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Investigating the Role for Dysbindin in Hippocampal-Dependent Learning and Memory: Glutamatergic Mechanisms

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Investigating the Role for Dysbindin in Hippocampal-Dependent Learning and Memory:

Glutamatergic Mechanisms

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor

of Philosophy in Psychology

by

Bryant Lance Horowitz

2012

ABSTRACT OF THE DISSERTATION

Investigating the Role for Dysbindin in Hippocampal-Dependent Learning and Memory:

Glutamatergic Mechanisms

By

Bryant Lance Horowitz

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2012

Professor J. David Jentsch, Chair

Schizophrenia is a complexly defined disorder with many genes contributing to the high

heritability, but the degree to which these genes contribute to the pathophysiology is unclear.

DTNBP1, which codes for the dysbindin protein, has been identified as one of the candidate risk

genes in schizophrenia. Dysbindin is one of eight proteins that make up the biogenesis of

lysosome-related organelles complex 1(BLOC-1), which includes pallidin, muted, snapin,

cappuccino, and BLOC-1 subunits 1, 2, and 3.The BLOC-1 complex is involved in trafficking of

vesicles. Variation in *DTNBP1* has been associated with increased risk of schizophrenia in

behavioral genetic studies of humans, as well as deficits in cognition and memory phenotypes.

Studies on the physiology of the dysbindin protein reveal reduction in expression within

forebrain glutamatergic neurons, playing a role in trafficking of vesicles in the BLOC-1 complex. The sandy mouse, which carries a mutation of the *DTNBP1* gene and does not code for the dysbindin protein, has been used to study behavioral, cellular and physiological processes; revealing compromised glutamatergic neurotransmission and deficits in working memory and cognitive function similar to patients with schizophrenia. Sandy mice were used to examine hippocampal-dependent tasks of memory. Homozygous sandy mice showed normal locomotor movement and some learning in a Morris water maze task compared to heterozygous and wild type controls, but were impaired in spatial memory. Sandy mice also showed deficits in recognition memory and contextual memory compared to heterozygous and wild type controls. To investigate the role of dysbindin as BLOC-1 dependent the pallid mouse, which has a null mutation in the Pldn gene which produces no pallidin protein, was examined in context and recognition memory tasks and showed deficits in fear generalization and recognition memory; but not identical to deficits shown in sandy mice. Taken together, this data indicates that dysbindin may be acting independently of BLOC-1.

The dissertation of Bryant Lance Horowitz is approved.

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2012

Dedicated to my parents, the late Joel and Judi Horowitz, to my wonderful wife Jessica, and to

my son, Carter Joel.

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Chapter 1

Introduction

Schizophrenia is a mental disorder that affects approximately 1% of the population, with a heritability of approximately 80% (Cardno & Gottesman, 2000). It is characterized by positive symptoms, such: as hallucinations, delusions, and thought disorders, and negative symptoms that include: flat affect, deficits in social interaction, emotion, and motivation, and cognitive deficits such as impairments in attention, memory, associative learning, and working memory, and associative learning.

Multiple hypotheses and lines of study reveal schizophrenia to be a multifactorial disorder influenced by genetic, neurodevelopmental, and social factors (Cannon, 2005; Cardno & Gottesman, 2000; Kumamoto et al., 2006). The neurodevelopmental hypothesis of schizophrenia posits that abnormalities of early brain development increase the risk for the subsequent emergence of clinical symptoms. This hypothesis points to abnormal brain development as a significant factor in schizophrenia. Brain abnormalities such as reduced gray matter and hippocampal volume are seen in imaging and postmortem studies, as well as abnormalities in neurotransmission (Talbot, 2004; Weickert et al, 2004; Weickert et al., 2008).

Although this disease is highly heritable, the pathophysiology is not fully known, nor are the specific genetic and environmental factors. Although genetic factors contribute substantially to risk for the disorder, specific disease-promoting alleles are elusive (Benson, Newey, Martin-Rendon, Hawkes, & Blake, 2004), due in part to the fact that the diagnostic phenotype is likely an inappropriate trait to use in gene finding analyses. Rather, key quantitative neurobehavioral features of the disorder may be more fruitful to use in discovering the genetic influences on the disorder.

Factors considered in cognitive impairment include: information processing, abstract categorization, executive function, cognitive flexibility, attention, memory, and visual

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processing. The deficits in these factors are of particular interest, as they are increasingly viewed as the core of problems associated with schizophrenia and may be predictive of functional outcome (O'Tuathigh et al., 2006; Ross, Margolis, Reading, Pletnikov, & Coyle, 2006). Additionally, they may represent important intermediate endophenotypes that can direct future mechanistic research meant to reveal the behavioral abnormalities in schizophrenia.

Endophenotypes

Endophenotypes are measurable neurobehavioral processes along the pathway between disease and distal genotype and have emerged as an important concept in the study of complex neuropsychiatric diseases, such as schizophrenia (Cannon, 2005; Cannon & Keller, 2006). Endophenotypes are heritable and always quantitative traits that may not be readily apparent in routine clinical exams of affected individuals yet may reflect neurobiological features underlying the disease and may be useful in genetic linkage studies (Ross et al., 2006). Endophenotypes represent clues to genetic underpinnings, other than the disease syndrome itself, because they theoretically share more variance with particular genes than does the complex multidimensional disease which probably depends on the contribution of many different genes.

Studies of schizophrenia have defined it as a complex disorder, likely involving multiple genes that contribute to a modest degree of risk (Bearden et al., 2007). There are however, certain conditions that confer a substantially elevated risk for schizophrenia and may therefore represent a simpler trait. Ideally, endophenotypes can serve as dissected components of the complex schizophrenia phenotype; reflecting fewer genes and thereby reducing the complexity of the genetic analyses required to identify contributing genes (Turetsky et al., 2007).

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There are several proposed endophenotypes that are seen in patients diagnosed with schizophrenia. Included amongst these are cognitive deficits attributable to dysfunction of the prefrontal cortex. There are numerous studies showing that working memory deficits, problems with executive function, as shown via the Wisconsin Card Sort Test (Cannon, 2005; Glahn et al., 2003), and associated abnormalities (Fallgatter et al., 2006; Turetsky et al., 2007) are heritable quantitative traits that are associated with disease liability. Spatial memory has also been explored as an endophenotype, using a spatial delayed response task (Glahn et al., 2003).

There is debate about whether behavioral or more biologically-grounded measures, (brain structure, metabolism, receptor expression), represent superior endophenotypes. Some camps have used behavioral expressions to identify them; others have argued that structural and functional measures of brain function are suggested endophenotypes.

Deficits in sensory motor gating and eye tracking have been identified as proposed endophenotypes in schizophrenia (Gottesman & Gould, 2003; Turetsky et al., 2007). Neuropsychological tests have been used to determine these deficiencies, such as assessments of P50 suppression and prepulse inhibition of the startle response, in conjunction with electrophysiological procedures such as evoke-related potential (ERPs) and electromyographic measures (Turetsky et al., 2007). Eye-tracking dysfunction has also long been identified in schizophrenia (Gottesman & Gould, 2003). The heritability of these deficiencies has been extensively addressed, and this line of work has led to linkage studies of gene and chromosomal regions involved.

Neuroimaging studies have been used to investigate genetic influences on brain structure (Bearden et al., 2007; Narr et al., 2009; Preston et al., 2005; Watson et al., 2012). For example, neuroanatomical abnormalities in schizophrenia include a reduction of neuropil in the prefrontal

cortex, which underlie hypofrontality (Talbot, 2004; Weickert et al., 2008). Hypofrontality is a marker of PFC dysfunction that arises during demanding cognitive tasks. This is seen particularly in the dorsolateral prefrontal cortex (DLPFC) by using functional magnetic resonance imaging (MRI) with patients or using electrophysiological studies while performing a working memory or executive functioning task (Cannon, 2005).

Other Brain Regions

Deficits seen in the temporal lobe in schizophrenia relate mostly to episodic and declarative memory problems (learning and recalling), emotional detachment and even problems associated with language (Cannon, 2005; Gottesman & Gould, 2003). Additionally, the hippocampus is shown to be heavily involved in the processing of declarative and spatial memory; while the amygdala is implicated in emotional learning and memory (Hanlon et al., 2006; Harrison & Eastwood, 2001; Watson et al., 2012). There are many genetic studies associated with these problems, seen in schizophrenic patients and their family members (Cannon, 2005; Gottesman & Gould, 2003).

Genetic Determinants of Schizophrenia

Several genes have been proposed as susceptibility genes for schizophrenia including: Disrupted-In-Schizophrenia 1 (DISC1), dysbindin (*DTNBP1*), catechol-O-methyltransferase (COMT), Dopamine receptor D4 (DRD4), neuregulin 1(NRG1), and G72 (Chiba et al., 2005; Collier& Lia, 2003). Dysbindin is among the strongest genetic associations to these symptoms; perhaps through its ability to modulate excitatory glutamatergic function in the medial temporal lobe (Cannon, 2005; Numakawa et al., 2004; Talbot et al., 2004).

DTNBP1

One of the leading susceptibility genes for schizophrenia is the *DTNBP1* gene which encodes for the dysbindin-1 protein is located at the chromosomal location 6p22.3 (Bray et al., 2005; Owen et al., 2004). This gene codes for a 40–50-kDa protein expressed in neurons in many areas of the mouse and human brain and is named for its capacity to bind α and β-dystrobrevins: proteins that are part of the dystrophin glycoprotein complex (DPC) (Benson et al., 2001; Kendler, 2004). The dysbindin protein is located in postsynaptic densities (PSD) in muscles. Dysfunction of the dystrobrevins in conjunction with the DPC in muscles has been implicated in Duchenne Muscular Dystrophy (Benson et al., 2001). Dysbindin also contains a coiled-coil domain (CCD) for interaction with other proteins.

There are numerous studies that have found an association between schizophrenia and single nucleotide polymorphisms (SNPs) within the *DTNBP1* gene in human populations including: Finnish, German, Irish, Australian, and Japanese patients (Numakawa et al., 2004; Schwab et al., 2004; Straub et al., 2002; Voisey et al., 2010; Williams, O'Donovan, & Owen, 2004). Bray et al. (2005) reported that certain risk variants for *DTNBP1* are associated with lowered expression of mRNA and protein, irrespective of diagnosis (Burdick et al., 2006), suggesting that genetic mechanisms that increase risk in schizophrenia function to lower protein expression. Dysbindin is believed to play a role in synaptic plasticity and signal transduction (Arnold, Talbot, & Hahn, 2005; Numakawa et al., 2004). This gene could be involved in endophenotype markers such as cognitive dysfunction (Gornick et al., 2005), and this phenotypic representation must be taken into account.

Variation in DTNBP1 and Schizophrenia

One way to *characterize* function of *DTNBP1* is to explore the relationship between haplotypes of the gene in schizophrenia and/or its endophenotypes. Haplotypes are distinctive sets of alleles that incorporate a group of markers (Kendler, 2004; Voisey et al., 2009). There is evidence showing that particular haplotypes spanning *DTNBP1* are associated with risk for schizophrenia and more specifically with decreased general cognitive ability in patients and in healthy volunteers. For example, dysbindin risk haplotypes in humans associate with significantly poorer performance in spatial working memory (Glahn et al., 2003) and in a go/no-go anteriorization task (Fallgatter et al., 2010), a phenomenon attributable to reduced activation in the dorsolateral prefrontal cortex (DLFPC) and hippocampal formation.

Postmortem evidence suggests that the dysbindin protein is expressed in regions of the brain that are critical to cognitive function, and that its expression is reduced in risk haplotype carriers (Burdick et al., 2006). Contrary to the finding by Tang et al. (2009), dysbindin protein expression is reduced in DLPFC, as well as the dentate granule and polymorph cells and in hippocampal cells in the dentate gyrus, CA2, and CA3 (Talbot et al., 2004; Weickert et al., 2004; Weickert et al., 2008). Given postmortem evidence that dysbindin is under-expressed in the dorsolateral prefrontal cortex, influence of dysbindin on this cortical region may be crucial for modulating cognitive function.

Neurotransmitter Function in Schizophrenia and Dysbindin

It has long been postulated that chemically, an overexpression of dopamine in the striatum, specifically up-regulation of D2 receptors, leads to the psychosis and positive symptoms. This is compounded by a downregulation of D1 dopamine receptors in the prefrontal

cortex, referred to as *hypofrontality*, may be responsible for the cognitive deficits and the negative, antisocial aspects of schizophrenia (Weinberger et al., 2001). Dopamine levels are suppressed by dysbindin-1 according to in vitro studies in rat PC-12 cells (Kumamoto et al., 2006). However, midbrain dopamine is reported to have increased release, as well as a higher turnover rate in both PFC and hippocampus (Muratoni, 2007). Knockdown of the dysbindin protein has also shown to affect internalization as well as signaling of D2 receptors, but has no effect on D1 receptors (Iizuka, Weinberger, & Straub, 2007). Reductions were observed in the DRD2 gene which encodes the D2 receptor.

   More recently, abnormalities in glutamatergic function seem to interact with dopaminergic dysfunction in this disease, with many studies reporting impaired glutamatergic release (Collier, et al., 2003; Kendler, 2004).

   Glutamate has complex interactions with dopamine and likely plays an active role in schizophrenia. Dysbindin is well positioned to impact glutamate neurotransmission, thereby influencing a number of cortical processes underpinning neurocognition, including hippocampal long-term potentiation and delay-related neural activity. Furthermore, dysbindin likely modulates glutamatergic presynaptic connections in the hippocampal formation and PFC (Owen et al., 2004; Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008), which can lead to an imbalance in these systems in a haploinsufficient condition. Knockdown of endogenous dysbindin causes a decrease in basal and release glutamate levels (Numakawa et al., 2004), showing a role in excitatory neurotransmission.

   The action of glutamate function is mediated at subtypes of ionotropic receptors; namely AMPA and NMDA. NMDA receptors play an important role in a variety of brain functions, including memory and learning and synaptic plasticity. Induction of LTP requires NMDA

receptor activation (Bliss & Collingridge, 1993; Tang et al., 2009). A reduction in NMDA receptors has been observed in the schizophrenic brain, where altered levels of glutamate affect neuronal activity and plasticity (Weickert et al., 2004; Karlsgodt et al., 2011). It was also discovered that lower expression of dysbindin correlated with an increase in Vglu-T1 in the hippocampus (Talbot et al., 2004). Therefore, there is conceptual and empirical evidence for a hypoglutamatergic state in schizophrenia.

In addition to receptor function, kinetics of glutamatergic release are compromised in the absence of dysbindin in a mouse model (Chen et al., 2008). Absence of dysbindin causes larger glutamate vesicle sizes, slower quantal release, lower release probability and a smaller population of the readily releasable pool within chromaffin cells in mice. This suggests a further role of dysbindin in the pathogenesis of schizophrenia, at the presynaptic level.

Neurobiology of Dysbindin-1

Human dysbindin transcripts are expressed ubiquitously throughout the brain including DLPFC and hippocampus, as well as cerebellum, substantia nigra, caudate nucleus, amygdala, thalamus, hypothalamus, pons, medulla, and spinal cord (Benson et al., 2001; Straub et al., 2002; Talbot, 2004). More specifically, dysbindin is also found in axons with large synaptic termini in the mossy fibers of the hippocampus, dorsal cochlear nuclei, and cerebellum (Benson et al., 2001).

Studies show the dysbindin protein is expressed both presynaptically and postsynaptically in the central nervous system (Fallgatter et al., 2006; Weickert et al., 2004). Postsynaptically dysbindin is expressed in postsynaptic densities, and is believed to be involved in signal transduction (Benson et al., 2001; Talbot et al., 2009). Presynaptically the protein is believed to

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be involved in intracellular mechanisms including vesicular trafficking and docking of glutamate (Chen et al., 2008; Talbot et al., 2004).

There are three major isoforms of dysbindin-1 expressed in the brain: dysbindin-1A, dysbindin-1B, and dysbindin-1C (Talbot et al., 2011; Tang et al., 2009). Dysbindin-1A is almost exclusively associated with postsynaptic densities (PSD), 1B almost exclusively with synaptic vesicles, and isoform 1C is involved with both pre and postsynaptic mechanisms, mostly seen in PSDs. These different isoforms of dysbindin-1 have different functions and they appear to be the most commonly expressed transcripts. Isoform 1A is the full length gene and differs from 1B only in the C-terminus region. 1A differs from 1C in the absence of an N terminus in front of the CCD. In the DLPFC of schizophrenic patients, reductions in isoform 1C were shown, with reductions up to 60% (Tang et al., 2009), possibly reflecting both pre and postsynaptic function; however, mRNA levels were shown to be increased for 1A and 1B, compared to control patients.

Additionally, dysbindin has been associated with the biogenesis of melaonsomes, lysosomes, and their related organelles as part of a group of proteins called the BLOC-1 complex in the presynaptic terminal (Falcón-Pérez & Dell'Angelica, 2002; Morris et al., 2008; Nazarian, Starcevic, Spencer, & Dell'Angelica, 2006). Dysbindin interacts with other proteins such as SNAP25, a SNARE protein involved in vesicular docking (Muratoni, 2006; Numakawa et al., 2004), as well as snapin (Feng et al., 2008; Talbot et al., 2008), pallidin, muted, and subunit 2 of the BLOC-1 complex (Li et al., 2003; Nazarian et al., 2006; Talbot et al., 2009).

BLOC-1

The BLOC-1 complex is located presynaptically on endosomes, and is made up of 8 protein subunits: dysbindin, muted, cappuccino, snapin, pallidin, and BLOC-1 subunits 1, 2, and

3, and these subunits are tightly bound to one another. It serves to regulate membrane protein targeting to synaptic vesicles, lysosomes, and lysosome-related organelles (Falcón-Pérez, Starcevic, Gaustam, & Dell'Angelica, 2002; Morris et al., 2008; Mullin et al., 2011; Ryder & Faundez, 2009). In addition to schizophrenia, BLOC-1 subunits are implicated in Hermansky-Pudlak Syndrome, a type of disease characterized by albinism, prolonged bleeding due to abnormal platelet dense granules, and bruising (Falcón-Pérez et al., 2002; Li, 2003; Nazarian et al., 2006). BLOC-1 may be necessary in the neonatal period for proper neurite outgrowth and normal cell development (Ghiani et al., 2010). Dysbindin is believed to be exerting its presynaptic function as part of this complex.

It is hypothesized that BLOC-1 plays a role in the pathophysiology of schizophrenia. First, the absence of any one subunit of BLOC-1 triggers the reduction of other BLOC-1 subunits (Falcón-Pérez et al., 2002; Ghiani et al., 2010). This is evidenced by null alleles in animal models, most notably mice, which show reduced expression of other BLOC-1 complex proteins. The BLOC-1 implication in schizophrenia has 3 predictions. First, BLOC-1 deficient mice should have behavioral phenotypes consistent with schizophrenia. Second, genetic polymorphisms in *DTNBP1* should lead to reduced dysbindin in the brains of individuals with schizophrenia; and third, brain tissue from postmortem patients should also possess reduced levels of other BLOC-1 subunits.

Pallidin Gene

Another subunit of BLOC-1 that directly interacts with the dysbindin protein is the pallidin gene (*PLDN*) (Falcón-Pérez & Dell'Angelica, 2002; Huang, Kuo, & Gitschier, 1999). Although the role of pallidin in schizophrenia is unknown, the pallidin protein is reduced in

schizophrenic patients and in mice with mutation of the *Dtnbp1* gene. This phenomenon is also observed for dysbindin protein expression in a mouse model which has no pallidin expression (Ghiani et al., 2010). The *PLDN* gene is ubiquitously expressed and encodes a novel 20 kDa protein, similar to the dysbindin protein in both function and location (Falcón-Pérez & Dell'Angelica, 2002; Moriyama & Bonifacino, 2002)

Animal Models of Schizophrenia

Animal experiments also demonstrate effects of cognitive dysfunction in the prefrontal cortex and hippocampus of nonhuman primates and rodents, similar to patients with schizophrenia (O'Tuathigh et al., 2006; Straub et al., 2002). Current animal models of schizophrenia are designed to test specific causative or mechanistic hypotheses regarding these abnormalities associated with the disease, namely cognitive impairment. With animal models, we can examine the causal relationship between genetic and environmental alterations and behavioral abnormalities, and examine intermediate endophenotypes (Amann et al., 2010). The most appropriate use of many current animal models is in the testing of narrowly focused hypotheses regarding specific aspects of the disorder. Animal experiments demonstrate many behavioral effects via pharmacological, behavioral, and physiological studies. For instance, treatment of rodents with NMDA receptor antagonists produces behaviors which model not only psychotic symptoms, but negative and cognitive deficit schizophrenic-like symptoms.

Genetic animal models involving targeted mutation have the potential to inform of the role of a given susceptibility gene on development and behavior of the whole organism and on whether disruptions of gene function is associated with schizophrenia-related structural and functional deficits (O'Tuathigh et al., 2006; Amann et al., 2010). One way to study this gene, and

its associated phenotypes, is to examine the behavioral and physiological functioning of mice that carry a null mutant dysbindin gene (Talbot et al., 2009). In mice, dysbindin is expressed in the axon terminals in the cerebellum and hippocampus in the adult, and in the PFC (Chiba et al., 2005; O'Tuathigh et al., 2006), similar to the human ortholog of the gene. Therefore, mice may represent a good tool with which to explore the functions of dysbindin.

Sandy Mouse as a Model

The "sandy" mouse carries a null mutation in *Dtnbp1* and has been used to model Hermansky-Pudlak Syndrome (Li et al., 2003), discovered as a spontaneous mutation in the DBA/6J strain of mouse. This mouse model becomes a tool to understand physiologic compositions in the brain and cognition modulated by dysbindin. The role of dysbindin has also been studied in the sandy mouse in relation to glutamate and dopamine neurotransmission (Chen et al., 2008; Jentsch et al., 2009; Iizuka et al., 2007; Karlsgodt et al., 2011; Murotani et al., 2007; Numakawa et al., 2004; Talbot et al., 2006). The mutation results in reduced levels of the protein in heterozygotes and is undetectable in homozygotes. The mutated gene is still transcribed, but the transcripts lack a string of amino acids, which result in the gene not coding for the dysbindin protein (Talbot et al., 2009).

Behavioral Abnormalities

The sandy mouse has been used extensively in numerous behavioral studies investigating both physical and cognitive function. The original DBA/6J strain was used in majority of the behavioral studies and the mice within this strain are normal in basic sensory and motor functions, but have deficits including: loss of audition and vision, abnormal irises, and enhanced responses to stress, as well as decreases in responding to dopamine agonists (Talbot et al., 2009)

Specifically, there are four main mutations in the DBA mouse, in the cadherin 23 gene which leads to age-related hearing loss, glycoprotein and tyrosine-related genes associated with pigmentary glaucoma, and hemolytic complement, which impairs inflammatory responses to infection (Talbot et al., 2009) .

Another strain of sandy mouse has been backcrossed to the C57Bl/6J strain. The C57Bl/6J strain appears to be normal in body characteristics, sensory abilities, neuromuscular strength, and sensorimotor reflexes, and does not have the mutations seen in the DBA/6J strain (Cox et al., 2009; Talbot et al., 2009); which represents a better mouse model to explore cognitive functions associated with dysbindin. Physically, sandy mice have produced both increases (Cox et al., 2009) and decreases in locomotor activity in an open field maze (Bhardwaj et al., 2009; Hattori et al., 2008; Takao et al., 2008). Other physical behaviors include decreases in sensitivity to thermal pain (Bhardwaj et al., 2009) and an increase in motor balance skills (Cox et al., 2009). Deficits have also been shown in negative behaviors, such as reduced contact with other mice in the social interaction task (Feng et al., 2008; Hattori et al., 2008), as well as abnormalities in working memory, as evidenced by the delayed-nonmatch-to-position task (Jentsch et al., 2009; Karlsgodt, et al., 2011).

Hippocampal function has also been studied in the sandy mouse. Structural and functional neuroimaging studies of the sandy mouse show decreased basal activity in CA1 as well as the dentate gyrus in sandy mice (Lutenkoff et al., 2012, which coincides with alterations in dysbindin protein expression in SCZ patients. Altered function of the dorsal hippocampus was also shown, which may affect sensory processing associated with hippocampal function. Deficits have been shown in memory retention and spatial skills that require the hippocampus (Bhardwaj et al., 2009; Cox et al., 2009; Feng et al., 2008; Hattori et al., 2008; Takao et al.,

2008), such as decreased entries in an elevated plus maze (Hattori et al., 2008). There are decreases in memory for intermediate (1-hour delay) and long-term (24-hour delay) memory tasks, as well.

What we do not know from these studies is whether the deficits in function and behavior of the null mutation dysbindin mice are definitively due to pre or postsynaptic phenomena. We also do not know if the effects are due solely to absence of dysbindin, or if the BLOC-1 complex is playing a role in the abnormalities seen within the sandy mouse.

Dissertation Aims

The studies reported in the current dissertation represent basic research in behavioral neuroscience. The specific aim was to observe behavior of intermediate and long-term memory in hippocampal function in mice with no expression of the dysbindin protein compared to their wild type and heterozygous littermates. Specifically, allocentric spatial long-term memory, intermediate recognition memory, and long-term contextual memory were examined. The secondary aim was to compare the sandy mice to another mouse with a null mutation for the pallid gene, expressing no pallid protein. The pallid protein interacts directly with the dysbindin protein within the BLOC-1 complex. The performances of both the sandy and pallid mice were then compared to determine whether the actions of dysbindin may be dependent or independent of the BLOC-1 complex.

Chapter 2

Dysbindin-Deficient Mice Exhibit Forms of Hippocampal-Dependent Memory Impairment

Abstract

Schizophrenia is complexly determined, involving multiple genetic and environmental factors,
yet the specific susceptibility genes remain poorly understood. One of the candidate genes for
SCZ that has been previously described is *DTNBP1*, which codes for the dysbindin protein.
Dysbindin-1 protein levels are reduced in the prefrontal cortex and hippocampus of SCZ
patients, an effect proposed to impair glutamatergic neurotransmission. Collectively, this chain
of events could explain some of the cognitive deficits observed in patients with schizophrenia.
To study the role dysbindin-1 in long-term memory function, we used homozygous mutant sandy
mice (-/-) which harbor a spontaneous genomic deletion resulting in a null-mutation of *DTNBP1*,
as well as heterozygous (-/+) and wild type littermate controls on a C57Bl/6J background. All
mice were assessed for spatial learning and memory in the Morris water maze, as well as
recognition memory in a novel object task and context memory in a contextual fear conditioning
task. Sandy mutant mice showed few differences during learning in the water maze, but did show
deficits in a memory retention probe trial. In the novel object recognition task, sandy mice
exhibited deficits in recognition memory for objects. Contextual fear conditioning revealed
deficits in contextual memory. Taken together, these data suggest disruption of dysbindin-1 leads
to deficits in hippocampal-dependent forms of memory

Introduction

　　Schizophrenia is a heritable, complexly determined disorder that involves genetic and environmental risk factors and affects approximately 1% of the population (Cannon, 2005; Kumamoto et al., 2006). The Dystrobrevin binding protein (dysbindin-1), which is coded for by the *DTNBP1* gene, has been identified as a promising molecular influence of risk for Schizophrenia and its cognitive endophenotypes, such as declarative memory, working memory, and executive function (Ross et al., 2006; Schwab et al., 2002; Straub et al., 2002) . DTNBP-1 risk haplotypes are with deficits in spatial working memory and associated tasks, as well as general cognitive decline (Glahn et al., 2003; Wolf, Jackson, Kissling, Thome, & Linden, 2009). These cognitive deficits can serve as a phenotypic marker to examine genotypic association as a cognitive endophenotype.

　　Dysbindin-1 protein expression in the brain is wide-spread (Benson et al., 2001; Talbot et al., 2004; Talbot, et al., 2006) and is localized to neuronal cell bodies in synaptic vesicles and postsynaptic densities, including in the axon terminals of glutamatergic synapses (Talbot, 2004). Through expression in these locations, it likely influences neurotransmission via influencing trafficking of vesicles (Talbot et al., 2004; Karlsgodt et al., 2010), and an absence of the protein affects the kinetics of neurotransmitter release (Chen et al., 2008; Numakawa et al., 2004). Because there is evidence for reduced protein expression in both mPFC and the hippocampus of postmortem tissue of schizophrenic patients (Straub et al., 2002; Talbot, 2004; Weickert et al., 2004), diminished dysbindin expression could affect glutamatergic neurons within the hippocampus leading to problems associated with hippocampal-dependent forms of memory, including spatial learning and recognition memory.

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Sandy mice harbor a spontaneous genomic deletion in the coding region of *DTNBP1* which does not code for the dysbindin protein (Li et al., 2003), providing a model system in which to examine the relationship between dysbindin expression and hippocampal function, as assessed by behavioral tasks, in order to draw inferences to patients with schizophrenia who show similar deficits (Bhardwaj, 2009; Cox et al., 2009; Feng et al., 2008; Hattori, et al., 2008; Jentsch et al., 2009; Takao et al., 2008).

In this study, we sought to investigate the role that dysbindin plays on hippocampal function by looking at allocentric spatial learning and memory using the Morris water maze task and its effect on long-term memory assessed 24 hours after training (Morris, 1984). We also looked at recognition memory using an intermediate delay of one hour after the familiarization of mice with the original object (Mumby, et al., 2001). We hypothesized that the null mutation of the dysbindin gene would result in deficits attributed to hippocampal function and thus impair both spatial and recognition memory.

Methods

Animals

All studies were performed on dysbindin mutant mice which had been backcrossed to the C57Bl/6J background (Jackson Laboratories, Bar Harbor, Maine). All mice were between 60 and 136 days old. Experimental mice were generated by heterozygote crosses, allowing for direct comparisons among homozygous mutants, heterozygotes, and wild type littermate control subjects. Genotypes were determined by polymerase chain reaction. The weight product [472 bp] was amplified with the following primers: TGAGCCATTAGGAGATAAGAGCA and AGCTCCACCTGCTGAACATT. The homozygous dysbindin (-/-) product [274 bp] was

amplified with the following primers: TCCTTGCTTCGTTCTCTGCT and

CTTGCCAGCCTTCGTATTGT). The mice were housed in same-sex groups in a room that was

controlled at a constant temperature of 71 +/- 3 degrees Fahrenheit. Animals were housed 2-4 per

cage and had free access to food and water. Animals were kept on a 12 hour regular light-dark

cycle.

Locomotor Task

A total of 70 Mice (n = 18 Wild type control, 21 Heterozygous, 31 Homozygous) were

transported directly from the vivarium to a testing room and were placed plastic (18 x 9 x 8 inch)

cages, the floor of which was covered with a layer of bedding sufficient to cover the ground. The

cages were placed in an apparatus that supplied six infrared beams equally spaced out throughout

the length of the cage. Each time a beam was broken by a mouse's body, it was counted as a

movement and recorded by OPTO M3 (supplier) program software on a PC laptop. The mice

were left in the cage for 30 minutes. The ambulatory movements were separated into 5-minute

bins of activity.

Morris Water Maze

A total of 70 mice (n = 25 Wild type, 26 Heterozygous, and 19 Homozygous mice) were

trained in a Morris Water Maze. 10 mice (n=5 wild type, 6 heterozygous, and 3 homozygous

mice) were omitted from the study due to floating. The water maze consisted of circular pool

made of white plastic, measuring 6 feet in diameter. The water was filled to 23 cm, and the water

was made opaque by mixing in nontoxic white paint. There was a Plexiglas platform that

measured 22 cm in height. This platform was placed in the northeast quadrant of the pool and

was hidden one cm below the water surface. The water was held at a constant temperature of 24 degrees Celsius using a heater. The water maze was in an isolated room, where it was lit by four lights underneath the pool. There were a number of distal, extra-maze cues on the walls of the pool. The cues were on a laminated 3" x 5" card attached to a wooden popsicle stick and taped to the outside of the pool, about an inch above the top of the pool wall. The researcher always stood the east side of the pool. The testing for the sandy mice spanned 11 consecutive days: 3 acclimation days, a pretrial day, 5 days of training trials, and a probe test.

The water maze was monitored from above by a video capture system (TopScan, Version 2.0), which was calibrated to the locations of the pool. From these data, the software extracted swim paths of the mice and quantified the swimming speed, path distance, and the amount of time it took the mouse took to find the platform (latency), as well as thigmotaxis (swimming within 2 cm of the wall).

Acclimation

Initially, the mice were acclimated to the sights and sounds of the room, as well as to handling over three consecutive days. The mice were taken from their housing room, placed on a cart and transported to the water maze room, where they were individually weighed, and tail-marked for identification purposes. The mice were then handled by the researcher for approximately two minutes. After all mice were handled, they were left alone in the room for an additional fifteen minutes before the training trials began.

Pretrial

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On the pretrial day, the mice were positioned with their front paws on the platform and their back paws in the water, to give them the experience with climbing up onto the platform. They then remained on the platform for 30 s. Subsequently, each mouse was placed in the water to swim for 30 s. Finally, each mouse was placed back on the platform for another 30 s. After this pretrial, the mouse was dried with paper towels and placed into a clean cage lined with paper towels. There was a space heater next to the cages. After all the mice in a group cage had run through the pretrial and were thoroughly dry, they were placed back in their original cage and returned to their housing room.

Water Maze Trials

The acquisition phase consisted of five daily sessions, with six trials per session; first each mouse experienced three consecutive trials followed by a one hour delay; then three more trials were run consecutively. The 6 trials/session involved the mouse being placed in the water at one of four different quadrants (SW, NW, SE, NE), in a semi-random order. For example, on Day 1, the starting positions were: trial 1 SW, trial 2 NW, trial 3 SE, and trial 4 SW, trial 5 SE, and trial 6 NW. Subsequent days involved a similar sequence of variable starting positions so that the mouse could not learn a simple response strategy to solve the task.

On each trial, mice were placed in the water with their heads pointed towards the inside wall of the pool and were allowed to swim until finding the submerged platform, or until they swam for a maximum of 60 s, whichever came first. In the case the mouse did not find the platform within 60 s, it was gently placed onto the platform at the end of the trial. In either case, the mouse was allowed to sit on the platform for an additional 60 s following the trial. As in the pretraining session, after each individual trial, the mice were dried off with paper towels and

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were placed into a clean cage lined with paper towels, located next to a heater. Once sufficiently dry, the mice were returned to their original cage. The home cages were changed every other day and only after testing.

As described above, the following parameters were measured for each trial: path length for each trial (in meters), the escape latency (time of trials in seconds) to find the hidden platform, the swim speed (in meters per second), and thigmotaxis was measured for each trial according to percentage of time per trial the mouse spent next to the wall. All of these parameters were calculated automatically by the TopScan 2.0 software.

Probe Trial

For the probe trial, the platform was removed in order to determine if the mice learned the location of the platform, using a true learning strategy. Good memory is shown, in this condition, if the mice spend most of their time swimming in the location where they expect the platform to be. This condition allows one to determine whether faster escape latencies in the training trials is attributed to a better search strategy, rather than to a better memory of the platform location. The probe trial was run 24 hours after the last session of training trials were run. The mice were placed into the water facing the wall in the southwest quadrant of the pool. Each mouse was then allowed to swim freely for 60 s, before being removed from the maze, dried off with a paper towel and placed in a clean cage lined with paper towels, next to a heater. When each group cage of mice was finished and sufficiently dry, they were placed back in their original cages.

The probe trial measures included the following parameters: amount of time spent in the quadrant that originally contained the platform (computed as a percentage of the total time in the

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target quadrant), the path length each mouse swam within the target quadrant, as well as the amount of time spent next to the wall of the trial (thigmotaxis, computed as a percentage of the total time).

Novel Object Recognition Task (NORT)

An open-field chamber ($L \times W \times H$: $30 \times 30 \times 30$ cm) made of opaque grey Plexiglas was placed in a quiet room. Mice of each genotype ($n = 10$ per genotype; 30 total) were individually placed in the arena for 20 min on two consecutive days in order to habituate them to the arena. On the third day, the mice were placed in the arena with two identical objects (black plastic pipe connectors) for 20 min to acclimate them with test condition. On the test day (Day 4), there were 3 phases: A 5 min habituation phase, a 5 min familiarization phase, and a 5 min test phase, with a 1 hr delay between the familiarization and test phases. In the habituation phase, mice were placed in the arena without any objects and allowed to explore freely for 5 minutes. The mice were then removed for the familiarization phase, and two identical objects were placed in the top right and bottom left corners of the arena, a few centimeters from each corner. The objects were grey metal pipe connectors slightly bigger than the black pipe connectors. The mice were allowed to explore the objects for 5 min. The mice were then removed and placed back into their respective home cage for 1 hr.

In the test phase, the mice were placed back into the arena with one familiar object (metal pipe connector) and a novel object (white plastic pipe connector) similar in size and shape of the familiar object. The familiar and novel objects were counterbalanced for location throughout the trials. Behavioral activity during the familiarization and testing phases were videotaped via an infrared surveillance camera, and recorded via a Lorex LW1001 infrared

surveillance camera connected to an HP laptop. A mouse was considered to be involved in object exploration when its head was oriented directly towards the object (at least 45 degrees) and within approximately 2–3cm from it. This includes rearing of the head. The time was also included if the mouse was directly interacting with the object by having at least one forepaw on the object, if the mouse stood on top of the object, or if the mouse was sniffing or licking the object. Object recognition memory was defined as the ratio of exploration time for the novel object (TN) over by the total exploration time for the novel and familiar (TF) objects [exploration ratio = TN/ (TF + TN)]. Rodents have a natural tendency to explore novel objects in their surrounding compared to objects already familiar. Increased time of exploration of novel object reveals recognition memory for objects (Trials were analyzed using the Ruby script program on an Apple Macbook to record the time spent exploring both objects. Time was recorded in seconds to the hundredths).

Statistical Analyses

Locomotor movement (total number of movements per five minute time bin) was analyzed using repeated measures ANOVA. Water maze training trials were analyzed using repeated measures ANOVA considering day and trial. Measures analyzed included latency (s), path length (m), swim speed, and thigmotaxis.

The probe trial data were analyzed using a separate one-way ANOVA. The measures analyzed included the amount of time spent in the same quadrant as the platform (target quadrant), the path length swam in the same quadrant as the platform, and the amount of time spent along the walls of the pool, thigmotaxis.

One-way ANOVA analysis was used to evaluate significant differences between genotypes for measures of total time spent exploring all objects, time spent exploring the familiar

object, time spent exploring the novel object, and the ratio of exploration of novel object/ total exploration time. On trials where a genotype or group effect or interaction was significant, simple contrasts were performed.

Results

Locomotor Task

There were no differences seen in locomotor activity indicating no increased or decreased activity in the heterozygous and homozygous mice, compared to wild type controls. This also indicates that heterozygous and homozygous mice were able to habituate to their environment just as well as controls.

Repeated measures ANOVA showed a significant main effect of bins (F5, 335 = 41.03; p <.01), but no significant main effect of genotype (F 2, 67 = 1.19; p >.05) or bin x genotype interaction (F 10, 335 = 1.65; p >.05) (Figure 2.1). The number of movements decreased in all genotypes over the 30 min interval for all genotypes, but there was no difference in the amount of activity between wild types, heterozygous, and their homozygous littermates. This suggests that the homozygous sandy mice showed equivalent rate of habituation to the open field environment compared to wild type and heterozygous mice.
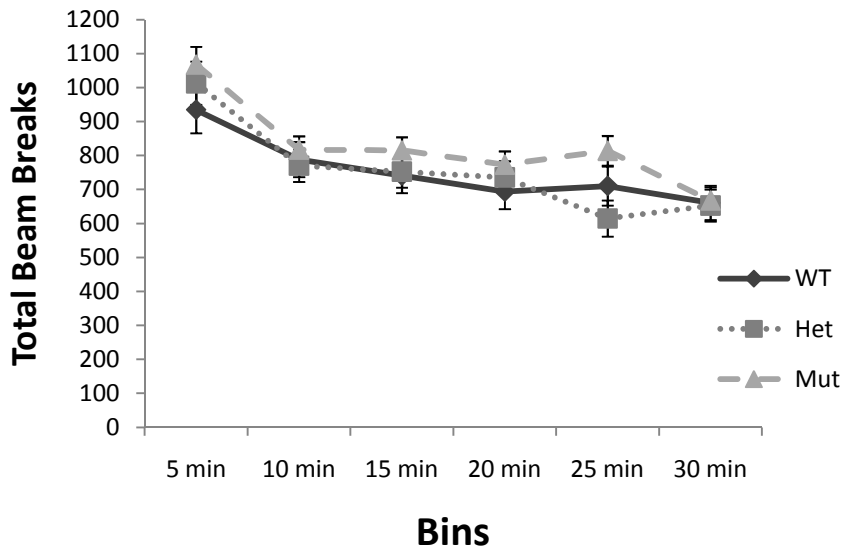
Figure 2.1. The total number of infrared beams broken over 30 minutes, measured in 5 minute bins for wild type (WT), heterozygous +/- (Het), and dysbindin mutant -/- (Mut) mice. There are no main effects of genotype.

Water maze task

Training trials

In the training trials, learning behavior was shown in all three groups, evidenced by decreased latency, path swam, and time spent near the walls over the days of the trials, indicating that heterozygous and homozygous mice showed the same rate of learning as their wild type littermates.

In the hidden platform training trials, repeated measures ANOVA revealed no differences between genotype groups for latency to reach the escape platform ($F_{2, 53} = 3.11$; $p > .05$) (Figure 2.2a). There was a main effect of day ($F_{4, 212} = 50.37$; $p < .01$) because latencies decreased over days, showing that all three groups showed learning. There was no genotype x day interaction ($F_{8, 212} = 1.79$; $p > .05$).

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The path lengths also revealed a significant main effect of day ($F_{4, 212} = 37.93$; $p < .05$) with paths getting shorter each day. Again, there was no significant effect of genotype ($F_{2, 53} = .947$; $p > .05$) or day x genotype interaction ($F_{8, 212} = 1.83$; $p > .05$). (Figure 2.2b)

Swim speed revealed no significant main effect of day ($F_{4, 212} = .337$; $p > .05$), nor was there a day x genotype interaction ($F_{8, 212} = .36$; $p > .05$), but there was a main effect of genotype ($F_{2, 53} = 4.34$; $p < .05$). Simple post hoc analyses revealed that homozygous sandy mice swam much slower (.179 m/s) than their wild type littermates (.201 m/s) (Figure 2.2c).

Thigmotaxis was measured as a percentage of the total trial. Repeated measures ANOVA showed a main effect of day ($F_{4, 212} = 133.57$; $p < .05$), with the percentage of time swimming the periphery decreasing over days. There was no main effect of genotype ($F_{2, 53} = .97$; $p > .05$), nor a day x genotype interaction ($F_{8, 212} = 1.32$; $p > .05$) (Figure 2.2d).

Figure 2.2a.Escape latencies each day averaged over 6 trials, measured in seconds (sec) for wild type (WT), heterozygous +/- (Het) and homozygous sandy mutant -/- (Mut) mice. There was a main effect of day, as all groups showed decreased latencies over days, but no main effect of genotype or genotype x day interaction.
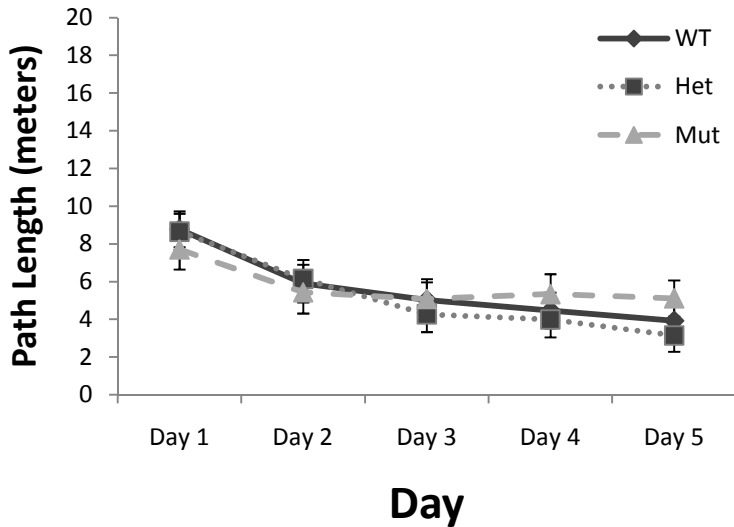
Figure 2.2b. Average length swam per trial with each day averaged over 6 trials, measured in meters (m) for wild type (WT), heterozygous +/- (Het) and homozygous sandy mutant -/- (Mut) mice. There was a main effect of day, as all groups showed decreased path lengths over days, but no main effect of genotype or genotype x day interaction.
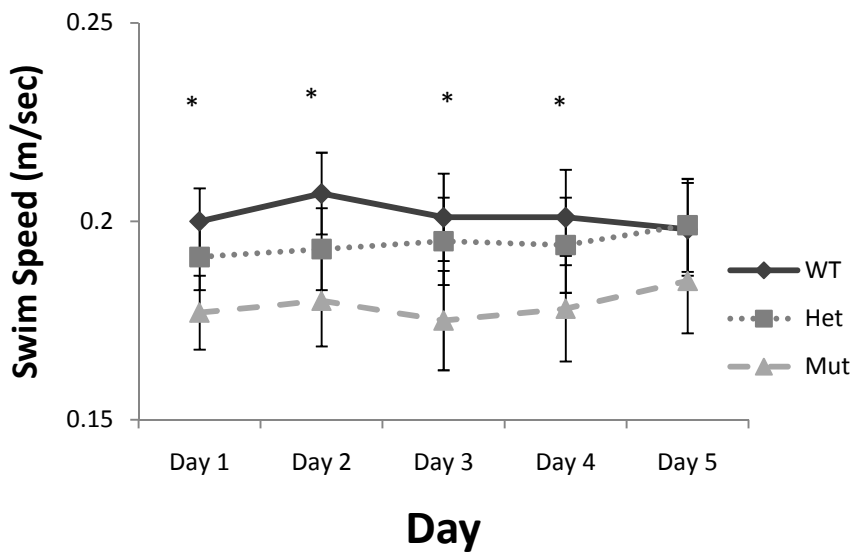
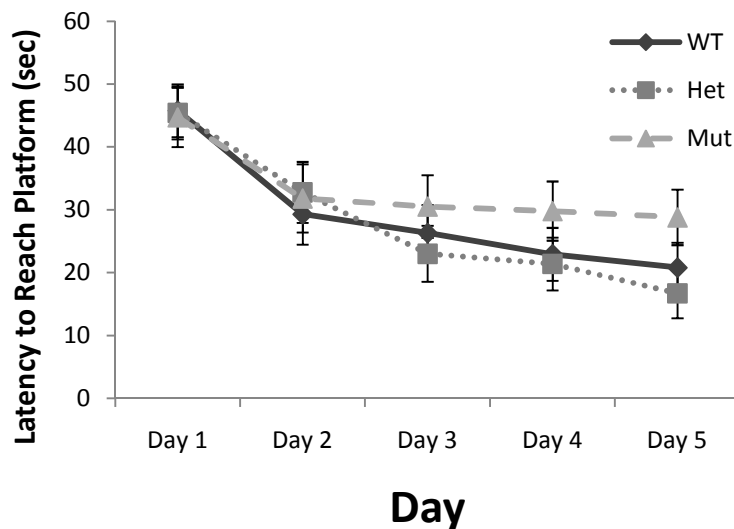Figure 2.2c. Average swim speed per trial with each day averaged over 6 trials, measured in meters/ second (m/sec) for wild type (WT), heterozygous +/- (Het) and homozygous sandy mutant -/- (Mut) mice. There was no main effect of day, but there was a main effect of genotype with homozygous mice swimming slower than wild type controls. There was no genotype x day interaction.
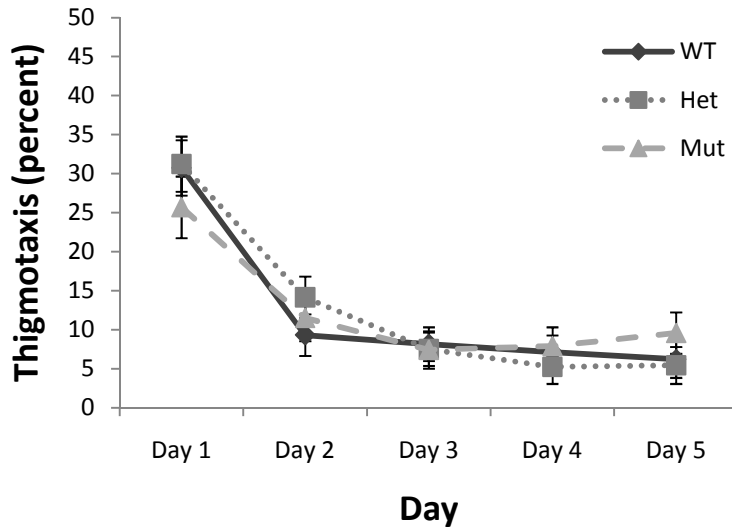
Figure 2.2d: Thigmotaxis, the amount of time swimming next to the walls (measured within 2 cm of the wall), is shown for each day, averaged over 6 trials, and measured by the percentage of the trial spent next to the wall (percent). There was a main effect of day with all groups decreasing over days, but no main effect of genotype and no genotype x day interaction.

Probe trial

Wild type mice spent more time in the target quadrant where the platform used to be, compared to both heterozygous and homozygous mice, which indicates these mice showed impaired memory for the platform location 24 hours after the training trials, or that they did not rely upon spatial cues in order to find the platform originally. If the mice only knew to swim a certain distance between the wall and center of the pool, this would be evident by swimming a continuous circle in the probe trial. The wild type mice also swam more within the target quadrant, turning around to actively look for the platform.

For the probe trial, there was a main effect of genotype for time spent in the target quadrant (F 2, 53 = 8.17; *p* <.01) (Figure 2.3a). Simple analyses showed that wild type mice (*M*= 32.05%) spent significantly more time in the target quadrant than both heterozygous (*M*= 24.52%) and homozygous sandy mice (*M*= 26.05%). Similarly, path length swam in the target

quadrant revealed a main effect of genotype ($F_{2, 53} = 4.78$; $p < .05$) (Figure 2.3b). Simple

analyses showed that wild type mice ($M = 4.23$ meters) swam more in the target quadrant than

heterozygous ($M = 3.36$ meters) and homozygous sandy mice ($M = 3.54$ meters) (figure 2.3b).

There were no differences seen in percentage of time spent near the walls ($F_{2, 53} = .21$; $p > .05$)

(not shown).
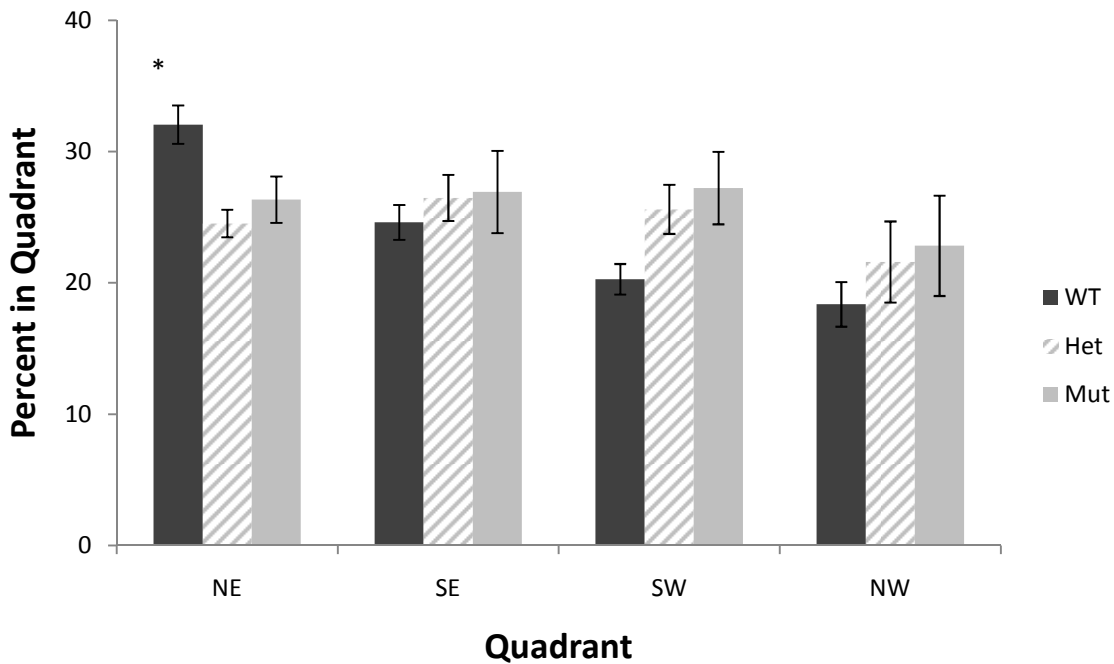
Figure 2.3a. Percentage of time spent in all quadrants for the probe trial for wild type (WT), heterozygous +/- (Het), and homozygous mutant -/- (Mut) mice. Northeast (NE) is the target quadrant. Wild type mice spent more time in the target quadrant than heterozygous and homozygous mice, and less time in the opposite quadrant.
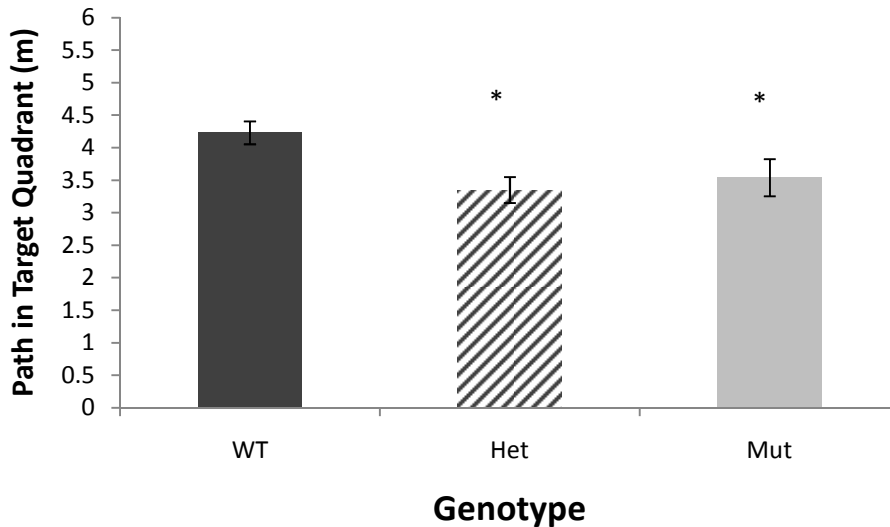
Figure 2.3b. Path length swam in the target quadrant during the probe trial for WT, heterozygous (Het), and homozygous sandy mutant mice (Mut), measured in meters. Wild type mice swam more in the target quadrant than heterozygous and homozygous sandy mice.

Novel Object Recognition

All three groups showed similar exploratory behavior, which coincides with the previous results of the spontaneous locomotor task. However, when looking at the ratio of preference for the novel object compared to the familiar one, wild type mice showed a preference for the novel object, whereas homozygous mice showed no preference for either object. As predicted, the heterozygous mice performed in between the wild type and homozygous mice.

A one-way ANOVA was used to look at differences between genotypes in the exploration of objects in the familiarization phase, which revealed no group differences in exploration ($F_{2, 27} = 0.01$; $p > .05$) (Figure 2.4a). An additional one-way ANOVA was used to look at exploration of a novel versus a familiar object in the test phase, ran one hour following the familiarization phase. We found no differences between genotypes in the total exploration of both objects ($F_{2, 27} = .21$, $p > .05$) (Figure 2.4b). Exploration of each object individually was

examined, starting with the familiar object and again no significant differences between

genotypes (F 2, 27 = 1.69; p >.05) were found (Figure 2.4c).

For the exploration of the novel object, a one way ANOVA revealed no significant

differences between genotypes (F 2, 27 = .172; p >.05). All 3 groups were almost identical in

time spent sniffing or orienting towards the novel object (Figure 2.4d).

With no differences seen between exploration of the individual objects, as well as the

total time spent exploring both objects, the ratio of exploration was examined by measuring the

preference of time spent for the novel object divided by the total amount of time spent exploring

both objects. This time a one way ANOVA revealed a significant difference between genotypes

for exploration ratio (F 2, 27 = 5.52; p <.05) (Figure 2.4e). Simple post-hoc analyses revealed

that wild types showed a significantly higher preference for the novel object than homozygous

sandy mutant mice. This indicates that the sandy mice showed no preference for either object and

spent about the same amount of time exploring each one, while the wild type mice had a much

higher preference for the novel object than the familiar one.
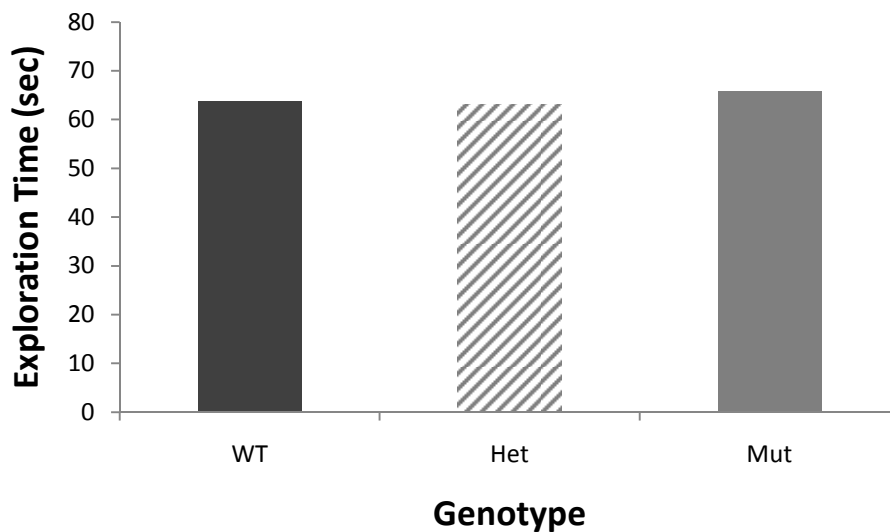
Figure 2.4a. Total time (sec) spent exploring objects in the familiarization phase for wild type
(WT), heterozygous (Het) and homozygous sandy mutant (Mut) mice.
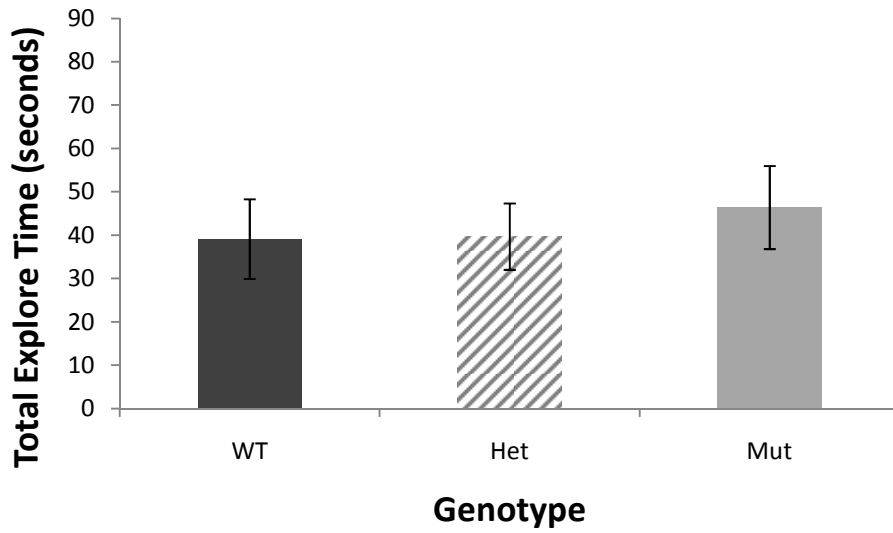
Figure 2.4b. Total time (sec) spent exploring both objects (novel and familiar) for wild type (WT), heterozygous (Het) and homozygous sandy mutant (Mut) mice. No differences were seen between groups.
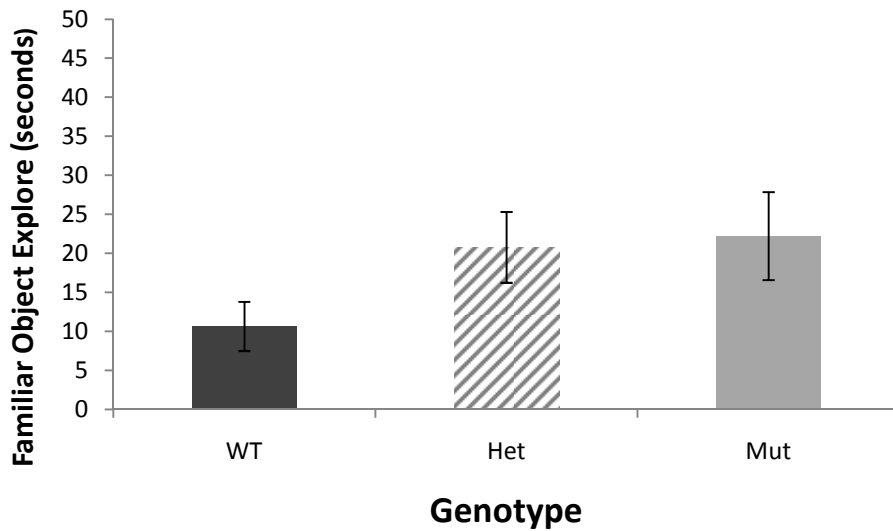
Figure 2.4c. Time spent exploring the familiar object in the test trial (sec) for wild type (WT), heterozygous (Het), and homozygous sandy mutant mice (Mut).There were no differences between groups
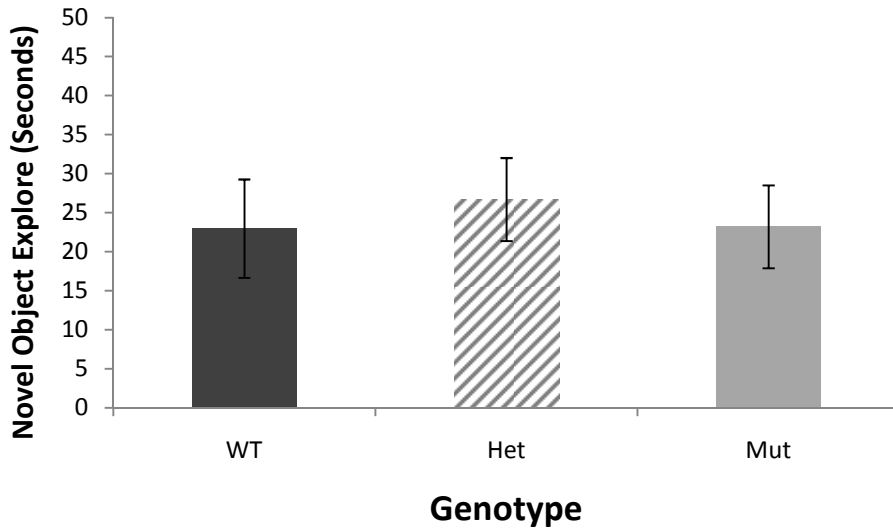
Figure 2.4d. Time spent exploring the novel object in the test trial (sec) for wild type (WT), heterozygous (Het), and homozygous sandy mutant mice (Mut). There were no differences between groups in exploration of the novel object.
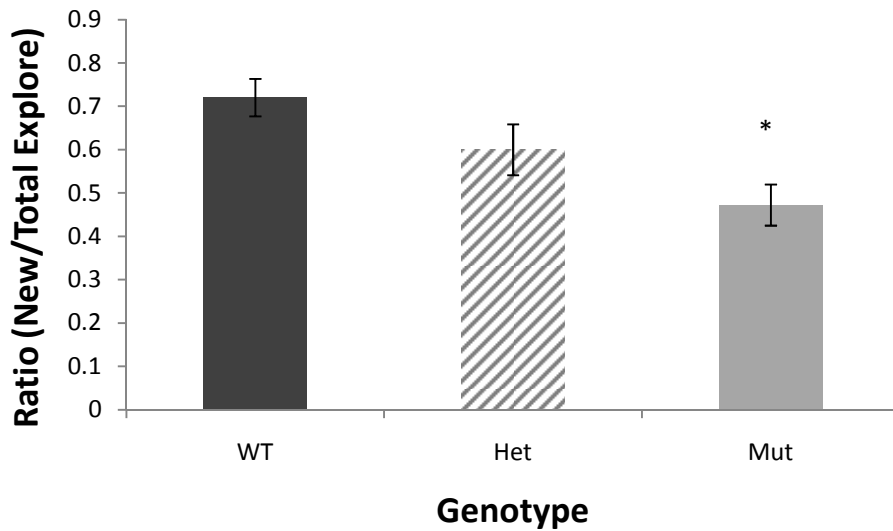
Figure 2.4e. Ratio of time spent exploring the novel object/ total amount of time exploring objects in the test trial for wild type (WT), heterozygous (Het), and homozygous sandy mutant (Mut) mice. Wild type mice had a significantly higher preference for the novel object than heterozygous and homozygous sandy mutant mice.

Discussion

Although the sandy mutation had arisen in the DBA/6J strain (Li et al., 2003; Talbot et al., 2009), we used sandy mice backcrossed to the C57Bl/6J strain in order to avoid other abnormalities inherent to the DBA strain, including age-related hearing loss and glaucoma (Cox et al., 2009). The null mutation of the dysbindin gene in the homozygous sandy mouse reveals specific behavioral functions, including spatial and recognition memory, assessed using the Morris water maze and novel object recognition tasks, respectively. Although sandy mice were as capable as heterozygous and wild type control mice in locomotor behavior and some forms of learning assessed by the water maze task, they exhibited difficulty with correctly recalling where the hidden platform was located in the probe trial. Furthermore, sandy mice showed normal exploration of novel objects in the NORT task but showed compromised recognition memory as evidenced by responses to objects they experienced in the past. The heterozygous mice showed normal recognition memory comparable to wild types but showed deficits in spatial memory in the probe trial. These data suggest that hippocampal-dependent spatial and recognition memory processes are the phenotypic result of the absence of dysbindin expression.

Hippocampus and Memory in SCZ

The hippocampus is critical for a variety of forms of learning and memory functions (Anagnostaros et al., 2001; Eichenbaum et al., 2004; Moses et al., 2005; Squire et al., 2003), and pathology in this brain region likely explains aspects of memory dysfunction observed in patients with schizophrenia, (Harrison & Eastwood, 2001; Preston et al., 2005). For example, imaging studies show bilateral reductions in hippocampal volume (Preston et al., 2005) and anomalous activation in memory tasks (Narr et al., 2009; Preston et al., 2005; Watson et al., 2012), and post

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mortem studies consistently identify signs of abnormal expression at the cellular neurochemical, and circuit dysfunction in the hippocampus of patients with schizophrenia (Talbot, 2004; Weickert et al., 2008). Imaging studies in the sandy mouse show reduced function in dorsal hippocampus, which may relate to the deficits seen in this study (Lutenkoff et al., 2012).

Recent work has implicated dysbindin as a potential cause of these phenomena; reductions in dysbindin protein expression have been detected in multiple brain regions including frontal cortex, midbrain, and multiple structures of the hippocampus (Burdick et al., 2006; Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008).

Evidence to support this causal influence derives from the study of sandy mice that exhibit reductions in expression of other proteins associated with the dysbindin protein as well as impairments in synaptic transmission of monoamines (Chen, 2008; Feng, 2008; Kobayashi et al., 2011; Numakawa, 2004; Talbot, 2006), which may relate to deficits of hippocampal-dependent memory processes reported here and in previous studies (Bhardwaj, et al., 2009; Cox et al., 2009; Jentsch et al., 2009; Karlsgodt et al., 2011; Takao et al., 2008).

Dysbindin and Glutamatergic Neurotransmission

One of the mechanisms by which loss of dysbindin expression may impair the function of hippocampus and other cortical regions involved in memory is through a dysregulation of glutamate neurotransmission. Impaired glutamatergic function has been previously reported in the sandy mouse (Chen et al., 2008; Jentsch, 2009; Karlsgodt, 2011; Talbot, 2004), although these mechanisms are poorly understood. These abnormalities include impaired kinetics of glutamate release (Chen et al., 2008), shown in CA1 of the sandy mouse. This is presumably due to abnormal packaging and trafficking of glutamate-containing synaptic vesicles, an influence of

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dysbindin that likely depends upon its role in the BLOC-1 complex (Dell'Angelica, 2004; Numakawa et al., 2004l; Ryder & Faundez, 2009). Hypofunction of NMDA receptors has also been previously described in the sandy mouse (Jeans et al., 2011; Karlsgodt et al., 2011), which suggests that the absence of dysbindin protein could impair neurotransmission via both pre and/or post-synaptic glutamatergic mechanisms.

Glutamate release onto NMDA and non-NMDA receptors in the hippocampus may be crucial for memory function, including spatial and recognition memory. Changes in NMDAR expression level have been associated with dysbindin-1 in the rat hippocampus (Jeans et al., 2011), demonstrating a relationship between levels of dysbindin and LTP, which is believed to be required in hippocampal memory processes (Bliss & Collingridge, 1993; Tang et al., 2009). Other studies have suggested that dysbindin exerts control of LTP by regulating surface expression of the NR2A subunit of the NMDA receptor in hippocampal neurons of sandy mice (Tang et al., 2009). Taken together, it seems that cognitive function and memory impairments examined in the dysbindin-deficient sandy mouse could be caused by the compromised function of glutamate within the hippocampus. Unfortunately, no experiments to date have directly evaluated this link via pharmacological or genetic rescue strategies.

Limitations and future directions

Although deficits in hippocampal-dependent context memory have been identified in the sandy mouse, there were some limitations to the study. No differences were observed between groups in the training trials of the water maze, although several mice were omitted from the study due to floating, which may have shown a lack of motivation in the mice to find the platform, leading to longer latencies overall. The results obtained in the training trials were lower

than previous studies with this mouse model (Cox et al., 2009), although it must be pointed out that a much stricter criterion was used in the former experiment. Similarly, percentage of time spent in the target quadrant, although significant, was much lower.

Impairments in object recognition memory have been attributed to hippocampal processes, but there are conflicting reports that recognition memory also requires perirhinal cortex, or is independent of the hippocampus altogether (Albasser et al., 2011; Moses et al., 2005; Mumby et al., 2001). In the latter case, this would not contribute to the literature on hippocampal-dependent processes, but it could account for dysbindin function outside of the hippocampus. Future studies should address other types of memory and cognition, perhaps incorporating the amygdala.

Previous studies of dysbindin have shown differing levels of protein expression within subregions of the hippocampus, with greater expression of dysbindin in CA2 and CA3, relative to CA1 (Feng et al., 2008; Talbot et al., 2006; Weickert et al., 2008). The different tasks used here rely, to some extent, on different parts of the hippocampus. Spatial memory, as assessed in the Morris water maze, is believed to be dependent upon the dorsal hippocampus (Fanselow & Hong Wei-Dong, 2010), and object recognition may be dependent upon CA3 (Clarke et al., 2010), which is also shown to be affected in patients with schizophrenia (Preston et al., 2005). Perhaps future studies can try and determine whether these subregions are differentially affected by loss of dysbindin.

It should also be noted that there is a small, but non-zero chance that there could be another mutation in close proximity to the spontaneous null mutation that could be responsible for the phenotypic results, because it is in linkage disequilibrium with the mutation that we are breeding for.

In conclusion, our results indicate that the sandy mouse shows behavioral deficits in spatial and recognition memory. This null mutation in the sandy mouse can lead to behavioral and cognitive impairments similar to those seen in patients with schizophrenia and may help us in understanding its role in the pathophysiology of this disease.

Chapter 3

Behavioral effects of dysbindin-deficient mice on hippocampus-dependent contextual fear

conditioning

Abstract

DTNBP1, which encodes for the dysbindin protein, has been identified as a candidate risk gene

for schizophrenia. Variations in *DTNBP1* are associated with increased risk of schizophrenia, as

well as deficits in cognitive ability and memory function. Reduced expression of the dysbindin-1

protein has been reported in the prefrontal cortex and hippocampus of schizophrenic patients. To

further study the influence of dysbindin-1 on memory functions, we used a contextual fear

conditioning task, which has both hippocampal and non-hippocampal dependent components, in

sandy mice that carry a null mutation of the *DTNBP1* gene. Homozygous sandy mice showed

normal tone-shock learning but were impaired in responding to context. The results of this study

indicate deficits in contextual fear memory, a hippocampal-dependent process, which may be

consistent with memory problems observed in patients with schizophrenia.

Introduction

Numerous genes have been identified as possible candidate genes that may contribute to the pathogenesis of schizophrenia, including NRG1, DISC1, and Dystrobrevin-binding-protein-1 (*DTNBP1*). Polymorphisms within these candidate genes have been associated with cognitive decline, especially in working memory, declarative memory, executive functioning, and tasks that require a higher cognitive load (Cannon, 2005). One of these genes, the *DTNBP1* gene, has been associated with increased risk for schizophrenia, as well as deficits in intellectual function and cognitive processes in schizophrenia patients (Benson et al., 2001; Ross et al., 2006).

Abnormal glutamate function in cerebral cortex has been hypothesized in patients with schizophrenia (Harrison & Eastwood, 2001; Weickert, et al., 2004), an effect that could be linked to reduced dysbindin protein expression (Talbot, 2004; Chen et al., 2008). Dysbindin seems to affect neurotransmitter release via vesicular exocytosis (Chen et al., 2008; Jentsch et al., 2009), resulting in disrupted glutamatergic release. Post synaptic glutamatergic receptors, particularly NMDA, exhibit dysregulation in the absence of dysbindin, as well (Karlsgodt et al., 2011).

Presently, we sought to further investigate the effects of dysbindin on memory functions of the hippocampus in mice with varying levels of dysbindin expression. Studies involving the homozygous sandy mouse, which does not code for the dysbindin protein due to a spontaneous null mutation in the coding region of the *DTNBP1* gene, have shown behavioral abnormalities using various memory tasks (Bhardwaj et al., 2009; Cox et al., 2009; Jentsch et al., 2009; Feng et al., 2008; Takao et al., 2008). In order to complement these studies, we evaluated contextual fear memory, which relies on the hippocampus in order to make the association of the environment in which the CS-US association takes place (Fanselow, 2005; Jacobs, Cushman, & Fanselow, 2010; Maren, 2008; Sanders et al., 2003), in dysbindin homozygous mice, and their heterozygous and wild type littermates.

It was hypothesized that sandy mice would show deficits in freezing to a trained context compared to wild type controls, consistent with deficits seen in other hippocampal-dependent memory processes, with heterozygous mice performing in between wild type and homozygous sandy mice. This is precisely what was found.

Methods

Animals

Subjects included 48 male mice backcrossed on the C57BL/6 background that were wild type, heterozygous, homozygous mice between 60 and 120 days old ($n = 16$ per group), (Jackson Laboratories, Bar Harbor, Maine). Experimental mice were generated by heterozygote crosses, allowing for direct comparisons among homozygous mutants, heterozygotes, and wild type littermate control subjects. Genotypes were determined by polymerase chain reaction. The weight product [472 bp] was amplified with the following primers: TGAGCCATTAGGAGATAAGAGCA and AGCTCCACCTGCTGAACATT. The homozygous dysbindin (-/-) product [274 bp] was amplified with the following primers: TCCTTGCTTCGTTCTCTGCT and CTTGCCAGCCTTCGTATTGT). The mice were housed in same-sex groups in a room that was controlled at a constant temperature of 71 +/- 3 degrees Fahrenheit. Animals were housed 2-4 per cage and had free access to food and water. Animals were kept on a 12 hour regular light-dark cycle.

Contextual-fear conditioning

All behavioral testing was assessed with MedAssociates Video Freeze fear-conditioning equipment. The fear-conditioning experiments were conducted in two different contexts. Context

A consisted of a fear-conditioning box in a well-lit room with rounded walls made from a 95cm by 20.5cm white plastic insert; the box was fitted with holes for the speakers and a grid floor with 36 individual stainless steel rods, each with a diameter of 0.32cm and spaced 0.64cm apart (center to center). The tray under the grid was scented with a 50% Windex solution, and the box was cleaned with a 10% ethanol solution between trials. The house lights were illuminated in this context.

Context B featured aluminum walls, and the grid floor was covered with a white plastic rectangular insert. On top of the plastic floor was an inverted V-shaped transparent plastic board inside the boxes, the tray underneath was scented with a 1% acetic acid solution. The boxes for context B were in a different room with low illumination, and inside the box the house lights were off.

Fear was measured as mean percent time spent freezing to a tone or context CS (percentage by component). Freezing is defined as complete cessation of movement and was measured by Med Associates, Near Infrared Video fear-conditioning system.

Habituation

Mice were habituated for 7 consecutive days. During this time they were transferred from the vivarium to the holding room for 1 hour. For the first 15 minutes, they were left alone in the room. Over the next few minutes, each mouse was picked up by their tail, handled for at least 10 seconds, and then placed into a 500ml glass beaker.

Acquisition—Day 1

The mice were transferred from the vivarium into a quiet anteroom. They were individually placed in the chamber into the context A room, one cage at a time, and tests began immediately. There was a 4-minute baseline followed by a 30 s tone, played at 70 db (2800 Hz). During the final 2 seconds of the tone, a 0.5 mA footshock was delivered to the mice. This was followed by a 2 min postshock period with no further programmed stimuli. After each session, the fear-conditioning boxes were thoroughly cleaned with 10% ethanol solution and dried

Context Fear Test—Day 2

The mice were taken from the vivarium and put in a quiet anteroom. They were then transferred to the context A chamber for 8 min. No tones or shocks were presented. After each session, the cages were cleaned and disinfected.

Fear Generalization Test—Day 3

The mice were taken from the vivarium and placed in a quiet anteroom. From there, they were placed into the context B chambers, for 8 minutes, in the absence of tone and shock. When each session was finished, the cages were be thoroughly cleaned with a 1% acetic acid solution.

Tone retention test—Day 4

The mice were taken from the vivarium and placed in a quiet anteroom. From there, they were placed in the context B chambers. In this test of tone fear retention, the first 90 s constituted the habituation period, with the next 30 s used as the baseline retention period. Subsequently, three 30 s, 70 db tones, each separated by a 1min interval were played. When each session was finished, the cages were cleaned with a 1% acetic acid solution.

Statistical Analysis

One-way ANOVAs were used to compare the percentage of time spent freezing in each context (A and B) between genotypes. Shock burst was measured as the maximum burst of activity captured the MedAssociates program. During the acquisition phase, freezing before and after the minutes after the footshock was measured. . During the context fear test, the overall percentage of time spent freezing was measured for each mouse. During the fear generalization test, the overall percentage of time spent freezing was measured for each mouse. Finally, during the tone test, the baseline freezing in context B was measured 30 s prior to the first tone. After that, freezing during each individual 30 s tone was measured.

Results

Contextual Fear Conditioning

There were no differences between groups in baseline, sensitivity level before and during the shock, or measures of fear generalization to the different context. There were no differences seen in the freezing behavior of the tone retention task, as well. However, homozygous sandy mice did show deficits in hippocampal-dependent context association, showing decreased freezing behavior in the same context in which they were shocked a day earlier, compared to wild type controls. Heterozygous mice showed freezing behavior in between wild type and sandy mice.

For the acquisition phase, a one way ANOVA examined baseline freezing during the four minutes prior to the CS-US pairing (Tone-shock). There was no significant differences between groups ($F 1, 32 = .085$, $p > .05$) (Figure 3.1a). Subsequently, to measure sensitivity of footshock,

ANOVA was used to examine the activity burst between genotypes. Repeated measures

ANOVA revealed a main effect of activity (F 1, 45 = 239.56), which showed an increase after

shock was administered, and revealed a main effect of genotype (F 2, 45 = 3.67; *p* >.05), but no

activity x genotype interaction (F 2, 45 = 1.29; *p* <.05) (Figure 3.1b). Simple contrasts for

genotype revealed that heterozygous mice showed a greater change in activity than wild type

controls, but neither individual activity differed from homozygous sandy mice and wild type

controls.

Finally, postshock freezing was evaluated with a one way ANOVA, revealing a main

effect of genotype (F 2, 45 = 37.44; *p* >.05) (Figure 3.1c). Simple comparisons revealed that wild

type mice froze significantly more than homozygous sandy mice (*M*= 4.82; *p* <.05), who froze

around baseline percentages, with heterozygous mice freezing similar to wild type mice.
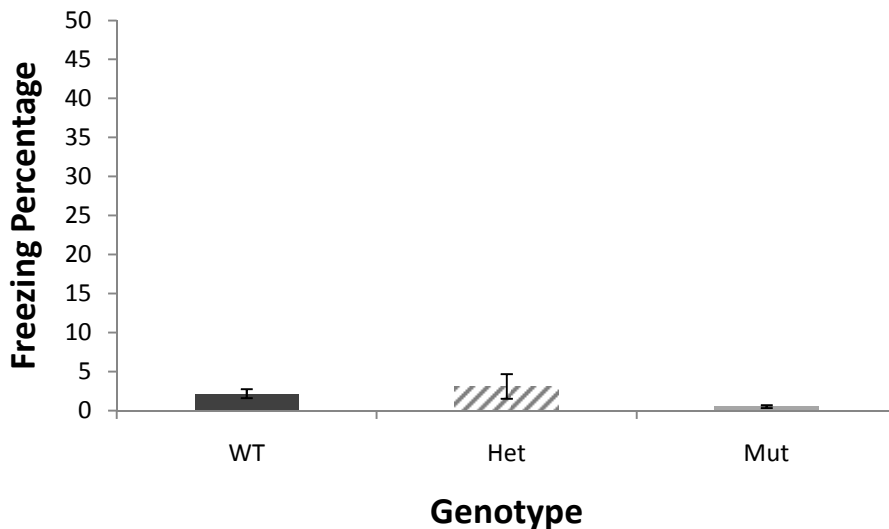
Figure 3.1a. Baseline freezing (percent) for acquisition trial. There were no differences between groups in percentage of baseline freezing.
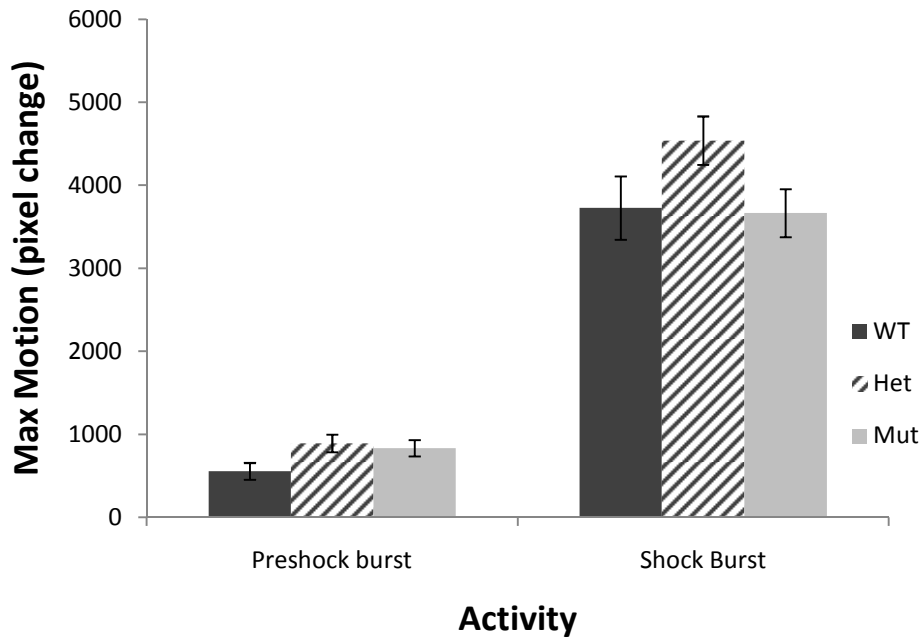
Figure 3.1b. Activity (pixel change) before the shock (2 sec) and during the shock (2 sec). There was a main effect of activity as it increased during the shock for all groups, but there was no main effect of genotype and no activity x genotype interaction.

Figure 3.1c. Postshock freezing (percent) in context A for wild type (WT), heterozygous (Het), and homozygous sandy mutant (Mut) mice. Homozygous sandy mice showed significantly less freezing compared to WT mice.

Twenty-four hours later, the mice were exposed again to context A in the absence of tone and shock (CS and US). A one way ANOVA revealed a main effect of genotype ($F_{2, 45} = 5.98$; $p < .01$) (Figure 3.2). Simple post hoc analyses revealed that WT mice froze significantly more ($M = 39.15\%$) than both heterozygous ($M = 28.35\%$; $p < .05$) and homozygous sandy mice ($M = 22.61\%$; $p < .01$).
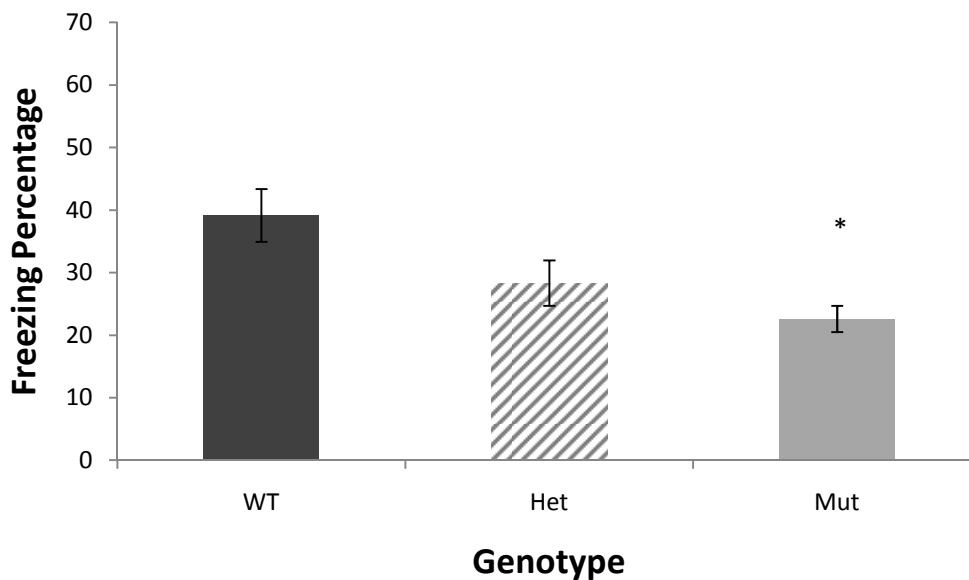
Figure 3.2: Freezing (percent) for the context association in context A. Wild type (WT), heterozygous (Het), and homozygous mutant (Mut) mice were placed back in the same context as the acquisition and percentage of the trial spent freezing was measured. Homozygous sandy mice froze significant less than WT mice, indicating a deficit in contextual processing.

To test for fear generalization, the mice were placed in context B for 8 minutes, on a separate day. A one way ANOVA revealed no main effect of genotype in the new context ($F_{2, 45} = 1.786$; $p > .05$) (Figure 3.3).
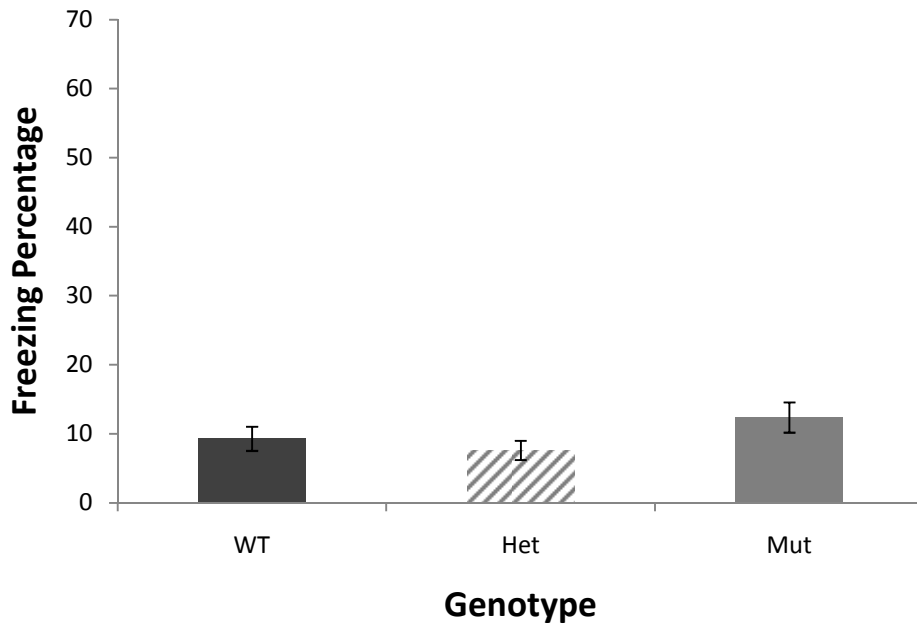
Figure 3.3: Freezing (percent) in context B for fear generalization. Wild type (WT), heterozygous (Het), and homozygous sandy mutant (Mut) mice were placed in a new context to test fear generalization (percent). There were no differences in context B, indicating no generalization to the new context

On the fourth day, the mice were tested for tone retention. A one way ANOVA revealed no main effect genotypes over the baseline period (F 2, 45 = 2.297; p >.05) (Figure 3.4a) and repeated measures ANOVA revealed no main effect of tone (F value), genotype (F 2, 45 = .225; p >.05), tone x genotype interaction (F 2, 45 =.95; p >.05) (Figure 3.4b). These statistical analyses suggest that not only may the sandy mouse have not learned the CS-US association, but there may have been context-dependent impairment, as well. Also, the fact that there were no differences in the tone test, which may indicate no deficit of amygdala-related fear memory, but may only implicate deficits in hippocampal-dependent contextual memory.
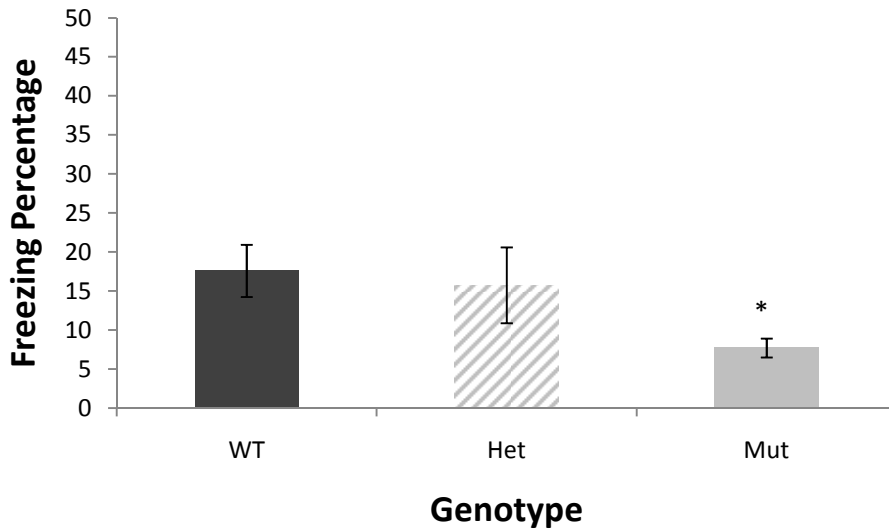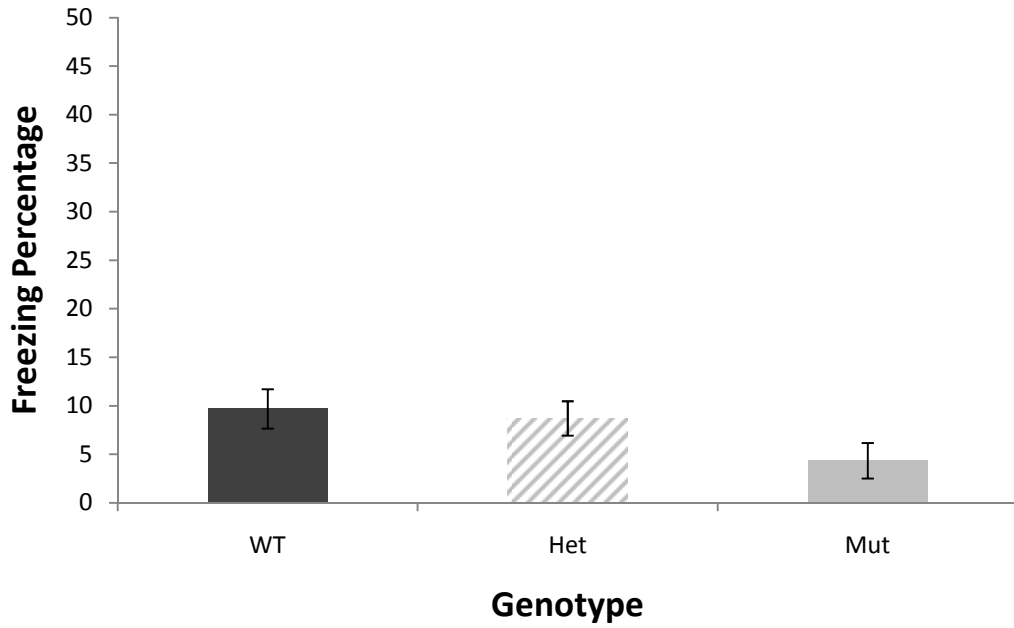
Figure 3.4a. Baseline freezing (percent) in context B for wild type (WT), heterozygous (Het), and homozygous sandy mutant (Mut) mice in the tone retention test. There were no differences between genotypes.
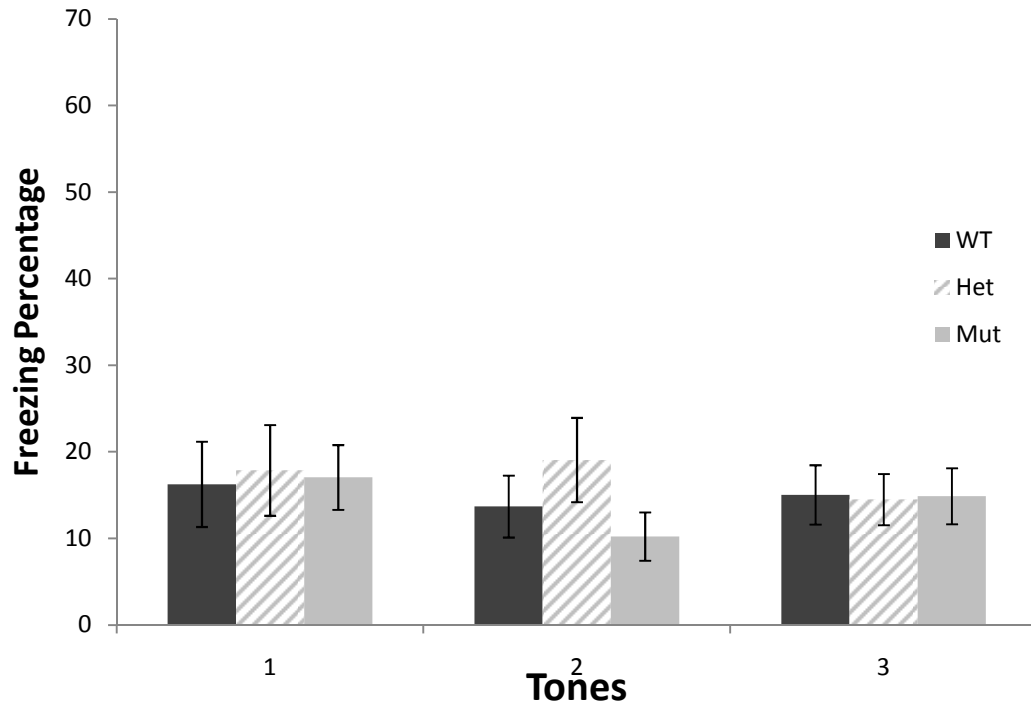
Figure 3.4b: Freezing (percent) during the tone retention test in context B on the fourth day. Wild type (WT), heterozygous (Het), and homozygous sandy mutant (Mut) mice showed no differences in freezing to the CS (tones)

Discussion

A null mutation of the gene that encodes for the protein dysbindin has led to a deficit in performance of a contextual fear conditioning task. The present results indicate that although homozygous sandy mice show similar freezing levels across baseline, fear generalization, and tone retention similar to wild type controls and heterozygous littermates, they do show deficits in processing of the context in which a tone-shock association was made, suggesting deficits in contextual memory brought about as a phenotypic result of the absence of dysbindin. Prior studies were performed in sandy mice on the DBA/6J background; these results showed no deficits in context association and showed homozygous sandy mice froze significantly more than

wild type and heterozygous mice during the tone retention test (Bhardwaj et al., 2009). The current study examined its effects backcrossed on a C57Bl/6J strain in order to avoid abnormalities inherent to the DBA strain (Cox et al., 2009).

Hippocampus and Memory in SCZ

The hippocampus is essential for various learning and memory functions, which include forming stimuli associations (Anagnostaras et al., 2001; Eichenbaum et al., 2004; Moses et al., 2005; Squire et al., 2003), and is required to form contextual memories (Fanselow & Poulos, 2005; Hemsley, 2005 Maren, 1992; Sanders et al., 2003). Studies involving lesions of the hippocampus in rats show impairment in a contextual fear conditioning task (Anagnostaras et al., 2001; Maren, 2008). Impairments in memory function, including associative learning, as well as contextually elicited responses have been observed in schizophrenia patients (Clarke et al., 2003; Hemsley, 2005). Imaging studies have revealed reduced activation in memory tasks as well as reductions in gray matter and volume of the brain of schizophrenia patients, including the hippocampus (Narr et al., 2009; Preston et al., 2005; Watson et al., 2012). Reductions in expression of the dysbindin protein have also been detected for multiple brain regions including frontal cortex, midbrain, and the hippocampus (Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008). Post mortem studies of SCZ patients show abnormal protein expression at the cellular level, leading to aberrant neurotransmission, as well as problems associated with circuitry in the hippocampus (Talbot, 2004; Weickert et al., 2004). Dysbindin has been implicated, in recent studies, as a potential cause of these anomalies; various brain regions including the hippocampus have revealed reductions in dysbindin protein (Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008).

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Sandy mice provide instrumental evidence for this, exhibiting impairments in synaptic transmission of monoamines (Chen, 2008; Feng, 2008; Kobayashi et al., 2011; Numakawa, 2004; Talbot, 2006), which may relate to deficits of hippocampal-dependent memory processes reported here and in previous studies (Bhardwaj, et al., 2009; Cox et al., 2009; Jentsch et al., 2009; Karlsgodt et al., 2011; Takao et al., 2008). Proteins that directly interact with dysbindin have also been shown to have reduced expression in the sandy mouse (Li et al., 2003; Nazarian et al., 2006).

Dysbindin and Glutamatergic Neurotransmission

Impaired glutamatergic neurotransmission has been previously reported in the sandy mouse (Chen et al., 2008; Jentsch, 2009; Karlsgodt, 2011; Talbot, 2004). *In vitro* studies of dysbindin have showed it may play a role in glutamate secretion (Numakawa et al., 2004). Abnormalities in glutamatergic neurons include larger vesicle size and slower quantal release in CA1 of the sandy mouse (Chen et al., 2008). Hypofunction of NMDA receptors has also been previously described in the sandy mouse (Jeans et al., 2011; Karlsgodt et al., 2011), suggesting an impairment in neurotransmission via pre and/or post-synaptic mechanisms in the absence of the dysbindin protein.

Limitations and future implications

Although deficits in hippocampal-dependent context memory have been identified in the sandy mouse, there were some limitations to the study. Freezing percentage after the footshock revealed sandy mice did not increase freezing over baseline levels, which could imply impairment in amygdala-dependent processing (Fanselow & Poulos, 2005). Previous studies of

sandy on the DBA/6J strain do not show differences between groups in learning the association between tone and shock (Bhardwaj et al., 2009). Although a single shock is sufficient to learn the association between tone and shock (Fanselow, 2009; Maren, 2008; Sanders et al., 2003), perhaps the use of 2 tone-shock associations could provide more robust results. Fear generalization could also be evident if multiple shocks were given.

There was no difference between groups in tone retention, where all groups showed no increases in freezing behavior to any of the tones. This is in opposition to Bhardwaj et al. (2009), which revealed an increase in freezing behavior of sandy mice over wild type controls. A possible explanation is that none of the mice made a proper association of the tone with the shock. The association of CS and US is dependent upon the amygdala (Fanselow & Poulos, 2005; Fanselow, 2009; Maren, 2008), which also contains the dysbindin protein (Benson et al., 2001). In addition, there may be differences in sensitivity to the shock. Bhardwaj (2009) showed that sandy mice had less sensitivity to a stimulus. Perhaps there is a differential in pain thresholds that could explain this phenomenon, and future studies could examine different shock values as well as multiple shocks in order to get more concrete results. The amygdala should be included specifically in future studies of the sandy mouse, both behaviorally and perhaps in studies involving LTP.

Another possibility is that the tone that was paired with the shock is a competing cue which could account for a lack of response in the sandy mouse. Future studies focused on the hippocampus could make use of multiple shocks without a competing cue to focus on contextual association memory. Finally, imaging studies with the sandy mouse reveal abnormalities that could be associated with sensory deficits in hippocampal circuitry, such as problems with

auditory cortex (Lutenkoff et al., 2012). This could account for the lack of association to the tone in retention trials, although all groups showed this absence of freezing behavior.

Majority of the information on the function of dysbindin has implicated its role in association with the BLOC-1 complex (Dell 'Angelica, 2002; Ghiani et al., 2010; Li et al., 2003; Mullins et al., 2011; Ryder & Faundez, 2009Starcevic et al., 2004). Dysbindin has also been shown to directly interact with other proteins in the BLOC-1 complex, such as muted and snapin pallid proteins (Nazarian et al., 2006). Studies of the sandy mouse brain have also revealed reductions in these proteins (Nazarian et al., 2006; Mullin et al., 2011). Future studies should examine other protein subunits of the BLOC-1 complex in order to determine if the mechanism of action of dysbindin is dependent or independent upon BLOC-1.

In conclusion, our results indicate that the sandy mouse shows behavioral deficits in spatial, recognition, and contextual memory. The null mutation which results in the absence of the dysbindin protein in the sandy mouse can lead to impairments in cognition and memory similar to those seen in patients with schizophrenia. The sandy mouse may thus be a useful tool for studying cognitive endophenotypes of schizophrenia.

Chapter 4

Behavioral Analysis of Hippocampal-Dependent Tasks on Pallidin-Deficient Mice: Implications

for BLOC-1 Dependence

Abstract

DTNBP1 has been identified as a candidate risk for schizophrenia. The protein coded by

DTNBP1, dysbindin, is part of a complex of proteins in glutamatergic cells within the

hippocampus called BLOC-1, and has direct interaction with other proteins in this complex. One

of the proteins that directly interacts with dysbindin is called the pallid protein. In order to

examine the function of the dysbindin protein within the BLOC-1 complex, we conducted

hippocampal-dependent contextual fear and recognition memory tasks on a mouse that harbors a

spontaneous deletion in the pallidin gene (Pldn), and therefore fails to produce the pallid protein,

in order to examine if the deficits were similar to the dysbindin-deficient sandy mouse. Pallid

mice showed increased freezing behavior in a contextual fear memory task and impaired

recognition memory. Previous studies using the sandy mouse show similar but not identical

deficits in recognition memory and differing results in contextual fear memory. Taken together,

these results suggest dysbindin may be working independent of BLOC-1 function.

Introduction

 DTNBP1, the gene that codes for the dystrobrevin binding protein, dysbindin, has been implicated as a candidate risk gene in the study of schizophrenia (Benson et al. 2001; Burdick et al., 2006; Ross et al., 2006; Schwab et al., 2004; Straub et al., 2002; Talbot et al., 2006). Genetic variants within *DTNBP1* have been associated with increased risk for schizophrenia (Straub et al., 2002; Voisey et al., 2009), as well as for intermediate phenotypes such as deficits in working and spatial memory function (Cannon, 2005; Wolf et al., 2009).

 Dysbindin is expressed widely in the brain, including in cortical regions often linked to memory function, namely the DLPFC and hippocampus (Talbot et al., 2004; Weickert et al., 2004). Reductions in dysbindin mRNA and its protein in these regions are observed in postmortem tissue from schizophrenia patients (Talbot, 2004; Weickert et al., 2008). The dysbindin protein is located in postsynaptic densities as well as synaptic vesicles and microtubules of glutamatergic neurons (Starcevic & Dell'Angelica, 2004; Talbot et al., 2006), though its primary function is associated with vesicular trafficking and fusion to the membrane for release (Chen et al., 2008). Dysbindin is also associated with hypofunction of postsynaptic glutamatergic NMDA receptors (Karlsgodt et al., 2011).

 Dysbindin acts to aid neurotransmitter release as part of a complex of proteins called the Biogenesis of Lysosome and related Organelles Complex, or BLOC-1, along with the proteins snapin, pallid, muted, cappuccino, and the BLOC-1 subunits 1, 2, and 3 (Dell'Angelica, 2004; Ryder & Faundez, 2009). Dysbindin, and other members of the BLOC-1 complex have been investigated in relation to Hermansky Pudlak Syndrome, a disease characterized by characterized by albinism, prolonged bleeding due to abnormal platelet dense granules, and bruising (Falcón-Pérez et al., 2002; Li, 2003; Nazarian et al., 2006). Much of this research on the dysbindin

protein identifies its physiological effects as BLOC-1 dependent. It has been theorized that the absence of any BLOC-1 subunit triggers the disappearance of all other BLOC-1 subunits (Falcón-Pérez, 2002; Li, 2003; Ghiani et al., 2010; Mullin et al., 2011).

The pallidin gene (*PLDN*), codes for the protein pallid, is expressed in the BLOC-1 complex (Falcón-Pérez et al., 2002; Ghiani et al., 2009; Starcevic & Dell'Angelica, 2004; Moriyama & Bonafacino, 2002). This 20-kDa protein has been shown to have a direct protein-protein interaction with the SNARE protein syntaxin-13, as well as with dysbindin (Ghiani et al., 2009).

Neurophysiological and behavioral abnormalities are seen in the sandy mouse, which harbors a deletion in the coding region of the *DTNBP1* gene, resulting in no production of the dysbindin protein (Li, 2003). In addition, pallidin protein expression is reduced in the dysbindin-deficient sandy mouse. Another BLOC-1 null mutant mouse model was discovered, in which a nonsense point mutation in codon 69 of the *Pldn* gene causes a null mutation in which this mouse does not produce the pallid protein (Huang et al., 1999). This pallid mouse has been used in the study of Hermansky Pudlak syndrome, a disorder that includes albinism and prolonged bleeding. Furthermore, levels of dysbindin are reduced in the pallid mouse, so it may be useful for further investigation of a relationship between BLOC-1 and schizophrenia.

In this study, the pallid mouse was examined using hippocampal-dependent contextual and recognition memory tasks. It was hypothesized that like the sandy mice, pallid mice would show behavioral deficits in these memory tasks, but that the deficits would not be identical to the sandy mice. Some of the deficits in memory exhibited in the pallid mice were similar but not identical to sandy mice, and some measures were not consistent, making a case for dysbindin acting independent of the BLOC-1 complex.

Methods

Animals

Subjects included male C57Bl/6J wild-type mice ($n = 15$ for fear conditioning; $n = 10$ for NORT) obtained from Jackson Labs and Pallidin null mutant mice ($n = 19$ for fear conditioning; $n = 10$ for NORT) on the C57Bl/6J strain between 60 and 120 days old, obtained from the Dell'Angelica lab at UCLA (Los Angeles, CA). Experimental mice were generated by homozygous to homozygous crosses (-/- crosses for the light coat colored mutant mice and +/+ for the Wild-type mice). A C→T substitution at nt787 results in a nonsense mutation at argentine codon 69, which results in deletion exon 2 and termination of translation after that site. The mice were housed in same-sex groups in a room that was controlled at a constant temperature of 71 +/- 3 degrees Fahrenheit. Animals were housed 2-4 per cage and had access to food and water ad libitum. Animals were kept on a 12 hour regular light-dark cycle.

Contextual Fear Conditioning

All behavioral testing was assessed with MedAssociates Video Freeze fear-conditioning equipment. The fear-conditioning experiments were conducted in two different contexts. Context A consisted of a fear-conditioning box in a well-lit room with rounded walls made from a 95cm by 20.5cm white plastic insert; the box was fitted with holes for the speakers and a grid floor with 36 individual stainless steel rods, each with a diameter of 0.32cm and spaced 0.64cm apart (center to center). The tray under the grid was scented with a 50% Windex solution, and the box was cleaned with a 10% ethanol solution between trials. The house lights were illuminated in this context.

Context B featured aluminum walls, and the grid floor was covered with a white plastic rectangular insert. On top of the plastic floor was an inverted V-shaped transparent plastic board inside the boxes, the tray underneath was scented with a 1% acetic acid solution. The boxes for context B were in a different room with low illumination, and inside the box the house lights were off.

Fear was measured as mean percent time spent freezing to a tone or context CS (percentage by component). Freezing is defined as complete cessation of movement and was measured by Med Associates, Near Infrared Video fear-conditioning system.

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Mice were habituated for 7 consecutive days. During this time they were transferred from the vivarium to the holding room for 1 hour. For the first 15 minutes, they were left alone in the room. Over the next few minutes, each mouse was picked up by their tail, handled for at least 10 seconds, and then placed into a 500ml glass beaker.

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The mice were transferred from the vivarium into a quiet anteroom. They were individually placed in the chamber into the context A room, one cage at a time, and tests began immediately. There was a 4-minute baseline followed by a 30 s tone, played at 70 db (2800 Hz). During the final 2 seconds of the tone, a 0.5 mA footshock was delivered to the mice. This was followed by a 2 min postshock period with no further programmed stimuli. After each session, the fear-conditioning boxes were thoroughly cleaned with 10% ethanol solution and dried

Context Fear Test - Day 2

The mice were taken from the vivarium and put in a quiet anteroom. They were then transferred to the context A chamber for 8 min. No tones or shocks were presented. After each session, the cages were cleaned and disinfected.

Fear Generalization Test - Day 3

The mice were taken from the vivarium and placed in a quiet anteroom. From there, they were placed into the context B chambers, for 8 minutes, in the absence of tone and shock. When each session was finished, the cages were be thoroughly cleaned with a 1% acetic acid solution.

Tone Retention Test - Day 4

The mice were taken from the vivarium and placed in a quiet anteroom. From there, they were placed in the context B chambers. In this test of tone fear retention, the first 90 s constituted the habituation period, with the next 30 s used as the baseline retention period. Subsequently, three 30 s, 70 db tones, each separated by a 1min interval were played. When each session was finished, the cages were cleaned with a 1% acetic acid solution.

Novel Object Recognition Task (NORT)

An open-field chamber ($L \times W \times H$: $30 \times 30 \times 30$ cm) made of opaque grey Plexiglas was placed in a quiet room. Mice of each genotype ($n = 10$ per genotype; 30 total) were individually placed in the arena for 20 min on two consecutive days in order to habituate them to the arena. On the third day, the mice were placed in the arena with two identical objects (black plastic pipe connectors) for 20 min to acclimate them with test condition. On the test day (Day 4), there were

3 phases: A 5 min habituation phase, a 5 min familiarization phase, and a 5 min test phase, with a 1 hr delay between the familiarization and test phases. In the habituation phase, mice were placed in the arena without any objects and allowed to explore freely for 5 minutes. The mice were then removed for the familiarization phase, and two identical objects were placed in the top right and bottom left corners of the arena, a few centimeters from each corner. The objects were grey metal pipe connectors slightly bigger than the black pipe connectors. The mice were allowed to explore the objects for 5 min. The mice were then removed and placed back into their respective home cage for 1 hr.

In the test phase, the mice were placed back into the arena with one familiar object (metal pipe connector) and a novel object (white plastic pipe connector) similar in size and shape of the familiar object. The familiar and novel objects were counterbalanced for location throughout the trials. Behavioral activity during the familiarization and testing phases were videotaped via an infrared surveillance camera, and recorded via a Lorex LW1001 infrared surveillance camera connected to an HP laptop. A mouse was considered to be involved in object exploration when its head was oriented directly towards the object (at least 45 degrees) and within approximately 2–3cm from it. This includes rearing of the head. The time was also included if the mouse was directly interacting with the object by having at least one forepaw on the object, if the mouse stood on top of the object, or if the mouse was sniffing or licking the object. Object recognition memory was defined as the ratio of exploration time for the novel object (TN) over by the total exploration time for the novel and familiar (TF) objects [exploration ratio = TN/ (TF + TN)]. Rodents have a natural tendency to explore novel objects in their surrounding compared to objects already familiar. Increased time of exploration of novel object reveals recognition memory for objects (Trials were analyzed using the Ruby script program on

an Apple Macbook to record the time spent exploring both objects. Time was recorded in seconds to the hundredths).

Statistical Analyses

One-way ANOVAs were used to compare the percentage of time spent freezing in each context (A and B) between genotypes. Shock burst was measured as the maximum burst of activity captured the MedAssociates program. During the acquisition phase, baseline freezing and freezing behavior after the tone-shock pairing were measured. . During the context fear test, the overall percentage of time spent freezing was measured for each mouse. During the fear generalization test, the overall percentage of time spent freezing was measured for each mouse. Finally, during the tone test, the baseline freezing in context B was measured 30 s prior to the first tone. After that, freezing during each individual 30 s tone was measured.

One-way ANOVA analysis was used to evaluate significant differences between genotypes for measures of total time spent exploring all objects, time spent exploring the familiar object, time spent exploring the novel object, and the ratio of exploration of novel object/ total exploration time.

Results

Contextual Fear Conditioning

There were no differences in baseline freezing and activity level before or during the shock observed in pallid mice and wild type controls. However, pallid mice showed much higher freezing percentage in measures of context association, fear generalization, and both baseline and

throughout the tone retention task. These results indicate that the pallid mice showed a sensitization effect after the shock was administered.

For the acquisition phase, a one way ANOVA examined baseline freezing during the four minutes prior to the CS-US pairing (Tone-shock). There was no significant differences between wild types and pallid mice (F 1, 32 = .085, p >.05) (Figure 4.1a). Subsequently, to measure sensitivity of footshock, ANOVA was used to examine the activity between genotypes. Repeated measures ANOVA showed a main effect of activity (F 1, 32 = 266.97; p <.01), but no main effect of genotype (F 1, 32 = .66, p >.05) or activity x genotype interaction (F 1, 32 = .29; p <.05) (Figure 4.1b).

Finally, postshock freezing was evaluated with a one way ANOVA, revealing a main effect of genotype (F 1, 32 = 5.80, p >.05), showing that pallid mice froze nearly twice as much as the C57/Bl6 wild-type mice (Figure 4.1c).
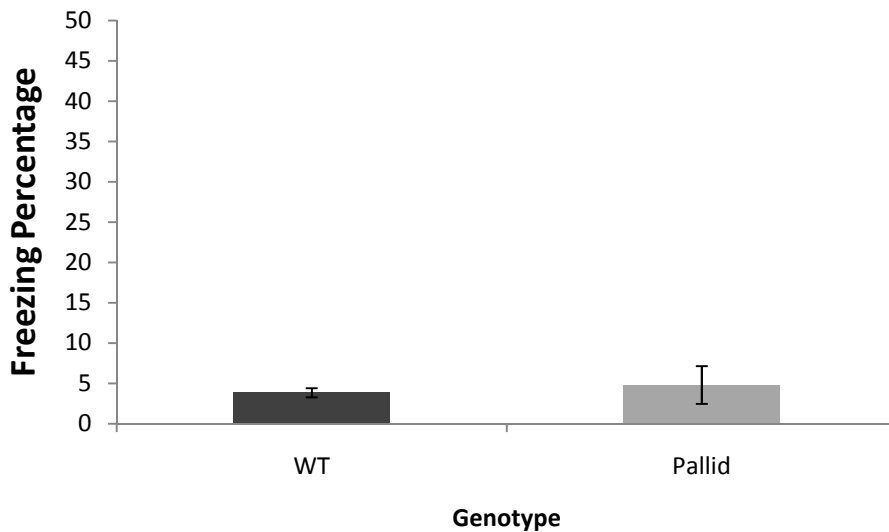
Figure 4.1a. Baseline freezing (percent) for acquisition trial of wild type (WT) and homozygous pallid mice. There were no differences between groups in percentage of baseline freezing.
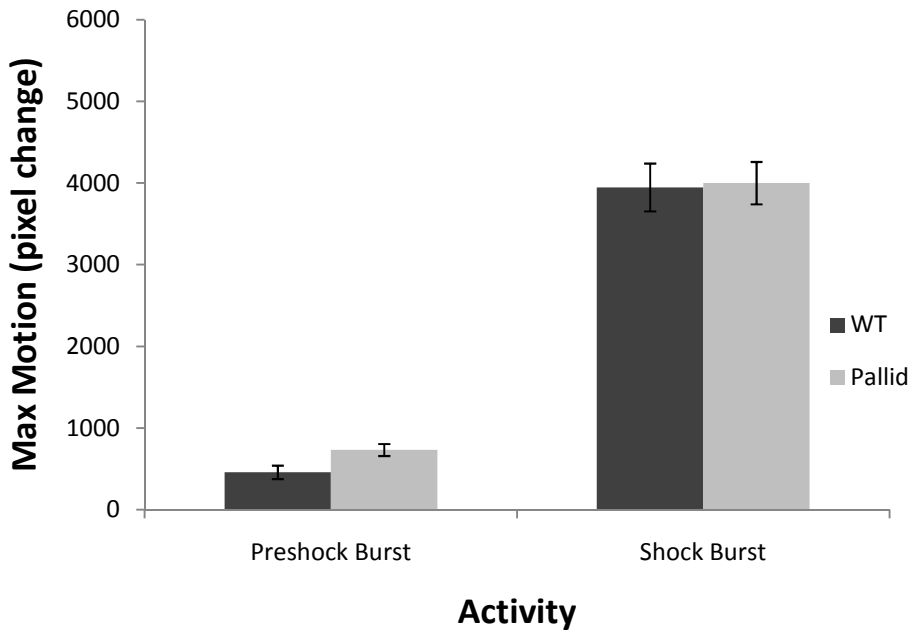
Figure 4.1b. Activity (pixel change) before the shock (2 sec) and during the shock (2 sec) for wild type (WT) and homozygous pallid mice. There was a main effect of activity as it increased during the shock for all groups, but there was no main effect of genotype and no activity x genotype interaction.
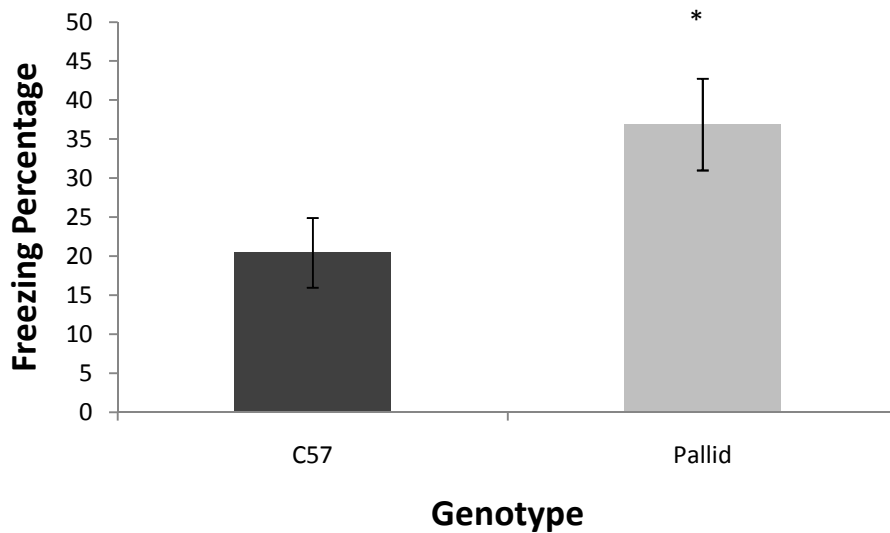
Figure 4.1c. Postshock freezing (percent) for wild type (WT) and homozygous pallid mice. Pallid mice showed much higher freezing compared to wild type mice.

Twenty-four hours later, the mice were exposed again to context A in the absence of tone and shock (CS and US). A one way ANOVA revealed a main effect of ($F_{1, 32} = 13.37$, $p < .01$) (Figure 3.2), with pallid mice freezing much higher than wild type controls.
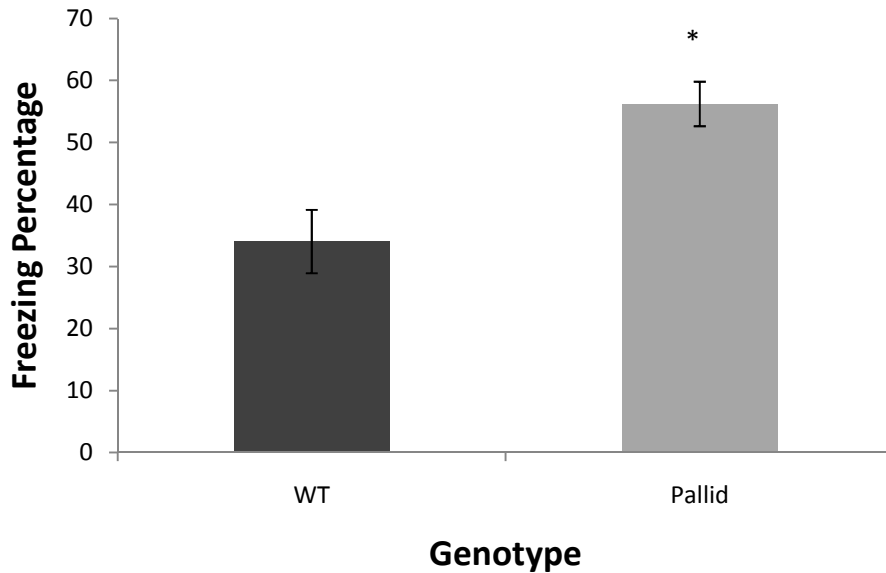
Figure 4.2. Freezing (percent) for the context test. Wild type (WT) and homozygous pallid mice were placed back in the same context as the acquisition and percentage of the trial spent freezing was measured. Pallid mice froze significant more than wild type mice.

To test for fear generalization, the mice were placed in context B for 8 minutes, on a separate day. A one way ANOVA revealed a main effect of genotype pallid mice in the new context ($F_{1, 32} = 35.88$, $p < .01$), with pallid mice ($M = 39.6\%$) freezing much higher than wild-type mice ($M = 7\%$) (Figure 4.3).
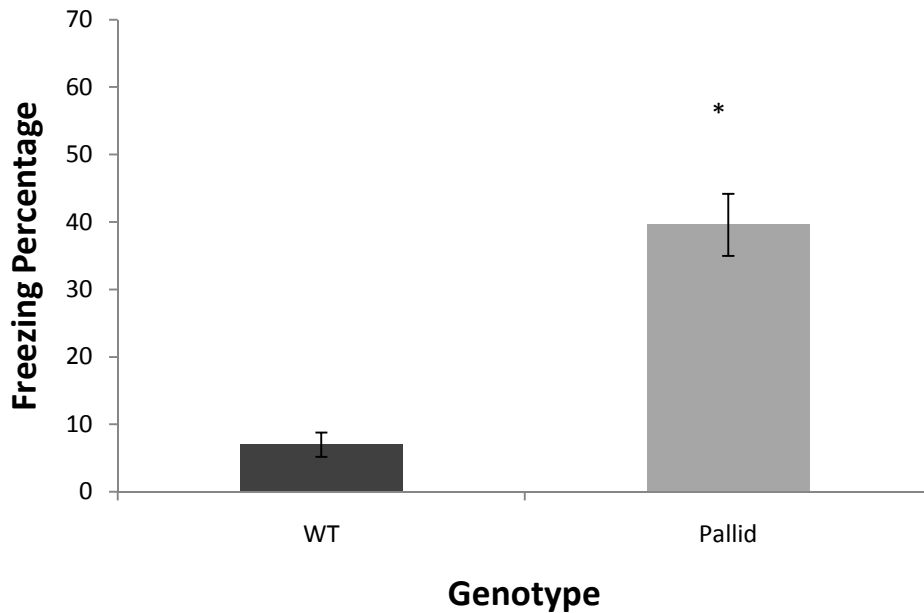
Figure 4.3. Freezing (percent) in a fear generalization test. Wild type (WT) and homozygous pallid mice were placed in a box with a different context, to see if the freezing response generalized to a new environment. Pallid mice froze significantly higher than WT mice, indicating an inability to generalize over contexts.

On the fourth day, the mice were tested for tone retention. A one way ANOVA revealed a main effect of genotype over the baseline period baseline ($F_{1, 32} = 37.196$; $p<.01$) (Figure 4.4a). For tone retention, repeated measures ANOVA revealed no main effect of tone ($F_{2, 64} = 1.945$; $>.05$), or tone x genotype interaction ($F_{1, 32} = 0.36$; $p >.05$), but did reveal a main effect of genotype ($F_{1, 32} = 35.179$, $p <.01$), with higher levels of freezing for pallid mice over all 3 CS presentations (Figure 4.4b). These statistical analyses suggest that the pallid mouse may be over sensitized to the footshock stimulus, as they showed increased freezing over all measures after the US was delivered.
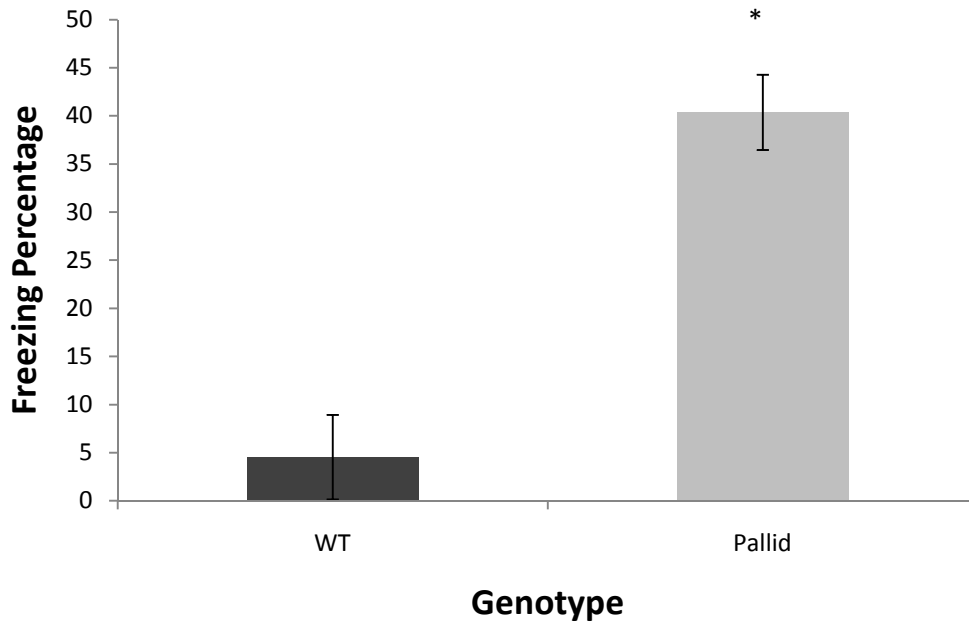
Figure 4.4a. Baseline freezing (percent) for wild type (WT) and homozygous pallid mice in the tone retention test. Pallid mice froze much more than wild type mice.
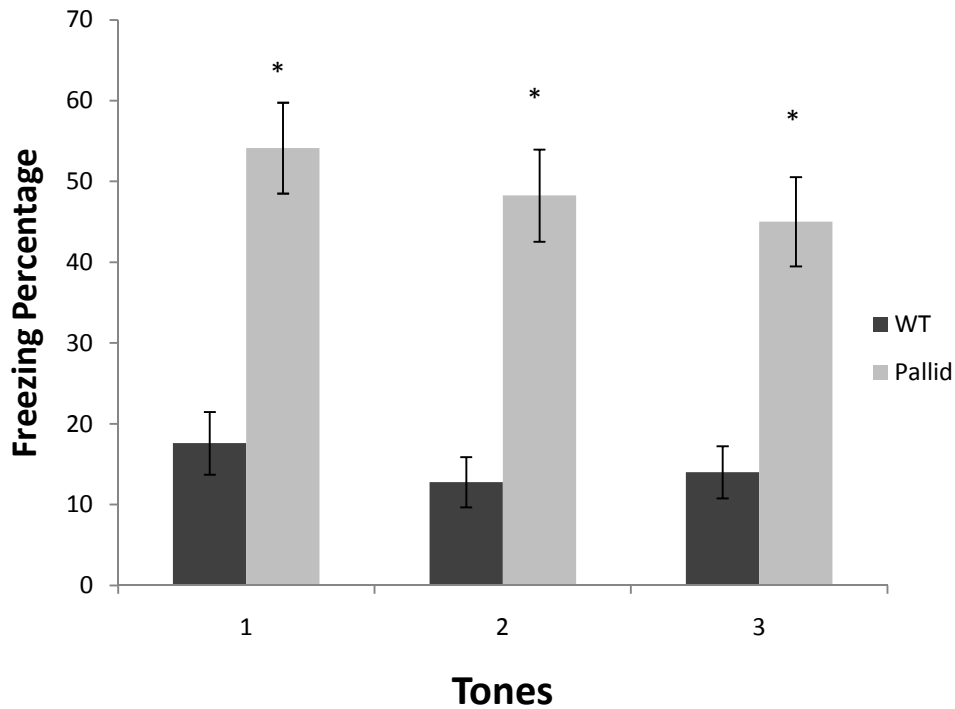
Figure 4.4b: Freezing (percent) during the tone retention test. Homozygous pallid mice froze significantly higher than wild types over each of the three CS presentations.

Novel Object Recognition Task

In this task, pallid mice showed a lack of exploratory behavior compared to wild type controls in exploratory behavior involving the objects. This effect was identical in both the familiarization and test phases, which rules out neophobia as an explanation. There is also a difference in ration of preference for the pallid mice compared to wild type mice, however it is hard to argue for a deficit in recognition memory with such a small amount of exploratory behavior in pallid mice.

The familiarization phase of this task looked at exploratory behavior of the original objects. A one-way ANOVA revealed a main effect of genotype (F 1, 18 = 17.73; p <.05), where wild type mice spent more time exploring the original objects compared to pallid mice, who spent very little time exploring the objects (Figure 4.5a).

A one-way ANOVA was used to look at differences between C57Bl/6J wild type mice and homozygous pallid mice in the exploration of a novel object versus a familiar object in the test phase, showing an effect comparable to the familiarization phase. A significant main effect for genotype was found in the total exploration of both objects (F 1, 18 = 14.75; p <.01) with the wild-type mice spending much more time exploring the objects (M = 70.72 seconds) than the pallid mutant mice (M = 9.49 seconds) (Figure 4.5b).

Next, exploration of each object individually was examined, and there were main effects of genotypes for both exploration of the familiar object (F 1, 18 = 9.4; p <.01) (Figure4.5c) and for exploration of the novel object (F 1, 18 = 17.44; p <.01) (Figure 4.5d), where the wild-type mice spent much more time exploring the objects.

Last, the ratio of exploration was examined by measuring the preference of time spent for the novel object divided by the total amount of time spent exploring both objects. A one way

ANOVA revealed a main effect of genotype for exploration ratio ($F_{1, 18} = 8.79$; $p < .05$) (Figure 4.5e). Wild type controls showed a much greater preference for the novel object than homozygous pallid mice, which showed no preference for either object by spending about the same amount of time exploring each one.
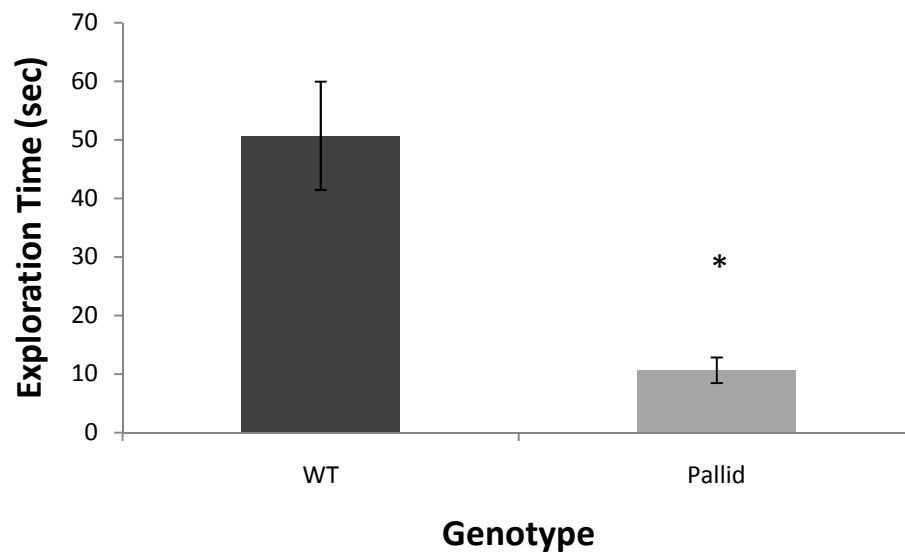
Figure 4.5a. Total time (sec) spent exploring objects in the familiarization phase for wild type (WT) and homozygous pallid mice. Wild type controls spent significantly more time exploring the objects.
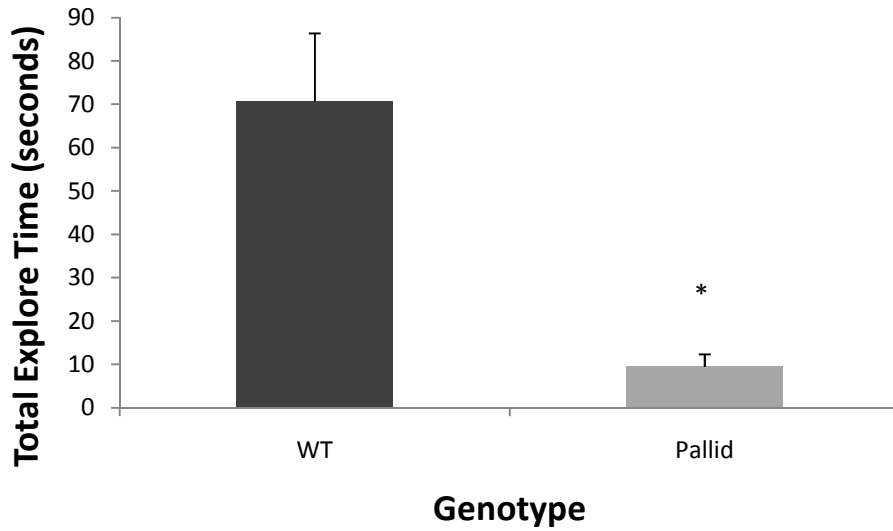
Figure 4.5b. Total time (sec) spent exploring both objects (novel and familiar) in the test phase for wild type (WT) and homozygous pallid mice. Wild type controls spent significantly more time exploring the objects.
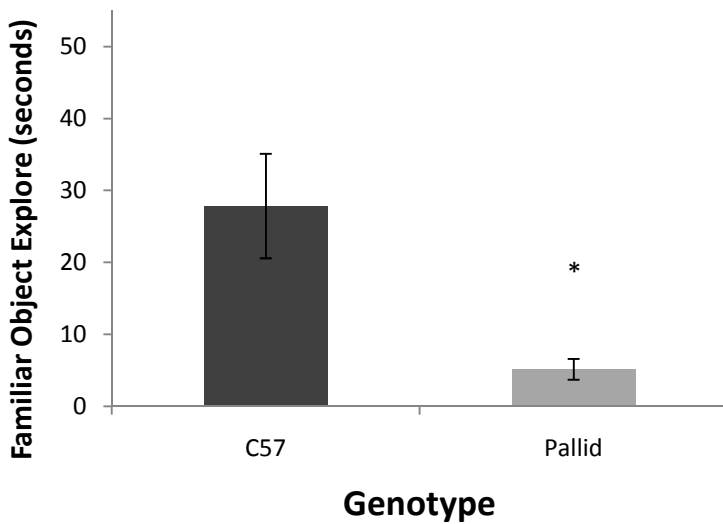
Figure 4.5c. Time spent exploring the familiar object in the test trial (sec) for wild type and homozygous pallid mice. Wild type controls spent more time exploring the familiar object.
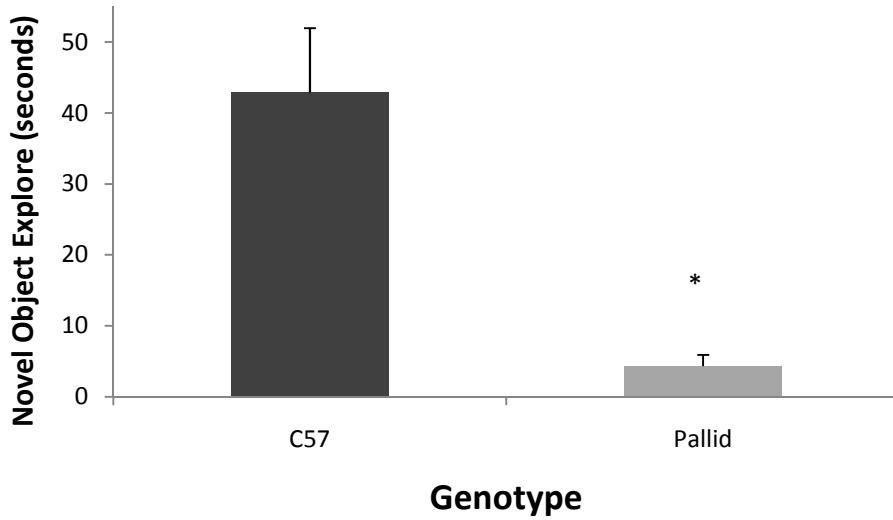
Figure 4.5d. Time spent exploring the novel object in the test trial (sec) for wild type (WT), and homozygous pallid mice. Wild type mice explored the novel object much longer than pallid mice.
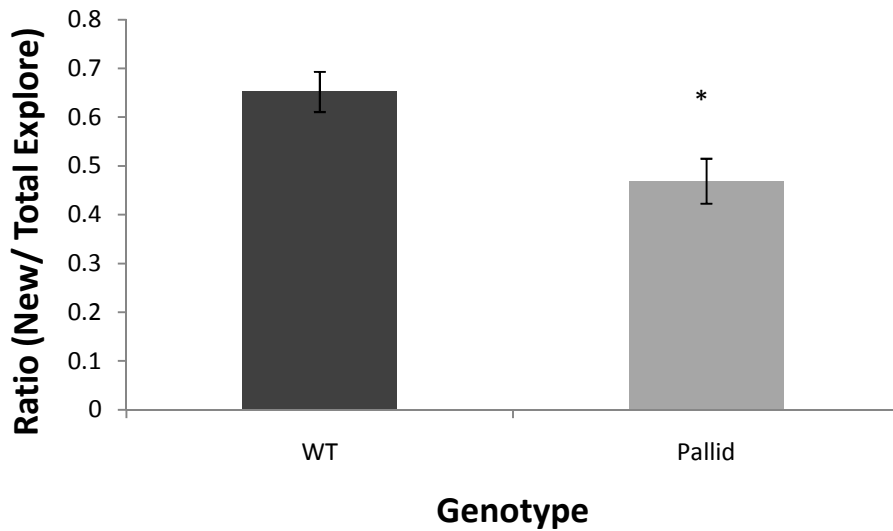
Figure 4.5e. Ratio of time spent exploring the novel object/ total amount of time exploring objects in the test trial for wild type (WT) and homozygous pallid mice. Wild type mice had a significantly higher preference for the novel object than pallid mice.

Discussion

The behavior of pallid mice was compared to wild type C57Bl/6J mice in hippocampal-dependent tasks of contextual and recognition memory. Although pallid mice showed similar baseline levels to wild type controls in a contextual fear conditioning task, they showed increased freezing in acquisition, context memory, and context generalization, as well as higher freezing behavior in a tone retention test. Pallid mice showed impaired recognition memory, with no preference for a novel object over a familiar one, as seen with wild type mice. Furthermore, pallid mice spent much less time exploring the objects than controls.

BLOC-1 Complex

The BLOC-1 complex is made up of 8 proteins: dysbindin, muted, cappuccino, snapin, pallidin, and BLOC-1 subunits 1, 2, and 3 (Falcón-Pérez et al., 2002; Morris et al., 2008; Mullin et al., 2011; Ryder & Faundez, 2009). The dysbindin and pallid proteins share a direct interaction (Nazarian et al., 2006; Mullin et al., 2011). The loss of one BLOC-1 protein subunit is believed to destabilize the complex, as it leads to a downregulation of other proteins (Falcón-Pérez, 2004; Feng et al., 2008; Mullin et al., 2011). The current hypothesis for dysbindin regarding the BLOC-1 complex is that alleles in BLOC-1 should influence disease risk and that reduction of dysbindin would lead to reductions in other BLOC-1 proteins (Morris et al., 2008; Mullin et al., 2011). Finally, the loss of one protein would lead to destabilization of the complex and silence the entire complex.

BLOC-1 mouse models

Previous studies on the sandy mouse reveal reduced expression of pallid, muted, and snapin proteins (Feng et al., 2008; Ghiani & Dell'Angelica, 2011; Li et al., 2003; Nazarian et al., 2006). Likewise, protein levels of dysbindin have been shown to be reduced in the pallid mouse (Ghiani et al., 2010; Ghiani & Dell'Angelica, 2011). Pallid mice and sandy mice also share phenotypic traits, such as albinism which is seen in their similar coat colors, and both mouse models have been used in the study of Hermansky-Pudlak syndrome (Falcón-Pérez et al., 2002; Li et al., 2003; Nazarian et al., 2006). According to the BLOC-1 hypothesis, the absence of either dysbindin or pallid would cause the disruption of the BLOC-1 complex. Therefore, if the actions of dysbindin are solely BLOC-1 dependent, then the pallid mouse should show identical impairments in these memory tasks.

In the contextual fear conditioning task, pallid mice showed continuous increased freezing behavior after receiving the footshock. This behavior carried over all 4 days of the task. In comparison, the sandy mice showed a decrease in freezing in the context association task, and show what appears to be increased generalization of fear. These results indicate the sandy mice show impaired contextual association, while the pallid mice show an increased response to generalize to a specific context. The increased freezing could be due to sensitization of freezing responses elicited by the single shock in the pallid mouse, so a recognition memory task was used, which eliminates the aversive stimulus and examines spontaneous behavior.

In the novel object recognition task, pallid and sandy mice did show similar impairments in the ratio of preference, both revealing no preference for either the novel or the familiar object. However, these mice showed marked differences in their time spent exploring the objects, as sandy explored the objects extensively, similar to their wild type and heterozygous littermates. The pallid mice spent almost no time exploring either object. This could be indicative of a lack

of interest in the objects, rather than a deficit in recognition memory. The pallid mice did not show effects of neophobia in this case, because the results were nearly identical to the earlier familiarization phase with the familiar objects. If neophobia could explain the behavior of the pallid mice, this would not be seen in the testing phase one hour later. The effect could be due to anxiety in the testing box, so further studies on the pallid mice should include an open field locomotor task to further investigate spontaneous behavior and look at anxious behavior in this mouse model.

Limitations and future implications

Although it has been shown that pallid and sandy mice show non-identical deficits in hippocampal-dependent tasks, there are a number of other issues to consider. It is possible that the impairments seen in the pallid mice are due to the consequences of their mutation and not due to impairments in cognitive processes. For example, this mouse has been used in previous experiments to characterize Hermansky Pudlak Syndrome (HPS), characterized by a lack of clotting factors causing prolonged bleeding, as well as pronounced albinism, giving these mice a white coat reminiscent of the sandy mouse (Li et al., 2003; Moriyama & Bonifacino, 2002). In this case, the abnormalities seen in the HPS phenotype could lead to physical deficits that are masked as deficits in cognition. In addition, it should be noted that the phenotypic results of the pallid mouse could be due to another mutation in close proximity to the pallid mutation, which would be in linkage disequilibrium with the original mutation. The chances of a mutation are small, but they are non-zero.

Furthermore, pallid mice have been born with a host of abnormalities including head tilt, inability to swim, and compromised pulmonary function, as shown by studies of the pallid mouse

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in emphysema (Falcón-Pérez & Dell'Angelica, 2002; Martorana, 1993) and a shorter lifespan than wild-type mice (Falcón-Pérez et al., 2002). Future studies with the pallid mice should include a battery of tasks in order to evaluate the range of physical abnormalities found in these mice and the contribution of these anomalies to their behavioral phenotypes. These types of anomalies could be consistent with the behavioral output in the pallid mice.

Although baseline and activity levels were similar to wild type controls, spontaneous locomotion was not measured in the pallid mice. This type of task may have given a better indication of whether the mouse displays natural hyperactivity or heightened levels of anxiety; or it may reveal hypolocomotion, which may contribute to the lack of exploration observed in the novel object recognition task. Further research involving the behavioral study of the pallid mouse should include a locomotor measurement.

In addition to behavioral abnormalities, to our knowledge, the pallid mouse has not been examined for physiological abnormalities, including glutamate neurotransmission, specifically. If the deficits in memory tasks are not congruent, perhaps the underlying cellular mechanisms are not the same as the ones seen in the sandy mouse. Future studies should attempt to examine the pallid mouse on a cellular level, as well as look for function of the pallidin protein outside of the BLOC-1 complex.

Another limitation is the fact that pallid mice are bred homozygous to homozygous, so wild type controls, although on the same background, are not littermates (Holmdahl & Mallison, 2012).. Differences in behavior could be different due to differential maternal patterns with the mice as pups. The genotypic variation between the C57Bl/6J wild types and the pallid mice in this study may be quite different, leading to such differences in the observed behavior. The use

of techniques such as cross-fostering, to see if the effects are due to genetic traits or the rituals of the environmental factors, can be employed in future scenarios.

Other genes involved in BLOC-1 have shown association to increased risk in schizophrenia, in association studies with humans, such as the muted gene (Guo et al., 2009; Morris et al., 2008; Ryder & Faundez, 2009). Future studies could incorporate mice with that are absent for the muted protein in BLOC-1, and compare the effects sandy and pallid mice.

It was hypothesized that the dysbindin protein is working independently of BLOC-1, at least in addition to its role within the complex. What was not accounted for is the possibility that the pallid protein has functional properties outside of its role within the BLOC-1 complex as well. The differences that the pallid mice showed in comparison to the sandy mice could be due to processes independent of the pallidin protein's direct interaction with dysbindin. The pallidin protein has been detected in other organs outside of the brain which have been implicated in Hermansky Pudlak syndrome, and pallidin binds directly to itself and syntaxin-13, in addition to dysbindin (Falcón-Pérez & Dell'Angelica, 2002; Huang et al., 1999; Moriyama & Bonifacino, 2002). However, none of these studies have found the presence of the pallid protein in the brain outside of its role in vesicular trafficking and docking to the membrane in either the human or mouse ortholog. More research should focus on a role for pallidin independent of the BLOC-1 complex.

In conclusion, the deficits seen in the pallid mice in hippocampal-dependent tasks are not identical to the deficits seen in the dysbindin-deficient sandy mouse, arguing against the idea that the effects of dysbindin are BLOC-1 dependent. Since this is the first attempt to use the pallid mouse in behavioral experiments, a more robust behavioral characterization profile of this mouse would be beneficial, in both cognitive and physical capacities, in order to lend support to the

differences observed in the present study. The Pallid mouse may also be seen as a useful tool in the ongoing study of the pathophysiology of schizophrenia.

Chapter 5

General Discussion, Conclusions, Future Implications

The work in the preceding chapters was aimed to examine the behavioral consequences of glutamatergic dysfunction of the hippocampus in the absence of *DTNBP1*, the gene which codes for the dysbindin protein (Benson et al., 2001; Li et al., 2003). This began with a history of theories and evidence for the role of dysbindin in schizophrenia, from association studies in humans that relate to increased risk for the disease, to phenotypic measures of physiological anomalies and cognitive deficits observed in mice.

Schizophrenia

Schizophrenia is a complex disorder that is thought to involve multiple genes interacting with some kind of environmental factors that combine to produce a devastating combination of symptoms. These include positive symptoms such as exaggerated motor skills, hallucinations, and delusions; and negative symptoms include lack of affect, changes in social behavior, and cognitive deficits in executive functioning and memory-related activities. Furthermore, there are brain abnormalities, such as enlarged ventricles and reduction of neuropil and grey matter in the brain, that are evidenced by imaging studies on patients diagnosed with schizophrenia compared to their matched controls (Burdick et al., 2006; Talbot, 2004; Weickert et al. 2004; Weickert et al, 2008). These physical abnormalities may be associated with atypical genetic makeup and measured by cognitive abilities and memory in behavioral tasks. Specifically, previous studies have shown reductions in hippocampal volume, as well as reductions of expression of *DTNBP1* and consequent dysbindin protein in the postmortem brains of schizophrenia patients (Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008).

Genetic association of DTNBP1

The overlap of genetic association of DNTBP1 and symptoms related to schizophrenia in humans comes from many studies of different populations (Bray, 2005; Fanous et al., 2006; Numakawa et al., 2004; Schwab et al., 2004; Straub et al., 2002; Voisey et al., 2010; Williams, O'Donovan, & Owen, 2004). Certain single nucleotide polymorphisms (SNPs) and haplotypes have been associated with both increased risk for schizophrenia and deficits in the cognitive function, although not all studies show this association. With evidence provided in association with respect to genetics, morphology, neurotransmission, and behavioral studies, this makes the dysbindin gene a promising candidate to study the pathophysiology of schizophrenia.

Glutamatergic Function in SCZ

Glutamatergic function has been implicated in schizophrenia in relation to the deficits seen in cognitive ability, memory, and executive functioning (Chen et al., 2008; Collier, 2003; Jentsch & Roth, 1999). The original hypothesis posited that abnormal transmission of dopamine was one of the underlying factors in the pathophysiology of schizophrenia, but more recently glutamate has become a main target, most likely in its interaction with dopamine. Studies involving pharmacological manipulation resulting in the inactivation of the N-Methyl-D-Aspartate (NMDA) receptor have shown psychosis-like symptoms in animal models, functionally similar to humans with schizophrenia (Jentsch & Roth, 1999). The sandy mouse is to have aberrant neurotransmission of glutamatergic function, primarily resulting in functional deficits in several cognitive tasks (Chen et al., 2008).

BLOC-1

Furthermore, glutamatergic neurotransmission has been posited to rely on the Biogenesis of Lysosome-Related Organelles complex 1 (BLOC-1), consisting of 8 proteins: dysbindin, pallid, muted, snapin, cappuccino, and BLOC-1 subunits 1, 2 and 3 (BLOS-1, 2, 3). Past research has implicated the role of the BLOC-1 complex in the trafficking and docking of glutamatergic vesicles for release (Chen et al., 2008; Dell'Angelica, 2004; Mullins, 2010; Ryder & Faundez, 2009; Starcevic & Dell'Angelica, 2004; Talbot et al., 2006), and the evidence from experiments involving human dysbindin and the sandy mouse model indicate its function as BLOC-1 dependent (Li, 2003; Nazarian et al., 2006; Ryder & Faundez, 2002; Talbot et al., 2006).

According to the functional hypotheses of BLOC-1, if one protein is absent from the complex, the entire complex is muted (Falcon-Pérez, 2002; Ghiani et al., 2009; Li, 2003; Mullin et al., 2011). If the main assumption of this hypothesis is correct, then any mouse that contains a mutation which eliminates any BLOC-1 protein should not only show deficits in these cognitive behavioral tasks, but the deficits seen in these mice should be nearly identical to one another.

Animal Models of SCZ

The use of animal models in the study of the pathophysiology of schizophrenia can be beneficial and relational to a number of symptoms that has been shown to be behaviorally similar to humans (Gottesman & Gould, 2003). Mouse models, therefore, provide promising potential for the ability to focus on intermediate characteristics of the disease; these endophenotypes may lead us towards the discovery of missing pieces of evidence that lie between a single genetic abnormality and the complexity of the disease as a whole (Cannon, 2005). In this method, we can make quantifiable inferences and have the ability to even identify relationships between

genetic variations and expression of traits (Amann et al., 2010; Cannon, 2005; Cannon & Keller, 2006).

We presented three experiments using the homozygous sandy and pallid mice. In the first two experiments we sought to observe hippocampal-dependent forms of memory in mice that produced no dysbindin protein, compared to heterozygous mice with one allele which produced a reduction in dysbindin protein levels, and their wild type control littermates. We confirmed there were deficits in spatial, recognition, and contextual memory in the sandy mouse (chapter 2 and 3), compared to wild types and heterozygous mice; although the heterozygous mice did show deficits as well in spatial memory (chapter 2).

With the deficits in memory confirmed in the sandy mouse, we then examined the effects of dysbindin within the BLOC-1 complex by comparing the behavior of the sandy mice to another BLOC-1 deficient mouse, the pallid mouse, which produces no pallid protein. There were deficits observed in the pallid mice compared to wild type controls in fear generalization and recognition memory; however, the deficits observed were not identical to the deficits observed in the sandy mouse (chapter 4).

Spatial and Recognition Memory in Sandy Mice

In chapter 2, homozygous sandy mice were compared to their heterozygous and wild type littermates and showed comparable abilities in locomotor activity, indicating no anxiety or lack in ability to habituate to their environment, as well as comparable abilities in learning, assessed in the training trials of the Morris water maze. We predicted that sandy mice would eventually learn to find the submerged platform based on the extramaze cues, but that they would take longer to learn, catching up by day 4 or 5. This was not the case. Sandy mice were similar to

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wild type controls and heterozygous mice in latency, path length swam, and in time spent next to the wall (thigmotaxis), only showing differences in swim speed, which was significantly lower than wild type controls. These results would not be attributed to problems in locomotor activity, since sandy mice performed similar to controls in the locomotor task. Sandy mice did show a lack of spatial memory in the probe trial twenty four hours later, when they performed at chance numbers, spending about 25% of the time in the quadrant where the platform had previously been located. Recognition memory was also impaired in the sandy mice, as wild type mice showed a distinct preference for a novel object compared to a familiar one (Mumby et al., 2001). Sandy mice showed no preference for either object, spending about the same amount of time exploring each one (chapter 2). Object recognition has been controversial as it has been implicated in different studies to be both dependent and independent of the hippocampus (Albasser et al., 2011; Moses et al., 2005; Mumby et al., 2001). To further study the sandy mouse in hippocampal-dependent memory, we examined another form of memory involving contextual association.

Context Memory in Sandy Mice

In chapter 3, we tested homozygous sandy mice and their heterozygous and homozygous littermates in a contextual fear conditioning task, and found that sandy mice showed a deficit in contextual memory when placed in same environment as the tone-shock association twenty four hours later, by showing much less freezing behavior than their littermates. This task confirmed impairment in another form of memory associated with the hippocampus.

Hippocampal-Dependent Memory in Pallid Mice

In the next chapter, we sought to assess whether the actions of dysbindin were solely dependent of BLOC-1, by comparing sandy mice to pallid mice in some of the same hippocampal-dependent memory tasks. The pallid mice showed increases in fear generalization in the contextual fear conditioning tasks; but also showed increased freezing behavior in the contextual association portion of the task compared to wild type controls. Furthermore, pallid mice spent almost no time exploring either object in the object recognition task and showed no preference for either object, possibly indicating deficits in recognition memory.

Future implications

It is worth noting that the heterozygous sandy mice differed in the amount of significance between groups, sometimes performing similar to the homozygous sandy mice, with deficits observed in the probe trial of the water maze, while other times they appeared to fall right in between the performance of the wild-type mice and the homozygous sandy mice, as witnessed in the contextual-fear component of the fear-conditioning task and the ratio of preference for objects in the NORT. An alternative explanation for this could be that this mouse does have one allele of the *DTNBP1* gene, which can lead to about 50% expression, conducive to behavior in between the wild-type and the homozygous mouse, which has no expression of dysbindin. Perhaps the deficits seen in the probe trial of the water maze show that the deficits in spatial memory are comparable to the homozygous mice, but perhaps there may be a compensatory mechanism in other types of hippocampal-dependent performance, namely recognition and contextual memory. The heterozygous mouse itself is an interesting aspect of the study since the amount of dysbindin expression seen in postmortem tissue is merely reduced and not absent

(Talbot, 2004; Weickert et al., 2004; Weickert et al., 2008), therefore the heterozygous phenotype may be the closest resemblance to humans. However, no previous study makes mention of the glutamatergic properties with respect to the heterozygous mice, so this may be a direction to look for in future research with the sandy mouse.

The comparison of results from chapter 4 with the results from chapters 2 and 3 indicate that, although there are deficits observed in both the sandy and pallid mouse models, these deficits are not identical to each other, and may be the first study to provide evidence to refute the hypothesis that the effects of dysbindin are solely dependent upon the BLOC-1complex. The dysbindin protein is not just confined to the BLOC-1 complex on the presynaptic side. Previous studies have also shown dysbindin to be located within the postsynaptic density (PSD) of glutamatergic neurons (Talbot, 2009). It would stand to reason that this could affect receptor density, receptor function or signal transduction in the post-synaptic cell.

Decreases in efficacy of the NMDA receptor have been associated with schizophrenia (Jentsch & Roth, 1999), and this may contribute to the deficits seen in the sandy mice as well. We have already seen decreases in NMDA evoked current in PFC pyramidal cells in the sandy mouse (Karlsgodt et al., 2011), so it stands to reason this same effect may apply to cells within the hippocampus. Perhaps further studies with sandy mice should concentrate on the exploration of the function of NMDA receptors within the hippocampus and PFC in order to expand upon the current research. Specifically, future research could also be directed towards trying to rescue the cognitive deficits associated with schizophrenia.

Studies using D-serine, an allosteric NMDA modulator (Ross et al., 2006; Tsai, Yang, Chung, Lange, & Coyle, 1998), could be used to try and rescue the effects of cognitive dysfunction by modulating glutamatergic activity at the level of the receptor. Another way to

regulate glutamate function would be to use Glycine transport inhibitors (GlyT-1), such as ALX-5407, which have been shown to work along with other antipsychotics (Atkinson et al., 2001; Talbot et al., 2006), and have been suggested for potential use as novel antipsychotics. GlyT-1 inhibitors have been shown to reverse the effects of PCP-induced psychosis in rodents (Javitt, Balla, Sershen, & Lajtha, 1998). In fact, ALX-5407 and D-serine have been shown to have similar effects to clozapine in mouse models of schizophrenia, rescuing the deficits seen in PPI and latent inhibition that were originally induced by MK-801 (Lipina, Labrie, Weiner, & Roder, 2005). A local elevation of glycine levels is expected to enhance NMDA receptor function (Yee et al., 2006). Perhaps these pharmacological agents could be incorporated in studies with the sandy mice in order to see if the genetic deficit can be rescued. In the previous chapters, we argued against the hypothesis of a purely BLOC-1 dependent function for dysbindin, and the pharmacologic treatments suggested here would bring focus to the glutamatergic receptor.

Two other proteins, muted and snapin, both directly interact with the dysbindin protein (Nazarian et al., 2006). The muted gene has been associated with increased risk of schizophrenia with dysbindin (Ryder & Faundez, 2009). It may be beneficial to test mice with the absence of other BLOC-1 subunit proteins for comparison, in order to further support or refute the idea that the function of the dysbindin protein is dependent upon the BLOC-1 complex.

Another possibility is to create a double knockout of the pallidin and *DTNBP1* genes, in which future researchers may be able to demonstrate additive effects in the complete absence of both proteins at once, and could be useful in determining BLOC-1 dependence versus independence.

Providing a way to regulate the glutamatergic function within the hippocampus specifically or globally within the entire brain may help to provide relief from at least some of

the devastating symptoms of schizophrenia. Treatments to improve cognitive processes could be combined, perhaps, with some of the other medications prescribed for patients with schizophrenia, such as atypical antipsychotics, which are prescribed to attempt to treat the positive symptoms and some of the negative symptoms associated with this complicated disease.

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