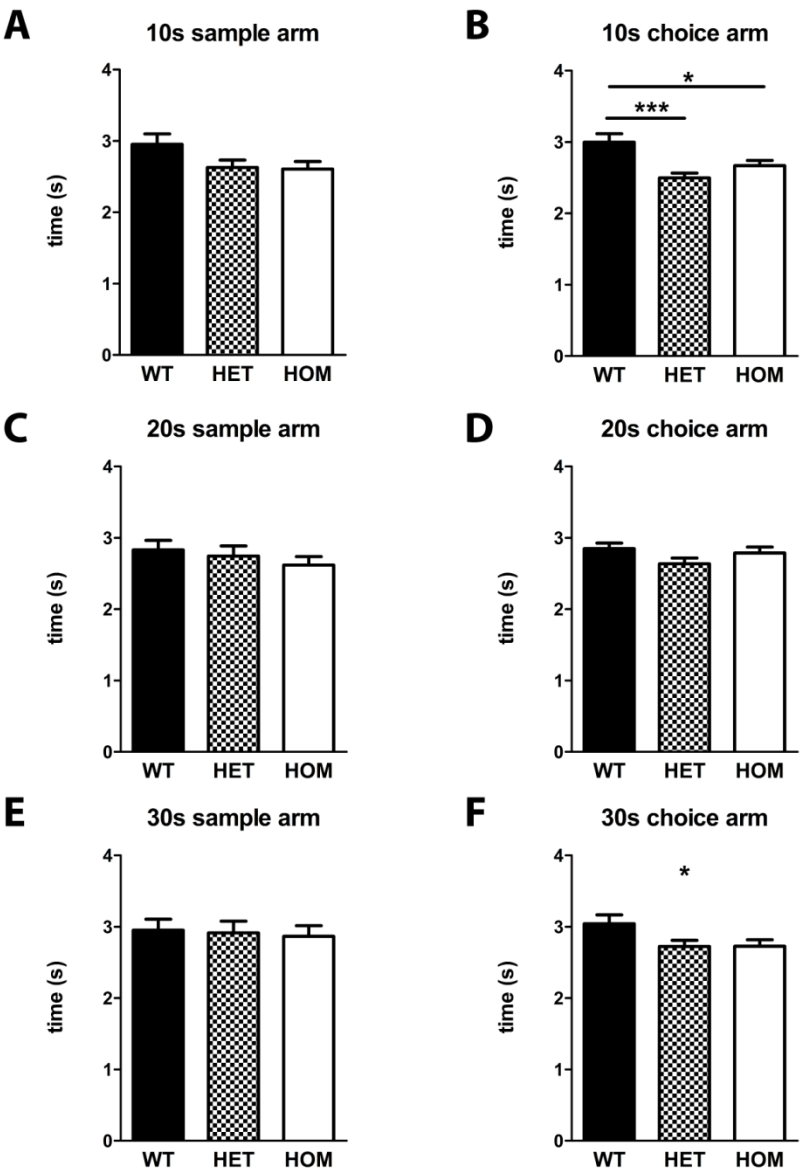


Figure 3.11 T maze speed. **A**,**C**,**E**) The time animals spent traversing the sample arm was not significantly affected by genotype at any intratrial delay. But choice arm time was reduced (speed was increased) in HET and HOM animals at **B**) 10s and there was also a significant effect of genotype at **F**) 30s delays.

arm was different (P=0.0003) with HET (P<0.001) and HOM (P<0.5) mice faster than WT (Figure 3.11A,B). There was no difference by genotype for time on the forced (P=0.53) or choice (P=0.16) arms with a 20s delay (Figure 3.11C,D). During the 30s delay trials, there was no genotype effect for time during the sample arm (P=0.93) but there was on the choice arm (P=0.04) with HET and HOM animals trending to be faster than WT although post-hoc testing was not significant (Figure 3.11E,F).

3.3.6 Sensorimotor gating

On the initial day of PPI testing, mice were exposed to a 120dB startle stimulus in order to determine baseline startle without prepulses. This baseline response was not affected by genotype (P=0.22), however there were 4 out of 17 HET and 5 out of 16 HOM mice that generated less than 2N of force while every WT animal generated more than 2N (Figure 3.12A). While this was not significant on Chi^2 testing by genotype (P=0.11), failure to startle excludes animals from PPI analysis. This reduction in startle to tone was not a result of diminished size since there was no genotype difference in weight (P=0.27) (Figure 3.12B). Interestingly, WT animal weight did not significantly correlate with force generated in response to a 120dB tone (P=0.31, Spearman r=0.31), both HET and HOM weights were correlated (P=0.046, r=0.49 and P=0.044, r=0.51 respectively) (Figure 3.12C).

While the distribution of non-startling compared with startling animals was not significantly affected by Chi^2 testing (P=0.11), it clearly influenced auditory startle since there was a highly significant effect of both genotype and tone volume (P<0.0001 for both) with significant post-hoc comparisons(P<0.05 HOM< WT at 110dB, P<0.01 HOM< HET at 118dB) (Figure 3.13A), yet with the most severely affected mice removed (3 HET, 4 HOM) the effect of genotype diminished but remained significant (P=0.04) while the effect of volume was unaffected (P<0.0001) and there were no longer any significant post-hoc comparisons (Figure 3.13B). Even without the most affected mice, genotype remained a significant factor and HOM animals still startled less and thus the most affected animals were not driving the entire statistical significance.

On PPI testing, when all animals were considered, there was a very significant effect of genotype and prepulse volume (P<0.0001 for both), with HOM PPI greater than HET at a

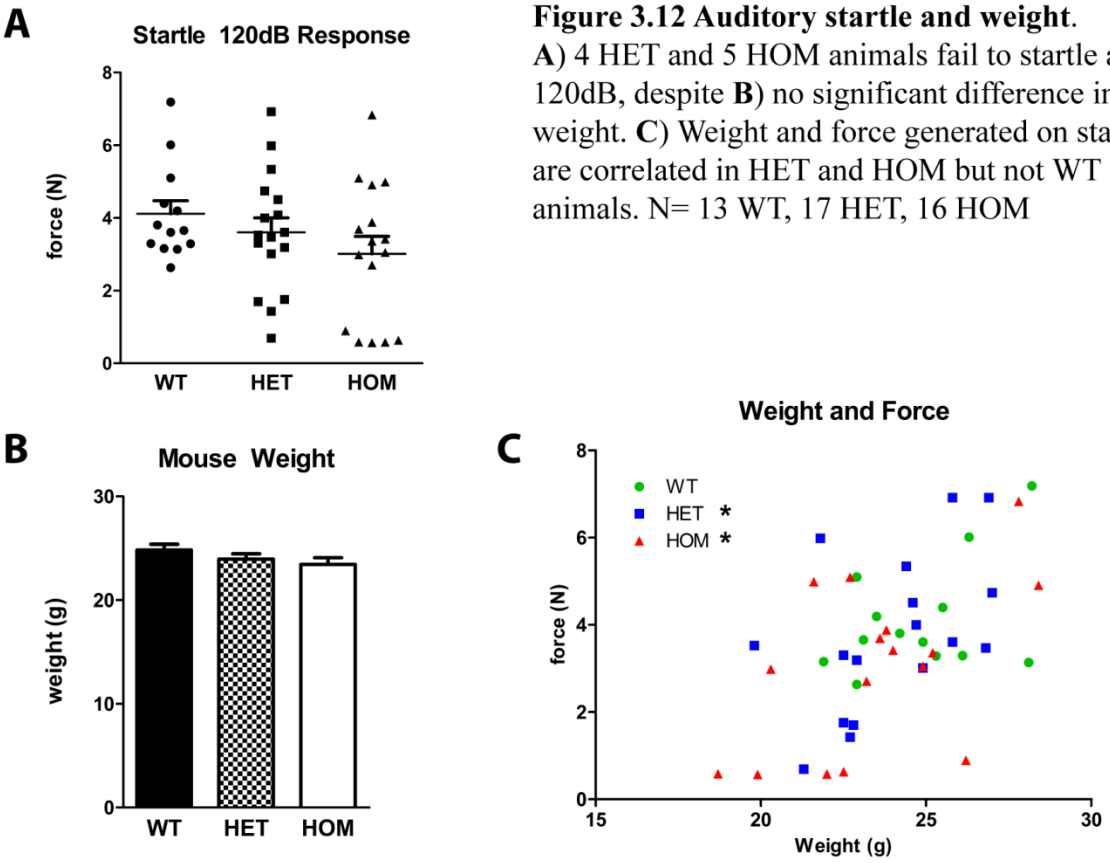


Figure 3.12 Auditory startle and weight. **A**) 4 HET and 5 HOM animals fail to startle at 120dB, despite **B**) no significant difference in weight. **C**) Weight and force generated on startle are correlated in HET and HOM but not WT animals. N= 13 WT, 17 HET, 16 HOM

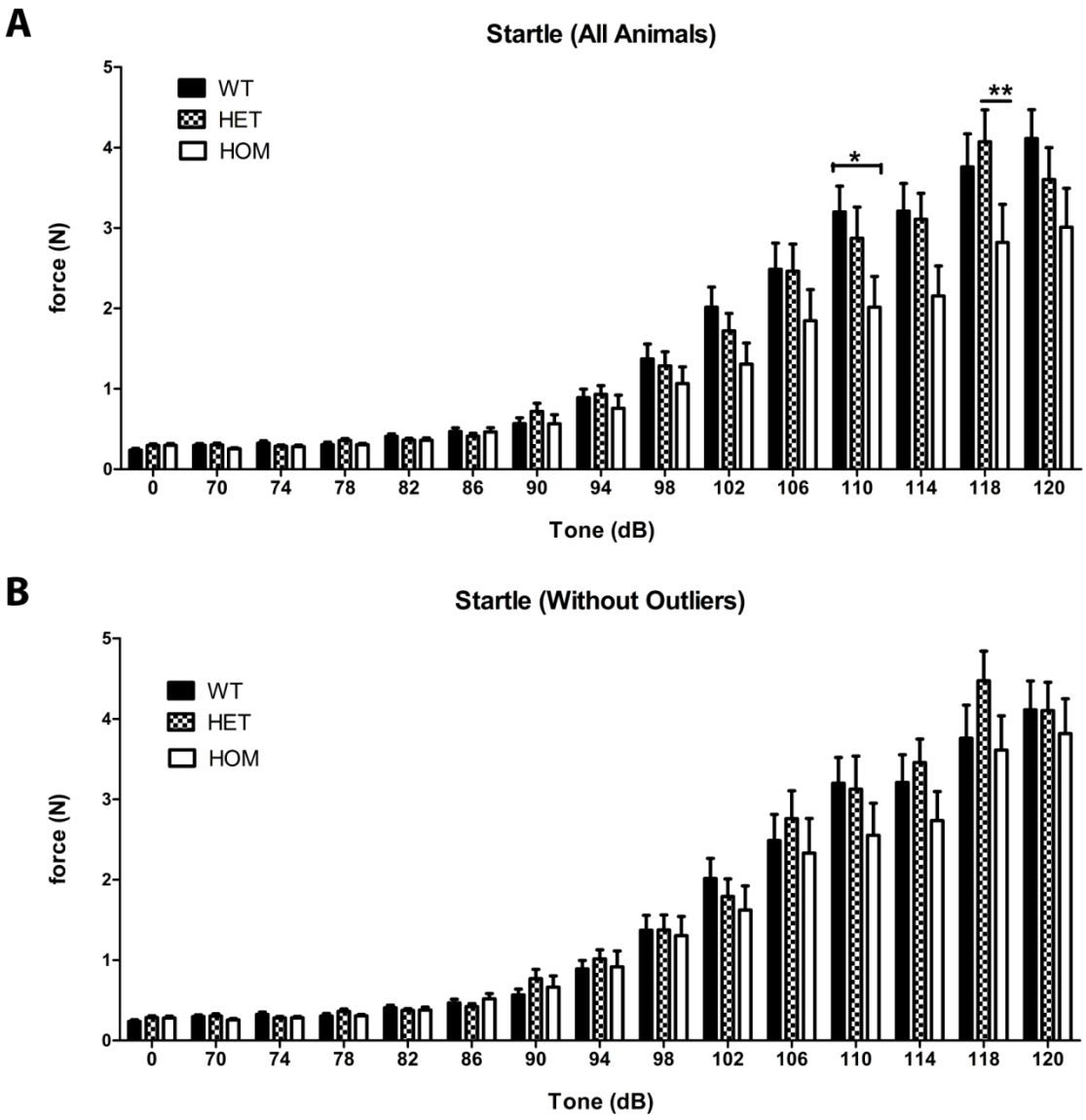


Figure 3.13 Auditory startle across pulse tones. **A**) When all animals are considered, there is a significant effect of genotype and volume on startle with HOM reduced compared to WT and HET. **B**) Upon exclusion of the animals that do not startle, there is still a significant effect of genotype on startle with HOM mice trending toward reduced startle.

prepulse volume of 74dB (Figure 3.14A). When the non-startling animals were excluded, prepulse volume was still significant ($P<0.0001$) and genotype remained significant to a lesser degree across all volumes ($P=0.002$) with no significant post-hoc comparisons at specific prepulse volumes (Figure 3.14B).

3.3.7 Auditory testing

Wei Dong, a postdoctoral candidate in Elizabeth Olson's laboratory at Columbia University, conducted auditory testing to determine if deafness were responsible for the reduced auditory startle. Two pairs of WT and HOM females were tested for distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) thresholds. CAP threshold reflects the minimum sound pressure level to cause a gross nerve action potential of a small group of neurons tuned to a single frequency. DPOAEs have been used for probing the active process of the cochlea. These animals were not tested for startle or PPI; the focus of auditory testing was to determine a baseline change in hearing since the tones used in determining PPI may cause auditory damage.

The amplitude of the outer hair cell response on DPOAE testing was not different across a range of paired primary tone frequencies in HOM compared with WT animals (Figure 3.15A). Not all animals survived the continuous anesthesia thus only one HOM had its compound action potential (CAP) threshold measured. This HOM animal was compared against other C57Bl/6 WT mice tested previously in the lab, with no difference in CAP threshold across sound frequencies noted (Figure 3.15B).

3.3.8 Fear conditioning

Two cohorts of mice completed fear conditioning as the terminal experiment in the behavioral battery. The prior protocols were open field and novel object recognition for both cohorts, but the first cohort also learned the water maze while the second did not. As the results were different from each cohort, it is possible the differences stem from prior behavioral training and handling. The cohorts are therefore presented here separately, with discussion to follow.

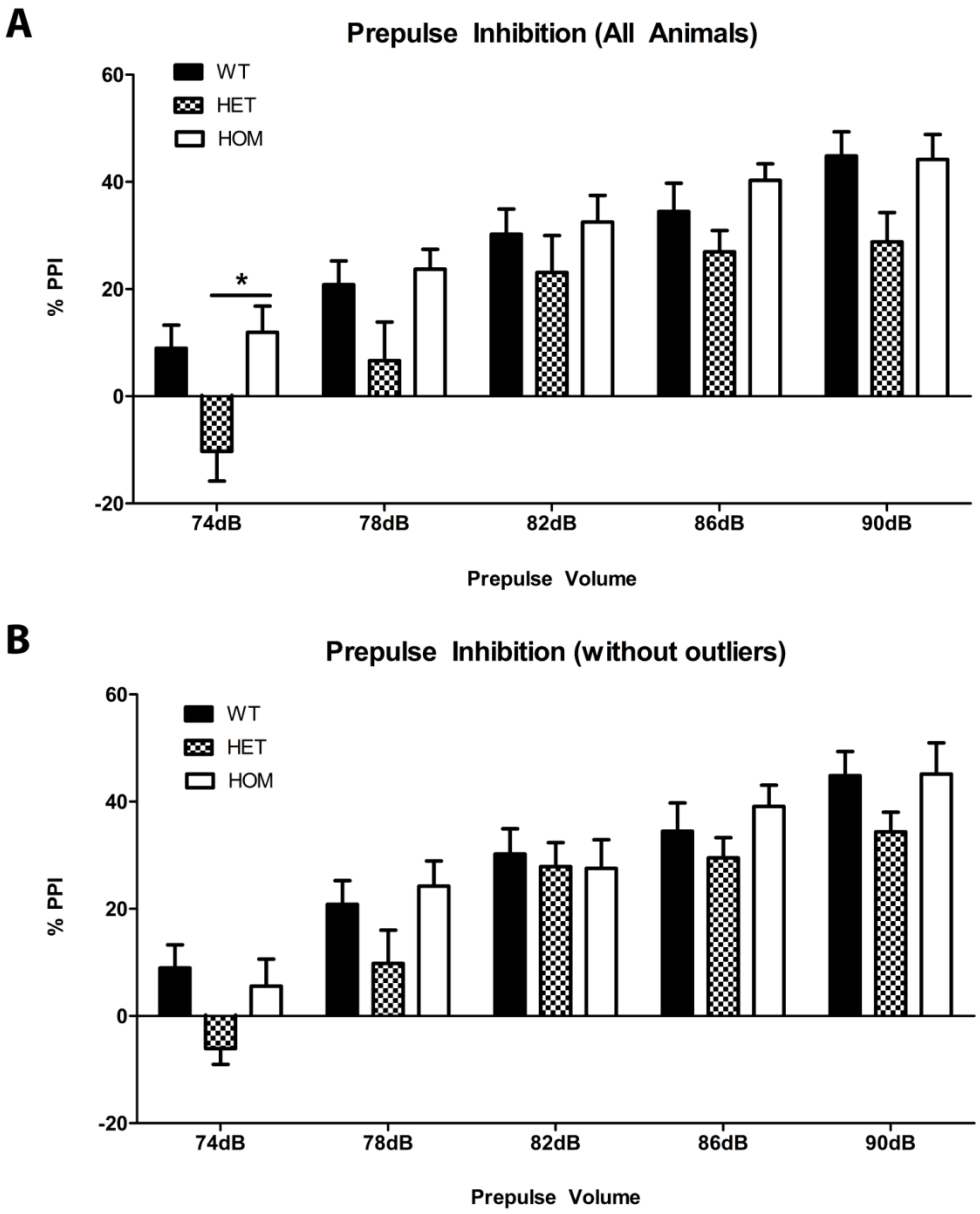


Figure 3.14 Prepulse inhibition. **A**) With all animals included, there is a significant effect of genotype and prepulse volume on startle with HET animals impaired. **B**) When nonstartling outliers are excluded, there is still a significant effect of genotype but only a trend toward HET impairment.

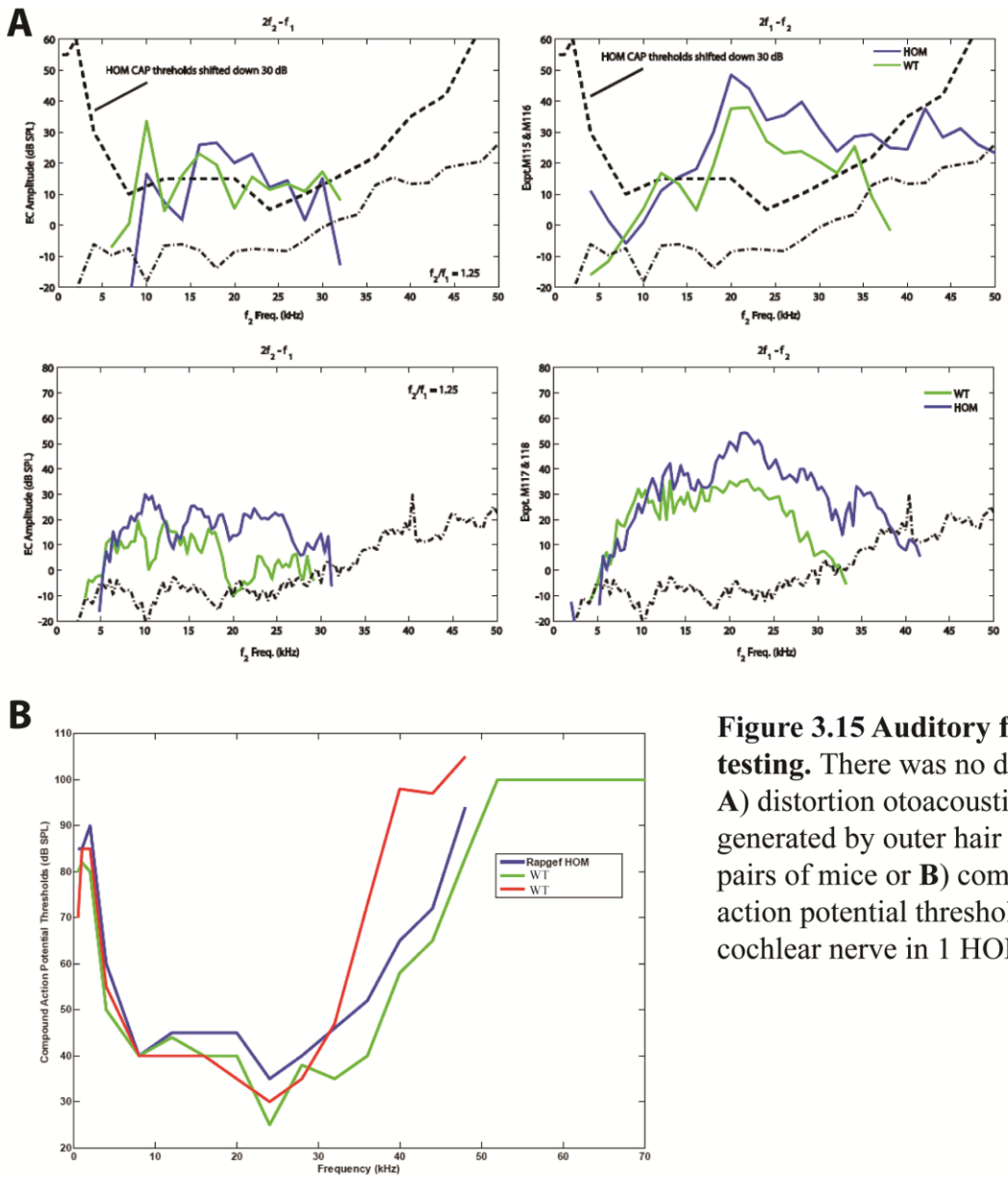


Figure 3.15 Auditory function testing. There was no difference in **A**) distortion otoacoustic emissions generated by outer hair cells in 2 pairs of mice or **B**) compound action potential threshold in the cochlear nerve in 1 HOM.

For the first cohort, during the initial 3 minutes of habituation to the conditioning chamber binned into 1 minute intervals, there was no significant effect of genotype (P=0.26) and a trend toward significance of test time (P=0.06) but there was a significant interaction of genotype and test time (P=0.02) (Figure 3.16A). When freezing response was averaged across the 3 minutes, there was no effect of genotype (P=0.49). Animals did not vary by genotype in

their response to the first unconditioned stimulus tone (P=0.34), the first shock (P=0.32), or the second tone (P=0.10) or shock (P=0.66).

On returning to the same context 24 hours later for 6 minutes, there was a nonsignificant trend for effects of genotype (P=0.06) but not test time (P=0.31) with HOM animals trending to freeze less in the context (Figure 3.16B) When contextual conditioning was analyzed as an average across the test time, genotype remained a trend (P=0.06). Two hours later, in a novel context for the first 3 minutes of cued testing, there was no effect of genotype (P=0.58) but surprisingly animals froze more across test time (P<0.0001); taken as an average across time, freezing response was still not affected by genotype (P=0.58) (Figure 3.16C). Once the 3 minute long tone began, all animals froze less as test time progressed (P<0.0001) but without regard to genotype (P=0.67), nor was this significant on analysis averaged across test time (P=0.67) (Figure 3.16D). During the initial 30s of the cued stimulus, there was no genotype effect (P=0.46).

For the second cohort, there was also no significant effect of genotype (P=0.27) or of test time (P=0.06) but there was a significant interaction of genotype and test time (P=0.005) on freezing during initial habituation (Figure 3.17A). The averaged freezing response showed no effect of genotype (P=0.27). Again, genotype did not influence how the mice responded to the first tone (P=0.56) or shock (P=0.71), or the second tone (P=0.44) or shock (P=0.36).

Upon contextual testing, there was an effect of genotype (P=0.016) but not test time (P=0.09) with HOM animals freezing less than WT in the 2^{nd}, 4^{th} and 5^{th}minutes (P<0.05) (Figure 3.17B). When averaged across the test time, genotype remained significant (P=0.016) and HOM animals froze less than WT on post-hoc testing (P<0.05). In the novel context there was an effect of genotype (P=0.03) and an expected reduction in freezing over test time

(P<0.0001) with HET and HOM animals freezing less than WT in the final minute (P<0.05) (Figure 3.17C).Taken as an average across time, freezing response was still affected by genotype (P=0.03) with no post-hoc comparisons significant. During the continuous tone, the effect of genotype on freezing was also significant (P=0.003); although all animals froze less as test time progressed as expected (P<0.0001), HOM animals froze less than WT at each time point (P<0.05 or <0.001) (Figure 3.17D). This effect remained significant on averaged analysis (P=0.003). Despite the strong effect across the entire 3 minute tone, during the initial 30 seconds of the cued stimulus, there was no genotype effect (P=0.24).

When freezing in the novel context is affected, one recommendation is to subtract the averaged freezing rate in the novel context from the cued response (see Discussion for further explanation). Upon performing this calculation, the previously significant effect of genotype became only a trend (P=0.066) (Figure 3.18A).

As mentioned before, there was a significant difference between the two cohorts independent of genotype for contextual (P=0.04) and cued (P=0.0002) conditioning. No post-hoc comparisons of contextual learning were significant, but WT from cohort 2 tended to freeze more than WT from cohort 1 while the reverse was true for HET mice (Figure 3.18B). On post-hoc testing of cued learning, WT from cohorts 1 and 2 were significantly different from each other in the last 2 minutes (P<0.001) while HOM and HET mice did not differ across cohorts (Figure 3.18C). Thus the WT animals were most different between the two cohorts and drove the majority of the discrepancy between the cohorts.

3.3.9 cFOS analysis of amygdala and hippocampus activation

To investigate the activation of the amygdala and hippocampus during fear conditioning, mice were exposed to conditioning with or without the unconditioned shocks then cFOS

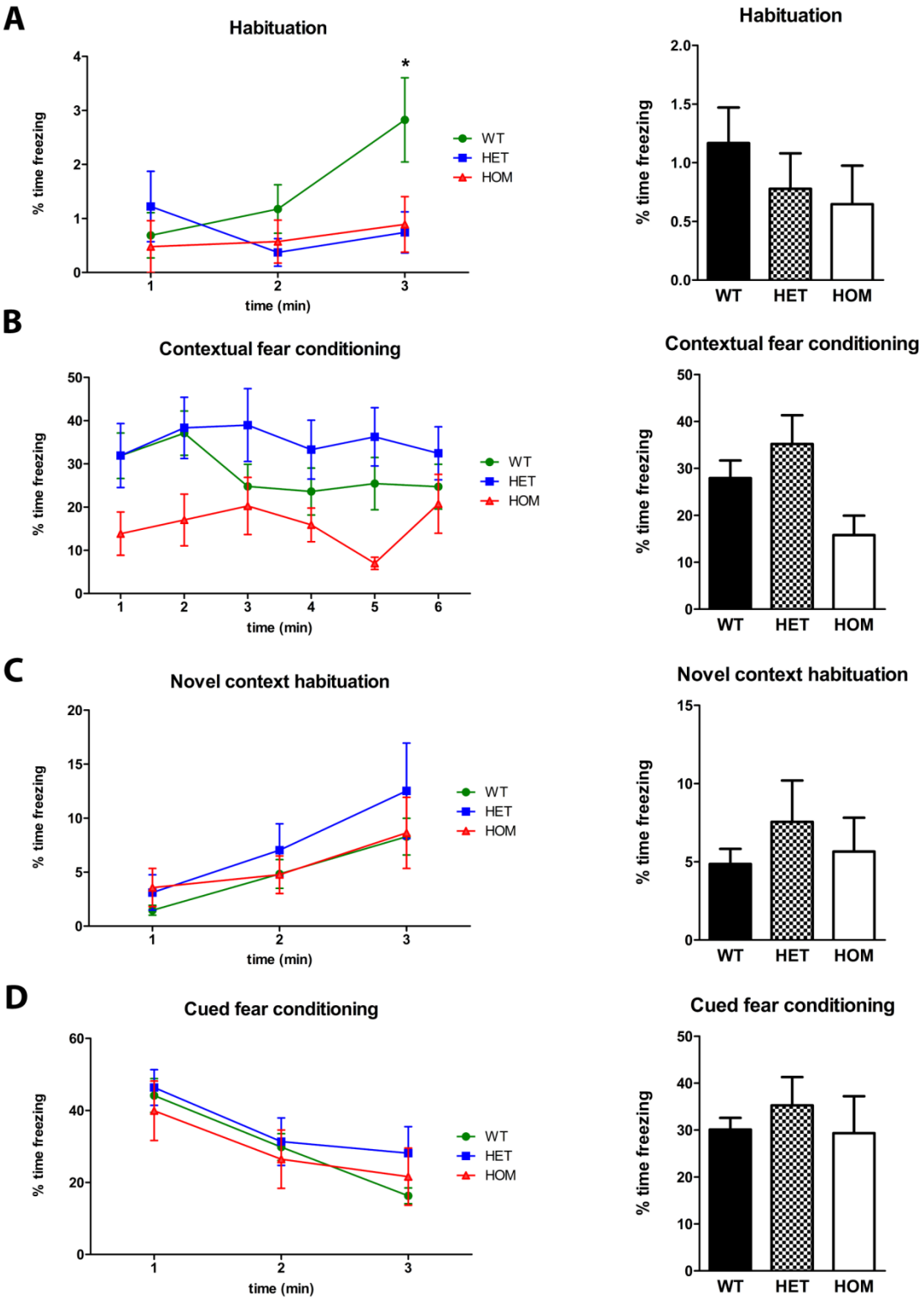


Figure 3.16 Fear conditioning cohort 1. **A**) On habituation WT animals froze more. There was no significant effect of genotype on **B**) contextual fear conditioning, **C**) habituation to the novel context, or **D**) cued fear conditioning. N= 14 WT, 12 HET, 7 HOM

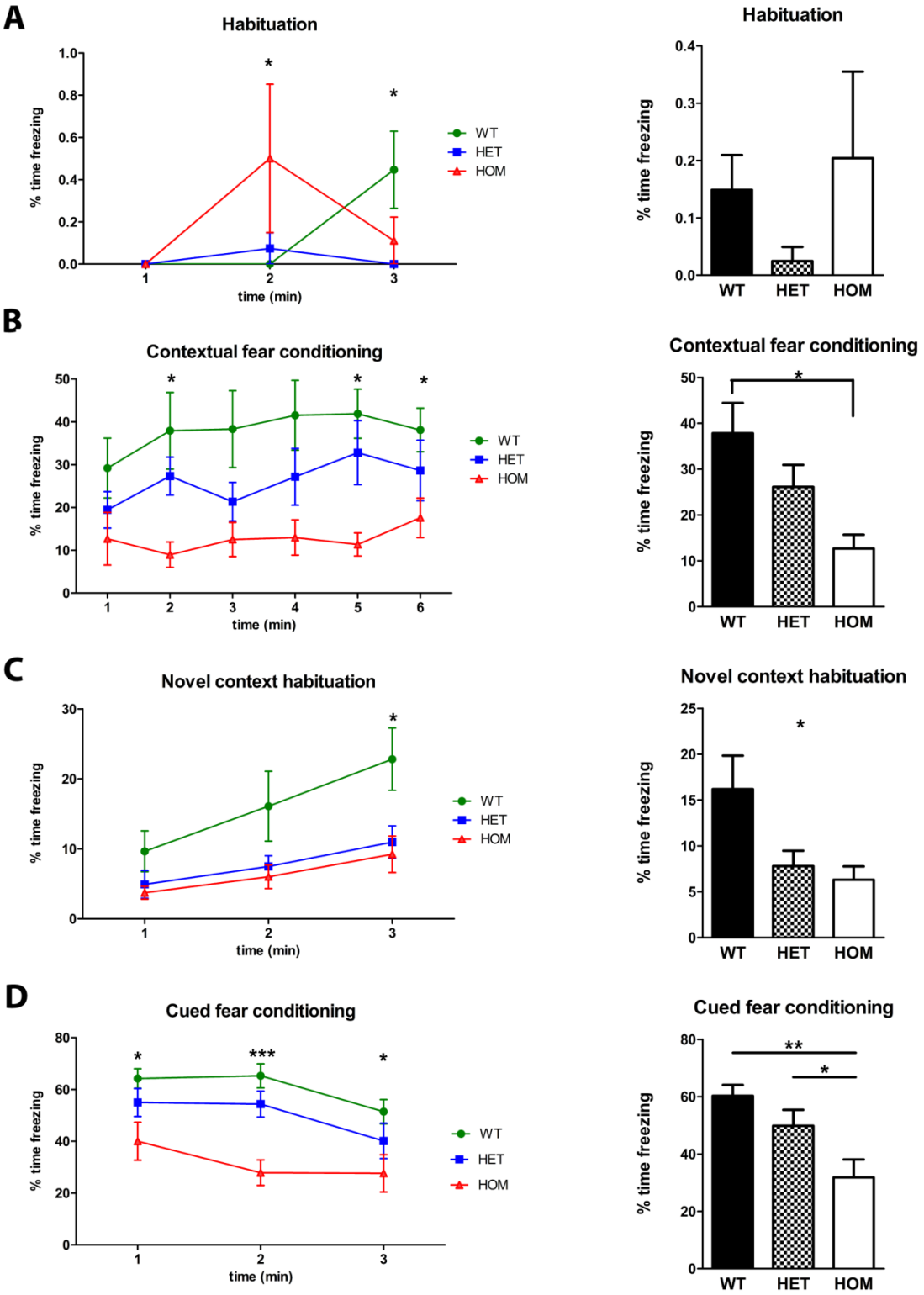


Figure 3.17 Fear conditioning cohort 2. **A**) On habituation WT and HOM froze more. HOM mice had a deficit in **B**) contextual fear, **C**) habituation to the novel context, and **D**) cued fear conditioning while HET animals had reduced cued fear. N= 12 WT, 12 HET, 9 HOM

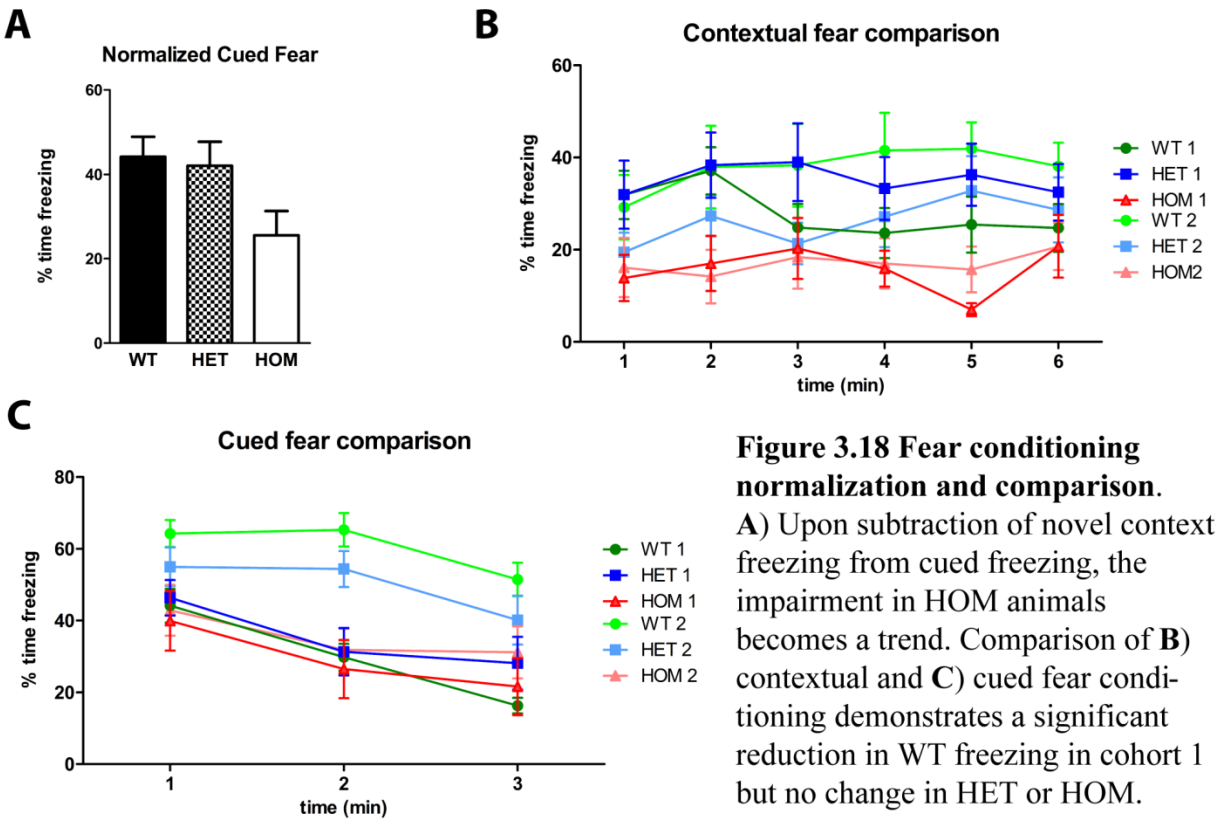


Figure 3.18 Fear conditioning normalization and comparison. **A**) Upon subtraction of novel context freezing from cued freezing, the impairment in HOM animals becomes a trend. Comparison of **B**) contextual and **C**) cued fear conditioning demonstrates a significant reduction in WT freezing in cohort 1 but no change in HET or HOM.

expression was assessed as a measure of neural activity. cFOS is an early component of the synaptic plasticity pathway and its expression is significantly upregulated in the amygdala, hippocampus, and cortex within 90 minutes after fear conditioning (Ressler et al. 2002). Moreover, cFOS staining pattern is also a reliable measure of neural activation and the number of cFOS positive neurons positively correlates with fear learning (Radulovic et al. 1998; Tronson et al. 2009).

As there were so few animals, analysis of behavioral outcomes from fear conditioning were not significant (data not shown), but there was an overall trend toward more freezing in the animals that experienced fear conditioning (FC) as opposed to controls without shocks (CTRL). Of the control animals, HOM mice tended to freeze less on all measures consistent with prior fear conditioning results, but there was no genotype trend on conditioned animals.

In the lateral amygdala (LA), there was a significant effect of condition (P=0.0001), but only a trend for WT and HOM but not HET to be higher after fear conditioned than in controls (Figure 3.19A). This suggests fear conditioning has a positive effect on cFOS activation in the LA, but no conclusions can be drawn about genotype effects of cFOS activation in this brain region during conditioning.

Downstream in the basolateral amygdala (BLA), there was a significant effect of condition (P<0.0001) with significantly increased cFOS protein levels in WT after conditioning (Figure 3.19B). HOM control cFOS levels were higher than WT, but this pattern did not persist after conditioning. Since WT cFOS activity increased with conditioning but HET and HOM did not, this suggests HET and HOM mice did not activate the BLA in response to conditioning.

In the central amygdala (CE), there was no significant effect (P=0.73) of conditioning or genotype (Figure 3.19C). If there was an effect of these factors on cFOS levels, this study was underpowered to detect it.

In the hippocampus, cFOS staining levels in the dentate gyrus (DG), CA3, and CA1 regions were all significantly affected by fear conditioning (P<0.0001, P=0.0004, P=0.02 respectively). Unexpectedly, in the DG cFOS neural activation tended to be lower in conditioned as opposed to control animals though no post-hoc comparisons were significant (Figure 3.19D). Among both conditioned and control mice, HOM had reduced cFOS activity compared to WT, with a downward trend in HET. Thus HET and HOM animals have consistently less DG activity, but this was unassociated with fear conditioning.

WT conditioned mice had significantly higher cFOS levels than WT control mice in the CA3 (Figure 3.19E). This pattern did not extend to conditioned HOM or HET, which were actually significantly reduced compared to conditioned WT (P<0.001). HET and HOM animals

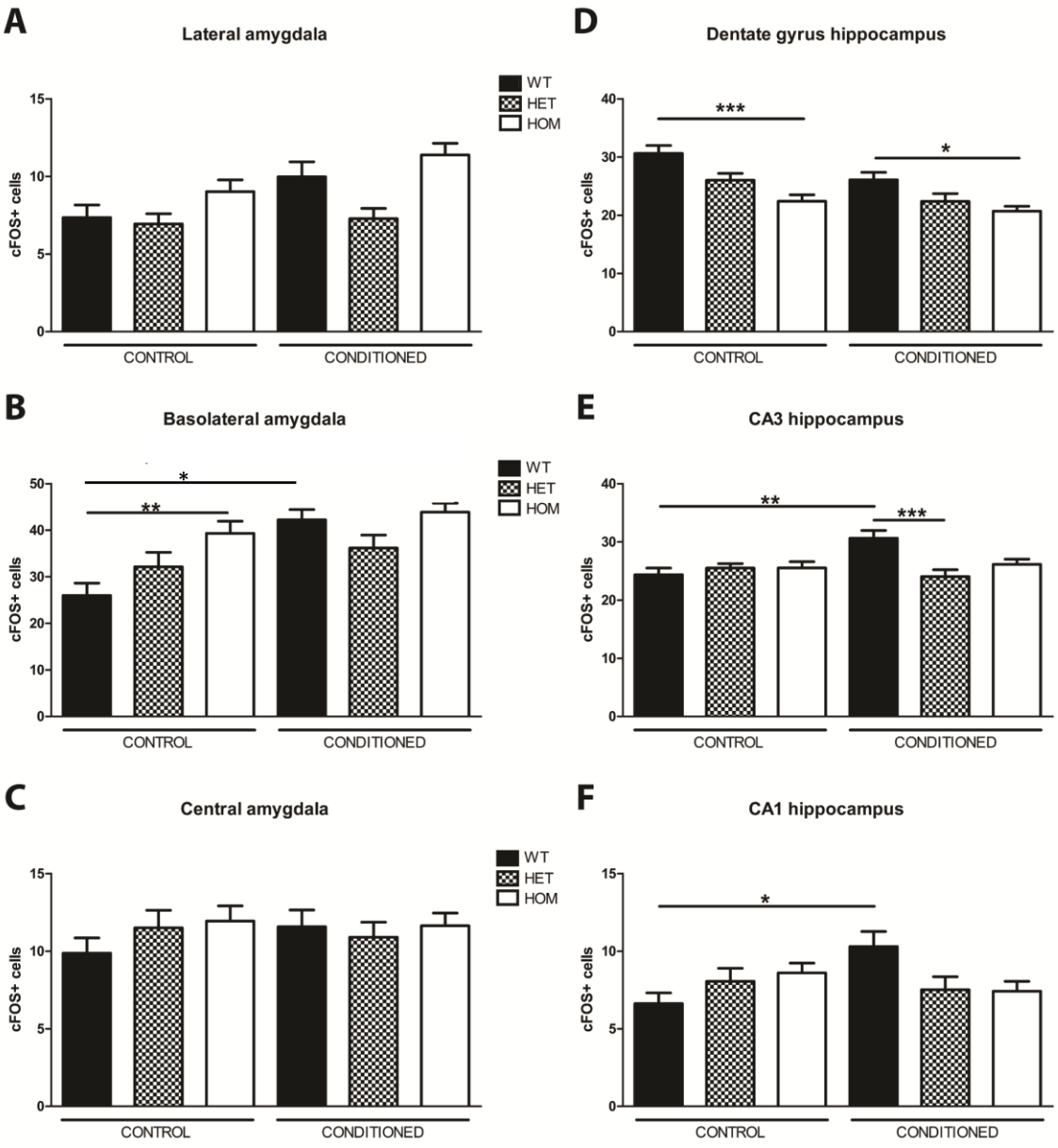


Figure 3.19 cFOS activity after fear conditioning. A) Lateral amygdala cFOS was elevated in HOM conditioned animals. **B**) Basolateral amygdala cFOS was increased by conditioning in WT, and in HOM over WT controls. **C**) Central amygdala cFOS was unchanged. **D**) Dentate gyrus cFOS was lower in HOM than WT regardless of conditioning, and further reduced by conditioning. **E**) CA3 cFOS levels were higher in conditioned WT over all controls. **F**) CA1 cFOS levels were increased in conditioned WT over controls. N=3,3,3 n=8 sections

appear to be impaired on CA3 activation after fear conditioning.

Finally in the CA1 WT animals had significantly more cFOS activity after conditioning, but this did not extend to HET or HOM animals (Figure 3.19F). Similar to the CA3, HET and HOM CA1 activation is also impaired during fear conditioning.

3.4 Summary of findings

Rapgef6 knock out mice demonstrated hyperactivity and anxiolysis on open field testing with increased rearing in HET and HOM animals on both days, increased margin distance in HETs on day 1, but increased total distance in HET and HOM on day 2 as well as increased center distance and center entries in HOMs on day 2. Rapgef6 HET and HOM animals did not show impairment in spatial learning or working memory since novel object recognition, water maze, and T maze findings were not significant. Despite normal audition, HET and HOM animals showed a variable penetrance for lack of startle response to 120dB. The remaining HOM animals with a startle response evidenced impaired startle across quieter tones. Prepulse inhibition was reduced in HET but not HOM animals, both with and without inclusion of the non-startling mice. Finally, the cohort of mice that were handled more had a trend toward impaired contextual fear conditioning, with a subsequent cohort handled less showing impaired freezing to contextual and cued conditions, but also generalizing this reduced fear to a novel context complicates interpretation of the data. cFOS activation studies following fear conditioning indicate a baseline hyperactivity in HOM BLA and hypoactivity in HOM DG, and impaired HET and HOM activation of the BLA, CA3, and CA1 after conditioning.

In summary, we have described evidence for hyperactivity, reduced anxiety, reduced startle and sensorimotor gating, and impaired fear conditioning in the context of normal spatial and working memory and audition. These behavior results imply that amygdala function is

impaired across several tested domains of innate and learned fear, that hippocampal function relative to fear but not spatial learning is also affected, and that mPFC-dependent working memory is intact.

3.5 Discussion

3.5.1 Behavioral domains and endophenotypes

As we are trying to develop a model that can be used to either inform or test new therapeutics, we started from strong human genetic findings and then determined the behavioral phenotype available for potential pharmacologic rescue. Given the high comorbidity of schizophrenia with other neuropsychiatric disorders, we cast a wide net for behaviors that are affected in other schizophrenia models plus unexpected phenotypes arising from pleiotropy. After completing the described behavioral assays, there are several questions that remain to be investigated.

3.5.1.1 Locomotion and exploration

Open field with laser tracking is an accurate way to measure locomotion and quantitate exploratory behavior such as rearing and anxiety behavior such as center avoidance (Bailey and Crawley 2009). Any drug or mutation which impairs motor ability or arousal will diminish locomotion, but open field center time or distance was increased by GABA agonists (barbiturates and benzodiazepines), serotonin receptor partial agonists and selective serotonin reuptake inhibitors, and dopamine receptor agonists (Brookshire and Jones 2009; Prut and Belzung 2003). Mice modeling the 22q11 deletion as a rare but recurrent genetic cause of schizophrenia were also hyperactive on open field similar to Rapgef6 mice, although male mice with the 22q11 deletion had increased anxiety and center avoidance (Stark et al. 2008). Conversely, deletion of *Disc1* or *Dgcr8* did not alter open field behavior (Koike et al. 2006; Stark et al. 2008).

Rearing in the open field is considered a form of nonspecific attention and exploration that is influenced heavily by dopamine and serotonin levels (Aspide et al. 2000). Rearing rates were increased by dopamine and serotonin receptor agonists interacting in the ventral striatum (Berridge 2006; Ikemoto 2002), but decreased in dominant negative E or N cadherin mutant mice (Edsbagge et al. 2004), deletion of dopamine D2 and D3 receptors (Vallone et al. 2002), and in the neurofibromatosis mouse model, where LDopa rescued the phenotype (Brown et al. 2010). Thus both rearing and overall locomotion are likely linked to schizophrenia and other neuropsychiatric diseases via the dopaminergic and serotonergic systems. Since *Rapgef6* KO mice had increased locomotion and rearing, this suggests that dopamine release or dopamine receptor expression are increased in the striatum, possibly analogous to findings in humans with schizophrenia (Abi-Dargham et al. 1998). While Rapgef6 is expressed in the striatum, there is no published evidence that it regulates dopaminergic function, so this hypothesis would have to be tested further.

3.5.1.2 Spatial and working memory

Novel object recognition (NOR) is a useful way to assess spatial hippocampal memory because it does not require positive or negative reinforcement, thus it is still informative in animals with altered reward circuitry or pain sensitivity (Sousa et al. 2006). Since neither of these two pathways was analyzed in this mouse model, NOR is an important addition to the maze results in demonstrating intact spatial memory. NOR is, however, sensitive to attention or activity phenotypes (Amann et al. 2010). Despite open field hyperactivity our KO animals did not differ from WT demonstrating there was no activity-related bias. NOR is known to rely on schizophrenia-related neurotransmitter systems such as glutamate receptors NMDA-R2b and mGlu1R, as well as GABA transporter, CREB, and calcineurin in the hippocampus and

parahippocampal cortex (Dere et al. 2007).

The Morris water maze tests hippocampal spatial memory. Moreover it also includes visible platform testing to assess visual perception and offers a chance to measure speed and locomotion independent from the open field (Sousa et al. 2006). C57Bl/6 mice excel at this task, so perhaps there is a ceiling effect masking a mild deficit, such as HET animals performing below WT at 24 hours, which would not be apparent in a background strain so proficient at the task (D'Hooge and De Deyn 2001). Among schizophrenia models, *Disc1* KO animals demonstrated normal water maze and novel object recognition testing (Kvajo et al. 2008). Despite clinical findings of hippocampal volume change (discussed in Chapter 4), mouse models of schizophrenia do not typically have water maze deficits.

T maze utilizes spatial as well as working memory. It is not considered the most accurate test of working memory, however, since it only involves one choice point, as opposed to an 8 arm maze or a more complicated delay paradigm, thus there may be a possible deficit on more complicated testing that has not yet been excluded (Sharma et al. 2010). Working memory tasks are known to rely on the medial PFC based on chemical lesion studies (Kellendonk et al. 2006), but also involve monosynaptic output from ventral CA1 to medial PFC (Wang and Cai 2006). Among schizophrenia models, Disc1 animals demonstrated specific deficits in working memory as measured on T maze and a delayed non-match to position task (Koike et al. 2006; Kvajo et al. 2008), while 22q11 and Dgcr8 models had an impairment in T maze acquisition (Stark et al. 2008). The striatal D2 receptor overexpression mouse model also had impaired working memory on the T and 8 arm radial mazes (Kellendonk et al. 2006).

3.5.1.3 Startle and prepulse inhibition

Prepulse inhibition involves cortical top-down processing to inhibit the startle reflex, thus

it is a measure of sensorimotor gating. The startle reflex is generated by a circuit from the cochlear root neuron to the nucleus reticularis pontis caudalis, then output via the facial and spinal motor neurons; this pathway is modulated in PPI by the striatum, pallidum, and hippocampus (Geyer and Dulawa 2003). PPI has been probed in most models of neuropsychiatric disorders because the protocol is highly analogous in humans and mice and individuals with schizophrenia and other disorders have PPI deficits yielding face validity (Powell et al. 2009). It is hypothesized that impaired sensory gating may contribute to positive symptoms such as hallucinations (Dulawa and Geyer 2000). Mice mimicking both the 22q11 deletion and heterozygous deletion of *Dgcr8* had impaired PPI with normal startle (Stark et al. 2008). Other genes within the 22q11 region also influence PPI; *Gnb1L* and *Tbx1* deletion both impaired PPI, but PPI was increased in deletion of 7 other genes in the region (Arguello et al. 2010). Disc1 KO mice were not affected (Kvajo et al. 2008) but Neuregulin1 KO mice showed PPI reduction (Chen et al. 2008). Genetic models of additional neuropsychiatric disorders had decreased PPI such as knock outs of Reelin, NCam, proline dehydrogenase, and Fabp7 (Amann et al. 2010).

Since PPI using auditory stimuli is dependent on hearing, any PPI or startle deficits merit auditory analysis. On compound action potential threshold measurements, the HOM mouse demonstrated no difference from other C57Bl/6 WT mice in activity in the cochlear nerve, implying no impairment in hearing threshold. During distortion product otoacoustic emissions testing, HOM animals did not differ from WT. This indicates the entire auditory afferent and efferent pathway, from the basilar membrane to the auditory cortex and back to the outer hair cells, remained intact and functional in HOM animals. Because the lack of startle response was not a robust aspect of the HOM mouse phenotype, the fact that the hearing was normal in the

tested mice does not rule out abnormal hearing in specific mice that lacked startle response. Auditory tests on startle-deficient animals should be done to probe this possibility further.

Beyond audition, the decrease in startle response in HOMs likely affected the calculation of PPI. While startle and PPI were initially considered to be independent (Paylor and Crawley 1997), recent experiments proved that changes in startle create artifacts when calculating PPI since startle response is part of the equation (Dulawa and Geyer 2000). Percent PPI (%PPI), which was used in this analysis, is considered the most accurate measurement since %PPI is somewhat independent of startle and significant %PPI results are still considered meaningful even if startle is also significantly different (Csomor et al. 2008). Experts agree that animals without any baseline startle cannot yield an interpretable %PPI, which is why our analyses were performed with and without non-startling animals. Since %PPI is a ratio, the lower the startle the higher the %PPI, both mathematically and experimentally (Csomor et al. 2008). This explains why HOM animals had normal %PPI despite reduced startle because PPI was biased upward by the startle value. Meanwhile, HET animals had decreased %PPI and no change in startle. There was possibly a reduction in PPI in HOM mice that was normalized up by the necessary calculations.

Several mouse models suggest that the dopamine, glutamate, and norepinephrine pathways might be involved in reduced startle. Clozapine and ketamine both increased startle and reduced PPI, implicating dopamine and glutamate NMDA receptors in startle and PPI modulation (Csomor et al. 2008). Similar to ketamine studies, deletion of the glutamate NR1 NMDA receptor subtype lead to increased startle and reduced PPI (Duncan et al. 2006). Knocking out the norepinephrine α2C receptor increased startle and reduced PPI, while

overexpressing the receptor increased PPI (Sallinen et al. 1998). These findings suggest the possibility that glutamate NMDA or norepinephrine α2C receptor activity may be elevated or dopamine systems may be depressed in the Rapgef6 mouse.

Data supporting amygdala impairments in this animal are discussed below and it is possible that amygdala hypofunction could explain the reduction in PPI. Lesion studies have shown that the basolateral amygdala (BLA) is important for proper PPI to occur, possibly by a pathway from the BLA to the nucleus accumbens or ventral pallidum, but that BLA lesions do not affect startle (Decker et al. 1995; Wan and Swerdlow 1997). Lesion of the medial amygdala leads to anxiolysis, increased startle, and reduced PPI that are hypothesized to be the result of diminished unconditioned fear and anxiety (Vinkers et al. 2010). This phenotype is reminiscent of the hyperactivity, anxiolysis, and PPI changes in our mouse, providing further support for a possible amygdala dysfunction as discussed below.

3.5.1.4 Anxiety and fear conditioning

Within the amygdala, thalamic and cortical sensory input to the lateral nucleus (LA), where the plasticity of fear learning is thought to occur, continues to the basolateral nucleus (BLA), and then outputs through the central nucleus (CE) and on to freezing centers in the brainstem (Johansen et al. 2011). Traditionally, contextual fear conditioning is thought to involve the hippocampus and amygdala, while cued conditioning is dependent on the amygdala alone (Maren 2008). Hippocampal lesion studies, however, demonstrate that the HPC is not essential to contextual conditioning, especially if it is lesioned prior to conditioning (Gerlai 2001). While fear conditioning is not a canonical endophenotype of schizophrenia, it is certainly relevant to other neuropsychiatric disorders. Moreover, there is growing interest in the amygdala in schizophrenia since some, but not all, MRI and fMRI studies have demonstrated reduced

amygdala volume and function in schizophrenia (Exner et al. 2004; Fahim et al. 2005; White et al. 2008). Among schizophrenia mouse models, both contextual and cued fear conditioning were impaired in 22q11 deletion animals but not in *Dgcr8* or *Disc1* animals (Kvajo et al. 2008; Stark et al. 2008).

Our results demonstrated a trend toward impaired contextual conditioning in one cohort, with impaired contextual and cued conditioning as well as reduced fear generalization in a second cohort. Since HPC function is normal on spatial testing and the amygdala is necessary for both contextual and cued learning, the results point to the likely possibility that amygdala dysfunction caused this phenotype. These behavior results are strengthened by cFOS analysis demonstrating impaired HET and HOM activation of the amygdala (BLA) and hippocampus (CA3, CA1) following conditioning. These cFOS activity results are in agreement with findings in the literature of an increase in CA1, BLA, and LA activity after fear conditioning (Radulovic et al. 1998; Reijmers et al. 2007; Tronson et al. 2009). Baseline hypoactivity noted as reduced cFOS staining in HOM dentate gyrus suggests that the dentate would be an appropriate region to study since neurogenesis promotes contextual fear conditioning (Drew et al. 2010; Saxe et al. 2006). Meanwhile, baseline hyperactivity in HOM BLA suggests there may be dysregulation of BLA input, perhaps analogous to the increase in baseline synaptic cortico-amygdala activity despite fear impairment in the Rap1 mouse model (Pan et al. 2008).

The cFOS results should be qualified by noting that changes in baseline cFOS expression, or even expression after conditioning, may be due to effects of Rapgef6 on other aspects of cFOS regulation than neural activity. For instance, changes in growth factors or even in Rap1 can alter cFOS directly and these pathways should be altered following *Rapgef6* deletion (Sakoda et al. 1992). While changes in cFOS expression typically suggest changes in neural activity, in this

case the overlapping nature of the regulatory pathways involved complicate the data interpretation. More direct analysis via electroporation or confirmation through other non-Rap1 regulated genes should be pursued.

While one study suggested there was no impact of the order of prior tests on fear conditioning (McIlwain et al. 2001), the only other study of inter-test interaction found that handling mice in order to perform tests lead to impaired contextual and increased cued fear conditioning, among other effects (Voikar et al. 2004). Mouse models of post-traumatic stress disorder found that stress increased fear learning and impaired extinction (Long and Fanselow 2012). Since the only difference between the two fear conditioning test cohorts was that the first cohort was subject to the stress and handling of the water maze, resulting in first cohort WT freezing levels lower than second cohort WT, these results do not correspond with PTSD models of stress but do align with handling effects on contextual fear conditioning. Notably, differences in handling failed to affect HOM animals on any fear measures or HET animals on contextual fear conditioning. This data suggests HOM and HET animals are resistant to the negative effects of stress and handling, perhaps due to amygdala hypofunction.

The second cohort displayed reduced freezing in the novel contest prior to the cued test in HOM relative to WT. This generalization of HOM contextual fear to the novel context is known to impact subsequent cued freezing measurement. Studies showed subtracting novel context fear from cued fear was the most appropriate mathematical method to correct for such differences, however the best overall solution would be to extinguish any novel context fear through habituation until this phenomenon is abolished, and then measure cued fear (Jacobs et al. 2010). We used this subtraction method, yielding a loss of significance on the second cohort cued conditioning, but as discussed below, future experiments could address this issue better.

Several other phenotypes can affect fear conditioning. If audition or pain threshold were impaired this would affect fear conditioning. Analysis above demonstrating all mice had the same response to the initial tone and after the first shock argues against impaired hearing or pain threshold. Changes in locomotion, anxiety, and exploration can all alter freezing and thus the output measured as fear (Maren 2008). Given *Rapgef6* KO mice have increased activity and decreased anxiety, this baseline phenotype might contribute to the reported reduction in fear conditioning by causing HET and HOM mice to freeze less in all stages of the test.

Acetylcholine receptor agonists injected into the nucleus accumbens led to impairments in startle and contextual fear conditioning and increased locomotion (Cousens et al. 2011). The startle deficit is hypothesized to contribute to the fear impairment in this animal as a result of pharmacologically strengthened input from the hippocampus and amygdala onto the nucleus accumbens. Similarly, abnormal activity in the Rapgef6 mouse HPC and/or amygdala, as evidenced by the behavior and cFOS activity data, may lead to the reduced startle and fear conditioning phenotypes observed here.

3.5.2 Evidence for neuropsychiatric model validity

The behavioral phenotype described in this chapter supports the utility of *Rapgef6* deletion as a model of neuropsychiatric disease, particularly schizophrenia. Our mouse demonstrated endophenotypes associated with schizophrenia including impaired PPI, hyperactivity, and amygdala dysfunction on fear conditioning and cFOS staining analysis. Reduced anxiety and fear learning could also represent imbalance in these affective circuits and thus a way to learn more about anxiety-related pathways in a mouse model of diminished responsiveness as opposed to increased fear.

3.5.3 Correlation with Rapgef and Rap mouse models

There are several ways in which these behavior results align with other Rap and Rapgef family mouse models. *Rapgef3* or *4* individual deletions had no behavioral effect on their own, suggesting there is a great potential for compensation or redundancy within this gene family that could account for the mild phenotype seen here. *Rap1* KO resulted in normal water maze performance, reduced cued fear, and reduced novel context fear similar to our mouse, which suggests *Rapgef6* KO also shares the reduced cortico-amygdala plasticity demonstrated in *Rap1* deletion. *Rap1* KO increased basal cortical activation of the lateral amygdala, which may be analogous to increased BLA cFOS activity in the Rapgef6 mouse. The *MRas* KO animal also had normal water maze performance, however our mouse had multiple behavioral deficits that were unaffected by *MRas* deletion. Although MRas was a major determinant of Rapgef6 localization in lymphocytes, our data suggested MRas and Rapgef6 behavioral phenotypes differ and therefore MRas may not direct Rapgef6 activity in neurons.

Unlike the *Rapgef6* KO animal, constitutively active Rap2 caused hyperactivity, impaired water maze performance, and normal fear conditioning. The *Rapgef3/4* double mutant had impaired water maze performance as well. *MRas* deletion led to normal fear conditioning. Thus deletion of *Rapgef6* overlapped phenotypically with *Rap1* deletion, but not as closely with *Rap2*, *MRas*, or *Rapgef3/4* models. Despite biochemical predictions, behavioral analysis suggests neural Rapgef6 may be independent of MRas and activating Rap1 more than Rap2.

3.6 Future directions

3.6.1 Behavioral paradigms

To completely rule out auditory dysfunction as a possible confounder on PPI studies, PPI could be performed using air puffs instead of tones. A reduction in startle and a reduction and/or

normalization in PPI to a tactile stimulus would reinforce the current findings.

As mentioned above, explicit testing of pain threshold is important to validate the fear conditioning results since if HOM animals did not feel the shock to the same extent as WT then they could not be equally conditioned. This assessment could be achieved with hot plate thresholding.

Rapgef6 KO animals demonstrated hyperactivity and anxiolysis on open field testing. This finding could be further explored in two directions, to examine more anxiety phenotypes and to quantify stimulant-induced hyperactivity. A more thorough assessment of anxiety would include the light/dark box assay and elevated plus maze testing, both of which measure anxiety-like preference for a dark or protected environment over the desire to explore a bright or exposed location. Just as patients with schizophrenia given stimulants demonstrated a significant increase in activity, so too can animals be tested by stimulant administration to explore the positive symptoms of schizophrenia; this phenomenon is thought to involve increased dopamine release from the striatum (Ujike 2002).

In order to better understand the fear conditioning impairments in this model, there are a variety of protocols that can dissect different aspects of the conditioning process to localize the specific deficit. First, as mentioned above, the confounding effect of decreased fear in the novel context on cued fear measurement could be resolved using novel context habituation. Next, habituation to both cue and context, as well as extinction and revival conditioning, could help determine if the decrease in fear conditioning persists across time and over multiple trials. Such experiments would further probe amygdala and hippocampal function, as well as prefrontal cortex regulation of extinction (Knapska and Maren 2009). Altering the strength of conditioning by increasing the shock strength and the number of tone-shock pairings could determine whether

the fear conditioning deficit can be overcome, as was seen in the *Rap1* knock out mouse. Finally, the cFOS activation paradigm used in this experiment was focused on cued conditioning, but this could be altered to analyze contextual conditioning to determine if amygdala and hippocampal activation deficits persist, or to evaluate any possible dysfunction of the PFC in fear conditioning and extinction (Knapska and Maren 2009; Morrow et al. 1999).

3.6.2 Genetic manipulations

It could be informative to cross the *Rapgef6* knock out onto other backgrounds or various mutations. Since individuals with schizophrenia likely carry a variety of both private and common risk variants contributing to the etiology of the disorder, a mouse model of one variant may be insufficient to fully recapitulate the human disorder (Girirajan et al. 2012). Crossing together models of different risk alleles can help determine if there is genetic redundancy of the two genes within the same pathway or epistatic interactions between different genes contributing to a larger phenotype (Beckers et al. 2009). Consider Rapgef3 and Rapgef4, each of which when individually knocked out had no behavioral phenotype but a double mutation did due to redundancy (Yang et al. 2012). Perhaps combining our *Rapgef6* knock out with either of these mice would reveal a reduction in compensation and a worsening of the behavioral phenotype.

Moreover, different genetic backgrounds contribute to epistasis in subtle ways that are frequently overlooked. Even though it is clear that backgrounds vary widely in their baseline behavioral phenotypes, not much effort has gone into determining what genetic factors cause these differences or how they contributes to mutant model phenotypes. Concerning the *Rapgef6* mouse, crossing onto backgrounds with different fear or startle thresholds could help reveal these phenotype differences. For example, C57Bl/6 is known to be a moderate background on anxiety measures thus making it the background of choice for most anxiety investigators (Crawley

2008), but given *Rapgef6* KO animals have reduced fear and anxiety they may be reaching the lower limit of test sensitivity and could be interesting to study on a more anxious background.

3.6.3 Pharmacologic interventions

As mentioned in the introduction, Rapgef3/4 agonist 8CPT can increase PPI without altering startle and strengthen fear conditioning by mimicking an increase in cAMP (Kelly et al. 2009) and 8CPT also compensates in vitro for *Rapgef6* knock down effects on cell connectivity (Dube et al. 2008). While Rapgef6 does not depend on cAMP, it would still be interesting to evaluate whether activating Rapgef3/4 could lead to behavioral compensation and thus rescue of PPI and fear impairments in the *Rapgef6* KO animal. This would be an interesting compliment to the suggestion above that crossing Rapgef knock out animals could diminish compensation and thereby aggravate behavioral phenotypes.

3.6.4 In the next chapter

In the subsequent chapter, we will consider neural morphology in brain regions associated with execution of these behavioral phenotypes in an attempt to correlate behavioral findings with cellular abnormalities or dysconnectivity.

Chapter 4: Morphology Findings

4.0 My role

I performed all experiments described here except for the Western blots, the design of the primers for cloning, and FACS which was performed by the Columbia University Medical Center FACS core.

4.1 Introduction

4.1.1 Alterations of neural morphology in schizophrenia

In a biologically based hypothesis, the symptoms of schizophrenia are due to alterations in circuits resulting from changes in neural morphology and connectivity. In order to identify these changes, there are numerous analyses of schizophrenia neural morphology and connectivity at the postmortem cellular and in vivo volumetric and functional imaging levels. Postmortem studies are complicated by many factors including the duration of illness, medication history, and time until tissue preservation. Although these methods are limited for technical reasons, postmortem analysis may still inform our understanding of schizophrenia pathophysiology. There are findings of prefrontal cortex specific impairment. In one study of the dorsolateral prefrontal cortex (dlPFC) there was a 23% reduction in layer III pyramidal neuron dendritic spine density in brains from individuals with schizophrenia compared to controls, and a 16% reduction compared to individuals with non-psychotic psychiatric disorders (Glantz and Lewis 2000). This finding was apparently specific to the dlPFC as it was not observed in samples of visual cortex. Further analysis of the same brains found that spine density, soma size, and dendritic length in layers V and VI of dlPFC were unaffected by diagnosis, but spine density in layer III was significantly lower than spine density in deeper layers among schizophrenic brains

only (Kolluri et al. 2005). Layer III receives mediodorsal thalamic and intracortical input suggesting there may be a reduction in activity from these regions.

In the amygdala, postmortem analysis found no changes in volume, neural density, or soma size in two studies (Berretta et al. 2007; Chance et al. 2002). Microarray analysis demonstrated alterations in genes involved in presynaptic function, myelination, and signaling, suggesting there may be more subtle dysregulation (Weidenhofer et al. 2006).

There are a variety of findings in postmortem hippocampus studies. Staining for the dendritic marker MAP2 identified an increase in dendritic length throughout the hippocampus and subiculum in schizophrenia cases, though this may be due to an increase in MAP2 expression (Cotter et al. 2000). The mossy fiber synapses from the dentate gyrus to the CA3 had reduced spine density onto smaller CA3 spines, known as thorny excrescences (Kolomeets et al. 2005; Kolomeets et al. 2007). Moreover, staining for immature neurons with Ki-67 found reduced adult neurogenesis in the dentate gyrus of patients with schizophrenia but not depression (Reif et al. 2006). The number of hippocampal inhibitory interneurons was reduced by multiple experimental techniques though pyramidal neuron number was unaffected (Benes et al. 2007; Konradi et al. 2011). Combined with studies of reduced dentate glutamatergic receptor expression, hippocampal morphology alterations and clinical findings of impaired hippocampal function contribute to a hippocampal hypothesis of schizophrenia in which reduced dentate glutamatergic release may dysregulate hippocampal circuitry, affecting pattern and associative memory and thus yielding psychosis and cognitive symptoms (Tamminga et al. 2010).

Changes in morphology may be connected to alterations in neural migration and extracellular matrix in schizophrenia. A number of studies of postmortem neuronal density, as reviewed recently, found evidence for increased neurons within white matter in various cortical

regions (Connor et al. 2011). This abnormality may be the result of reduced migration, altered neurogenesis or apoptosis balance, or cortical inflammation. Evidence in favor of altered migration came from analysis of the extracellular matrix, in which pro-migration factors such as Reelin, chondroitin containing molecules, and the Neuregulin1-ErbB pathway were affected in schizophrenia (Guidotti et al. 2000).

In vivo analyses of brain volume and function have yielded a variety of results depending on the experimental technique, sample size, and patient sample type (first episode psychosis, chronic, unmedicated, or longitudinal schizophrenia). One recent meta-review of meta-analyses concluded that there is sufficient evidence to confirm a reduction in whole brain volume, increase in lateral ventricular size, and volume reductions in subregions of the frontal and temporal lobes, most significantly within the superior temporal gyrus, anterior cingulate cortex, parahippocampus, hippocampus, and amygdala, as well as reduced midline thalamic volume (Shepherd et al. 2012). Additionally, there was also a reduction in corpus callosum cross-section area and in frontal and temporal connectivity.

Postmortem changes do not inform how the disease begins neurobiologically. Clinical and in vivo imaging results suggest that many of these anatomic changes are already present at the time of first psychosis and that symptoms can present as early as adolescence or childhood, a time when loss of synapse density, termed synaptic pruning, normally occurs (Keshavan et al. 1994). In the 1980s, long before much supporting data was available, neuroscientists first proposed that schizophrenia may be a neurodevelopmental disorder due to increased synaptic pruning in adolescence (Keshavan et al. 1994). More recent studies of gene expression, imaging, and epidemiology all support the idea that synaptic dysfunction is a common endpoint for many schizophrenia risk factors (Faludi and Mirnics 2011; Hayashi-Takagi et al. 2011). Analysis of the

effects of schizophrenia risk alleles on neural morphology and spine density, critical factors that determine synaptic function, is therefore important in order to assess whether there is evidence for neurodevelopmental dysfunction that contributes to adult symptoms and pathophysiology.

4.1.2 Importance of neural development and morphology in circuits

Neurons transmit information as a direct consequence of their connectivity. Thus neural migration and form determine neural function as cells can only influence what they can reach. Cortical projection neurons migrate linearly from the ventricular zone through deeper structures toward the pia, while inhibitory interneurons migrate radially from the ganglionic eminences. Though cell-autonomous signaling contributes to migration, extrinsic signal pathways such as Reelin, Notch, and Semaphorins for projection neurons and Neuropilins, Semaphorins, and Robos for interneurons are essential to migration targeting (Huang 2009; Nakajima 2007).

Neural morphology is important at two levels; the outgrowth of the axonal and dendritic arbors and the density and shape of spines. While traditionally neuroscience has been focused on axon guidance as a means to understand connectivity, there is growing interest in dendritic guidance and spine dynamics as two novel determinants of cellular function. Moreover, we now understand that dendritic morphology, beyond putting neurons in contact with each other, also shapes neurotransmission by affecting integration of synaptic input en route to the soma (Whitford et al. 2002). Analyzing neural morphology in vivo and in vitro is therefore an important aspect in understanding neural function.

Most neurons, such as cortical pyramidal neurons, are polarized, with an apical dendritic arbor and a basal axon. Even though the dendrites and axons tend to grow in opposite directions, they respond to the same trophic molecular cues using similar receptors. Experiments show that dendrites have opposite growth responses to axons to guidance cues such as Semaphorins,

Netrin, and Slits via Neuropilin, frazzled/DCC, and Robo receptors, respectively (Kim and Chiba 2004). Other important extrinsic signals include IGF, BDNF, NT3, and NT4; these factors have varying effects within different cortical layers, apical vs. basal dendritic arbors, and across brain regions (McAllister et al. 1995; Niblock et al. 2000). These divergent growth responses seem to result from differential cytosolic signaling pathway activity in distinct cellular subdomains, whether at the level of timed expression (axons develop before dendrites), sequestration of different signal pathways through cytoskeletal and motor protein polarization, differences in local protein synthesis, or diffusion barriers such as the initial axon hillock. Extrinsic signals that are shared between axons and dendrites likely evolved to increase cell connectivity and synaptic convergence by guiding pre- and postsynaptic processes toward each other (Kim and Chiba 2004).

Careful in vivo analysis demonstrated that all cortical neurons start with the same basic morphology before diverging into layer- and region-specific morphology patterns; therefore neurons have intrinsic growth pathways that are genetically predetermined which are later modified by extrinsic signals such as those mentioned above to determine their mature form (Whitford et al. 2002). Intrinsic signals are hypothesized to derive from elevated levels of Notch, Numb, and β-catenin (Whitford et al. 2002). Using in vitro culture systems permits analysis of these intrinsic patterning signals that guide polarization since extrinsic signals are not available unless experimentally added. Hippocampal neural culture in particular is a widely used robust technique to study neural morphology development because cultured neurons undergo four reproducible stages of neurite growth and axon specification (Bradke and Dotti 2000).

The last major force shaping neural morphology is electrochemical synaptic activity. Synaptic transmission is important for strengthening individual synapses and spines, but also for

the maintenance of dendritic arbors. Calcium influx during neural activity is hypothesized to activate various targets including Creb, CaMKIV, and eventually lead to activation of Rho GTPases that modify actin structure to influence dendritic branches (Wong and Ghosh 2002).

We will describe morphology analysis both in vivo and in vitro in an effort to understand effects of Rapgef6 on intrinsic, extrinsic, and activity driven neural morphological development.

4.1.3 Morphological role of Raps and Rapgefs

Nothing is currently known about the role of Rapgef6 in neural morphology, however its effect on cell adhesion pathways may alter neural development. In lymphocytes, Rapgef6 was brought to the membrane by MRas to mediate TNFα signal transduction, leading to Rap1 activation which then stimulated RapL, Riam, and integrins such as LFA1 to promote cell-cell adhesion (Yoshikawa et al. 2007). Rapgef6 also increased cadherin and catenin-dependent adherens junction maturation as well as E-cadherin expression in epithelial cells (Dube et al. 2008). In vitro epithelial migration via Jam-a activating Afadin could not promote β1-integrin adhesion unless Rapgef6 was present to activate Rap1a (Severson et al. 2009). Rapgef6 bound Bag3, an actin-associated protein at the leading edge of migrating cells, and was sufficient for the effect of Bag3 on promoting cell motility and adhesion via integrin recycling (Iwasaki et al. 2010). Integrins and cadherins are essential to radial and tangential neural migration in the brain, as well as neurite outgrowth via adhesion to extracellular matrix (Clegg 2000; Suzuki and Takeichi 2008). β1-integrin was especially key in radial glia development and thus radial neural migration (Graus-Porta et al. 2001). Given its role in promoting cell-cell and cell-extracellular adhesion, we expect that Rapgef6 could play a significant role in neural adhesion and migration as well as morphology.

Multiple Rapgef and Rap family members were shown to affect neural morphology and

related developmental processes such as adhesion and migration. *Rapgef1* deletion was embryonic lethal due to failure of vasculogenesis, so initial studies focused on embryonic fibroblasts, which displayed increased motility and reduced cell spreading in culture, suggesting reduced cell adhesion (Ohba et al. 2001). Further experiments proved Rapgef1 was essential in fibroblast focal adhesion formation involving paxillin and β1-integrin (Voss et al. 2003). A Rapgef1 hypomorph mouse model displayed increased cells in the neuroepithelium due to failure of progenitors to stop symmetric mitosis rather than asymmetric division to yield neurons (Voss et al. 2006). Neurons that did develop failed to migrate appropriately in vivo because radial glia were abnormally oriented and also did not migrate in vitro in slice culture or cell culture (Voss et al. 2008). It appears that Reelin activated Rapgef1 to activate Rap1 and that this pathway is critical in the development of proper neural cytoarchitecture (Voss et al. 2008).

Rapgef2 promoted neurite outgrowth in PC12 cells in response to NGF stimulating TrkA receptors by activating Rap1 and the MEK-ERK-MAPK pathway (Hisata et al. 2007). Knock down of both *Rapgef1* and *Rapgef2* caused a reduction in NGF induced neurite outgrowth in these experiments. More evidence for the role of Rapgef2 in promoting neural growth came from the cortical delayed knock out mouse which had widespread neuronal migration abnormalities including heterotopias (mislocalized, functional neurons) and failure of white matter tracts (Bilasy et al. 2009). Axons that were normally destined to decussate across the corpus callosum were instead bunched near the midline (Bilasy et al. 2011).

Rapgef3/4 activation increased β1-integrin mediated cell adhesion via Rap1 and the MAPK pathway (Bos 2006; Roscioni et al. 2008). In vitro, Rapgef3/4 activation via specific agonist 8CPT caused reduced spine area, increased spine motility, reduced synaptic AMPA receptor density, and diminished neurotransmission (Woolfrey et al. 2009). In comparison, while

individual deletion of *Rapgef3* or *4* had no effect on neural morphology, behavior, or electrophysiology, a double *Rapgef3/4* knock out mouse had no change in spine density but a reduction in CA1 EPSC frequency and LTP as well as behavioral changes discussed previously (Yang et al. 2012).

Rap1, the downstream target of Rapgefs, is well documented to promote cadherin mediated cell-cell junctions and integrin mediated cell-extracellular matrix adhesion by activating RapL, Riam, and Rac (Bos 2005; Retta et al. 2006). In vitro, inhibiting Rap1 via a dominant negative transcript diminished increases in dendritic complexity after neural depolarization, suggesting Rap1 was essential in activity-induced neurite outgrowth (Chen et al. 2005). Rap1 activity permitted long term depression by facilitating removal of synaptic AMPA receptors (Xie et al. 2005; Zhu et al. 2002). Via Cdc42, active Rap1b directed axon polarity in vitro (Schwamborn and Puschel 2004), and in slice culture Rap1 was required for maintaining neural polarity and orientation during migration as a downstream effector of Reelin (Franco et al. 2011; Jossin and Cooper 2011).

In contrast to the growth promoting effects of Rap1, in vitro constitutively active Rap2 caused a retraction of the axonal and dendritic arbors and a reduction in spine density (Fu et al. 2007). Rap2 activation also depotentiated synapses by facilitating the removal of AMPA receptors from the synapse in vitro (Zhu et al. 2005). This finding extended in vivo to a constitutively active overexpression Rap2 mouse that had reduced spine density in CA1 pyramidal neurons and increased hippocampal long term depression (Ryu et al. 2008).

MRas is thought to be upstream of Rapgef6 (Gao et al. 2001). MRas activation by growth factors in fibroblasts increased filopodia and actin organization (Matsumoto et al. 1997). Despite in vitro findings of MRas promoting neurite outgrowth (Sun et al. 2006), MRas deletion had no

effect on neural morphology in the hippocampus or cortex in vivo or in culture (Nunez Rodriguez et al. 2006).

The role of Rapgefs in integrin-mediated cell adhesion is conserved across species. *Drosophila* Rapgef homolog Dizzy promoted cell protrusion and migration in a process presumed to involve integrin activation (Huelsmann et al. 2006). Rapgef1 homolog C3G was critical for muscle cell adhesion via integrins (Shirinian et al. 2010).

In summary, Rapgef1 and 2 family members were involved in promoting cell adhesion and migration as well as neurite outgrowth, while Rapgef3/4 had no effect on morphology in vivo. Rapgef target Rap1 promoted axon polarity and neurite outgrowth, while Rap2 inhibited axon and dendrite outgrowth in vitro and spine dynamics in vivo. MRas increased neurite outgrowth in vitro, but had no effect in vivo. All of these effects appeared to be mediated via integrins, cadherins, and consequent actin restructuring. Since Rapgef6 activates Rap2 more than Rap1 (Kuiperij et al. 2003), it may have a growth restricting effect, thus the knock out phenotype may include increased neurite growth. This hypothesis is complicated, however, since Rapgef6 activity appeared to promote lymphocyte adhesion, implying that while Rap2 is biochemically in vitro the most important effector, biologically the overall impact may be dominated by Rap1 effects.

4.2 Methods

4.2.1 Western blot

Following lethal anesthesia with carbon dioxide, mouse brain regions were excised according to the standard mouse brain atlas (Paxinos and Franklin 2004). Crude synaptosomal preparations were made by homogenizing in buffer containing 5 mM Hepes/10% sucrose (pH 7.5). Homogenates were spun down at 1000 × g, and the supernatant was further centrifuged at

12,000 × g. The pellet was resuspended in the same buffer, then the protein concentration was determined using a DC protein assay kit (BioRad). The protein homogenate was mixed with NuPage LDS loading dye (Invitrogen) with reducing reagent and boiled for 10 minutes, then loaded onto a 4-12% gradient polyacrylamide gel and run and transferred onto a nitrocellulose membrane according to the manufacturer's instructions (Invitrogen).

Rapgef6 protein is predicted to be 177.9 kDa. Antiserum was generated in rabbits against the C-terminal synthetic peptide CLEPRDTTDPVYKTVTSSTD, then affinity purified against this peptide to isolate antibodies (Yoshikawa et al. 2007). Primary antibody rabbit anti-Rapgef6 was used at 1:100.

4.2.2 Cresyl violet staining

5 week old Rapgef6 littermates were lethally anesthetized with carbon dioxide and perfused with PBS and 4% paraformaldehyde (PFA), then the brains were post-fixed, cut into 40um sections on a vibratome, and mounted. After washing in 0.5% TritonX and water, the slides were immersed in cresyl violet with neutral red for 4 minutes, then dehydrated in ethanol and xylene and mounted.

4.2.3 In vivo knock out morphology

Rapgef6 animals were crossed with the Thy1-M-GFP mouse line, which expresses GFP sporadically in a mosaic fashion in pyramidal neurons. Male littermates of Rapgef6$^{+/-}$ x Rapgef6$^{+/-}$ GFP^{+} matings were lethally anesthetized with carbon dioxide at 10-12 weeks, perfused with PBS followed by 4% PFA, and the brains extracted. The brains were sliced 100µm thick on a vibratome, washed 3x5min in PBS, and stained with TOPRO 1:2500 in PBS for 10 minutes. Finally the slices were mounted in ProLong Gold.

Regions of interest were defined using a mouse brain atlas to set anatomic boundaries

(Paxinos and Franklin 2004). The dorsal hippocampus CA3 was defined by the flexure of the mossy fiber pathway to its end. Dorsal CA1 began at the end of the mossy fiber pathway and terminated at the midline. Both of these were imaged from approximately Bregma coordinates -1.3 to -1.9. Medial PFC was imaged from Bregma +2 to +1.5, with pre- and infralimbic subregions defined as the upper and lower halves of the tissue medial to the forceps minor of the corpus callosum. The lateral/basolateral nuclei of the amygdala were defined as the region within the forking of the external capsule from Bregma -1.1 to -1.9.

For dendritic arbors, only neurons in the middle of the tissue with intact arbors were imaged. In the mPFC and HPC, the basal dendritic arbors were identified by their distinctive morphology and placement relative to structural landmarks, for instance HPC basal arbors lie outside the mossy fiber and Schaeffer collateral pathways.

4.2.4 Morphology imaging and analysis

For in vivo and in vitro analysis, slides were imaged on a confocal microscope (Zeiss) at 20x optical zoom for neurites and 63x optical zoom for spines to capture images of fluorescent neurons. For spines, images were taken after the first branchpoint along primary dendrites. A maximum intensity projection image was generated from the 3D image stack. This image was saved as a TIFF for analysis. In assessing neurites, images were loaded into the NeuronJ plug-in for ImageJ, where the neural processes were manually traced and labeled. NeuronJ automatically calculated the length and number of each neurite subtype, while branchpoints were manually counted after neurite tracing. For spine assessment, spines were manually counted and measured using LSM software (Zeiss) if there was a visible neck connecting to the dendrite. Spine morphology was assessed according to head shape and neck measurements as previously published (Chakravarthy et al. 2006).

For Sholl analysis, neurite tracings were saved and a macro was written in ImageJ to generate concentric circles 50μm apart, which was initiated over the center of the soma. Crossings of neurite tracings over circles were then manually counted.

Data was analyzed by t test, one-way or two-way ANOVA with Bonferroni post-hoc testing in Prism (GraphPad). The genotype or knock down status was not known until after imaging and analysis was complete.

4.2.5 In vitro hippocampal neural culture

Timed matings of either C57Bl/6 or Rapgef6$^{+/-}$ mice were arranged and plugs were noted as day embryonic (E) 0.5. Pregnant females were lethally anesthetized with carbon dioxide at E17.5 and the embryos were extracted. Hippocampi were excised bilaterally under a dissecting microscope in HBSS/Hepes solution and placed on ice. The remainder of the brain was used for genotyping if necessary. The hippocampal tissue was digested in 1.2mL 0.025% Trypsin/EDTA (Gibco) for 10 minutes at 37^{o}C, then the digestion was halted by adding 12μL FBS (Gibco) and incubating for 5 min at 37^{o}C. The tissue was then triturated 20 times with a flame-polished glass pipette. After determination of cell density via hematocytometer counting, neurons were plated at a density of 4x10^{8} cells/mL onto polyornithine coated coverslips in plating medium (DMEM, 10% FBS, penicillin, streptomycin), which was exchanged for NB (NeuroBasal Medium (Gibco), B27, glutamine, penicillin, streptomycin) after 4 hours of incubation. Neuron cultures were maintained in a 37^{o}C incubator with 5% CO_2. The day of culture plating was counted as day in vitro (DIV) 0.

4.2.6 In vitro knock out of Rapgef6

Neurons from *Rapgef6*$^{+/-}$ timed mating cultures were transfected on DIV2 via incubation with Lipofectamine 2000 (Invitrogen) for 3 hours in NB without antibiotics with 1.2μg of

plasmid per coverslip of βactin-GFP. On DIV5 the coverslips were then fixed for 20 minutes in 4% PFA. For spine analysis, cultures were transfected at DIV7 and fixed at DIV21. As the GFP fluorescence was sufficient for imaging, the coverslips were then washed 3 x 5 minutes in 1x PBS, incubated with TOPRO 1:2500 in PBS for 10 minutes, then washed again in PBS 3 times and mounted with ProLong Gold. Retained embryonic brain tissue from the cultures was genotyped for *Rapgef6*.

4.2.7 Assessment of shRNA efficacy

shRNAs directed against mouse *Rapgef6* with turbo-GFP coexpression were purchased (OpenBiosystems). These shRNAs were transfected into N18 cells via Lipofectamine2000 (Invitrogen), 8μg DNA per 6cm plate. As transfection efficiency was quite low, Rapgef6 levels could not be determined via Western blot, staining and quantification, or qPCR on an entire culture. Instead, transfected cells were isolated through FACS sorting for GFP expression, then subjected to qPCR. N18 cells were transfected for 48 hours, then trypsinized, washed, and resuspended in 10% FBS in PBS. Fluorescent cells were sorted at the Columbia University FACS core on a FACSCalibur machine, then RNA was extracted via Qiazol (Qiagen). Following reverse transcription PCR (Ambion), cDNA was combined with the customized primer set and probes below directed toward exon 1 of *Rapgef6* using the TaqMan reagents and GAPDH control (ABI).

Table 4.1 *Rapgef6* qPCR primers

qPCR Primer and Probe sets	Sequence
Forward primer Rapgef6 exon 1	GGCAGGCACTGAGGAAGAAG
Reverse primer Rapgef6 exon 3	AAGCATTTGATTGCCACTGTATC
Probe Rapgef6 exons 1-2	FAM-CGCCCGAGCGGACTCCTGAG-BHQ1

4.2.8 In vitro knock down of Rapgef6

For Rapgef6 shRNA knock down neurite experiments, neurons from C57Bl/6 timed matings were transfected at DIV2 with either 1.2µg/well of scrambled or shRNA87843. Neurons were fixed in 4% PFA at DIV4 or DIV5. For spine experiments, the cultures were transfected at DIV7 and fixed at DIV21. To enhance the fluorescent signal from the turbo-GFP on the shRNA construct, all DIV2-transfected coverslips were stained with rabbit anti-tGFP 1:7500 (Evrogen), as well as mouse anti-Tau1 1:3000 (Chemicon), while DIV7-21 neurons were stained with rabbit anti tGFP 1:7500 and mouse anti-PSD95 1:100 (Millipore).

4.2.9 Rapgef6 expression vectors

Rapgef6 human and mouse cDNA was purchased (Open Biosystems). PCR primers were designed to incorporate unique restriction enzyme cut sites as well as the hemaglutinin (HA) exogenous tag at the 3' end. Additional primers were designed to amplify PCR product that for ligation in order to delete the GEF domain (*RAPGEF6*ΔGEF), analogous to the mouse deletion.

PCR products were amplified using these primers, then isolated on an agarose gel, purified, ligated with T4 (NE Biolabs) into pcDNA vector, and transformed into competent XL Gold cells (Stratagene). Cells were grown with antibiotic selection and surviving colonies were isolated for growth followed by DNA extraction via mini prep (Qiagen). This DNA was sent for sequencing from the vector T7 region to verify the resulting plasmid orientation. Only vectors containing human *RAPGEF6* or *RAPGEF6*ΔGEF were successfully cloned.

The Kataoka lab gifted plasmids of human *RAPGEF6* with 5' FLAG and eGFP tags under the CMV promoter (pFLAG-CMV2-RAPGEF and pFLAG-CMV2-EGFP-RAPGEF), as well as *hRAPGEF6* with the MRas binding RA domain (amino acids 749-779) deleted (pFLAG-CMV2-RAPGEFΔRA and pFLAG-CMV2-EGFP-RAPGEFΔRA) (Gao et al. 2001).

Table 4.2 *Rapgef6* cloning primers

Species	End	Primer Name	Primer Sequence
Human	5’	5’ XhoI hRAPGEF6	TATCTCGAGACCATGAACTCACCCGTGGACCCT
Human	3’	3’ hRAPGEF6 HA HindIII	GCTAAGCTTAAGCGTAATCTGGAACATCGTATGGGT AGCTGGTGACTGCTGAAACTTGTTCATTTTC
Human	3’	3’ hRAPGEF6 HindIII	GCTAAGCTTAGACTGCTGAAACTTGTTCATTTTC
Mouse	5’	5’ XhoI mRAPGEF6	TATCTCGAGACCATGAACTCGCCCGTGGACCC
Mouse	3’	3’ mRAPGEF6 XhoI	GCTCTCGAGTTAGACTGCTGACACCTGCTCGTT
Mouse	3’	3’ mouse exon 21 deletion	TTTGTGCTGCCTTGACTCAATGATTCATGGAGAAATG TCATATCTTTC
Mouse	5’	5’ mouse exon 21 deletion	GAAAGATATGACATTTCTCCATGAATCATTGAGTCA AGGCAGCACAAA
Human	3’	3’ hRAPGEF6 deletion	TCTTGGTCAAGGTACCATATGCGGATAGCTGGCTTTC CTTAACTA (used with 5’XhoI-hRAPGEF6)
Human	5’	5’ hRAPGEF6 deletion	TAGTTAAGGAAAGCCAGCTATCCGCATATGGTACCT TGACCAAGA (used with 3’hRAPGEF6-HindIII)

4.2.10 In vitro overexpression neural morphology

C57Bl/6 hippocampal cultured neurons were transfected at DIV2 with pcDNA-GFP, RAPGEF6-GFP, or RA-RAPGEF6-GFP, then fixed at DIV4 or DIV5 and stained with rabbit anti-GFP 1:1000 (Invitrogen), mouse anti-Tau 1:3000, and chicken anti-Map2 antibodies (Abcam). GFP^+ neurons were then imaged and analyzed as above.

4.2.11 N18 overexpression morphology

N18 cells were grown on glass coverslips in DMEM (Gibco) with 10% FBS, penicillin, and streptomycin. When cells were 50-75% confluent, they were transfected via Lipofectamine with plasmids containing either βactin-GFP, human RAPGEF6, or *RAPGEF6* with the GEF domain deleted (RAPGEF6ΔGEF). Twenty-four hours later, N18 cells were fixed with 4% PFA, then stained against Rapgef6 (1:150) and mounted onto slides. GFP+ controls were not stained.

These slides were imaged on a fluorescent microscope (Nikon) using SPOT software (Diagnostic Instruments). Resulting images were then qualitatively assessed for the morphology of green cells based on whether cells were round or a simple spindle shape, had short, uncomplicated processes, longer processes, or highly complex processes, blind to transfection status. Four independent rounds of transfection were analyzed and the percent of cells of each morphology category were compared

4.3 Results

4.3.1 Localization of Rapgef6

The Allen Brain Atlas (www.brain-map.org) predicted that *Rapgef6* mRNA is expressed at very low levels diffusely throughout the mouse cortex, with higher expression in the hippocampus particularly within CA3. We sought to verify this finding at the protein level using antibodies to detect and quantify Rapgef6 via Western blot and immunohistochemistry.

Commercial antibodies (Santa Cruz, AbCam) were nonspecific for Rapgef6 detection. The Kataoka laboratory generously gifted a custom-made antiserum raised against the C terminus that reliably detected the absence or presence of Rapgef6 in HOM or WT mice on Western blot. However Western blots using this antibody detected additional numerous smaller bands in both HOM and WT mouse extracts. These bands likely represent cross-reactivity of the antibody with other family members or proteins; therefore its usefulness is limited for immunohistochemistry or immunocytochemistry.

By careful dissection, we isolated various brain regions and did comparative Western blotting. Two isoforms of Rapgef6 were expressed in the hippocampus, striatum, and at a lower level in the cerebellum (Figure 4.1A). Brain expression seemed to be higher than in the spleen, a region previously reported to have high expression (Yoshikawa et al. 2007), despite the Allen

Atlas prediction of low brain expression. Developmentally, Rapgef6 was expressed in pups as early as P10 and DIV8 neurons in culture, as well as in the N18 neural-like cell line. Rapgef6 levels were higher in synaptosomal preparations than in whole brain extracts, suggesting it may be localized at the membrane in spines. Within the hippocampus, Rapgef6 was expressed at relatively equal levels in the dentate gyrus, CA3, and CA1 (Figure 4.1B). While hippocampal expression appeared to be higher than that of the prefrontal cortex (Figure 4.1B), amygdala expression was higher still (Figure 4.1C).

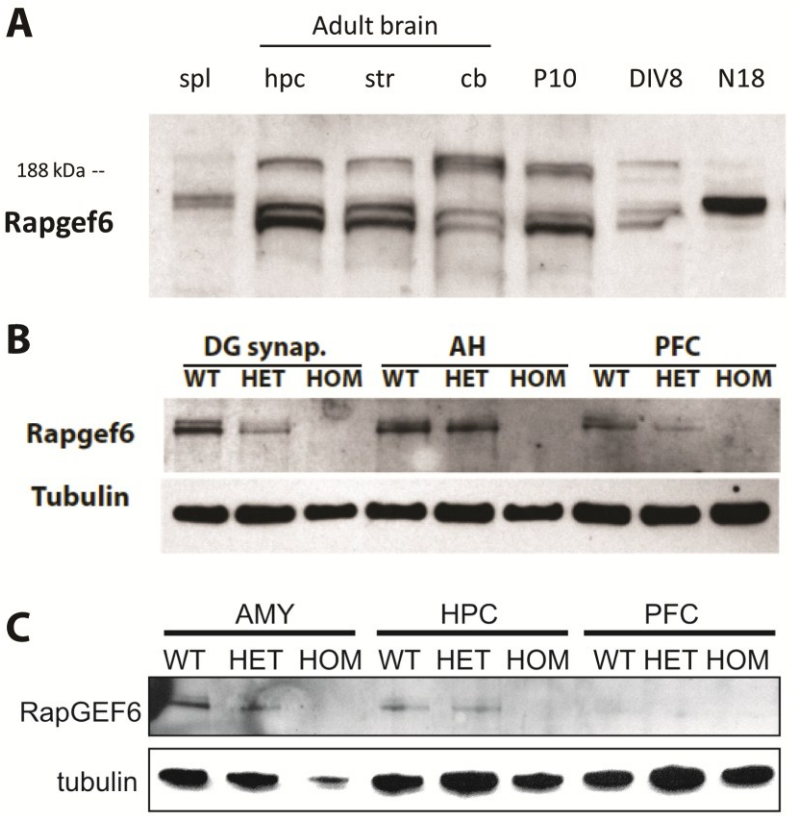


Figure 4.1 Rapgef6 protein expression on Western blot. **A**) Rapgef6 was expressed in spleen, adult hippocampus, striatum, and cerebellum, as well as P10 pups, DIV8 cultured neurons, and N18 cells. **B**) Rapgef6 was enriched in the synaptosomal fraction of hippocampal subregions dentate gyrus, Ammon horn (CA3+CA1), and prefrontal cortex. **C**) Rapgef6 expression was higher in amygdala than in hippocampus or prefrontal cortex. On all blots, HOM brains did not express any Rapgef6 at 178kDa.

4.3.2 Knock out neural morphology in vivo

Cresyl violet staining of brains demonstrated that Rapgef6 HOM and HET animals were not grossly different from WT littermates in their neural architecture (Figure 4.2). No heterotopias, aberrant white matter tracts, or absence of brain regions were noted.

Basal dendritic arbors of CA3 and CA1 subregions of the hippocampus were analyzed (Figure 4.3A). In CA3, there was no effect of genotype on total dendritic length (P=0.27), nor on the number of segments (P=0.48) or branchpoints (P=0.45) (Figure 4.3B-D). While the number of primary segments was not different (P=0.15), the primary segment length was significantly reduced in HET (P<0.05) with a trend in HOM (Figure 4.3E,F). There was no effect of genotype on secondary, tertiary, or quaternary dendrite lengths (P=0.90, P=0.17, P=0.98 respectively).

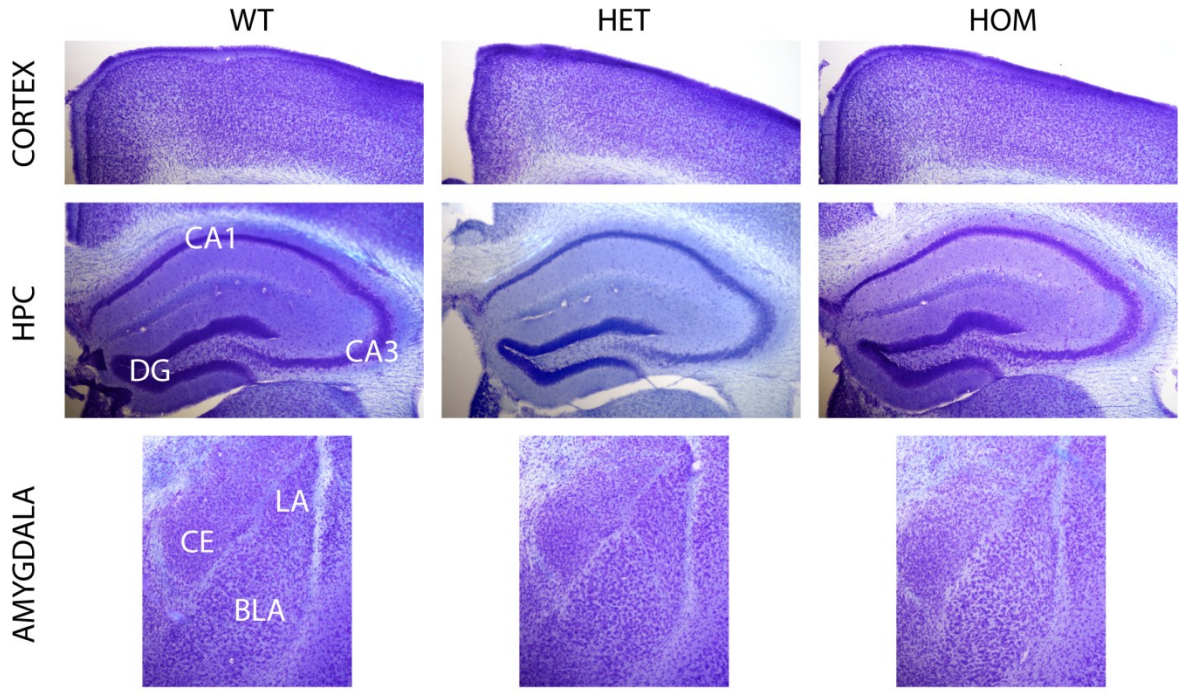


Figure 4.2 Rapgef6 mouse cresyl violet stain. Knock out of Rapgef6 did not grossly affect brain anatomy, particularly in the somatosensory cortex, hippocampus, or amygdala. The dentate gyrus, CA3, and CA1 regions are indicated in the hippocampus, and the lateral, baso-lateral, and central nuclei of the amygdala are labeled.

On Sholl analysis of CA3, there were no significant differences in crossings close to the soma although there was a trend toward reduced HET and HOM crossings (Figure 4.4). From 450µm from the soma onward this trend reversed; there was a significant effect of genotype at 450µm, 650µm, and 700µm (P=0.04, P=0.003, P=0.002 respectively) with a trend at 500µm and 600um. HOM neurons had more crossings than HET and WT (HOM>HET P<0.05 at 450,

HOM>WT P<0.05 and HOM>HET P<0.01 at 650, HOM>WT and HET P<0.01 for both at 700).

At the next step of the trisynaptic pathway in CA1, there were no significant effects of genotype on morphology. Total basal dendritic length (P=0.49), number of dendritic segments (P=0.31), number of branchpoints (P=0.83), and primary dendritic number (P=0.81) and length (P=0.14) were all equivalent among genotypes (Figure 4.5). Higher order secondary through quaternary branch lengths were also unaffected (P=0.83, P=0.19, P=0.87 respectively).

Sholl analysis of CA1 neurons did not yield any differences by genotype (Figure 4.6). Crossings close to the soma (P=0.67, P=0.78, P=0.77, P=0.53, P=0.53, P=0.8 at 0µm to 250µm) were, unlike CA3, no different by genotype from those far from the soma (P=0.42, P=0.31, P=0.5, P=0.23, P=0.66, P=0.74 at 300µm to 550µm).

CA3 basal dendritic spine density was significantly affected by genotype (P=0.007) (Figure 4.7A). HOM spine density was reduced about 20% relative to both WT and HET density (P<0.05 for both). In comparison, CA1 basal spine density did not differ by genotype (P=0.15) (Figure 4.7B).

In the basolateral amygdala of these animals, spine density was counted along the apical and basal dendritic trees of pyramidal neurons (Figure 4.8A). Neither basal (P=0.46) nor apical (P=0.71) dendritic spine density were affected by genotype (Figure 4.8B,C). Dendritic morphology could not be assessed due to technical limitations.

Finally, the pre- and infralimbic subregions of the medial prefrontal cortex layer V pyramidal neurons were analyzed for basal dendritic morphology (Figure 4.9A). Basal dendritic length (P=0.5), number of segments (P=0.58), and branchpoints (P=0.62) were all not significantly affected by genotype (Figure 4.9B-D). Primary dendritic number (P=0.11) and length (P=0.21) were also unaffected (Figure 4.9E,F). On Sholl analysis there was a significant

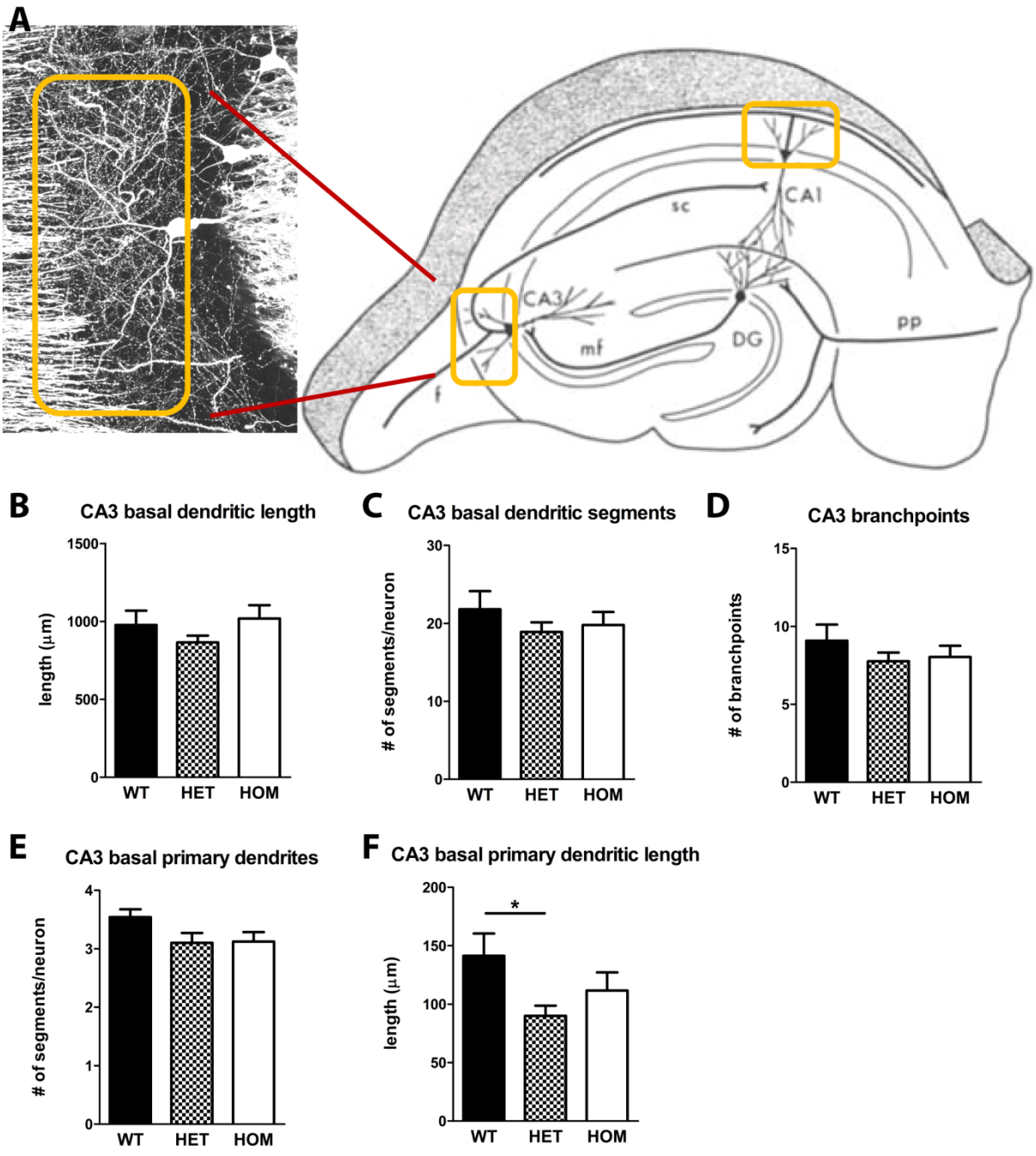


Figure 4.3 Rapgef6 KO in vivo CA3 morphology. **A**) Rapgef6 mice were crossed with Thy1-GFP to label neurons for tracing, including the basal arbor of CA3 and CA1 pyramidal neurons. There was no effect of genotype on **B**) total basal dendritic length, **C**) number of basal dendritic segments, **D**) number of dendritic branchpoints, or **E**) number of primary dendrites, but **F**) there was a reduction in HET and a trend in HOM in the length of primary dendrites. N= 4 WT, 6 HET, 5 HOM n= 26, 40, 33

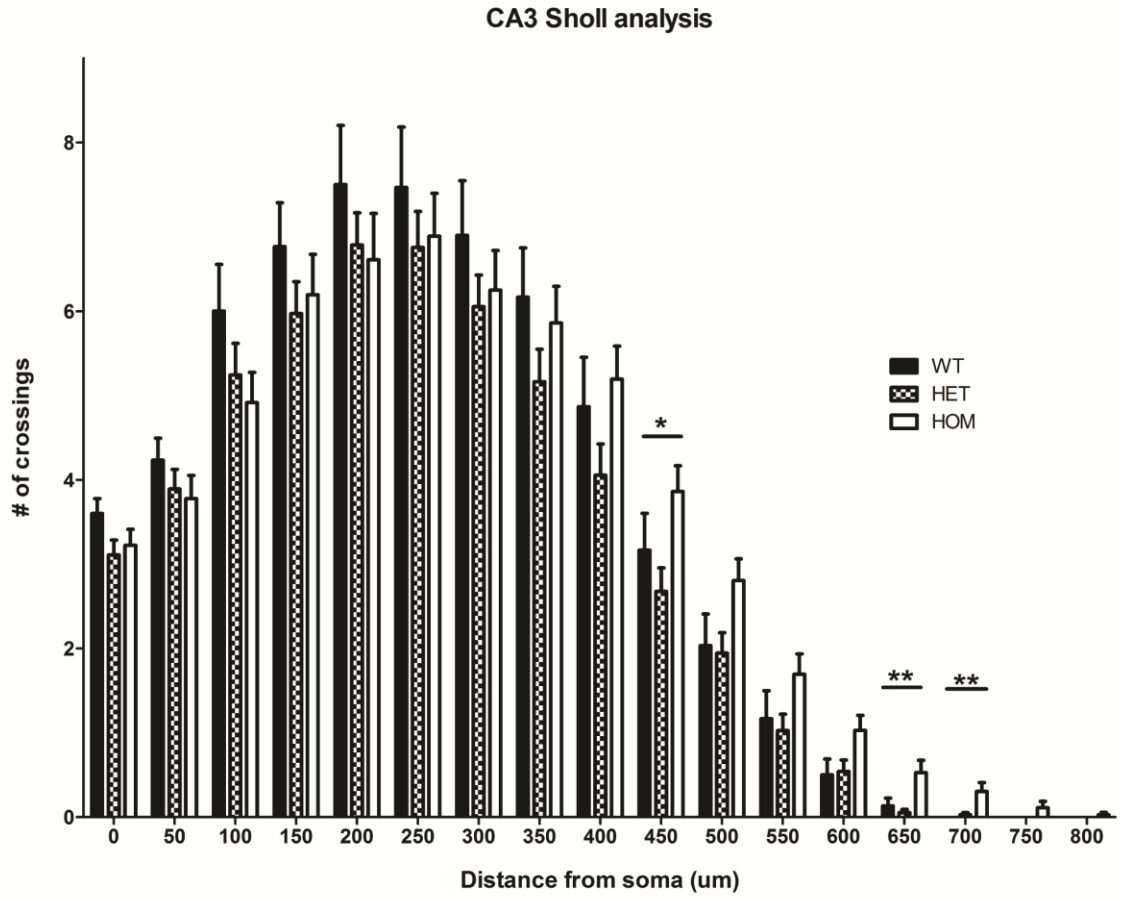


Figure 4.4 CA3 Sholl analysis of in vivo knock out. Sholl analysis of the CA3 basal dendritic arbor demonstrated an increase in HOM neuron complexity further from the soma, especially an increase in HOM over HET at 450, 650, and 700um distance.

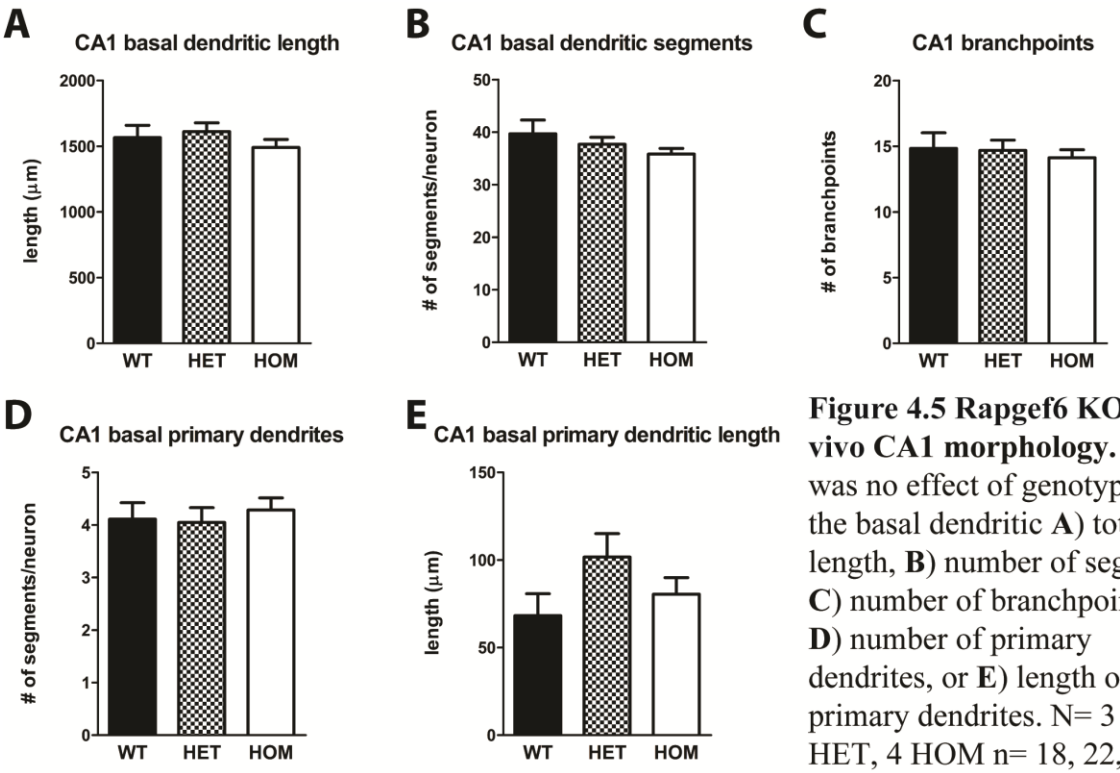


Figure 4.5 Rapgef6 KO in vivo CA1 morphology. There was no effect of genotype on the basal dendritic **A**) total length, **B**) number of segments, **C**) number of branchpoints, **D**) number of primary dendrites, or **E**) length of primary dendrites. N= 3 WT, 4 HET, 4 HOM n= 18, 22, 21

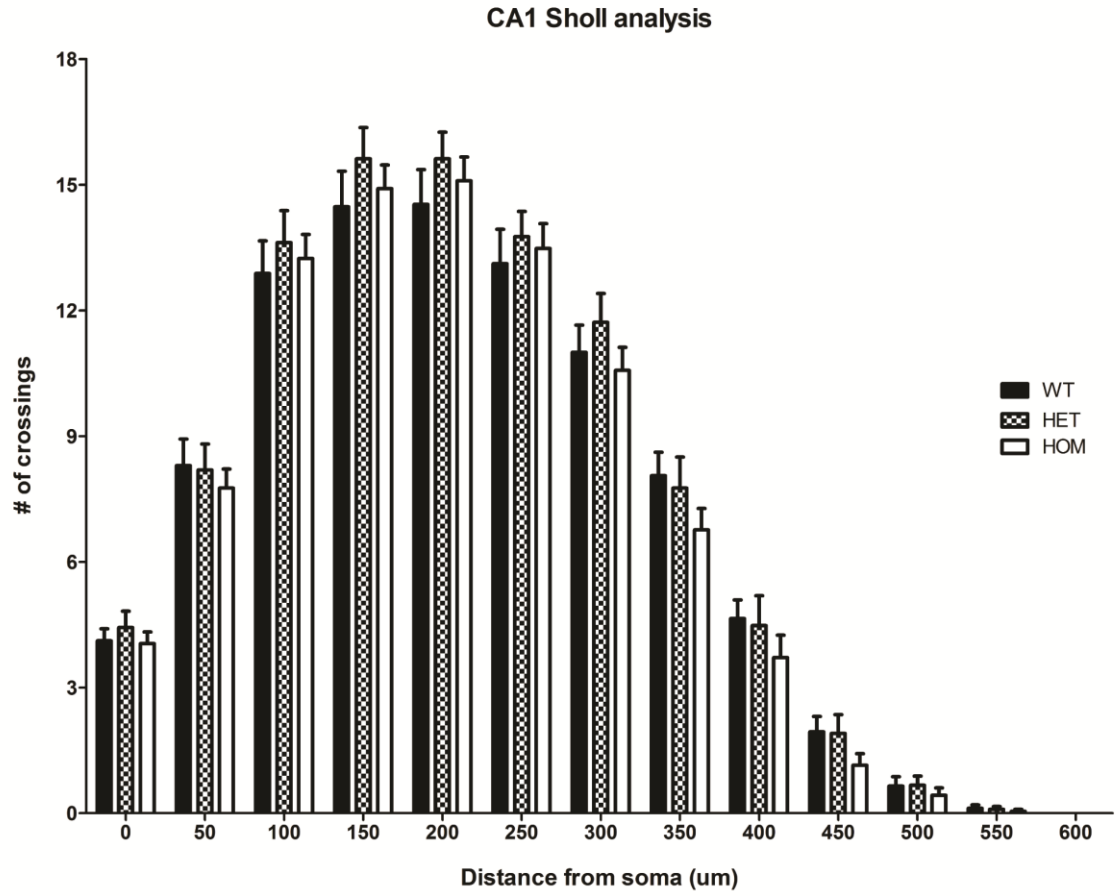


Figure 4.6 CA1 Sholl analysis of in vivo knock out. There was no effect of genotype on the complexity of the CA1 basal dendritic arbor as measured by Sholl analysis.

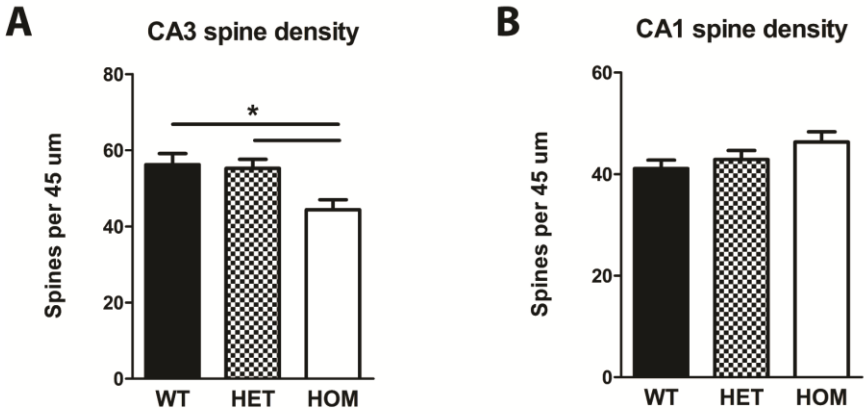


Figure 4.7 Rapgef6 KO in vivo hippocampal spine density. A) CA3 spine density was significantly reduced in HOM relative to both WT and HET. N= 3 WT, 4 HET, 4 HOM n= 32, 34, 29 **B**) CA1 spine density was not significantly affected by genotype. N= 3 WT, 4 HET, 4 HOM n= 26, 37, 35

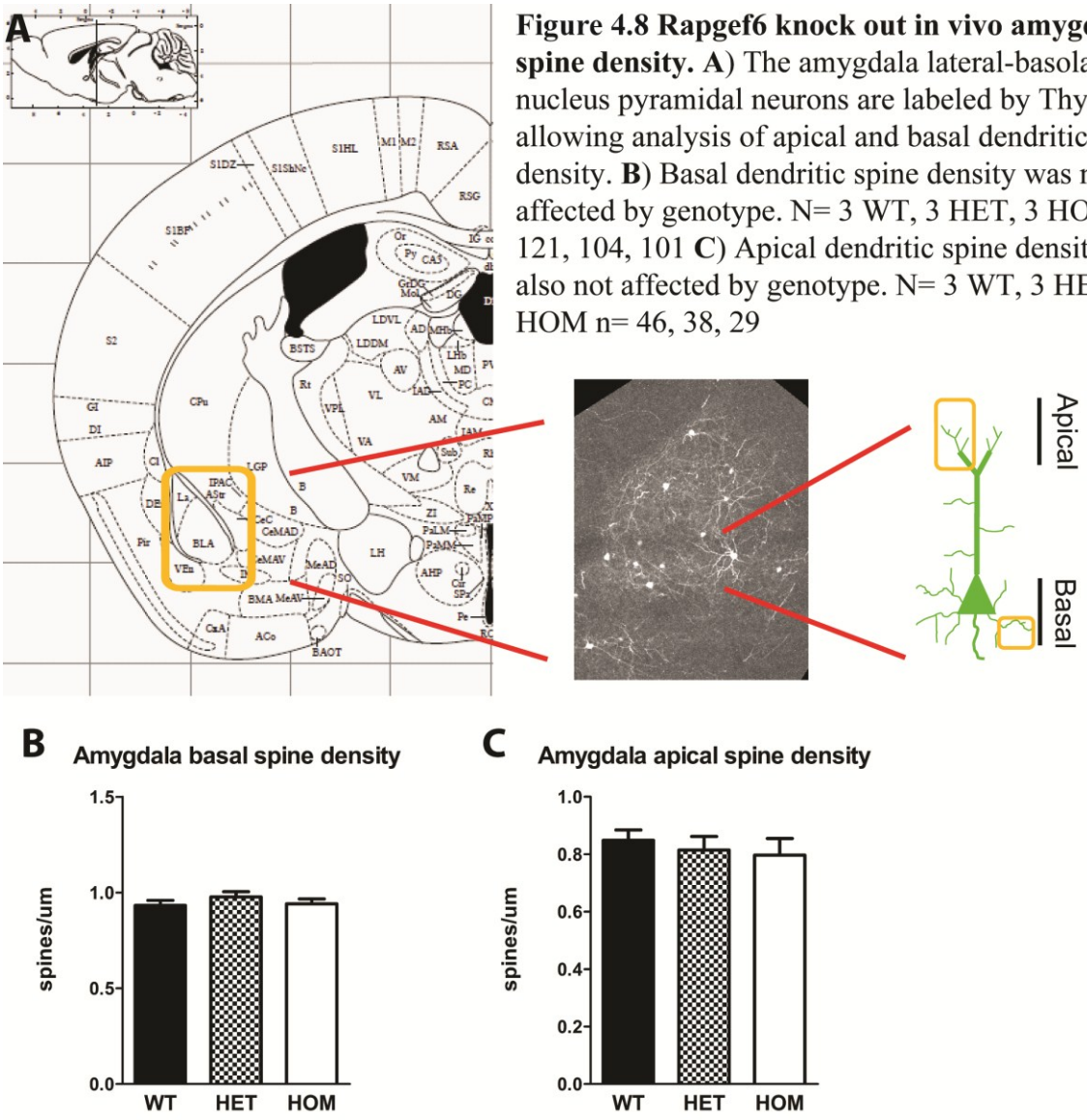
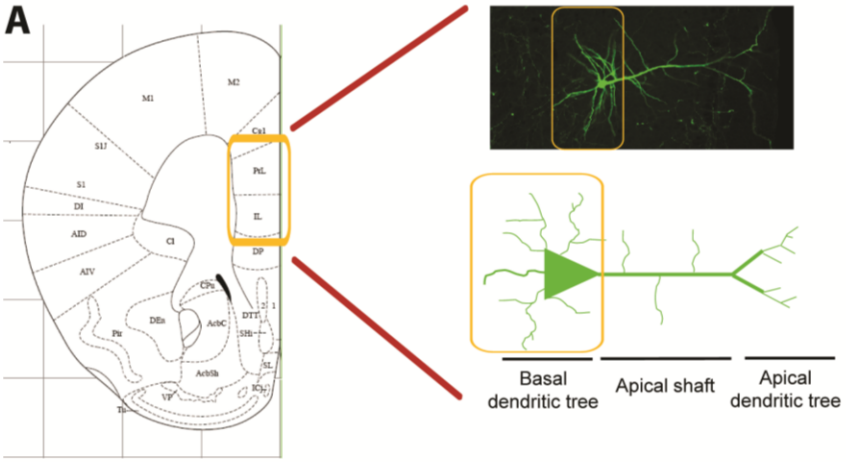
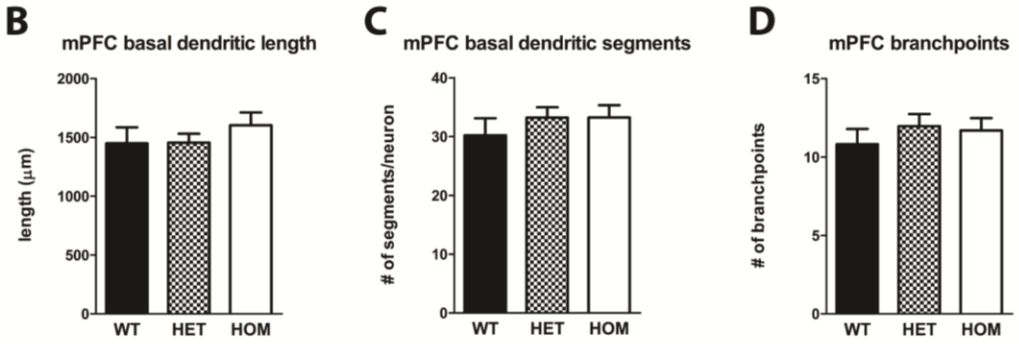


Figure 4.8 Rapgef6 knock out in vivo amygdala spine density. A) The amygdala lateral-basolateral nucleus pyramidal neurons are labeled by Thy1-GFP, allowing analysis of apical and basal dendritic spine density. **B**) Basal dendritic spine density was not affected by genotype. N= 3 WT, 3 HET, 3 HOM n= 121, 104, 101 **C**) Apical dendritic spine density was also not affected by genotype. N= 3 WT, 3 HET, 3 HOM n= 46, 38, 29





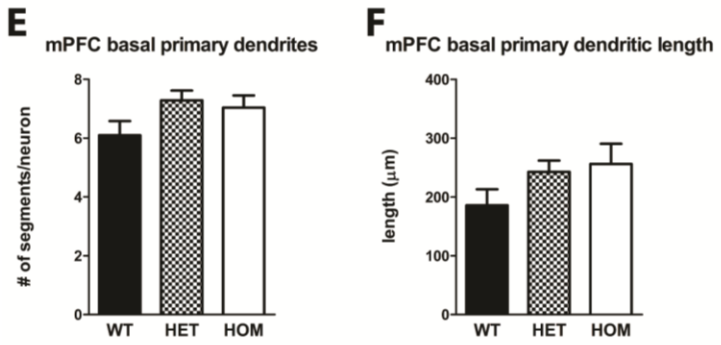


Figure 4.9 Rapgef6 knock out in vivo medial prefrontal cortex layer V morphology. A) The pre- and infralimbic mPFC neurons are Thy1-GFP+ allowing analysis. There were no differences in **B)** basal dendritic length, **C**) segments, **D**) branchpoints, or the primary dendritic **E**) number or **F**) length. N= 3 WT, 6 HET, 5 HOM n= 21, 35, 26

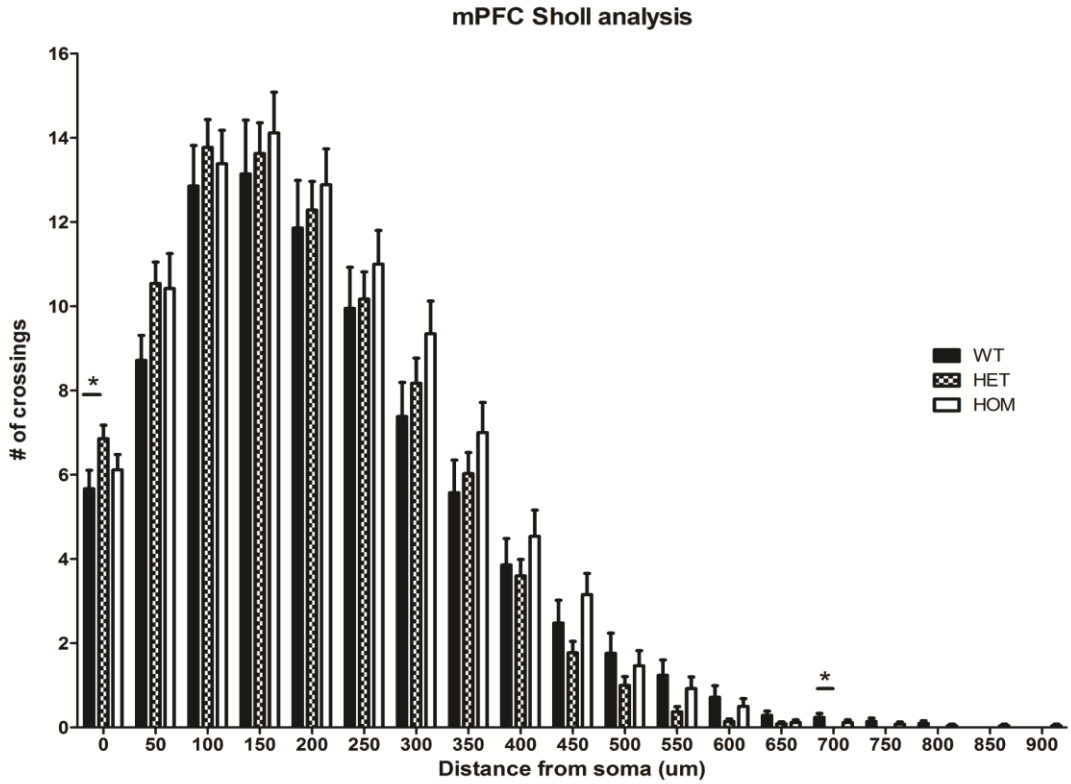


Figure 4.10 mPFC Sholl analysis of in vivo knock out. Sholl analysis of the medial prefrontal cortex layer V pyramidal neuron basal dendritic arbor demonstrated minimal effect of genotype on neural complexity. HET crossings were increased over WT at the soma, and decreased compared to WT at 700um.

effect of genotype at 0μm (P=0.03) and 700μm (P=0.01) from the soma where post-hoc testing demonstrated an increase in HET crossings over WT at the soma and a decrease in HET compared to WT at 700μm (P<0.05 for both) (Figure 4.10).

4.3.3 Knock out neural morphology in vitro

Cultures of Rapgef6 HET x HET embryos at E17.5 yielded hippocampal neurons for analysis of early neural morphology at DIV5. Total axon length and dendritic length were both not significantly affected by genotype (P=0.10, P=0.79) (Figure 4.11A,B). Dendritic complexity as measured by the number of segments (P=0.13) and number of branchpoints (P=0.84) were

also not different (Figure 4.11C,D). Similar to CA3 neurons in vivo, there was an effect of genotype on primary dendrite number (P=0.002) and length (P=0.048); on post-hoc testing HET primary dendrites were less numerous (P<0.001) and shorter (P<0.05) than WT, with a trend in a reduction in HOM (Figure 4.11E,F). Secondary and higher order branches were not significantly different by genotype.

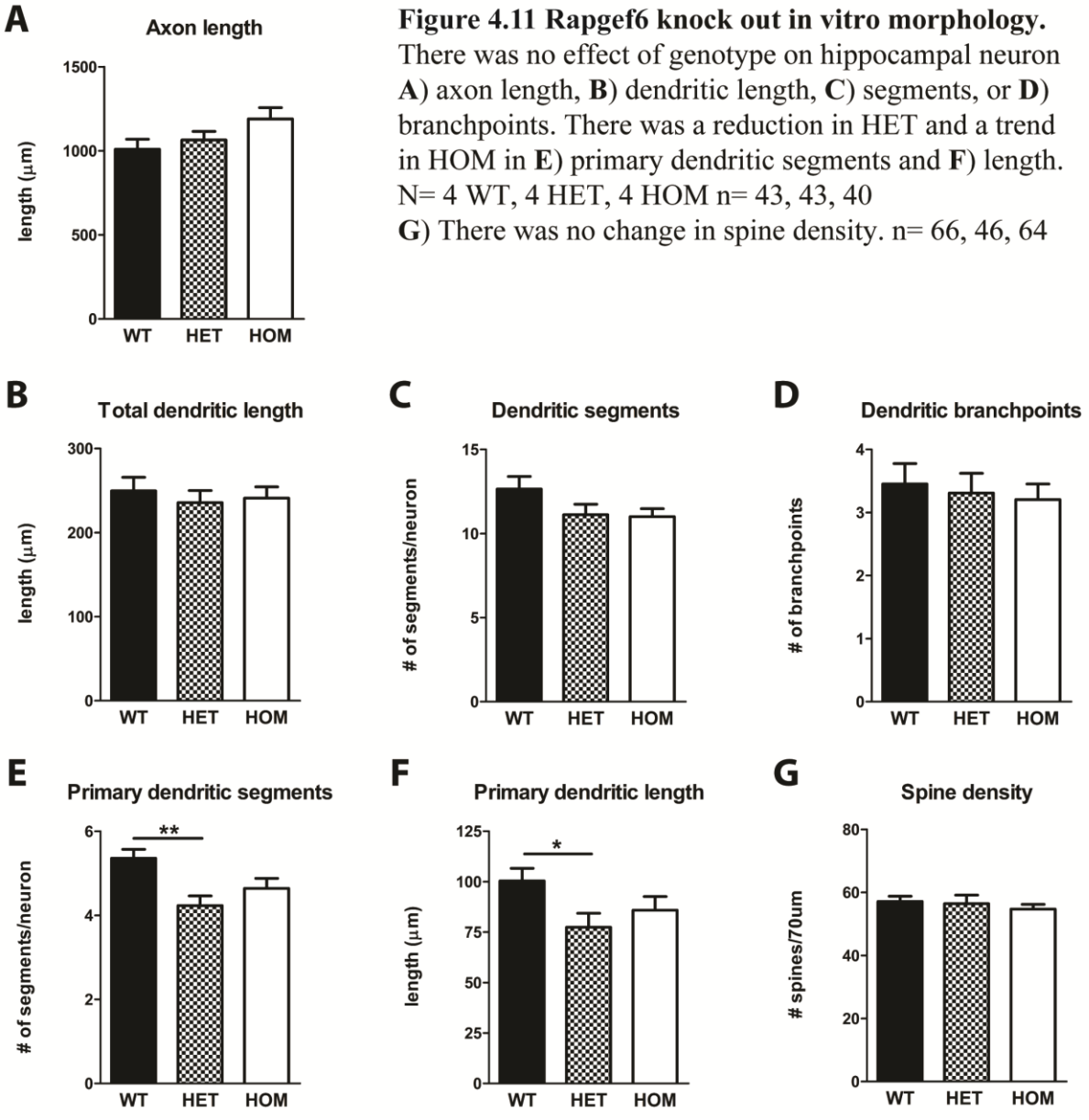


Figure 4.11 Rapgef6 knock out in vitro morphology. There was no effect of genotype on hippocampal neuron **A**) axon length, **B**) dendritic length, **C**) segments, or **D**) branchpoints. There was a reduction in HET and a trend in HOM in **E**) primary dendritic segments and **F**) length. N= 4 WT, 4 HET, 4 HOM n= 43, 43, 40
G) There was no change in spine density. n= 66, 46, 64

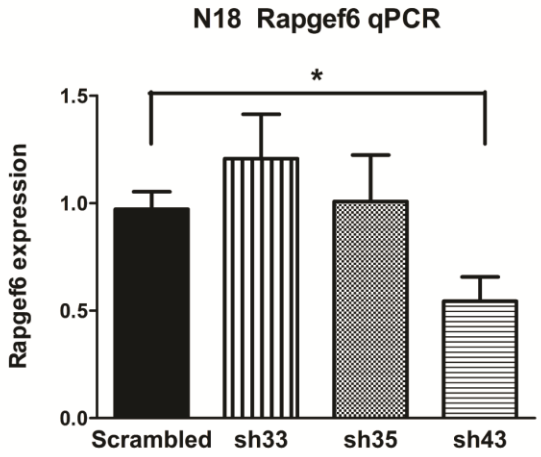


Figure 4.12 Rapgef6 shRNA knock down validation via qPCR. N18 cells transfected with shRNAs and enriched by FACS demonstrated no change in *Rapgef6* expression from scrambled shRNA except a significant 45% reduction in expression by shRNA43.

On analysis later in development at DIV21 of dendritic spines, there was no significant effect of genotype on spine density (P=0.62) (Figure 4.11G).

4.3.4 Validation of Rapgef6 shRNA

N18 cells transfected with GFP expressing shRNAs directed against *Rapgef6*, as well as scrambled shRNA, were enriched via FACS for GFP and cDNA was generated. On qPCR, only shRNA87843 (sh43) was significantly effective at reducing *Rapgef6* expression by approximately 45% compared to scrambled (P=0.015) (Figure 4.12).

4.3.5 Knock down neural morphology in vitro

shRNA43 validated above was then used to examine the effects of acute *Rapgef6* knock down on WT hippocampal neural morphology in culture. Two time points of knock down were analyzed, DIV2-4 and DIV2-5. DIV2-4 neurons did not significantly differ by transfection. Axon length (P=0.52) and branchpoints (P=0.76) (Figure 4.13A,B), as well as dendritic total length (P=0.73), number of segments (P=0.38), and branchpoints (P=0.37) (Figure 4.13C-E) were no different between scrambled and shRNA43 transfected neurons.

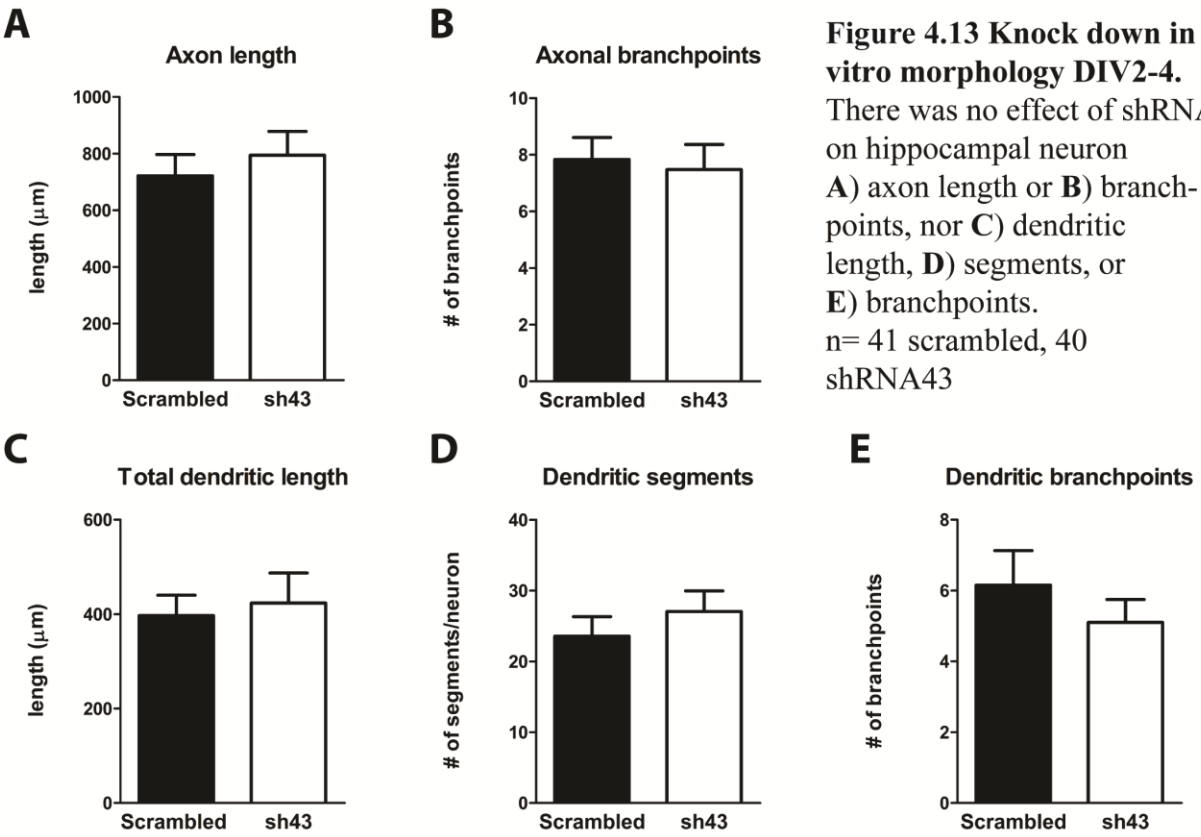


Figure 4.13 Knock down in vitro morphology DIV2-4. There was no effect of shRNA on hippocampal neuron **A**) axon length or **B**) branch-points, nor **C**) dendritic length, **D**) segments, or **E**) branchpoints. n= 41 scrambled, 40 shRNA43

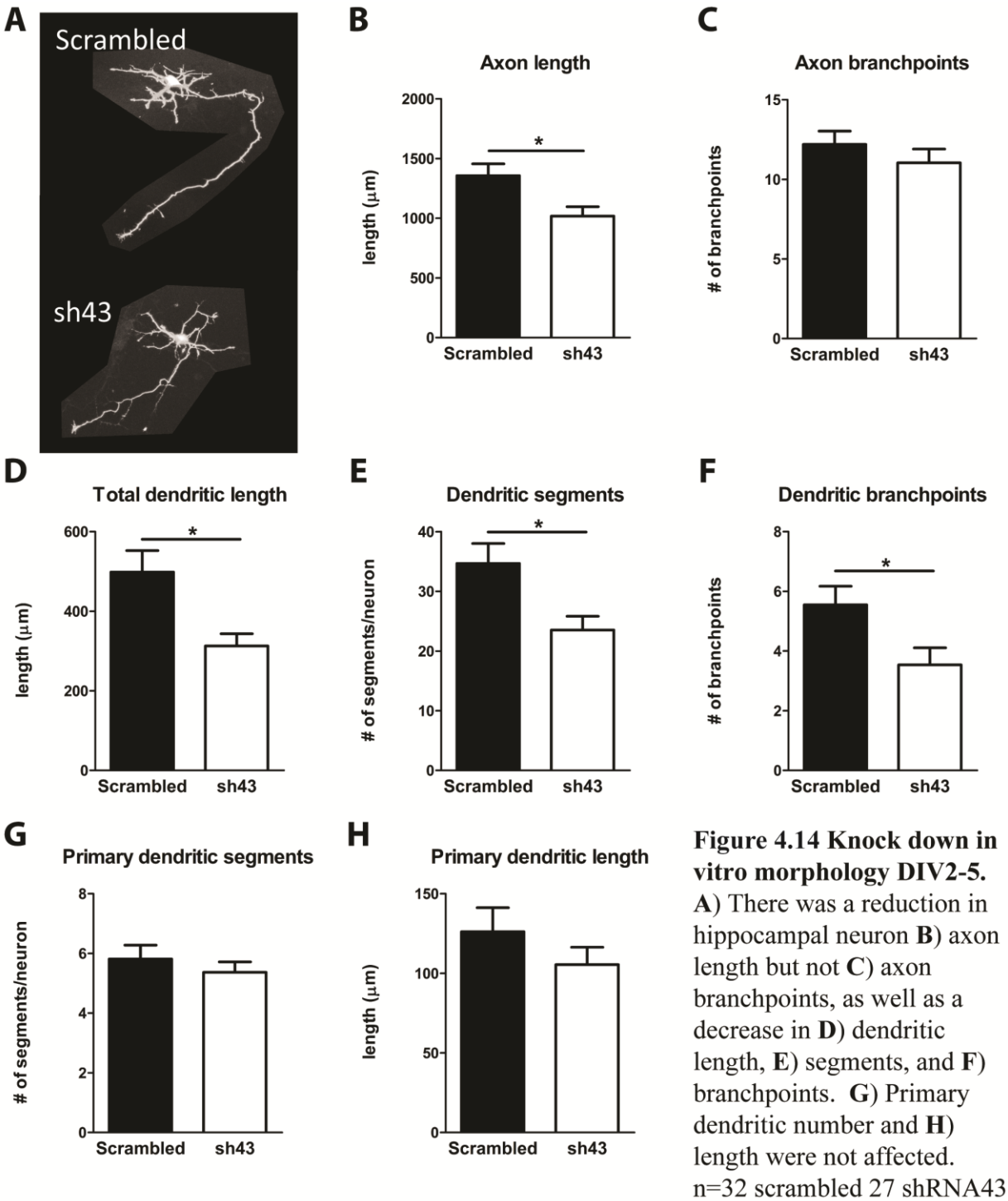


Figure 4.14 Knock down in vitro morphology DIV2-5. **A**) There was a reduction in hippocampal neuron **B**) axon length but not **C**) axon branchpoints, as well as a decrease in **D**) dendritic length, **E**) segments, and **F**) branchpoints. **G**) Primary dendritic number and **H**) length were not affected. n=32 scrambled 27 shRNA43

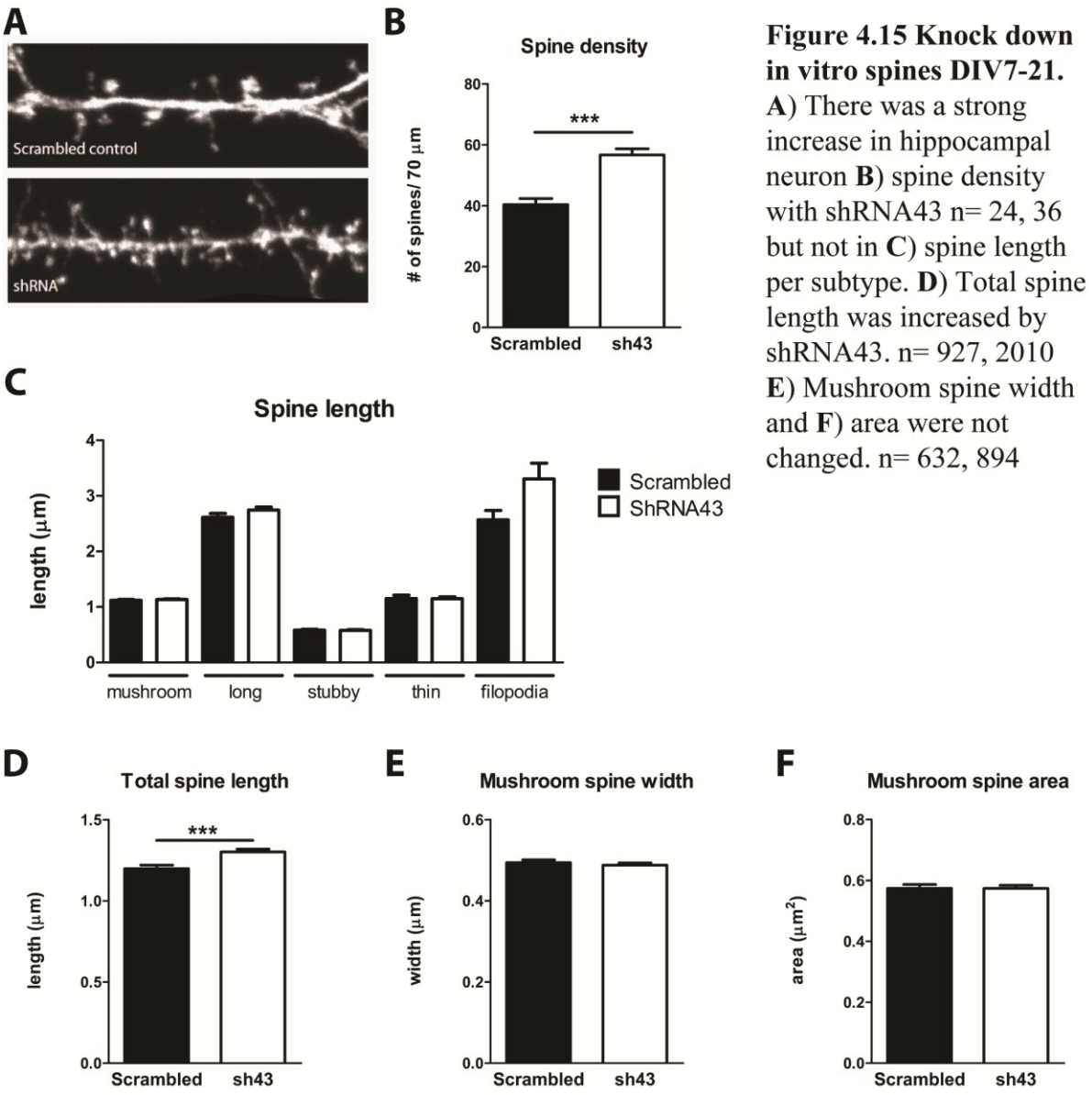


Figure 4.15 Knock down in vitro spines DIV7-21. **A**) There was a strong increase in hippocampal neuron **B**) spine density with shRNA43 n= 24, 36 but not in **C**) spine length per subtype. **D**) Total spine length was increased by shRNA43. n= 927, 2010 **E**) Mushroom spine width and **F**) area were not changed. n= 632, 894

Transfection from DIV2-5, however, yielded significant results. Axon length was significantly reduced 25% by shRNA43 (P=0.012) but not branchpoints (P=0.34) (Figure 4.14B,C). shRNA43 also led to a significant, consistently 35-40% reduction in total dendritic length (P=0.006), branchpoints (P=0.02), and segments (P=0.01) compared to scrambled shRNA (Figure 4.14D-F). Analysis of primary dendrite number (P=0.46) and length (P=0.29) were not

significantly different, nor were higher order dendritic branches (Figure 4.14G,H).

Dendritic spines analyzed at DIV21 were also affected by *Rapgef6* knock down. Spine density was increased 40% by shRNA43 ($P<0.0001$) (Figure 4.15B). When spines were assigned morphologies (Chakravarthy et al. 2006), there were no morphology-specific changes in spine length (mushroom $P=0.43$, long $P=0.18$, stubby $P=0.9$, thin $P=0.96$, filopodia $P=0.2$), but there was an overall 8.5% increase in total spine length ($P=0.0006$) (Figure 4.15C,D). Given that mushroom spines are the most mature subtype, the width and area of these spines was also measured, but there was no difference ($P=0.48$, $P=0.99$ respectively) (Figure 4.15E,F).

4.3.6 Overexpression in vitro neural morphology

Human RAPGEF6 clones of both the intact cDNA (RG6) and a form with the RA domain that interacts with MRas for localization deleted (RA) were generated in the Kataoka lab with eGFP tags. These proved more convenient for morphology analysis than HA-tagged clones produced in our lab. As for knock down analysis, neurons were transfected at DIV2 and analyzed at DIV4 and 5.

On DIV4 there were no differences between RG6, RA, or control GFP transfected neurons (Figure 4.16). Axon length ($P=0.41$) and branchpoints ($P=0.16$), as well as dendritic length ($P=0.94$), segments ($P=0.62$), and branchpoints ($P=0.42$) were all equivalent between conditions.

Similarly, on DIV5 there were no significant effects of transfected plasmid on morphology (Figure 4.17). Axon length ($P=0.34$) and branchpoints ($P=0.94$) were unchanged by overexpression of *RAPGEF6* or mutant *RAPGEF6*, as were dendritic length ($P=0.87$), number of segments ($P=0.93$), and number of branchpoints ($P=0.83$).

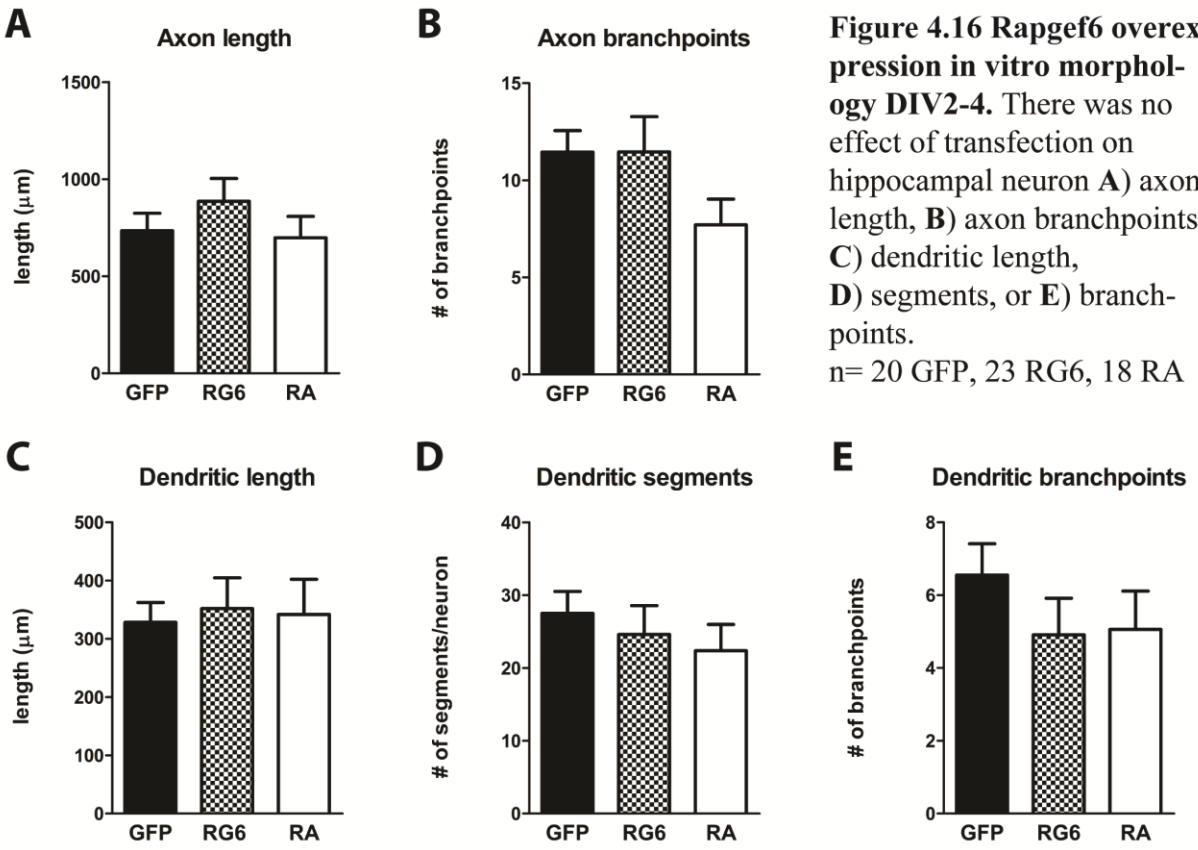


Figure 4.16 Rapgef6 overexpression in vitro morphology DIV2-4. There was no effect of transfection on hippocampal neuron **A**) axon length, **B**) axon branchpoints, **C**) dendritic length, **D**) segments, or **E**) branchpoints.
n= 20 GFP, 23 RG6, 18 RA

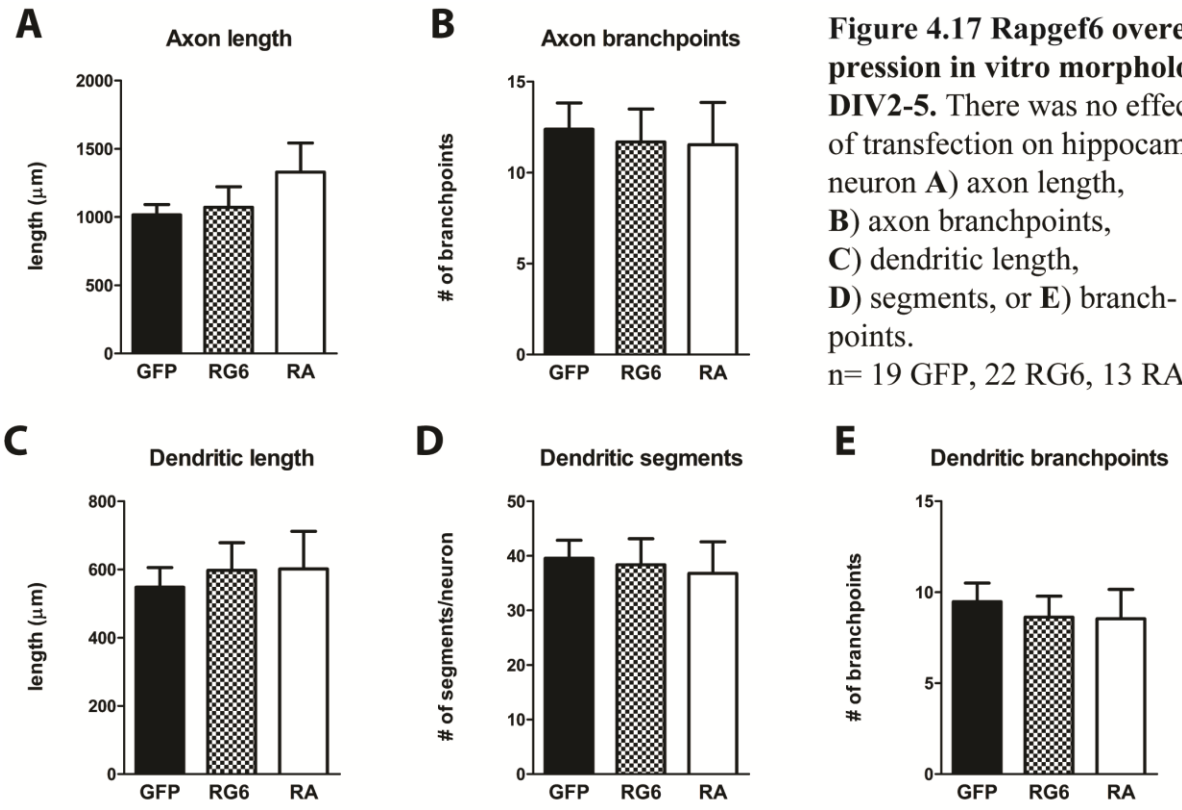


Figure 4.17 Rapgef6 overexpression in vitro morphology DIV2-5. There was no effect of transfection on hippocampal neuron **A**) axon length, **B**) axon branchpoints, **C**) dendritic length, **D**) segments, or **E**) branchpoints. n= 19 GFP, 22 RG6, 13 RA

4.3.7 Overexpression N18 morphology

N18 cells are derived from neuroblastoma tissue and are widely considered neural-like in their cellular responses. Initial tests of the effect of *RAPGEF6* overexpression involved transfecting GFP, human *RAPGEF6*, or *RAPGEF6* with the GEF domain deleted (ΔGEF) into N18 cells and qualifying the resulting morphology. There were very striking and overall significant effects of transfection ($P<0.0001$) (Figure 4.18A,B).

When analyzed by transfection plasmid, among GFP+ control N18 cells there was a significant difference of morphology ($P<0.0001$) with more small processes cells than round/spindle ($P<0.001$), large processes ($P<0.0001$), or complex ($P<0.0001$). The number of round cells was also greater than complex ($P<0.001$). After *RAPGEF6* transfection there was a

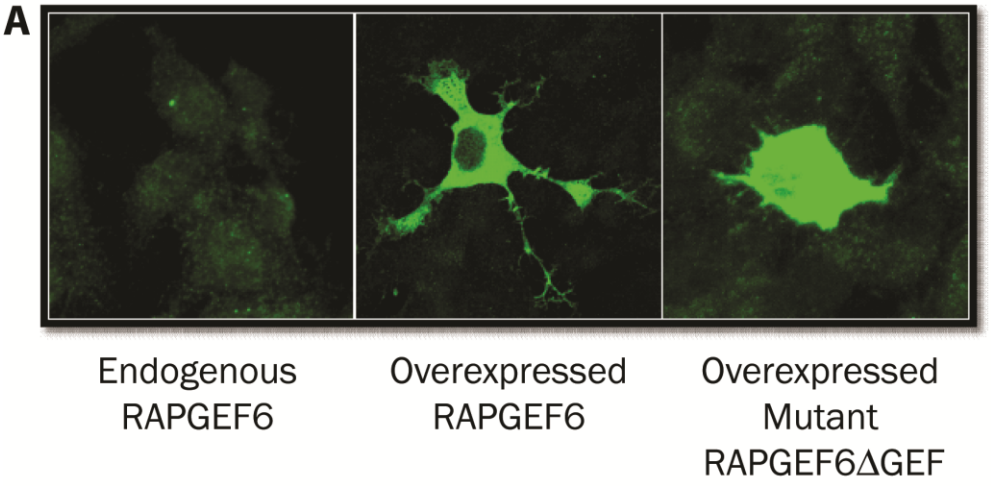


Figure 4.18 Rapgef6 overexpression N18 morphology. **A**) Staining against RAPGEF6. Transfection of *hRAPGEF6* or mutant *hRAPGEF6* without the GEF domain caused significant changes in N18 morphology. **B**) Qualitative morphology was more complex in mutant transfected cells than control and even more complex in *RAPGEF6* overexpressing cells. N= 4 cultures

significant difference (P<0.0001) consisting of more complex cells than round (P<0.0001), small (P<0.0001), or large (P<0.0001). There were also more small (P<0.01) and large (P<0.05) processes than round cells. When the nonfunctional *RAPGEF6* mutant was transfected the effect on morphology was still significant (P=0.0002), but post-hoc testing was less significant with more small processes than round (P<0.001) or complex (P<0.001) and more large than round (P<0.05).

Analysis in terms of the morphology category demonstrated the same significant differences. Considering round/spindle cells (P=0.0005), there were more found in GFP

(P<0.001) and ΔGEF (P<0.05) than *RAPGEF6* transfected cells. Among cells with small processes (P<0.0001), the same finding held with a higher percentage found in GFP (P<0.001) and ΔGEF (P<0.001) than *RAPGEF6* transfected cells. Cells with large processes were differentially distributed (P=0.0003) with more in ΔGEF transfected cells than GFP (P<0.01) or *RAPGEF6* (P<0.001). Complex cell morphology differences between transfected plasmids was highly significant (P<0.0001); these cells were most populous in *RAPGEF6* transfected cultures than GFP or ΔGEF (both P<0.0001). Overall, *RAPGEF6* transfection shifted cell morphology toward a more complex state, while ΔGEF transfection had an intermediate effect on complexity.

4.4 Summary of findings

In vivo, Rapgef6 KO resulted in a reduction in CA3 spine density and primary dendrite length with an increase in cell complexity far from the soma. No significant changes were noted in CA1 arbors or spines, mPFC layer V neural basal arbors, or amygdala spine density. In vitro knock out neurons demonstrated only a reduction in primary dendrite number and length. These knock out findings were far more subtle than in vitro knock down neural and N18 overexpression findings, which strongly supported a role for Rapgef6 in promoting neurite outgrowth. In vitro hippocampal neuron knock down led to dramatic reductions in axon length and dendritic length and dendritic complexity, as well as an increase in spine density. While in vitro neural overexpression had no effect, overexpression in N18 cells of mutant *hRAPGEF6* mildly increased neurite complexity, while *hRAPGEF6* overexpression strongly increased N18 neurite outgrowth and complexity.

In vivo there was moderate evidence that Rapgef6 promoted hippocampal primary dendrite growth and spine density, yet in vitro Rapgef6 strongly promoted neurite outgrowth, suggesting experimental differences in culture might have exaggerated the role of Rapgef6.

4.5 Discussion

4.5.1 Localization of Rapgef6

While Allen Brain Atlas suggested Rapgef6 expression was limited in the brain, we detected Rapgef6 protein throughout the brain particularly in the amygdala, prefrontal cortex, and all regions of the hippocampus. These localization results suggested that these brain regions would be relevant to evaluate neural morphology and behavior effects of *Rapgef6* deletion.

4.5.2 Discussion of brain regions and results

4.5.2.1 Hippocampus

Analysis of hippocampal neurons in vivo and in vitro from knock out mice confirmed a reduction in primary neurites in HOM neurons, but no other consistent effect on hippocampal neural morphology. Interestingly, we observed a reduction in spine density in CA3 HOM neurons in vivo but not in vitro. Experiments in wild type hippocampal cultures using shRNA against Rapgef6 were far more affected, probably due to a variety of effects discussed below. Surprisingly, despite reduced dendritic and axonal arbor growth, spine density was increased in knock down neurons. This may represent a compensatory change to normalize synaptic input along a shorter arbor.

It is important to note, as mentioned later, that hippocampal neural morphology is rather different in vivo than in vitro thus analysis comparisons are not necessarily analogous. In vivo we imaged the basal dendritic arbor of CA1 and CA3 due to technical limitations that made the apical dendritic arbor inaccessible, but it is the apical arbors which participate in the trisynaptic HPC circuit while the basal arbors gather additional input from entorhinal cortex. Results reported here are therefore not as relevant to classic HPC function. Cultured neurons from HOM, HET, and WT littermates were overall decreased in length and complexity compared to cultured

neurons from knock down analysis. This growth discrepancy makes comparison between knock out and knock down cultures difficult.

Abnormalities in the HPC have been confirmed in schizophrenia. Contrasting with our results, one postmortem study found an increase in dendritic length throughout the hippocampus (Cotter et al. 2000). Mossy fiber synapses from the dentate gyrus onto CA3 were reduced in density, similar to our in vivo reduction in CA3 spine density (Kolomeets et al. 2007). Though pyramidal hippocampal neurons were not affected in postmortem tissue, inhibitory interneuron number was reduced in schizophrenia (Konradi et al. 2011). Meta-analyses found bilateral volume reductions of up to 8% in first episode psychosis (Steen et al. 2006; Vita et al. 2006) and in individuals with chronic schizophrenia after correcting for illness duration (Adriano et al. 2012). Since postmortem studies find a reduction in interneurons but not pyramidal neurons in the hippocampus, the reduction in volume may represent a decrease in dendritic arbors. Compared with the literature, our findings concur on reduced CA3 spine density but do not show evidence for either reduced hippocampal volume or increased dendritic arbors.

In mouse models of schizophrenia there are many reports of altered hippocampal anatomy. Mice mimicking the 22q11 deletion had reduced mushroom spine density and width and decreased basal dendritic complexity in CA1 in vivo and HPC neurons in vitro (Stark et al. 2008). This finding appeared to be partially due to deletion of *Dgcr8* since its deletion alone decreased CA1 spine width and basal dendritic complexity in vivo and in vitro. Neonatal PCP in mice caused a reduction in spine density in the hippocampus as well as in other regions (Nakatani-Pawlak et al. 2009). In line with the synaptic overpruning hypothesis of schizophrenia, prenatal stress caused increased CA1 spine density and decreased CA3 spine density at prepubertal ages in rats, but decreased spine density in both regions after puberty (Martinez-

Tellez et al. 2009). Our spine density results are in line with other schizophrenia mouse models.

Behaviorally, the hippocampus is linked to spatial memory. In Chapter 3, this was tested via novel object recognition, Morris water maze, and T maze paradigms, all of which demonstrated no genotype dependent impairments. Contextual impairment was noted on fear conditioning, possibly suggesting hippocampal dysfunction, however it is possible this was due to amygdala dysfunction since cued conditioning was also affected.

4.5.2.2 Prefrontal cortex

In vivo, Rapgef6 had no effect on the basal dendritic arbor of layer V pyramidal neurons in the prefrontal cortex. Due to technical limitations, we were unable to image the apical dendritic arbor. Cortical culture was not performed. The basal dendritic arbor in the deep layers of PFC receives heavy dopaminergic input from the ventral tegmental area that is hypothesized to modulate working memory (Krimer et al. 1997; Miller and Cohen 2001), thus the medial PFC is highly relevant to schizophrenia research.

Dorsolateral PFC activity was decreased in individuals with schizophrenia during an n-back working memory task upon meta-analysis, but these patients had an increase in activation of the anterior cingulate and left frontal pole indicating a malfunctioning cortical circuit (Glahn et al. 2005). Interventions hypothesized to mimic to schizophrenia including L-methionine administration and basolateral amygdala and hippocampal lesions all reduced PFC spine density (Marquis et al. 2008; Solis et al. 2009; Tueting et al. 2010). Among genetic models of schizophrenia, *Dgcr8* deletion reduced layer II-IV neural density and layer V basal dendritic spine density, while *Disc1* deletion caused diminished PFC volume and shorter apical dendritic length (Kvajo et al. 2008; Stark et al. 2008). Since we did not assess spine density, our findings cannot be compared with the literature.

The prefrontal cortex mediates working memory and attention control. We tested working memory using the T maze and did not find any effect of *Rapgef6* deletion.

4.5.2.3 Amygdala

There was no effect of *Rapgef6* deletion on apical or basal dendritic spine density in the lateral/basolateral amygdala pyramidal neurons in vivo. The lateral nucleus dendritic arbors receive processed sensory information about conditioned and unconditioned stimuli, initiating Hebbian plasticity, then this nucleus sends synapses onto the dendritic arbor of the basolateral nucleus neurons that are essential for fear conditioning (Johansen et al. 2011). We did not anatomically discriminate between the lateral and basolateral nuclei, thus it is possible there may be a change in one nucleus that is masked by contributions from the other.

Amygdala activation compared between neutral and negative emotional stimuli was reduced in individuals with schizophrenia bilaterally in a meta-analysis, although amygdala response to negative stimuli alone was not different (Anticevic et al. 2012). Moreover, postmortem microarray analysis demonstrated altered expression in schizophrenia amygdalae of multiple genes involved in presynaptic function, myelination, and signaling (Weidenhofer et al. 2006). Amygdala morphology is not routinely assessed in rodent models of schizophrenia, however anxiety and depression models such as serotonin transporter deletion demonstrated reduced PFC arbors and increased amygdala pyramidal neuron spine density (Wellman et al. 2007).

We examined amygdala behavioral function directly in fear conditioning and indirectly in anxiolysis measures and unlearned fear in the open field paradigm. Both measures of amygdala function were impaired, as was cFOS activation within amygdala neurons during fear conditioning.

4.5.2.4 Overexpression of Rapgef6

Overexpression of *hRAPGEF6* and a mutant with the membrane targeting RA domain deleted to prevent MRas interaction had no detectable effect on neural morphology in vitro. In the course of overexpression experiments, we had difficulty achieving measurable levels of Rapgef6 protein expression. Furthermore, biochemical studies suggested Rapgef6 may be autoinhibitory (Kuiperij et al. 2003) and difficult to express unless the C terminus was truncated (Kuiperij et al. 2006). It is possible that overexpression results were falsely negative due to poor protein expression below the threshold of morphological effects.

N18 cells derived from mouse neuroblastoma are commonly used as an in vitro model of neural-like survival, morphology, and other cellular pathways. While nascent N18 cells are round or spindle shaped, many manipulations cause an increase in cellular complexity due to tubulin rearrangement (Morgan and Seeds 1975; Seeds and Maccioni 1978), reflecting a neuronal phenotype. Based on this, we used N18 cells as a preliminary assessment of the role of *hRAPGEF6* overexpression on morphology and found a strong effect upon increased neurite growth and complexity. Curiously the GEF deleted mutant increased neurite outgrowth somewhat over control transfection suggesting either an off-target effect of plasmid transfection or a dominant negative effect of the catalytically dysfunctional protein. N18 cells were more successfully transfected with *hRAPGEF6* and qualitatively appeared to express the construct more effectively when stained for RAPGEF6, therefore N18 cells may have been a more successful expression system than primary neural culture and thus yielded more significant morphology results.

4.5.3 Differences between in vitro and in vivo systems

There are three major differences in neural culture as opposed to in native tissue:

developmental stage, growth signaling, and cell autonomous or non-autonomous conditions. In vitro axon specification is different from in vivo, where extracellular cues limit axon development to only one process (Whitford et al. 2002). Activity-dependent changes in morphology are completely different in vitro where connectivity is grossly disorganized and altered compared to in vivo. Neurons in vitro are restricted in their development and connections, lacking in native extracellular matrix and extrinsic signals. But transfection in vitro is an effective way to test cell-autonomous effects.

Next, as mentioned in the introduction, neural growth is regulated by extrinsic and intrinsic signaling. Culture systems mostly rely on intrinsic directions because native extrinsic signals and activity-based triggers are disrupted by tissue digestion. Comparisons of in vivo and in vitro axon polarization demonstrate different signaling programs at work in each system. In vitro there is an intrinsically programmed growth of several neurites, one of which stochastically becomes the axon (Bradke and Dotti 1999; Bradke and Dotti 2000). In vivo, one process grows initially in an oriented polarization repulsed by extracellular cues such as Sema3A (Polleux et al. 1998). Thus in vitro systems highlight effects of or changes to internally programmed cell growth, whereas in vivo systems elucidate changes in extracellular influences (Whitford et al. 2002).

Third, in vitro and in vivo differ in the role of cell non/autonomous effects. Although knock out cultures are genetically homogeneous, transfection of shRNA or overexpression vector leads to expression heterogeneity since only a fraction of neurons take up and express the plasmid. As a result, cells in culture reflect mostly cell autonomous functions since they are isolated among unaffected neighboring cells, while neurons in tissue are also subject to non-autonomous influences from the effects of deletion in all of the surrounding cells. These three

technical differences between culture and tissue may help explain why we found different results in each experimental preparation.

There is also a biological distinction between knock down and knock out experiments, most importantly concerning temporal (acute as opposed to chronic expression changes) and off-target effects. Knock down by shRNA is by nature far more acute and less successful in reducing protein levels than a constitutive knock out such as we used for Rapgef6. Because shRNA works so acutely, there may not be time for compensatory effects, such as those discussed below, to arise as they normally would in vivo across development in the chronic absence of a protein. Additionally, shRNA can have off-target effects potentially due to reduction of unintended gene expression. Supporting this conclusion, analysis of morphology after transfection with a control shRNA demonstrated reduced dendrites and spine density (Alvarez et al. 2006). However spines were increased in our findings therefore suggesting this may not be a typical off-target effect. Methods to identify or eliminate off-target effects include repeating results with additional targeted shRNAs and rescuing knock down with overexpression of an shRNA-resistant cDNA to prove results are indeed target gene dependent.

Two sources of compensation are most likely to diminish any phenotype resulting from *Rapgef6* deletion in vivo. First, there may be remaining isoforms unaffected by either the shRNA or deletion of exon 21. The Genome Browser does not report any meaningful predicted isoforms unaffected by our interventions, nor is it likely that an isoform missing the catalytic domain would be functional, however it is possible that unreported isoforms exist. Next, there may be compensation by redundancy from other gene family members. For instance, Rapgef3 and 4 presumably compensate for each other in vivo since mouse models showed there was no phenotype unless they were both deleted (Yang et al. 2012). Rapgef2 is most homologous to

Rapgef6 suggesting it might be the most likely redundancy candidate, although it is also possible that Rap1 or Rap2 are also upregulated to compensate for Rapgef6 knock out.

Due to these and other technical issues, it is not uncommon for mouse models to yield discrepancies between in vitro and in vivo results or for in vivo morphology to poorly predict functional behavioral findings. For example, in the stathmin deletion animal, there were no in vivo morphology changes in the amygdala to correlate with reduced fear conditioning, however electrophysiology demonstrated reduced long term potentiation in the lateral amygdala, indicating altered function in the absence of morphological changes (Shumyatsky et al. 2005).

4.5.4 Primary dendrite morphology

We demonstrated a reproducible finding of decreased primary dendritic number and length in the setting of unchanged total dendritic length and complexity in vivo and in vitro. This was also found to be the case for another novel schizophrenia candidate gene mouse model in our lab (B. Xu, in press). There is little known about the mechanism of primary dendrite reduction, but it has been shown that excess BDNF and NT4 worked via Rac1 and Rho (GTPases similar to Raps) to specifically increase primary dendrites more than the total dendritic arbor (McAllister et al. 1995). Perhaps Rapgef6 activation is also functionally downstream of one of these trophic factors leading to diminished primary dendrite growth. Presumably changes in the primary dendritic arbor could influence synaptic integration in a manner detectable by electrophysiology assays.

4.5.5 Role of Raps and Rapgefs compared to Rapgef6

In comparison with other family members' effects on neural morphology, *Rapgef6* deletion is far less deleterious. As mentioned above, this lack of effect may be similar to the minimal effects of *Rapgef3/4* deletion individually due to functional redundancy (Yang et al.

2012). Overexpression of constitutively active Rap2 caused reduced CA1 spine density in vivo which is similar to the reduction in CA3 spine density we observed (Ryu et al. 2008), however this is a contradictory result as *Rapgef6* deletion should tend to reduce Rap2 activity. MRas overexpression in vitro increased PC12 neurite outgrowth whereas MRas deletion caused no morphology changes in vivo or in vitro (Nunez Rodriguez et al. 2006; Sun et al. 2006); this is the most analogous situation to our model in terms of results and biology given that MRas localized Rapgef6 to the membrane in lymphocytes and hypothetically in neurons. Overall our results suggest that while Rap2 is biochemically predicted to be the stronger target of Rapgef6, the neural process growth promoting effect of Rapgef6 expression and inhibiting effect of its deletion on neurites suggest that the neurite promoting activity of Rap1 may be the more relevant Rapgef6 target in neurons.

4.5.6 Morphology results in light of behavior phenotype

As mentioned above, the behavioral findings do not correlate with the minimal neural morphology changes observed. There were changes in hippocampal morphology but only a possible correlating behavioral deficit on contextual fear, whereas amygdala functional deficits on fear conditioning and perhaps impacting anxiolysis and startle did not correlate with amygdala pyramidal neuron spine changes. There may still be changes at the synaptic or circuitry wiring level that our analysis did not detect, or in second messenger pathways such as those mediated by GTPases, to explain these behavioral results. Possible experiments to detect changes at these levels are discussed below.

4.6 Future directions

4.6.1 Electrophysiology

Electrophysiology assessment of the entire hippocampus would be helpful to determine whether there is a functional impact of reduced CA3 spine density. This change may lead to diminished transmission through CA3 neurons, or there may be effects we cannot predict based on the reduced cFOS results and potential synaptic changes in the apical dendritic arbor that were not assessed for morphology. Amygdala electrophysiology of cortical and thalamic inputs to the lateral nucleus would be useful to see if there is further functional overlap between our Rapgef6 mouse and the Rap1 KO mouse that had reduced fear conditioning and altered cortico-amygdala synaptic strength (Pan et al. 2008) and to support the significance of the cFOS finding suggestive of reduced amygdala activation. Whole cell responses and firing properties from neurons in culture could permit assessment of whether synaptic integration is altered as a result of primary neurite morphological changes.

4.6.2 Morphology and cellular techniques

There are many experiments by which we could try to assess integrin and cadherin function in the absence of Rapgef6. First, we could determine expression via qPCR or Western blot since knock down of *RAPGEF6* decreased integrin and cadherin expression (Dube et al. 2008; Severson et al. 2009). Since key functions of these pathways include cell migration and adhesion, we could assess these parameters in neurons. Neural migration can be quantified in ex vivo slice cultures with video capture of the movement of GFP+ neurons, or through incorporation of BrdU or electroporation of GFP into newly generated neurons at various time points in development. When combined with cortical layer-specific immunohistochemistry, these techniques can identify improper migration. Neural adhesion in vitro assays exist to assess

binding to various integrin substrates (Denda and Reichardt 2007).

In order to further assess the discrepancy between in vitro knock down and knock out results, we should validate and transfect additional shRNAs to recapitulate the knock down finding for Rapgef6 specificity. We should also demonstrate Rapgef6 specificity by rescuing the knock down phenotype with shRNA-resistant *Rapgef6* overexpression, however initial rescue trials were unsuccessful due to difficulty with both overexpression and cotransfection. Impairing synaptic activity dependent growth by culturing neurons in TTx could determine whether the increase in spine density upon in vitro knock down is a compensatory response to upregulated activity onto a shorter arbor, as opposed to a cell autonomous effect.

As Rapgef6 was increased in gliogenesis and decreased in neurogenesis on microarray (Kempermann et al. 2006) and the dentate gyrus had reduced cFOS activation, we could assess these processes in the knock out mouse. BrdU injections followed by staining and cell counting would identify alterations in dentate gyrus adult neurogenesis, perhaps finding increased neurogenesis given Rapgef6 might inhibit this pathway. Gliogenesis may be reduced on BrdU counts, or we could assess overall glia density via specific staining and cell counting. BrdU studies would also permit analysis of neural and glial migration.

In vivo, it would be interesting to try to rescue the spine deficit in CA3 with Rap1 and Rap2 overexpression or 8CPT administration via slice culture. Alternatively, BDNF could be used to attempt a rescue of primary dendrite growth in knock out cultures or on slice culture. This would help to determine what ligands are upstream of Rapgef6 signaling in the brain.

4.6.3 Testing Rapgef6 activity

We attempted to quantify Rap1-GTP activity both in vitro and in cellular extracts using a commercial assay (Pierce). Unfortunately, the assay was not specific or sensitive enough to

consistently detect changes in Rap activity in our samples. Further optimization of this assay would be incredibly useful in order to determine if the absence of Rapgef6 leads to a predicted decrease in Rap1 or Rap2 activity, thereby providing direct evidence for a functionally significant affected pathway.

In order to determine the binding partners of Rapgef6 in neurons, we could attempt coimmunoprecipitation (coIP) followed by mass spectroscopy identification. We attempted to validate TNIK as a potential binding partner through coIP assays but were unable to get reliable results (Hussain et al. 2010). Other potential coIP targets could be JamA, Bag3, and Afadin using brain homogenates since these are known binding partners outside the brain (Dube et al. 2008; Severson et al. 2009).

In order to determine if other Rapgefs are compensating for the loss of Rapgef6, we could run qPCR to measure increased expression of other Rapgef family members or downstream target Raps. qPCR might be more sensitive than protein level analysis since protein upregulation may be mild. Microarray analysis from brain regions of KO animals could detect changes in Rapgef or Rap genes and altered expression of other interacting genes, perhaps identifying novel Rapgef6-dependent pathways.

4.6.4 In the next chapter

We will next reconsider all of the experimental findings together and discuss the overall effect of *Rapgef6* knock out, as well as the implications of these results for understanding neuropsychiatric disorders.

Chapter 5: Summary and Conclusion

5.1 Summary of results

In human genetic studies, we discovered CNVs impacting *RAPGEF6* and *RAPGEF2*. Behavioral analysis of a mouse modeling *Rapgef6* deletion determined that amygdala function was the most impaired behavioral domain as measured by reduced fear conditioning and anxiolysis. More disseminated behavioral functions such as startle and prepulse inhibition were also reduced, while locomotion was increased. Hippocampal-dependent spatial memory was intact, as was prefrontal cortex function on a working memory task. Neural activation as measured by cFOS levels demonstrated a reduction in hippocampal and amygdala activation after fear conditioning. In vivo neural morphology assessment found CA3 spine density and primary dendrite number were reduced in knock out animals but additional hippocampal measurements were unaffected. Furthermore, amygdala spine density and prefrontal cortex dendrites were not changed. Considering all levels of analysis, the Rapgef6 mouse was most impaired in hippocampal and amygdala function. These brain regions are particularly significant for schizophrenia research, as discussed further in section 5.2.

5.1.1 *RAPGEF6* is a genetic and functional schizophrenia candidate gene

Though schizophrenia is up to 81% heritable (Sullivan et al. 2003), attempts to identify causal mutations have yielded very few penetrant, non-private, replicated loci. Though it was statistically unlikely to find two mutations affecting the same small gene family, we discover two CNVs affecting *RAPGEF* family members within an Afrikaner population: a de novo deletion of exons 2-11 of *RAPGEF6* in a sporadic case of schizophrenia and an inherited duplication involving *RAPGEF2* in two siblings concordant for schizophrenia as well as their unaffected

mother (Xu et al. 2008; Xu et al. 2009). Whole exome sequencing of the same population identified two additional inherited mutations in *RAPGEF2* (Xu et al. 2012). Moreover, *RAPGEF6* is within the chromosome 5q31 locus implicated in schizophrenia by multiple karyotype, linkage, and association studies (Bennett et al. 1997; Chen et al. 2006b; Lewis et al. 2003). Together, these genetic results converge on the *RAPGEF* family with particularly strong evidence for *RAPGEF6* as a candidate gene in the development of schizophrenia.

There are no published studies on the direct role of RAPGEF6 in the nervous system, therefore we focused on other RAPGEF family-dependent pathways with neural relevance. One mouse study found *Rapgef6* expression was positively correlated with astrocyte gliogenesis and negatively correlated with adult neurogenesis (Kempermann et al. 2006). The role of Rapgef6 in these processes remains unclear. In vitro knock down and overexpression experiments demonstrated that RAPGEF6 activated Rap2 more than Rap1, was localized to the membrane by MRas, and promoted cadherin and catenin dependent cellular junctions (Dube et al. 2008; Gao et al. 2001; Kuiperij et al. 2006; Severson et al. 2009). A mouse model with *Rapgef6* deleted had no gross anatomical abnormalities, but immune B cells failed to activate integrin signaling for cellular adhesion to the extracellular matrix in response to TNFα (Yoshikawa et al. 2007). Since integrins and cadherins are also key elements in neural migration and neurite outgrowth (Clegg et al. 2003; Suzuki and Takeichi 2008), these findings suggest that Rapgef6 may also participate in neural development and morphology.

The other members of the Rapgef family and their downstream targets, Rap1 and Rap2, have key roles in the nervous system, implying Rapgef6 may be neurally important as well. Rapgef1 was activated by neurotrophins and Reelin in neuroepithelial development and neural radial migration, respectively (Voss et al. 2008; Voss et al. 2006). Deletion of *Rapgef2* resulted

in cortical heterotopia and failure of cortical axons to decussate at the midline within rostral commissures (Bilasy et al. 2011; Bilasy et al. 2009). SNPs within *RAPGEF3* were associated with depression (Middeldorp et al. 2009), while SNPs within *RAPGEF4* were associated with autism (Bacchelli et al. 2003). Introduction of Rapgef3/4 agonist 8CPT into the hippocampus improved contextual fear conditioning in wild type mice (Ma et al. 2009). Although deletion of either gene independently produced no demonstrable phenotype, deletion of both *Rapgef3* and *Rapgef4* caused impairments in spatial memory and social interaction as well as a reduction in hippocampal long-term potentiation (Yang et al. 2012). Rapgef5 was downregulated during neural differentiation (Rebhun et al. 2000).

There are multiple conflicting reports of Rap1 and Rap2 effects on neural development and synaptic function in vitro. In animal models, targeted cortical deletion of Rap1 caused cued fear conditioning impairments and disturbed cortico-amygdala synaptic function (Pan et al. 2008). Constitutively active Rap2 led to hyperactivity, spatial memory deficit, and decreased contextual fear extinction on behavioral assessment with reduced CA1 spine density and increased hippocampal long-term depression (Ryu et al. 2008). Postmortem brain analysis found that Rap1 expression was reduced in individuals with schizophrenia and depression (Yuan et al. 2010), while Rap2 expression was reduced in the anterior cingulate in patients with schizophrenia (Funk et al. 2012). Rapgef and Rap family members are involved in a variety of neurodevelopmental, synaptic transmission, and behavior phenotypes. Overall, the convergence of genetic data and phenotypes in the literature suggest that Rapgef6 is both a positional and functional candidate gene for schizophrenia etiology.

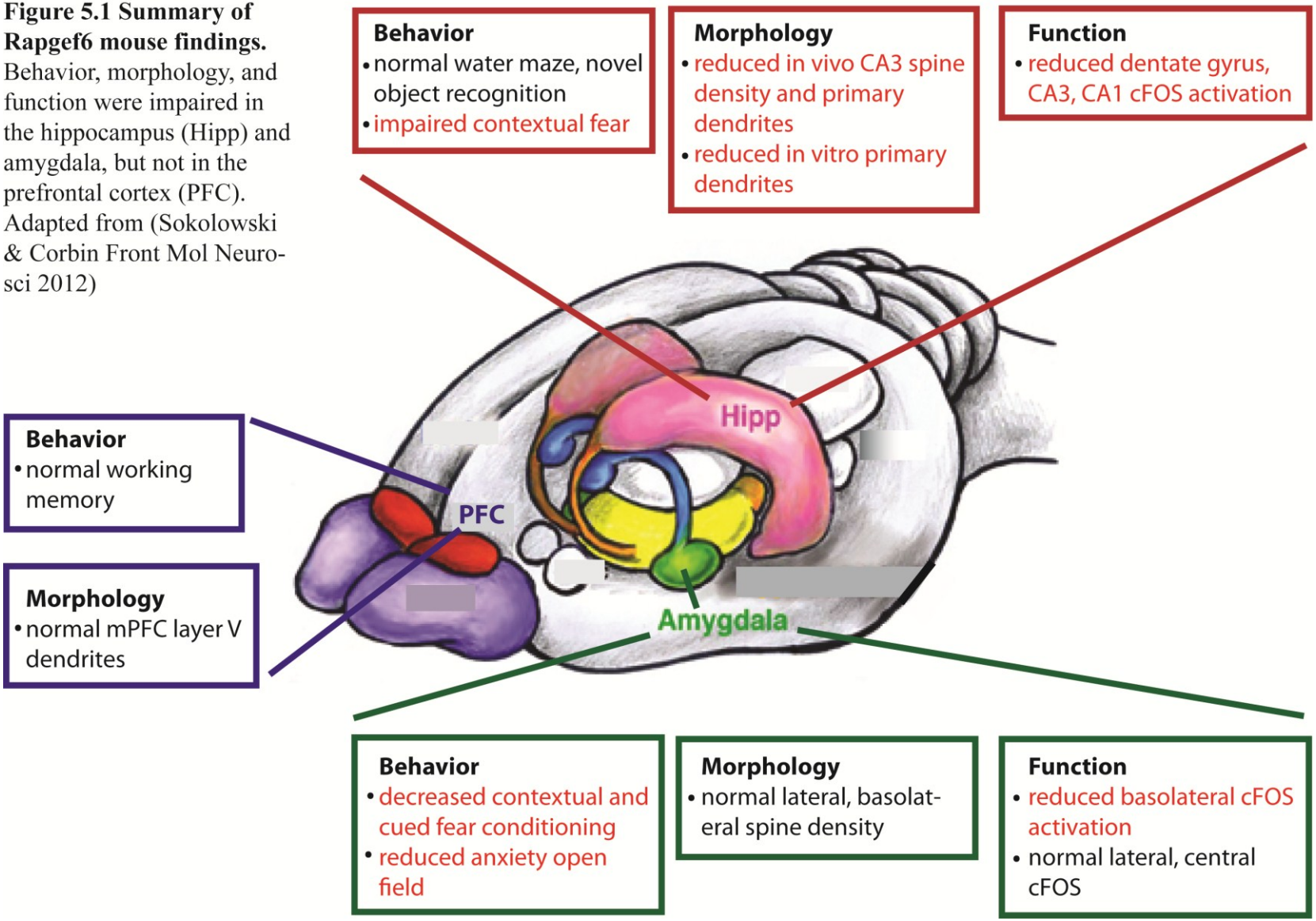


Figure 5.1 Summary of Rapgef6 mouse findings. Behavior, morphology, and function were impaired in the hippocampus (Hipp) and amygdala, but not in the prefrontal cortex (PFC). Adapted from (Sokolowski & Corbin Front Mol Neurosci 2012)

5.1.2 Rapgef6 deletion mice demonstrated hyperlocomotion and reduced anxiety

We acquired the *Rapgef6* deletion mouse model, in which the exon coding for the GEF catalytic domain was deleted (Yoshikawa et al. 2007). We performed a comprehensive behavioral and morphological assessment to determine the effect of *Rapgef6* deletion on neuropsychiatrically relevant phenotypes (Figure 5.1). HET and HOM animals were born at Mendelian frequencies and were grossly normal for motor abilities and lifespan. Cresyl violet staining confirmed that there were no obvious neuroanatomical changes, including no heterotopias, and the commissures and hippocampus were intact (Figure 4.2).

To understand basic motor function and locomotion, we first performed the open field test. On open field testing, HOM and HET animals were hyperactive and showed increased rearing, which is considered both a measure of activity and nonspecific attention (Figures 3.3-3.5). Increased activity in the center of the field demonstrated a lack of anxiety-like behavior in HET and HOM animals (Figure 3.4). Dopamine stimulation can increase both locomotion and rearing (Berridge 2006; Brookshire and Jones 2009), suggesting that Rapgef6 mice may have increased striatal dopamine release or receptor expression, similar to findings in individuals with schizophrenia (Abi-Dargham et al. 1998).

5.1.3 Rapgef6 deletion mice demonstrated mildly impaired hippocampal function and morphology

We sought to understand the role of Rapgef6 in the hippocampus. While characterization of spatial memory did not find many hippocampal-dependent behavioral deficits, there were subtle morphological changes in the hippocampus in vivo and in knock out hippocampal neurons in vitro, as well as striking reductions in hippocampal neural arbors upon knock down. Novel object recognition and Morris water maze, two measures of hippocampal-dependent spatial

memory, were both unaffected in HOM animals (Figures 3.6-3.9) (Sousa et al. 2006). Contextual fear conditioning was impaired in HOM animals in the second cohort with a trend toward impairment in the first cohort (Figures 3.16, 3.17). Contextual conditioning involves the amygdala as well as hippocampus, although hippocampal function is not necessary (Gerlai 2001; Maren 2008).

In line with mildly altered hippocampal performance on contextual fear conditioning but not spatial memory tasks, in vivo morphology of the basal dendritic arbor was not significantly altered in hippocampal area CA1 (Figures 4.5, 4.6). In CA3, the only significant changes were a reduction in the primary dendritic length in HET animals and an increase in dendritic complexity far from the soma on Sholl analysis in HOM neurons (Figures 4.3, 4.4). When spine density was measured, density was reduced in CA3 but not CA1 in HOM neurons (Figure 4.7). Similar to in vivo results, cultured hippocampal neurons had no significant changes in axon or dendritic measures except a reduction in primary dendrite number and length in HET neurons with a trend in HOM neurons (Figure 4.11). Spine density was not affected in vitro. As BDNF and NT4 are neurotrophic factors that increased primary dendritic outgrowth via GTPases (McAllister et al. 1995), it is possible that Rapgef6 might be downstream of these ligands, accounting for the specific deficit in primary dendrites. Upstream Rapgef6 mediators are currently unknown in the brain.

In contrast, hippocampal neurons transfected with shRNA to knock down *Rapgef6* had reduced axon length as well as dendritic length and complexity but greatly increased spine density (Figures 4.14, 4.15). Discrepancies between knock out and knock down results are probably due to a combination of off-target shRNA effects and differences between culture and

tissue conditions. Since in vivo morphology is more relevant to animal behavior and more analogous to human neuroanatomy, we emphasize in vivo results over in vitro findings.

In order to better understand hippocampal function during a behavioral task, we analyzed cFOS immureactivity as a measure of neural activation after cued fear conditioning. cFOS activation demonstrated impaired CA3 and CA1 neural activation following cued fear conditioning recall. In the dentate gyrus, HOM animal cFOS levels were reduced regardless of conditioning (Figure 3.19). Though loss of Rapgef6 only subtly affected hippocampal morphology (and the trisynaptic pathway was not directly assessed), our cFOS results imply there may be synaptic dysfunction throughout the hippocampus during fear conditioning. If this phenotype extends to other behavioral tasks, then the hippocampus may by hypoactive and inefficient at processing and recalling memories in the absence of Rapgef6.

In summary, spatial memory was intact and contextual fear memory impaired in the setting of reduced primary dendrites and CA3 spine density as well as decreased cFOS activation throughout the hippocampus. The small effect of *Rapgef6* deletion on dendritic growth and spine formation in vivo may influence neurotransmission, leading to the deficit we observed in certain hippocampal behavioral functions.

5.1.4 Rapgef6 deletion impaired amygdala function but not spine density

Both fear conditioning cohorts demonstrated impairments in contextual and cued fear learning to varying extents, although the two cohorts differed in their pretest training (Figures 3.16-17). Fear conditioning is the canonical paradigm to probe amygdala function in learning and memory (Maren 2008). Anxiety as measured by open field testing and sensory motor gating as assessed by prepulse inhibition are phenotypes that are modulated by the amygdala and were also impaired in the Rapgef6 KO mouse (Vinkers et al. 2010; Wan and Swerdlow 1997). While

amygdala lesion studies do not support a role for the amygdala in modulating baseline startle response, some mouse models report overlapping fear and startle deficits as amygdala dependent. For example, injection of acetylcholine receptor agonist into the nucleus accumbens led to deficits in startle and contextual fear conditioning as well as hyperlocomotion, a behavioral profile matching *Rapgef6* deletion (Cousens et al. 2011). This suggests that analysis of acetylcholine signaling and the nucleus accumbens may help explain the mechanism of Rapgef6 effects on the amygdala. Thus amygdala dysfunction was present in the Rapgef6 mouse across many behavioral paradigms including learned and unlearned fear.

We investigated the pathology leading to this behavior result by examining spine density in the amygdala. Spine density on the basal and apical dendritic arbors of lateral and basolateral pyramidal neurons was not affected in vivo upon Rapgef6 KO (Figure 4.8). Yet there was a significant failure of Rapgef6 HET and HOM animals to activate cFOS in the basolateral nucleus following fear conditioning (Figure 3.19). There are other examples of mouse models, such as the Stathmin knock out, with fear deficits, normal amygdala morphology, but altered amygdala neurotransmission (Shumyatsky et al. 2005). Given the strong behavioral phenotype of fear impairment with cFOS evidence for amygdala hypoactivity, either the morphology study was underpowered to detect spine density changes or the pathophysiology lies at the level of synaptic transmission strength.

5.1.5 Rapgef6 deletion did not affect prefrontal cortex

Although Rapgef6 is expressed in the prefrontal cortex, we found no evidence for dysfunctional behavior or morphology in this brain region. Rapgef6 KO animals had no significant deficits on the T maze protocol assessing prefrontal cortex dependent working memory (Figures 3.10-11) (Kellendonk et al. 2006). Moreover, in vivo analysis of the basal

dendritic arbor of layer V pyramidal neurons in the medial prefrontal cortex found no significant genotype differences (Figures 4.9-10). Our paradigms may have missed the pathology, for example a more complicated maze task or analysis of layer II/III neural morphology might be affected. Alternatively, while Rapgef6 is widely expressed it might be redundant in certain circuits but necessary in others, such as the amygdala.

5.2 Validity of *Rapgef6* deletion as a model of schizophrenia

The evidence summarized above demonstrates the validity of this mouse as a schizophrenia model. At the genetic level, recent research has validated the finding that rare CNVs are significantly associated with schizophrenia etiology (Malhotra and Sebat 2012). We identified a rare CNV affecting *RAPGEF6* and chose to model the deletion because the convergent genetic data at this gene locus make it a strong candidate for schizophrenia risk.

At the behavioral and morphological levels, the Rapgef6 KO mouse demonstrated many but not all of the archetypal schizophrenia endophenotypes amenable to rodent modeling. The Rapgef6 mouse demonstrated impairment in fear, anxiety-like behavior, and prepulse inhibition, an increase in locomotion, and diminished activation in the hippocampus and amygdala all of which are clinically relevant to neuropsychiatric disease. KO animals were hyperactive and this may represent psychomotor agitation in patients (Powell and Miyakawa 2006). Both locomotion and rearing rates have been positively linked to dopamine function, suggesting these mice may have dopaminergic imbalances in striatal circuits (Berridge 2006; Brookshire and Jones 2009). Prepulse inhibition deficit is a well-recognized and defined schizophrenia endophenotype (Powell et al. 2009). Such sensory disturbances might form the basis for hallucinations and delusions (Dulawa and Geyer 2000). HET animals had decreased prepulse inhibition, while reduced startle probably skewed HOM prepulse inhibition back to WT levels.

Schizophrenia patient meta-analyses found amygdala volume was significantly reduced (Shepherd et al. 2012) and amygdala function was diminished in fMRI studies during emotional tasks (Anticevic et al. 2012). Although postmortem analysis found no change in amygdala volume on histological analysis, amygdala gene expression results found altered synaptic and signaling pathways in patients (Berretta et al. 2007; Chance et al. 2002; Weidenhofer et al. 2006). These results have led to the proposal that amygdala dysfunction may underlie negative schizophrenia symptoms (Pietersen et al. 2007). Reduced anxiety on the open field test and diminished cued and contextual fear conditioning, in light of reduced basolateral amygdala cFOS activation, strongly suggest that there is amygdala dysfunction in Rapgef6 animals which is not captured by our spine density analysis but which may be relevant to schizophrenia pathophysiology.

Studies of the hippocampus in individuals with schizophrenia consistently yield reductions in volume, even in first episode psychosis (Adriano et al. 2012; Steen et al. 2006) and impairments in hippocampal memory tasks that contribute to cognitive symptoms (Tamminga et al. 2010). Postmortem, patient brains had decreased interneurons and fewer mossy fiber synapses (Kolomeets et al. 2007; Konradi et al. 2011). While we found that Rapgef6 KO mice had no significant changes on spatial memory tasks such as novel object recognition or the Morris water maze, they showed impairment in contextual fear conditioning, aligning with less cFOS activation in the CA3 and CA1 after fear conditioning and less cFOS in the dentate gyrus independent of conditioning. CA3 spine density and primary dendrite number were also reduced in vivo.

We did not find evidence for working memory deficits or structural changes in layer V of the medial prefrontal cortex. Postmortem analysis found layer V and VI were intact in PFC of

schizophrenic patients, limiting reduced spine density findings to layer II/III only (Glantz and Lewis 2000; Kolluri et al. 2005). Similarly, functional studies confirmed reduced PFC activation during working memory tasks (Glahn et al. 2005) and diminished working memory performance across a range of testing paradigms (Lee and Park 2005). These domains appear to be unaffected by *Rapgef6* deletion.

In comparison with other rare mutation models of schizophrenia, the Rapgef6 mouse demonstrates a certain degree of phenotypic overlap. 22q11 mice were hyperactive but had increased anxiety (Stark et al. 2008). While no schizophrenia models had impaired baseline startle, PPI was reduced following deletion of 22q11 and Dgcr8 but not Disc1 (Koike et al. 2006; Stark et al. 2008). Of those three models, only the 22q11 model displayed fear conditioning impairments, but all three had working memory deficits on the T maze and other tasks (Koike et al. 2006; Kvajo et al. 2008; Stark et al. 2008). Disc1 mice had dysmorphic adult-born neurons in the dentate gyrus, while 22q11 and Dgcr8 mice had reduced CA1 arbors and spine densities in vivo, and this held true in vitro for the 22q11 mouse as well (Kvajo et al. 2008; Mukai et al. 2008; Stark et al. 2008). Considering other neuropsychiatric disease models, we have not yet identified any mouse with reduced startle and impaired PPI in the setting of normal audition. Our results overlap somewhat, but not entirely, with the amygdala deficit in Rap1 cortical deletion mouse (Pan et al. 2008). As described above, our behavior results resemble those of nucleus accumbens injection of acetylcholine receptor agonist (Cousens et al. 2011). Thus the Rapgef6 mouse model possessed a unique behavioral and morphological phenotype.

Deletion of *Rapgef6* possibly subtly reduces neurotrophic signaling and neural adhesion for connectivity, leading to small changes in synapse number in CA3 and diminished synaptic strength or transmission in the hippocampus and amygdala. Reduced connectivity within, and

possibly between, these regions negatively impacts fear-related learning as well as unlearned fear or anxiety. The hyperlocomotion and reduced startle observed in this mouse may represent a disinhibition of exploration and a lessening of reflexive fear responses in the setting of reduced anxiety. Alternatively, these phenotypes may indicate that connectivity changes extend to the dopaminergic system. Comparison with other animal models suggests that acetylcholine and norepinephrine function could also be affected in this model and should be examined in order to better understand the pathology.

Our aim in analyzing this Rapgef6 model is to contribute to our understanding of schizophrenia pathogenesis and the development of novel treatments that target disease mechanisms. Our model is based on human genetics results, leading to etiologic validity. Rapgef6 KO mice demonstrated schizophrenia related endophenotypes such as hyperactivity, impaired prepulse inhibition, diminished fear conditioning and anxiety, and cFOS functional reductions in hippocampal and amygdala activity, yielding face validity. As these phenotypes are not specific to schizophrenia, this mouse may also inform our comprehension of anxiety disorders through the role of the amygdala in generating learned and unlearned fear, or interactions between the amygdala and hippocampus. Whether this mouse is useful for therapeutic testing and predictive validity remains to be seen.

In conclusion, this model contributes to our understanding of schizophrenia in a specific domain. Though the prefrontal cortex and hippocampus are major research targets in schizophrenia, we do not find changes in the prefrontal cortex and hippocampal deficits are minimal. Strikingly, the major phenotypes in this mouse model are reduced anxiety and fear learning, indicating the amygdala is the structure most affected by *Rapgef6* deletion. Rapgef6 may contribute to disease pathophysiology through diminished amygdala function, parallel to

findings of decreased amygdala size and function in patients with schizophrenia. Amygdala dysfunction could contribute to negative schizophrenia symptoms. Therefore, this model provides evidence about the involvement of the amygdala in negative symptoms, as opposed to the positive or cognitive symptoms typically targeted in animal systems.

5.3 Model limitations

There are several reasons why the rodent phenotype of *Rapgef6* deletion was less severe than we had predicted based on other mouse models of the Rapgef family. First, the effect of the *RAPGEF6* CNV in the patient with schizophrenia may have been modulated by additional, unidentified mutations. Such phenotype modulation is known as the two-hit hypothesis for rare mutations (Coe et al. 2012). There is direct evidence that having additional rare CNVs correlated with worsening developmental delay phenotype from the recurrent 16p12.1 deletion (Girirajan et al. 2010). This result can be interpreted more broadly to suggest that other rare or even common alleles may modify the phenotypic outcome caused by a large scale rare mutation. As discussed below, these extra hits could be modeled by crossing our line with other modifying mutations, either identified from patients or based on candidate interacting genes. For example, we could reduce compensation by crossing into deletion or hypomorph lines of other Rapgefs or Raps. There may be a variety of additional mutations or risk alleles not assessed in the CNV study that contribute to the human phenotype but are not currently modeled in the mouse.

Furthermore, the mouse mutation and genetic background may contribute to reduced penetrance. This mouse mutation does not exactly mimic the human CNV in terms of which gene regions were deleted; instead the mutation in the mouse models the effect of a catalytically dead protein that Western blotting demonstrates is not expressed (Yoshikawa et al. 2007). Though the Genome Browser does not predict any remaining functional *Rapgef6* isoforms in the

human or mouse genomes after the respective deletions, it is possible there are unidentified isoforms in each species that partially compensate for the deletion effects. Concerning the genetic background, this mutation was generated in 129Sv oocytes and then backcrossed onto the C57Bl/6 background (Yoshikawa et al. 2007). As mentioned in Chapter 3, inbred strains have varying phenotypes at baseline, with C57Bl/6 considered moderate on anxiety measures and strong on maze tasks (Crawley 2008; D'Hooge and De Deyn 2001). Crossing the *Rapgef6* deletion onto another background would be interesting as a method for dissecting the effects of the deletion from the background. For example, a more anxious background might ameliorate the fear conditioning phenotype, while a background with worse hippocampal function might uncover spatial memory deficits. Thus, the accuracy of how the mutation mimics the human genetic findings and the subtle genome-wide influences of background strain may limit schizophrenia-related endophenotype expression.

Interestingly, the phenotypes of HET and HOM animals did not always align. For example, while both genotypes had reduced neural activity on cFOS analysis, HET animals did not demonstrate reduction in CA3 spine density or some behavioral deficits. Therefore there is dissociation between the phenotypes by genotype, implying the behavioral and morphological deficits following complete *Rapgef6* deletion may not be entirely due to neural activity changes. Alternatively, compensation mechanisms are more likely to occur in complete deletion as opposed to the heterozygous state. This could explain why on certain phenotypes HET but not HOM animals were significantly affected. A careful comparison of genotype results helps inform potential mechanisms in this mouse model.

Finally, the evolutionary differences in brain development between humans and mice may affect mutation penetrance and relevance. Mice have been used to model human disease for

decades because there is high genetic homology between mice and humans as well as strong similarity between brain region specific gene expression profiles (Strand et al. 2007), however, this does not guarantee that individual genes will serve analogous purposes in development and function. Though human and mouse Rapgef6 proteins share 90% amino acid identity (blast.ncbi.nlm.nih.gov), it is possible they do not share the same expression pattern or functionality in both species, leading to differing phenotypes upon deletion. Moreover, we aim to model schizophrenia, a uniquely human disease state that is challenging to translate into animal models. While there are endophenotypes of cognitive and negative symptoms discussed above that can be accurately measured in both species, many symptoms are not amenable to modeling. Interspecies differences may be masking or invalidating phenotypes in our model.

5.4 Future directions

5.4.1 Human genetics

With neuropsychiatric genetics progressing at a rapid pace, it is likely that more mutations in *RAPGEF* gene family members will be discovered. We intend to monitor results from exome and genome sequencing as well as future CNV studies for additional mutations affecting this gene family. To date, CNV results suggest large scale mutations of *RAPGEFs* in schizophrenia are private and very rare as our laboratory was the only one to uncover such CNVs in the literature. While there were no de novo pathological mutations affecting RAPGEFs in published exome sequence data, there will be far more genetic data available shortly as more consortia and laboratories invest in sequencing technology. Identification of additional mutations would strengthen the association of this gene family with disease and thus bolster the etiological validity of Rapgef models. Moreover, smaller scale mutations could identify critical protein regions or regulatory domains for RAPGEF function.

5.4.2 Animal behavior

Although we have assessed the *Rapgef6* deletion mouse for phenotypes related to locomotion, spatial and fear learning, and sensory processing, behavioral testing in this mouse line is far from exhausted. Additional behavioral phenotyping would clarify known deficits and possibly identify novel dysfunction.

One of the domains to further explore is anxiety-like and fear behavior. While our open field results suggested reduced anxiety, more validated paradigms to pursue are the elevated plus maze and light-dark box (Bailey and Crawley 2009). Furthermore, the fear conditioning impairment could be more accurately assessed by adding habituation to remove the novel contextual fear generalization and extinction training to test prefrontal cortex contributions (Jacobs et al. 2010; Knapska and Maren 2009). Anxiety is an important behavioral trait to assess because there is high comorbidity between schizophrenia and anxiety disorders (Achim et al. 2011). It would be useful to determine more accurately whether the Rapgef6 mouse phenotype also includes comorbid behavioral changes and the extent of amygdala dysfunction.

There are no Rapgef6-specific agonists or antagonists we can use to alter function, nor are there any pharmaceutical options that target up- or downstream pathways that are specific to Rapgef6. There is a specific agonist of Rapgef3 and 4, the cAMP mimic 8CPT (Bos 2006). Administering this compound to Rapgef6 KO mice would test whether Rapgef3/4 can compensate to rescue behavioral deficits. Since 8CPT increased prepulse inhibition and fear learning in wild type animals and these domains are affected in Rapgef6 mice, it is likely such an intervention would be beneficial (Kelly et al. 2009). While a positive response would not prove that Rapgef3/4 and their associated pathways are affected following *Rapgef6* deletion, it would at least offer a treatment and suggest pathways that can compensate for a dearth of Rapgef6.

Given the two-hit hypothesis of neurodevelopmental disease, it might be useful to cross the Rapgef6 line with other modifying mutations (Coe et al. 2012). Additional rare CNVs were not identified in individuals harboring *RAPGEF6* and *2* CNVs, however they may carry more common large scale mutations or rare single nucleotide variants that could interact with the *RAPGEF* loci to increase the genetic risk of schizophrenia. Modifying mutations would presumably affect genes that interact with RAPGEF6, thereby informing our understanding of its pathways. Alternatively, we could test compensation by crossing Rapgef6 KO animals with those lacking Rapgef3 or Rapgef4. A worsening of phenotype would indicate that other family members typically counterbalance the loss of Rapgef6 function.

5.4.3 Cellular pathophysiology

Currently, we cannot explain which neurobiological alterations led to behavioral impairments in the Rapgef6 mouse model since neural morphology was not greatly altered and cFOS activation findings only apply to cued fear conditioning. We are still seeking the level of affected function. Our behavioral results were similar to the Rap1 mouse model that had increased cortico-amygdala synaptic transmission (Pan et al. 2008), in addition spine density was reduced in the hippocampus yet overall morphology was intact; therefore we predict the dysfunction occurs at the synaptic level. This hypothesis could be tested via slice electrophysiology recording in the hippocampus and amygdala. Furthermore, to test specific neurotransmitter systems such as dopamine and acetylcholine, which might be influenced by Rapgef6 based on comparison of our phenotype with the literature, we could employ specific receptor agonists and antagonists during slice electrophysiology. Schizophrenia does not have dramatic neuropathological findings, supporting the idea that it is a disease of circuit connectivity (Arguello and Gogos 2012). If *Rapgef6* deletion affects synaptic function and

circuitry in schizophrenia-relevant regions such as the hippocampus and amygdala, that would be further evidence for etiological validity.

Rapgef6 modulates cadherin and integrin signaling in lymphocytes, therefore we predict these adhesion pathways are also altered in neurons (Dube et al. 2008; Yoshikawa et al. 2007). Integrin and cadherin families are critical to neural migration and morphology (Clegg et al. 2003; Suzuki and Takeichi 2008). To assess these pathways, we could analyze cortical migration using BrdU or electroporation labeling or cortical layer immunohistochemistry. Functional impairment of cadherins and integrins can be easily quantitated via neural adhesion assays in vitro (Denda and Reichardt 2007). These experiments could determine whether integrin and cadherin expression and function are altered, providing clues to a mechanism by which *Rapgef6* deletion leads to cellular phenotypes.

5.5 Conclusion

In conclusion, we have described human mutations affecting the *RAPGEF* family and have translated one of these mutations into a mouse model with *Rapgef6* deleted. Behavioral and morphological experiments demonstrated deficits in the amygdala and hippocampus, brain regions implicated in schizophrenia pathophysiology at a variety of levels. The exact cause of Rapgef6 pathology has not yet been determined, but the dysfunction appears to be due to subtle spine density changes as well as synaptic hypoactivity. Continued investigation may yield a deeper understanding of amygdala and hippocampal pathophysiology as well as novel therapeutic targets in schizophrenia.

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