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Behavioral Phenotyping of VMAT1 Knockout Mice: Relevance to Neuropsychiatric Disorders

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BEHAVIORAL PHENOTYPING OF VMAT1 KNOCKOUT MICE: RELEVANCE TO

NEUROPSYCHIATRIC DISORDERS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Schizophrenia is a debilitating mental disorder that causes a large economic burden and is

prevalent across all cultures and countries around the world. Although both environmental

factors and genetics are known to play an important role in the etiology of schizophrenia, the

exact role of genetics and its interaction with environmental factors in an individual's

predisposition to develop schizophrenia is poorly understood. Schizophrenia is characterized by

symptoms that include positive symptoms (e.g. delusions, hallucinations, disorganized thinking

and speech), negative symptoms (e.g. avolition, anhedonia, depressive-like behavior), and

cognitive dysfunctions (e.g. executive functioning deficits in learning and memory, attention,

and vigilance). Genomic screening has identified polymorphisms of the vesicular monoamine

transporter 1 (VMAT1) gene (SLC18A1) that are associated with schizophrenia and bipolar

disorder. The current study represents the first extensive phenotyping of both young and aged

mice in which the VMAT1 gene (SLC18A1) has been deleted. The results demonstrated

behavioral effects of deleting the VMAT1 gene that may relate to aspects of schizophrenic-like

behavioral changes in this model. Specifically, young VMAT1 knockout mice displayed significant deficits in sensorimotor gating in the prepulse inhibition (PPI) task and in the acquisition of operant learning in the autoshaping task. When exposed to a mild stressor (24 hours of food deprivation), young VMAT1 knockout mice displayed a significant reduction in locomotor activity that was not evident under free-feeding conditions. Thus, young VMAT1 knockout mice showed deficits in tasks that model positive symptoms and cognitive deficits seen in schizophrenia; however, they did not display differences in behaviors related to models of the negative symptoms of schizophrenia or deficits in tasks designed to measure motor skills. While less extensive phenotyping was conducted in aged VMAT1 knockout mice, there were no significant deficits evident in any of the assays conducted in older animals. These findings demonstrated that deletion of the VMAT1 gene has behavioral effects that appear to be mediated by changes in brain monoamine function and changes in response to stressors (i.e. food deprivation) that may reflect changes in adrenal gland monoamine function.

Behavioral Phenotyping of VMAT1 Knockout Mice: Relevance to Neuropsychiatric Disorders

Schizophrenia

        Brief History of Schizophrenia and Treatment. Schizophrenia is a truly unique

disease. Eugene Bleuler was the first to recognize this when he termed what had previously been

known as *dementia praecox*, schizophrenia (Fusar-Poli & Politi, 2008). By using 'split-mind'

Bleuler hoped to accentuate the departure between the real world and the internal world of those

suffering from what Emile Kraepelin had previously called 'young dementia'. However Bleuler

and Kraepelin were not the first to identify this debilitating set of symptoms; descriptions of

symptoms matching schizophrenia are seen as early as 1500 BCE in the Egyptian *Book of*

Hearts, an ancient medical text describing the physical and mental illness of the heart (the source

of thought to ancient Egyptians) (Kyziridis, 2005). Treatment of schizophrenia was largely the

same as other mental disorders until the mid-1950s, when the first chemical treatment for

schizophrenia was discovered quite by accident. Chlorpromazine was developed for use in a

sedative cocktail; however, in early clinical tests Henri Laborit suggested that this new

compound could be useful in treating psychotic agitation. Jacques LH was the first human

patient to receive chlorpromazine and, after 20 days of treatment was "ready to resume normal

life" (Delay, Deniker, & Harl, 1952). Chemical management of schizophrenia advanced quickly

and less than 15 years later clozapine began clinical trials. Clozapine was the first of the second

generation antipsychotics, which help to alleviate a larger range of psychotic symptoms and lack

the major motor side effects associated with first generation drugs (Ramachandraiah,

Subramaniam, & Tancer, 2009). Just as the discovery of chlorpromazine ushered in a new era in

the treatment and understanding of schizophrenia as a mental disorder, the relatively recent

advancement of genetic and genomic testing have uncovered more about the development and

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course of the disorder. As these technologies have allowed greater insight, each answered

questions asks two more, and those who study the disorder began to realize how truly complex

this disorder is. As one of the biggest hopes of the 'genomic era', that a single "schizophrenia

gene" could be identified, has been proven fruitless (Gershon, Alliey-Rodriguez, & Liu, 2011),

researchers have now turned their focus to how each schizophrenia risk gene contributes to the

cacophony of symptoms that plague these patients' minds.

  Prevalence and Etiology. Currently, an estimated 0.7% of the worldwide population is

afflicted with schizophrenia across all countries and cultures (Saha, Chant, Welham, & McGrath,

2005). Schizophrenic symptoms usually begin to manifest in early to mid-20s, and although

symptom management is possible through pharmacological interventions, a cure does not exist,

nor is one on the horizon. Unfortunately, nearly two thirds of affected individuals have persisting

or fluctuating symptoms (American Psychiatric Association, 2013). Those who develop

schizophrenia are often left to manage symptoms of the disorder for the duration of their lives,

which is highlighted in the difference between the prevalence and incidence for the disorder.

Prevalence of a disease is how many people have the disorder at one point in time or how likely

it is that someone will develop the disorder in their life time. Incidence is the number of new

cases reported in a specific time span. A meta-analysis of 158 incidence studies of schizophrenia

shows a median rate of 15.2 new cases annually per 100,000 people (McGrath et al., 2004).

Although this number is less than .02% of the population, the fact that schizophrenic symptoms

will be with the patient through their life easily compounds case numbers over the years.

  Studies on incidence and prevalence also reveal interesting demographic data on who in

the population develops schizophrenia. Men have a higher incidence of the disorder;

approximately 1.4 men will develop schizophrenia for every woman that does (McGrath et al.,

2004). Interestingly, there is no statistical difference in the prevalence of schizophrenia between the sexes, a median ratio of 1.11 men have this disorder for each woman that has it, possibly hinting at a difference in the course of the disease (Saha et al., 2005). Data were inconclusive or non-significant for differences between urban and rural dwelling schizophrenics. This review also reports a significantly increased incidence and prevalence of schizophrenia in migrant populations; median incidence in the migrant population is 4.6 new cases for every new case in the native born population, and median prevalence reveals a ratio of 1.84 migrant schizophrenics for every native born schizophrenic (McGrath et al., 2004). The prevalence of schizophrenia also has been reported to be lower in less developed countries, with median rates of 2.62 per 1000 people. While rates in emerging economic countries are higher (median rates 4.69 per 1000 people) than developed countries (median 3.30 per 1000 people), the two are not significantly different (Saha et al., 2005).

Studies of affected people's family members also show that the higher the genetic homology with the affected individual, the higher the likelihood of that individual developing this disorder. Third degree relatives (e.g., first cousins) of schizophrenics have a 2% likelihood of displaying symptoms, while second degree relatives have a 2-6% chance of being afflicted, and those most closely related, first degree relatives, have as high as a 17% likelihood of also developing schizophrenia (Gottesman, 1991). Twin studies have shown the heritability, i.e. how much of phenotypic variability can be accounted for by genotypic variability, of these disorders to be in the realm of 80% (Cardno & Gottesman, 2000; McGuffin, Tandon, & Corsico, 2003). Another interesting line of evidence for the genetic influence of schizophrenia comes from the discordant incidence rates between monozygotic and dizygotic twins. Dizygotic twins, who share no more genetic similarity than any two siblings, have a similar concordance rate as any two first

degree relatives. Monozygotic twins, who outside of epigenetic differences that may occur in their life (Fraga et al., 2005), have extremely high genetic homology (much higher than dizygotic twins) and a concordance rate for developing schizophrenia closer to 50% (Gottesman, 1991). The difference between heritability and concordance rates underlies the notion that while schizophrenia does have a very strong genetic component to it, environmental factors still play a key role in the development of psychotic symptoms. Finally, the astounding case study of the Genain quadruplets highlights the interplay of heritability and environment. These identical quadruplets developed varying functional degrees of schizophrenia. It was later reported that the sister with the best functional prognosis was the 'favored child' and often received excessive praise and admiration from her parents. The least functional quadruplet was believed to be verbally and emotionally abused by both the mother and father (Rosenthal, 1963).

While genetic factors have been a recent target for research on the cause and development of schizophrenia as a disease, the interplay of environmental factors was one of the first targets for identifying who would develop the disorder and trying to determine if external sources could be to blame for the development and/or functional prognosis of schizophrenia. Possible environmental influences range from neonatal disease, weakened immune system function, and early life stress, to more unique environmental influences such as season of birth (Torrey, Miller, Rawlings, & Yolken, 1997). However, almost as a foreshadowing of the hopes and later discoveries of the genomic era, no single environmental risk was found to be the cause of schizophrenia, and researchers turned their focus to determining which types of environmental risk are most detrimental, as well as how they can be mitigated, corrected, or avoided.

Schizophrenia is a disease that affects not only the life of the afflicted individual and their immediate caretakers, but the country as a whole. Such a pervasive and debilitating disorder

costs billions of dollars not only in direct care to the family and patient: cost of medication, travel, etc.; but also to the state and community at large: work loss, reduced resources for other diseases, societal stigmatization. Two review studies in 1985 and 1990 show the total direct and indirect costs of schizophrenia to be 22.8 and 32.5 billion dollars respectively (Rice & Miller, 1996a, 1996b). In 2002, direct and indirect costs of schizophrenia were estimated to be $62.7 billion dollars in the United States alone (Wu et al., 2005), while a study from the United Kingdom estimates the cost of schizophrenia in 2005 to be £ 6.7 billion (Mangalore & Knapp, 2007) (approximately 12.06 billion USD in 2005), with the majority of the difference between the two countries coming from the number of affected individuals.

While pharmacological interventions revolutionized the management of schizophrenic symptoms, little progress has been made since the introduction and use of typical and atypical antipsychotic drugs. Additionally, an estimated 20-33% of schizophrenic patients will experience psychotic symptoms despite two or more treatment trials with different antipsychotics (Lieberman, 1999). While clozapine is approved for use in treatment-resistant schizophrenics, the atypical antipsychotic only maintains 30-60% efficacy in these treatment resistant patients (Conley & Kelly, 2001; Kane, Honigfeld, Singer, & Meltzer, 1988). Additionally chronic clozapine usage has been associated with agranulocytosis, a potentially life threatening reduction of white blood cells, which requires the addition of regular blood testing in schizophrenic patients (Griffith & Saameli, 1975).

Symptomology. Schizophrenia is a debilitating mental disorder characterized by abnormal behavior, disorganized thought patterns and speech, as well as deteriorating cognitive and social skills. With the recent release of the *Diagnostic and Statistical Manual 5* (DSM-5), schizophrenia has under-gone minor revisions including elimination of difficult to diagnose

criteria as well as a restructuring of dubiously defined 'first-rank' symptoms. The DSM-5 defines schizophrenia and related psychotic disorders as having 5 main categories of symptoms: delusions, hallucinations, disorganized thinking, disorganized or abnormal motor behavior, and negative symptoms.

The first four major symptoms (delusions, hallucinations, disorganized thinking, and disorganized or abnormal motor behavior) are often identified in research literature as 'positive symptoms' of schizophrenia (American Psychiatric Association 2013). This broad class identifies symptoms that are present in the schizophrenic, but absent in the non-affected population. Delusions are defined as "fixed beliefs that are not amenable to change in light of conflicting evidence" (American Psychiatric Association 2013) and often follow a number of themes including, but not limited to, delusions of persecution, referential, grandiose, erotomanic, nihilism, and body or somatic delusions. Delusions can also be classified as bizarre or non-bizarre, with delusions that are outside of the realm of reason to same-culture peers or delusions not derived from ordinary life experience classified as bizarre. Hallucinations are perceived sensory events that have no external stimuli. While these hallucinations can take any sensory form, auditory hallucinations are the most common with 60-90% of schizophrenic patients reporting hearing voices, music, or other auditory sensations without stimuli being present (Cummings JL, 2003). Visual hallucinations are the second most common type of hallucination in schizophrenia and occur in 16% (Mueser, Bellack, & Brady, 1990) to 20% of patients (Bowman & Raymond, 1931), although children seem to have a higher prevalence of visual hallucinations than adults (David et al., 2011). Olfactory, gustatory, tactile, and somatic hallucinations have also been reported, but at much lower incidence rates than auditory and visual hallucinations (Ali et al., 2011). Although disorganized thought is not a directly

observable set of symptoms, abnormal thought processes can be inferred through disorganized speech. Severity of the disorganization can range from frequently switching topics of conversation to providing semi- or non-related answers to questions. In extreme cases of disorganized thought/speech, symptoms can resemble receptive aphasia, colloquially referred to as "word salad". In these extreme cases the syntax of speech remains intact; however the semantics of the phrase is completely lost. Linguist Noam Chomsky composed the sentence "Colorless green ideas sleep furiously" to exemplify this idea. While the syntax and grammar of the sentence are correct, it is devoid of all meaning, similar to how those with extreme thought disorganization will sound to others. Disorganized thought is a common symptom of many psychiatric disorders, however, even the DSM-5 warns that "the symptom must be severe enough to substantially impair effective communications" (American Psychiatric Association 2013). Finally, disorganized or abnormal motor behavior contains a wide range of behavioral abnormalities. The most obvious of these are catatonic like behavior classified as a marked decrease in reactivity to external stimuli. Resistance to instruction, maintaining abnormal or rigid postures, purposeless motor activity, and stereotypic or repetitive behaviors are all examples of catatonia.

The last major set of symptoms in the DSM-5 is broadly defined as negative symptoms. These symptoms are characterized by a lack of behavior in the schizophrenic that would be present in the normal population. These symptoms are not only harder to detect, but also more difficult to treat, as first generation or typical antipsychotic drugs often do not alleviate the negative symptoms of schizophrenia (Dunlop & Brandon, 2015). The DSM-5 identifies diminished emotional expression and avolition as the two most prevalent negative symptoms. Diminished emotional expression is, as the moniker implies, a lack of emotion in facial

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movements, vocal patterning (intonation), and a lack of body language and extremity usage that often is associated with implied emotions. Avolition is a deficit in the self-ability to initiate actions. The individual may have an interest in participating in work or social situations but is metaphorically glued to their chair. Other, less pervasive, negative symptoms include deficits in speech (alogia), deficits in pleasure derived from activities (anhedonia), and lack of interest in social contact (asociality).

Genetics of Schizophrenia

Heredity of Schizophrenia. From anecdotal evidence like the Genain quadruplets to the work of Gottsman and colleagues with the prevalence of schizophrenics' relative's risk of developing disorders, genetics has a clear contributory role to the development and functional prognosis of schizophrenia. Schizophrenia is also unique among neurological disorders because it has a nearly uniform prevalence in all cultures and areas of the world (McGrath et al., 2004). It is so pervasive that some have gone so far as to suggest that schizophrenia could represent a genetic abnormality that made humans the creative, unique hominids they are (Horrobin, 1998). It is interesting that, with all we do know about the interplay of genetics and schizophrenia, we are still no closer to a causal link between genetic abnormalities and the function of the disease state itself. Genomic areas as small as single nucleotide polymorphisms to entire 'hot' regions or even arms of chromosomes have been suggested to increase the chance of developing the disorder.

One of the primary methods for identifying potential risk genes in schizophrenia and other genetically driven disease states is through genome wide association studies (GWAS). These studies require the examination of polymorphisms in a population on a single nucleotide level. These sequenced polymorphisms are then compared to the polymorphisms seen in healthy

individuals, and inconsistencies in the nucleotide present are noted. If one allele of a gene is more prevalent in the afflicted population than the healthy controls, that gene is then considered to be associated with the disease. This GWAS approach is called a phenotype-first approach, as it is used to look for genetic differences based on clinical manifestations of the disorder/disease in question. This method is more exploratory, as it looks at the entire genome, compared to candidate gene-specific studies. GWAS studies also say nothing about the function of the gene/polymorphism associated with the disorder and, by the genetic nature of neuropsychiatric disorders, since each specific genetic abnormality only increases likelihood of disease development by a small percentage, the statistical strength required causes type II statistical errors to be abundant. In fact a single polymorphism must reach a staggering statistical threshold in order to be significantly associated with the disorder ($p<10^{-8}$) (Dudbridge & Gusnanto, 2008; Risch & Merikangas, 1996). Since each gene contributes only a small percent change in the development of a neuropsychiatric disorder, the individual power, or more accurately effect size (the amount of variance in disease state accounted for by change in genetic expression) of each gene can also lead it to be overlooked by large scale analyses like GWAS (Manolio et al 2009; Visscher, Brown, McCarthy, & Yang 2012). A study recently highlighted this by reporting that approximately 45% of the variance in height of humans could be accounted for by some 300,00 polymorphisms compared to the mere 5% effect size seen by any single polymorphisms that have a significant influence from a GWAS (Yang et al., 2010). Similarly with schizophrenia, where no single gene has shown to have a large effect on heritability, a collection of many 'non-significant' single nucleotide polymorphisms (SNPs) allowed researchers to account for 33% of the hereditary variability in schizophrenia (Purcell et al., 2009).

Another method for identifying risk genes associated with schizophrenia, or any genetically driven disorder, is to investigate possible dysfunctions in genes that have a biological mechanism important to the disease. Here the function of the gene is more important to its investigation as a risk gene rather than the location or the prevalence of the gene in the affected population. Although this approach looks at a much smaller genomic region compared to GWAS they are less exploratory and ask a more functional question. This method of identifying risk genes draws a more direct hypothetical link between gene dysfunction and disorder (Kwon & Phile 2000). One difficulty with this method of gene identification, especially for a disorder like schizophrenia, is that the etiology and thus the biological mechanisms for the disorder are poorly understood. Since clinical presentation of the disorder is so varied between patients, and clear and persistent biomarkers for the disorder makes it difficult for causal genes to be identified using this method (Tsuang, Stone, & Faraone 2000). Once a candidate gene has been proposed case control studies are used to determine if this gene polymorphism is more prevalent in the affected population than the healthy controls (Zhu & Zhao 2007).

One approach for studying the functional roles of specific candidate genes in schizophrenia is to generate genetically modified mice in which the candidate genes has been disrupted (knockout mice), or variant alleles have been introduced (transgenic mice). Such knockout/transgenic models fall into two general categories: models looking at potential pathophysiological mechanisms, typically genes dealing with neurodevelopmental processes or genes relating to 'known' neurotransmitter systems associated with schizophrenia (Guillin, Abi-Dargham, & Laruelle, 2007; Waddington, Scully, Quinn, Meagher, & Morgan, 2001); and models that examine the role of clinically determined risk genes. Why employ two methods of model selection when it comes to genetic models of schizophrenia? Again, it seems to be due

largely in part to the extreme complexity and lack of concrete data about how the disease is actually working. Many genome wide association scans will identify genes that have an increased or decreased probability of existing in the affected population, with little attention paid to the functional significance of these genes (Harrison & Owen, 2003; Harrison & Weinberger, 2005). This, combined with the heterogeneity of symptoms and genetics, makes it important to explore as many models as possible. Knockout mice for two genes identified by GWAS studies in particular, *DISC-1* (disrupted-in-schizophrenia-1) and *NRG1* (neuregulin-1), have received a large amount of attention (Farrell et al., 2015; Lipina & Roder, 2014; Mouri, Nagai, Ibi, & Yamada, 2013). While a large variety of behavioral models have been used for testing these knockout animals, none produce a full or complete phenotype for schizophrenia. Knockout of SLC18A1, the gene encoding VMAT1, fits both the clinically significant model, and the pathological model of determining risk genes: a number of genome wide association scans have linked it to increased prevalence of schizophrenia, bipolar, and other mood disorders; and VMAT1 expression has been detected in developing CNS and PNS cells, suggesting that the transporter's systemic expression may play a larger role in development than it does in postnatal functioning (Hansson, Mezey, & Hoffman, 1998).

Two-Hit Model of Schizophrenia. One of the most baffling hallmarks of schizophrenia is the interplay of genetic predisposition and environmental influences in relation to the incidence of the disorder in an individual. Originally proposed as a two-hit model of inheritance, where genetic predisposition and environmental influences interact (T. A. Bayer, P. Falkai, & W. Maier, 1999), some researchers now propose a three-hit model of inheritance, which delineates environmental influences into early life, and late life factors (Ellenbroek, 2003). Although both the two-hit and three-hit models propose a genetic vulnerability compounded by environmental

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factors, Bayer's original proposal of a two hit model lumped "viral infection(s), birth complications, social stressors" into the single category, while three-hit models suggest distinct influences of early life and late life stressors (Thomas A Bayer, Peter Falkai, & Wolfgang Maier, 1999; Ellenbroek, 2003; Tsuang, 2000). As there is irrefutable evidence of the non-genetic factors of schizophrenia (Hoek, Brown, & Susser, 1998; Jablensky & Kalaydjieva, 2003; Koenig et al., 2005; Takei, Van Os, & Murray, 1995), and evidence that environmental factors change protein expression genetic models also have sought to include potential environmental assaults to develop a more etiologically sound model of schizophrenia.

Many of these non-genetic factors are prenatal or perinatal. Viral exposure and obstetric complications, for example, both cause structural and chemical changes in the brain consistent with schizophrenia (Lazar, Neufeld, & Cain, 2011). One postnatal animal model that has received a large amount of attention and support is post-weaning social isolation. Social interaction is an important neurodevelopmental event for all mammals and even as early as the 1960s and 70s researchers were beginning to identify a number of behavioral abnormalities associated with animals who were raised in isolation (Harlow, Dodsworth, & Harlow, 1965; Valzelli, 1973). This model has garnered even more support since social isolation produces behavioral and neurochemical alterations that are transitionally relevant to schizophrenia. Social isolation produces an extremely robust reduction in prepulse inhibition of acoustic startle, a model of sensory-gating deficits seen in schizophrenia (Sakaue, Ago, Baba, & Matsuda, 2003; Varty, Powell, Lehmann-Masten, Buell, & Geyer, 2006), and is reversed by atypical antipsychotics like quetiapine, olanzapine, and clozapine (Bakshi & Geyer, 1998; Cilia, Reavill, Hagan, & Jones, 2001; Varty & Higgins, 1995; Wilkinson et al., 1994). Social isolation also produces neophobia, social withdrawal, and cognitive inflexibility (Fone & Porkess, 2008),

representing deficits in positive symptoms (sensorimotor gating), negative symptoms (social withdrawal neophobia), and cognition (cognitive inflexibility) – the three core symptom classes of schizophrenia. While social isolation is not a complete model (like every other behavioral, genetic, neurochemical, and developmental model of schizophrenia), the robustness, repeatability, and diverse classes of abnormal behaviors it produces makes it a good model for investigating neurodevelopmental etiology, identifying longitudinal biomarkers, and serves as a screen for novel therapeutic drugs. Although there are a high number of tasks that have been proposed to measure varying aspects of schizophrenic-like-behavior in animals only those relevant to the present project are introduced below.

Vesicular Monoamine Transporter 1 & 2. The vesicular monoamine transporter (VMAT) is a protein pump located in the lipid bilayer membrane of an intracellular vesicle. The vesicle stores monoamines (dopamine, serotonin, norepinephrine), and it is binding of the vesicle to the presynaptic terminal membrane that allows these neurotransmitters to be released into the synapse or released into blood in the case of the adrenal medullary cells that store and release catecholamines. Monoamine neurotransmitters can either be transported into vesicles after synthesis or after neurotransmitter reuptake, which allows the vesicle to store the monoamines until they are released. There are two isoforms of VMAT, aptly named VMAT1 & VMAT2. They are structurally similar, each with 12 transmembrane domains and both tails located in the cytoplasm outside of the vesicle (Liu et al., 1992). Both VMAT 1 & VMAT 2 use proton gradients generated by vesicular H^+-ATPase to exchange H^+ for monoamine. Both VMATs are anti-porters that exchange two H^+ ions in efflux while the monoamine is brought into the cell (Wimalasena, 2011). VMAT1 differs from VMAT2 in pharmacology and expression. VMAT1 has a higher affinity for serotonin (Brunk et al., 2006), while VMAT2 has the added capability of

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transporting histamine (Erickson, Schafer, Bonner, Eiden, & Weihe, 1996). Additionally the VMATs differ in their expression. VMAT2 is more prevalent in the human and rodent brain than VMAT1, while VMAT1 is more commonly expressed in the adrenal medulla (Erickson et al., 1996). Recently, however, VMAT1 expression has been detected in human, rat, and mouse brain (Ashe et al., 2011; Hansson S.R., Hoffman B.J., & Mezey, 1998; Hansson et al., 1998; Ibanez-Sandoval et al., 2010).

Genome wide association scans (GWAS) and other genetic screens of patients and controls have identified several single nucleotide polymorphisms (SNPs) of VMAT1 that are associated with a number of psychiatric disorders including schizophrenia in people of Japanese descent, especially women (Richards et al., 2006), schizophrenia in people of European descent (Bly, 2005; F. W. Lohoff et al., 2008), bipolar disorder in people of European descent (F. W. Lohoff et al., 2006), and bipolar disorder in people of German descent (F. W. Lohoff et al., 2008). Figure 1 depicts the proposed structure of the VMAT1 protein as well as the location of known polymorphisms that have been implicated with neuropsychiatric disorders. As indicated by the amino acid variants shown in the figure, all of these human polymorphisms result from missense genetic polymorphisms, meaning the SNPs alter the amino acid they code for, which is more likely to lead to disrupted functioning in the polymorphic proteins. Table 1 illustrates identification of three VMAT1 SNPs in the GenBank data base. The SNPs in Table 1 alter amino acid positions 4, 98, and 136 in the VMAT1 protein and occur in 20-36% of a European American population, whereas other amino acid variants shown in Figure 1 are rare in this population. Table 1 lists SNP ID number in the data base, the base

Figure 1: Structure of the Human Vesicular Monoamine Transport 1 (VMAT1) transporter protein
Adapted from (Falk W Lohoff et al., 2014)
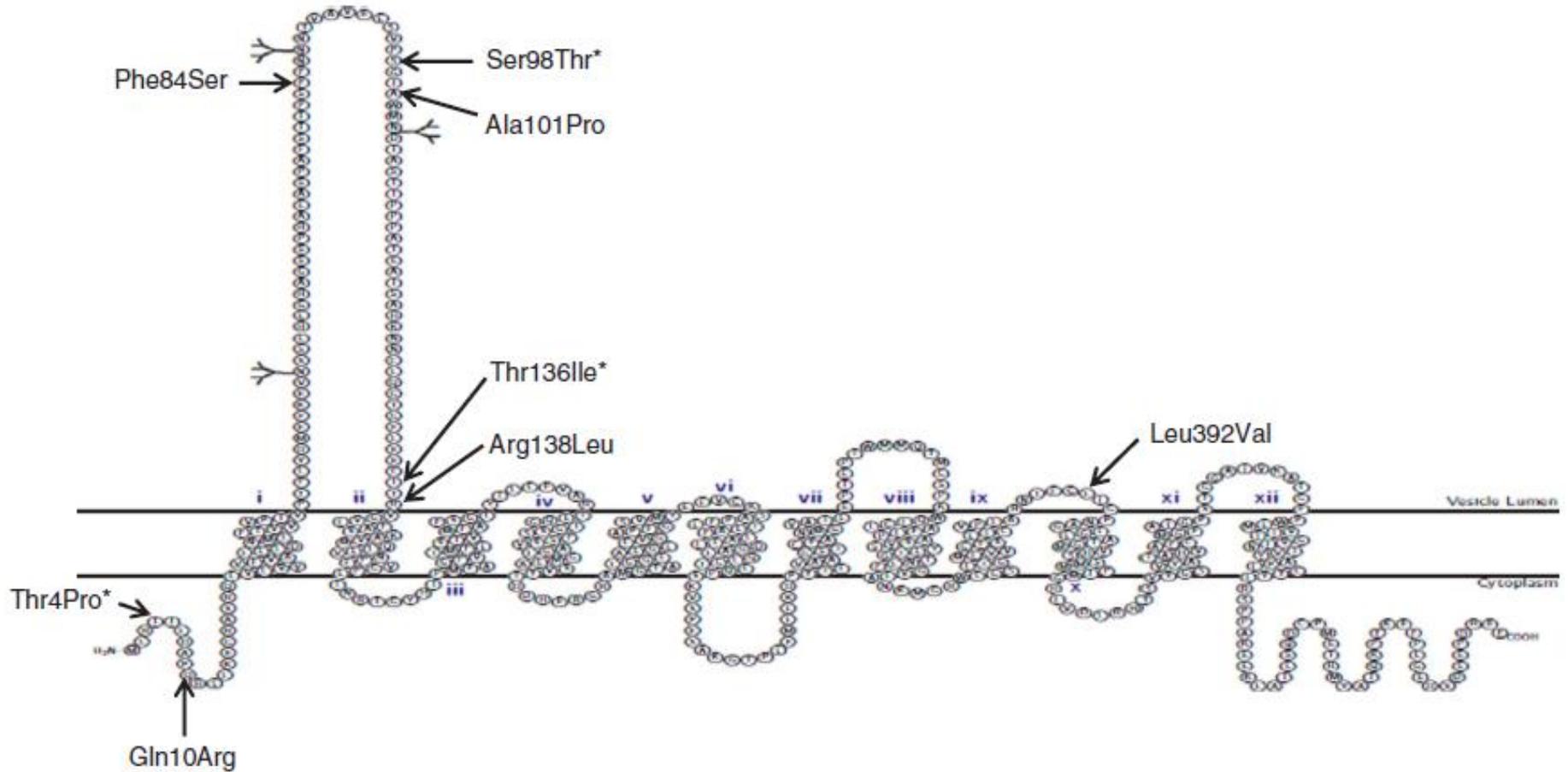
Table 1. Three Single Nucleotide Polymorphisms in the Human VMAT1 gene SLC18A1

*SNP ID	+mRNA Bases Amino acids	SNP Alleles	Major SNP Allele	Major Amino mRNA → Acid
rs2270641	A277C Thr4Pro	T/G	T	A→Thr
rs2270637	G560C Ser98Thr	C/G	C	G→Ser
rs1390938	T674C Thr136Ile	A/G	G	C→Thr

*SNP ID from the NCBI SNP data base. +mRNA base numbers 277,560, and 647 are based on the human VMAT1 mRNA sequence in the GenBank mRNA data base (Accession Number NM_003053.3), and amino acid numbers 4, 98, 136 are based on the human VMAT1 protein sequence (GenBank Protein Accession Number NP_003044).

position of each SNP in the VMAT1 mRNA sequence (such as base # 277), the alternate

nucleotide bases at that location (as Adenine =A or cytosine=C), the resulting amino acid

position in the VMAT1 protein sequence (as amino acid # 4) and the alternate amino acids at that

positon (as Threonine or Proline). Table 1 also shows the SNP allele (the DNA base) and the

major (most frequently occurring) allele observed in the human population, resulting in the most

frequently occurring amino acid at that position in the VMAT1 protein.

Recent studies have examined functional effects of these polymorphisms in the VMAT1

protein. Ahmed Khalifa and colleagues here at VCU transfected cells with variant human

VMAT1 sequences, extracted protein from the cells, and were the first to demonstrate reduced *in

vitro* transport of radiolabeled serotonin when the transfected gene coded for a threonine at

position 136 in the VMAT1 protein (Khalifa et al. 2012), Subsequently Lohoff and colleagues

confirmed that Thr-136 is associated with lower transporter activity, but they also clarified that

an isoleucine at position 136 in the VMAT1 protein actually results in excessive transport of

serotonin, dopamine, and norepinephrine *in vitro* (Lohoff et al., 2013). In 2006 in a study of

more than 500 bipolar patients and 500 control subjects, Lohoff et al found an association of

Thr-136 with bipolar disorder (Lohoff et al, 2006). They later stated that a genome wide study

failed to confirm this association, and they demonstrated that subjects with the 136-Ile were less

reactive to negative words than were those with the Thr-136 (Lohoff et al., 2013). Nevertheless,

Lohoff and his colleagues observed abnormally high in vitro dopamine uptake by VMAT1

protein with a phenylalanine at position 84, which is caused by a rare VMAT1 SNP found only

in patients with bipolar disorder. These findings suggest further investigation of the functional

effects of human VMAT polymorphisms is warranted. Deficits or increases in storage and

release of monoamines, even to modest degrees, could cause larger downstream effects and could contribute to the development of psychotic-like behaviors and thoughts.

SLC18A1, the gene that encodes the VMAT1 protein, has been ranked as a strong candidate for schizophrenia risk genes (Ross, Margolis, Reading, Pletnikov, & Coyle, 2006). It was ranked 55th in a meta-analysis examining aggregate risk odds ratio from multiple databases of schizophrenia risk genes (Sun et al 2010). Unfortunately, the szgene database was shut down in 2011, and although no new data was added after October 2010, SLC18A1 had 6 positive case-control studies (Betcheva et al., 2009; Bly, 2005; Chen et al., 2007; Lohoff et al., 2008; Richards et al., 2006; Talkowski et al., 2008) and one negative case-control study (Betcheva et al., 2009). An analysis of schizophrenia risk with SNPs and endophenotypes in schizophrenia linked the Val392Leu polymorphism in SLC18A1 to antisaccade (Greenwood et al., 2014), which is a task controlled by the frontal cortex and requires a participant to focus their vision away from a stimuli when looking at a fixed point. Since the SZ gene (Allen et al. 2008) database has closed, other studies have identified an increased prevalence and increased risk of schizophrenia in a Bashkortostan population in a case controlled study (Galaktionova, Gareeva, Khusnutdinova, & Nasedkina, 2014). Other than these studies, no prominent research has been published on the risk associated with SLC18A1 polymorphism in humans.

Knockout Mouse Models. As genetic engineering technologies advanced, the ability to manipulate genetic expression in living organisms quickly became a vital tool for discovering how naturally occurring abnormalities could affect whole organism behavior. The use of genetically altered mouse models has been spurred at least in part, by the aforementioned technological advances in generating mutant mouse genomes (Arguello & Gogos, 2006; J. Chen, Lipska, & Weinberger, 2006); replicable evidence from clinical association studies for

schizophrenia candidate genes (N. C. Allen et al., 2008; Gogos, 2007; Harrison & Weinberger, 2005); and greater understanding of the pathophysiology of schizophrenia (Tandon, Keshavan, & Nasrallah, 2008a, 2008b). One of the largest advances made recently in the genomic era is the completion of the human genome project in 2002. With the entirety of the human genome laid out, researchers now had a map with which to explore and identify abnormalities in disease states with clear genetic connections. Knockout and transgenic animal models have expanded the knowledge of genetic etiology of many psychiatric disorders. However, these models are just that, models of the disorders, and while they have helped to make advances in understanding, they are not without their own limitations.

In biopsychology's not so distant past, Carl Lashley and others attempted to correlate brain structure to function with brain lesion studies. The general concept is that if removal or damage to an area of the brain brought about absence or deficits in function, there was likely some correlative link between that region and that function. Genetic knockout mouse models are in essence the same idea with a microscopic scalpel. However, as with all loss of function studies, it becomes difficult to delineate whether behavioral changes are caused by the lack of the specific protein itself or if it is because another mechanism has developed in its place (D. N. Stephens, A. N. Mead, & T. L. Ripley, 2002). Proteins do not work alone, but in large biochemical complexes and cascades, where each step is directly dependent on many other simultaneous molecular events (Crawley et al., 1997). Although genotypic plasticity may help alleviate the lack of a specific protein, it is no guarantee that changes in expression will serve the exact same purpose in the exact same fashion. For example: research has demonstrated that dopamine D_2 Receptor knockout mice show altered adulthood glutamatergic transmission, caused by developmental changes in maturation of excitatory synapses in the striatum (Tang,

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Low, Grandy, & Lovinger, 2001). Furthermore, a reduction of striatal glutamatergic N-methyl-D-aspartate (NMDA) receptors and increased expression of mGluR1 AMPA receptor proteins in the same area were observed in dopamine D_1 receptor knockout mice (Ariano, Drago, Sibley, & Levine, 1998). Differences in dopaminergic response in these knockout models is therefore difficult to interpret, as it is unclear whether reduced signaling is caused by the lack of dopamine receptors, or changes in other downstream receptor systems. Although these knockout mouse models make it difficult to interpret the molecular mechanism of these differences, these studies provide important information to how dysfunction, or lack of a system or protein, causes changes in whole organism behavior, an analog of how these dysfunctions may contribute to changing behavior and contributing to the overall symptomology of schizophrenia.

Developing genetically manipulated mice also poses a unique problem that researchers must pay special attention to when designing their studies. More basic model organisms for genetic disorders, like *C. Elegans* or yeast, can be mutated by exposure to mutagenic substrates, such as ethyl methanesulfonate, and through sequencing and phenotyping be narrowed down into the desired genotype or behavioral phenotype (Bettinger, Leung, Bolling, Goldsmith, & Davies, 2012). Developing an acceptable genotype/phenotype is relatively easy as both organisms have a short life cycle and multiple generations can be easily produced and maintained. This would make it seem as though we should be studying yeast for developing schizophrenic models, however the differences in homology of behavior would make it near impossible to study 'schizophrenic-like-behavior' in these model organism. While they may be fruitful for studying biological mechanisms on their own the focus of behavioral phenotyping studies is to look at whole organismal behavior. Developing a genetically altered mouse model is much more complex (See Figure 2). The genetic manipulation is developed *in vitro* and inserted into

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Figure 2: Development of Transgenic and Knockout Mouse models

Adapted from: (Stephens, Mead, & Ripley, 2002)
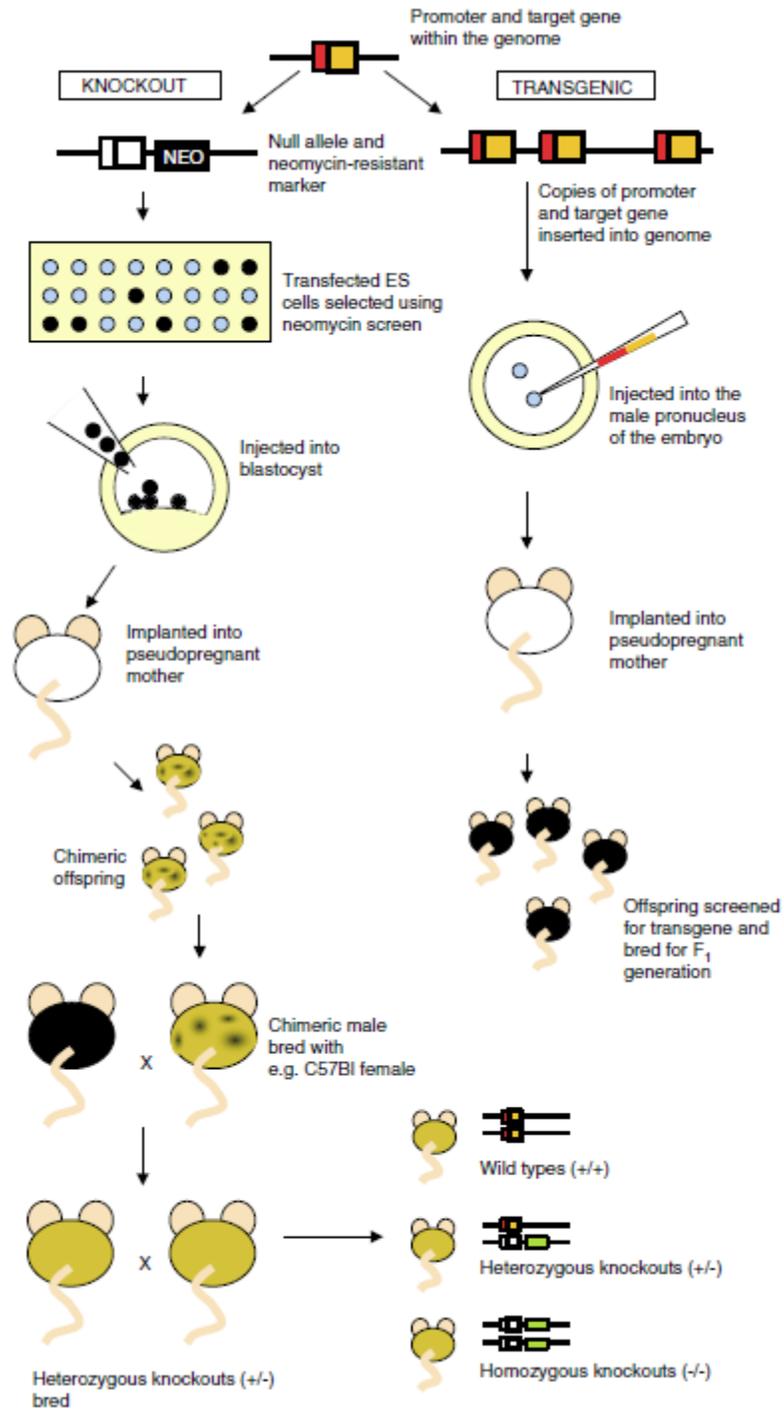
embryonic stem cells. Once the manipulation has stabilized and confirmed gene knockout/transgenic expression is established the stem cell is inserted into a new mouse embryo. This surrogate mother gives birth to chimeric mice that express both the manipulated genotype and the wildtype genome from the surrogate mother. This chimeric mouse is then back-crossed, or bred with non-manipulated mice of the same inbred mouse strain, to produce a stable animal genome with only the desired gene missing.

Knockout mice, in which a gene is made inoperative, have been shown to have a higher global *variability* in gene expression (Eraly, 2014) suggesting that complete removal/inactivation of a gene causes a compensatory increase in other gene expression. Studies have also shown that in gene knockout mice there is a higher frequency of genomic changes in genes surrounding (flanking) the targeted gene, whether this is due to the method of mutagenesis or a compensatory effect is unclear(Valor & Grant, 2007). Regardless, homology and overlap in genomic expression is high in both mice and humans, with as much as 50% of the mouse genome having a related/redundant partner (Chinwalla et al., 2002) and 50% of the human genome having a functional family member (Venter et al., 2001). In fact, the two isoforms of VMAT share more similarities than differences, only diverging in expression of location and preference for which monoamines they readily transport. One hypothesis is that an evolutionary mechanism is in place so that random mutagenic polymorphisms that occur in an organism do not completely disable that organism (Gu, 2003).

Schizophrenia is often classified as a neurodevelopmental disorder although neurodegenerative structural changes in the brain have been identified and correlated with functional outcome of the disorder (Veijola et al., 2014). Schizophrenia also has been associated with marked reductions in size and volume of the hippocampus (Carol A Tamminga, Stan, &

Wagner, 2010; Zierhut et al., 2013), cortex (van Haren et al., 2011; Vita, De Peri, Deste, & Sacchetti, 2012), enlarged ventricles (Kempton, Stahl, Williams, & DeLisi, 2010) and other brain regions.

The strain of mice used to develop genetically manipulated mice can be almost as important as the gene being manipulated. C57BL/6 is one of the most commonly used strains in behavioral research (Deacon, Thomas, Rawlins, & Morley, 2007), and was chosen as the second mammal to have its complete genome sequenced because of its varied and pervasive applications in biomedical research (Battey, Jordan, Cox, & Dove, 1999). As more researchers began to use different mouse strains, it became clear that differences not only in behavior, but also central nervous system chemistry, and later gene expression patterns, could differ between inbred strains of mice. With this in mind, building a phenotype database has become a primary concern of researchers. While projects like the Mouse Phenome Database (MPD) have made progress (Grubb, Churchill, & Bogue, 2004), the sheer number of inbred strains used in behavioral research, as well as the time it takes to conduct some of the complex studies, has made this a project that will continue for the foreseeable future. A database like this not only helps to make a searchable and indexed collection of a large number of studies, but also helps to normalize and collate the wide variety and scope of these types of projects. For example, in 2003 Willott published a paper phenotyping 40 inbred strains of mice in acoustic startle and prepulse inhibition (Willott et al., 2003). Brooks and colleagues produced 2 papers in 2005 looking at a number of behavioral studies in 7 inbred mouse strains (Brooks, Pask, Jones, & Dunnett, 2005; Brooks, Pask, Jones, & Dunnett, 2005). Collating all of these diverse and separate articles into a single cross-study searchable database makes finding the information that much easier and decreases the likelihood that an improper background strain will be used.

Animal Models of Psychotic Behavior

One of the biggest hurdles in conducting non-human animal research for disorders like schizophrenia is that mice are not humans, and they do not develop schizophrenia. Many characteristics of the disease are not directly observable in animals since they cannot self-report hallucinations, disorganized thoughts, and other features that are primary targets for clinical diagnosis. Although schizophrenia is a uniquely human disorder, researchers have identified a number of animal behavioral models that can either model or mimic specific symptoms and etiological theories of the disease. Although these clinical and ethologically modeled tasks seek to model the etiology of these complex neuropsychological disorders, even in the case of schizophrenia, a problem arises as researchers do not know the actual etiology of many psychological disorders. With that, behavioral assays modeling schizophrenia, or any psychological disorder, fall into two categories: tasks that model putative etiological function or, more accurately, tasks that measure the clinical manifestation of certain symptoms of schizophrenia; and tasks that measure endophenotypes of schizophrenia. Endophenotypes can be defined as discrete, quantitative, genetically determined features that may be part of a complex illness but are not necessarily part of the clinical presentation (Braff, Freedman, Schork, & Gottesman, 2007). Unfortunately, being such a complex disease, discovery and validation of endophenotypes of schizophrenia have met with difficulty. High etiological heterogeneity, differential course and presentation of symptoms between patients, poorly defined and inconsistent neuropathology, and a lack of clear biomarkers all contribute to difficulty in defining endophenotypes of schizophrenia (Arguello & Gogos, 2006).

Tasks Modeling the Positive Symptoms of Schizophrenia. The positive symptoms of schizophrenia seem to be mediated by dopamine hyperactivity, specifically dopamine D_2 in the

mesolimbic dopamine pathways, one of the major dopamine pathways extending from the ventral tegmental area to the nucleus accumbens (Davis, Kahn, Ko, & Davidson, 1991; Crow, 1980). Both typical and atypical antipsychotics are effective at treating the positive symptoms of schizophrenia and both classes of drugs share an action at mesolimbic dopamine D_2 (Meltzer, 1989, 1991). Typical antipsychotics are more selective for dopamine D_2 and D_3 receptors, and appear to treat the positive symptoms of schizophrenia, but not the negative symptoms. Although receptor action does not necessarily imply behavior, this is a prevailing theory in schizophrenia research (Meltzer & Stahl, 1976; Seeman, 1987; Snyder, 1976).

Although the dopamine hypothesis is one of the most prevalent and widely accepted neurochemical theories of schizophrenia, many researchers believe dopamine imbalance is not the only responsible mechanism. In all drugs used to manage schizophrenia, dopamine is the primary pharmacological target; however, these drugs are far from completely effective in managing all symptoms of the disorder (Kapur & Mamo, 2003). Some researchers believe it could be a potential 'driveway' through which the myriad of genetic, environmental, and developmental effects lead, and dopamine imbalance may be more an effect of schizophrenia than a direct cause (Di Forti, Lappin, & Murray, 2007). However, dopamine imbalances continue to be one of the most robust and replicable effects for modeling schizophrenia-like symptoms in animals. One common animal model is dopamine-induced hyperactivity in the locomotor open field task. While it could be argued that it is a translational model of psychomotor agitation seen in schizophrenic patients, it more accurately models etiological over-expression and disrupted signaling of dopamine. Locomotor activity is also a relatively straightforward task that is easily quantified, and the relation of dopamine and movement is relatively well defined with clear pharmacology. Enhanced dopaminergic activity leads to enhanced motor activity, be it horizontal

locomotion, rearing, and at high doses stereotypic-like behaviors (van den Buuse, 2010).

Although there are many dopamine agonists, amphetamine is commonly used because of its specificity to the mesolimbic dopamine pathway, the same pathway thought to be involved in schizophrenia (Lodge & Grace 2008).

Another approach for achieving locomotor hyperactivity in rodents comes from the use of dissociative NMDA antagonists. Although these drugs, like ketamine and phencyclidine, indirectly interact with dopamine systems, the production of hyperactive behavior is independent of dopamine activation (Adams & Moghaddam, 1998). Modeling symptoms of schizophrenia using these drugs has stronger construct validity as antagonism of NMDA receptors has been reported to cause hallucinations in humans similar to those seen in schizophrenics (Moghaddam, 2003; C. A. Tamminga, Lahti, Medoff, Gao, & Holcomb, 2003). NMDA receptor antagonism also exacerbates psychotic related symptoms in schizophrenics, decreases cognitive function in schizophrenics (Malhotra et al., 1997), and causes non-schizophrenic patients to present with behaviors that could be classified as positive or negative symptoms of schizophrenia (Allen & young, 1978).

Prepulse inhibition (PPI) is a commonly used as a model of a positive symptom of schizophrenia and PPI is often referred to as an endophenotype of the disorder. PPI measures deficits in sensorimotor gating, the process by which irrelevant or excessive sensory input is disregarded, allowing the individual to attenuate to more salient sources of sensory input. Schizophrenics show a significant deficit in sensorimotor gating, which leads to sensory overload causing disorganized thoughts and distractibility that mimic some of the positive symptoms of schizophrenia (Braff, Grillon, & Geyer, 1992). Sensorimotor gating deficits have been reported in human schizophrenics as well as many animal models (Roussos et al., 2008). If

a weaker non-startling prepulse precedes the startling stimulus by a short time interval (~100 ms), the startle response will be reduced in healthy controls (Dawson, Schell, Hazlett, Nuechterlein, & Filion, 2000). Deficits in PPI have been reported in first episode schizophrenics, medicated patients with acute psychosis, and unaffected relatives (Braff et al., 1992; Braff, Swerdlow, & Geyer, 1999; Cadenhead, Swerdlow, Shafer, Diaz, & Braff, 2000; Ludewig, Geyer, & Vollenweider, 2003), and also has been shown to correlate with severity of positive and negative symptoms (Dawson et al., 2000; Ludewig & Vollenweider, 2002; Swerdlow et al., 1999). Dopamine agonists, specifically *d*-amphetamine, cause a significant reduction in prepulse inhibition in rats, which can be reversed using typical and atypical antipsychotics (Andersen & Pouzet, 2001).

Tasks Modeling the Negative Symptoms of Schizophrenia. Many of the negative symptoms of schizophrenia closely resemble or directly overlap with the core symptoms of depression. Even the National Institute of Mental Health description of the negative symptoms of schizophrenia warns that "these symptoms are harder to recognize as part of the disorder and can be mistaken for depression" (NIMH, 2014). Additionally, depression is a common co-morbid disorder with schizophrenia and rates of co-morbid depression with schizophrenia range from 23% to 57%, although a modal comorbidity of 25% is believed to be the most accurate and methodologically inclusive figure (Buckley, Miller, Lehrer, & Castle, 2009). One model of depressive-like behavior that has high face validity is the forced swim test, although there is some debate as to whether it is a model with high predictive validity (Willner, 1984).Taken as a predictive model for antidepressant-like effects of drugs, forced swim has little place as a model for schizophrenia given that most antipsychotic drugs do not produce behavioral differences in the forced swim task, except for the atypicals clozapine and sulpiride (Castagne, Moser, Roux, &

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Porsolt, 2011), and the atypical antipsychotic quetiapine (Guan & Zhu 2000) which have known antidepressant-like-effects or facilitate antidepressant medication. However, new research suggests that there may be more overlap in risk-loci for a number of adult-onset psychotic disorders, including schizophrenia and depression ("Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis," 2013); thus, investigation of a genetic effect of depressive-like behavior could prove fruitful and lead to new insights about the overlapping symptomology and etiology of both disorders.

Another common negative symptom in schizophrenia is anhedonia. Anhedonia is often defined as an overall decrease in the joy of living (i.e. not experiencing pleasure) and has been a core symptoms of both depression and schizophrenia for as long as each has been a defined disorder (Carpenter, Heinrichs, & Wagman 1988; van Praag, Uleman, & Spitz 1965). One problem with measuring anhedonia in rodents returns to one of the core problems of using animal models; how do we measure an internal state or thought process possessed by an animal? Do mice and rats even have the capacity to experience pleasure or hedonic cognition, and if so, how could we measure it? Behavior that is voluntarily initiated and repeated is often considered an expression of a rewarding or hedonic behavior. Given a choice between plain water and a weak concentration sucrose solution, rodents will show a strong preference for the sucrose (Towell, Muscat, & Willner, 1986). Food reinforced lever pressing is a common operant manipulation and both mice and rats will readily acquire lever pressing behavior under a fixed ratio 1 schedule of reinforcement. Progressive ratio is a commonly used operant schedule in which animals are required to lever press for a reinforcer (e.g., a low concentration milk/sucrose solution). With each successive reward, the number of lever presses required for presentation of the sucrose solution is increased. As lever pressing requirements increase, the reinforcing value

of the reinforcer is outweighed by the effort required to give a presentation of that reward. The

"breakpoint" is defined as the ratio at which animals no longer show the willingness for this

reward (defined as a decrease or stoppage of self-initiated behavior), and has been suggested to

be a measure of "wanting" behavior (Uematsu, Tsurugizawa, Kitamura, Ichikawa, Iwatsuki,

Uneyama, Torri 2011). An animal that has a lower breakpoint can suggest that animal is less

willing to produce hedonic behaviors; the work required for the reward outweighs the reinforcing

value of the reward itself. This behavioral model also shares face validity for the motivational

deficits seen in schizophrenia; if motivation is defined as willingness to work for a reward,

animals that cease responding at a lower breakpoint show less motivation-like-behavior to obtain

that reward.

Tasks Modeling the Cognitive Symptoms of Schizophrenia. Although not formally

included in the DSM-V, cognitive deficits have been noted and debated to as either criteria for

inclusion in the diagnosis of schizophrenia or as a predictive symptom of those at risk (Green

1996; Nuechterlein, Barch, Gold, Goldberg, Green, Heaton 2004). Cognitive deficits are

common in patients with schizophrenia (Elvevag & Goldberg, 2000; P. S. Goldman-Rakic &

Selemon, 1997), and research has shown that there is a positive correlation between severity of

cognitive symptoms and the functional prognosis of the schizophrenia (Lazar et al., 2011).

Additionally, multiple studies have shown deficits in working memory in schizophrenics and

unaffected siblings (Broga & Neufeld, 1981; Patricia S Goldman-Rakic, 1994; Spalletta et al.,

2008). Despite this evidence, the research community continues to debate the inclusion of

cognitive deficits as a symptom of schizophrenia. Some researchers believe their inclusion would

raise awareness of the cognitive dysfunctions, leading to better treatment and potentially more

specific diagnosis (Keefe, 2008; Keefe & Fenton, 2007), while others call for its inclusion to

help differentiate some disorders with overlapping symptomology, specially mood disorders (Keefe, 2008). However, not all are convinced, and most opponents of the inclusion of cognitive deficits cite the large variability in data and a lack of clear understanding of how these dysfunctions and overall schizophrenic symptoms are related (Dickinson, Ragland, Gold, & Gur, 2008).

Just as positive and negative symptoms of schizophrenia are a broad classification for a number of different symptoms, so is the symptom header of cognitive deficits. This symptom classification includes information processing, abstract categorization, executive function, cognitive flexibility, attention, memory, and visual processing (NIMH, 2014). Spatial memory is easily assessed in rodent models with the Morris water maze, a task in which mice swim in a pool containing opaque water and try to locate a hidden platform that sits just below the water's surface. Visual cues along the edge of the pool and on the walls provide the animal with reference points, so that as it learns the location of the platform, the time required for the location of the platform decreases across trials. Certain models, like DISC-1 knockout mice (Jaaro-Peled, 2009), gestational Methylazoxymethanol (MAM) (Lodge, Behrens, & Grace, 2009; Moore, Jentsch, Ghajarnia, Geyer, & Grace, 2006), and neonatal ventral hippocampal lesions (Lipska, 2004; Tseng, Chambers, & Lipska, 2009) all reduce acquisition of spatial learning in the Morris water maze. Some of these cognitive deficits may be caused by reduced behavioral flexibility or enhanced perseveration, the inability to switch from a previously learned solution. In the standard Morris water maze task, the platform is in a fixed position for the duration of all trials. One manipulation that can be done to examine either of these potential deficits is repeated acquisition learning, where the location of the platform changes daily. Post-weaning social isolation (Fone & Porkess, 2008; Lapiz et al., 2003), which is a commonly used

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neurodevelopmental model of schizophrenia, produces deficits in repeated acquisition learning in the Morris water maze.

Operant behavior schedules also may be a potent tool for assessing learning deficits in mouse models of schizophrenic-like behavior. All operant tasks require some degree of learning, especially with lever pressing, which is not as inherent of a behavior in rodents as, for example, nose poking. Specifically, interval schedules of operant reinforcement, as opposed to ratio schedules of operant reinforcement, can measure two aspects of learning simultaneously. Interval schedules of reinforcement require animals to make an operant response to receive reinforcers, but the inter-reinforcer-interval is much less dependent on the presence of the animal's behavior and more dependent on the interval of the operant schedule, as set by the experimenter. Because the animal's behavior is not strongly controlled by the operant response it makes, comparing the number of total lever presses and the number of reinforced lever presses can highlight not only how quickly the animal learns the relation of the operandum and the presence of reinforcers, but also how quickly the animal can become efficient, making fewer non-reinforced lever presses in the task. Autoshaping of the lever press response is a task that requires animals to learn the association between reinforcement of a lever press with visual and auditory cues. The task is normally conducted over two-days. The first day involves testing the animals with an instrumental-Pavlovian condition in which termination of a cued period (visual and auditory cues indicate a lever press will deliver reinforcers) results in delivery of the reward, regardless of whether the animal makes an operant response during this cued period (instrumental learning) or not. The second day is an instrumental reinforcement condition only, in which reinforcers are only presented if the appropriate operant response is made during the signaled period. Drugs given before or after the instrumental/Pavlovian training period can highlight how different

aspects of learning are related to pharmacological manipulation (Vanover & Barrett, 1998). In a variation of this task pioneered by our lab, we have identified that having multiple days of instrumental training can highlight the speed of acquisition and changes in efficiency of animals responding, thus measuring different aspects of operant learning.

Behavioral Tasks measuring muscle/motor ability. Catatonia is considered a positive symptom of schizophrenia and is classified by an extreme loss of motor skills or the presence of constant, repetitive/stereotypic, motor movements (American Psychiatric Association, 2013). Thus, measures of motor skills are important for assessing this aspect of the schizophrenia phenotype. Further, it should be noted that several of the behavioral tasks designed to measure other aspects of the phenotype, (e.g., level pressing or swimming) depend upon muscle strength/motor ability. Thus, in any animal model employing these assays, it is important to ensure that the experimental manipulation (whether it is a gene knockout, drug treatment, etc), does not also impact muscle strength/motor ability.

Rotarod is a task that measures balance and motor coordination. Briefly, animals are placed on a slowly rotating rod that is large enough for the mouse to comfortably stand on. During the session the rotational speed of the rod increases, requiring increased motor coordination to maintain balance. The accelerated version of the rotarod, as opposed to the static version in which the speed of the rod's rotation does not change, can be used as a measure of motor skill learning as rats and mice tend to perform better both within a rotarod session and between sessions (Buitrago, Schulz, Dichgans, & Luft, 2004; Shiotsuki et al., 2010). Finally, although it is not used as a direct model of schizophrenic phenotypes, it has been used to examine and complement pharmacologically induced catalepsy, a common side effect of antipsychotic drugs (Kirschbaum, Hiemke, & Schmitt, 2009). While measuring muscle

coordination can help rule out differences or deficits seen in tasks like locomotor activity, the accelerating rotarod is not sufficient to rule out differences seen in operant assays like progressive ratio and autoshaping. Therefor measuring animal grip strength will assist in drawing a clearer line of evidence that deficits seen in these assays are caused by cognitive dysfunction and not simply motor impairments.

Grip strength is another basic phenotyping assay that measures limb strength of rodents. Both forelimb and hind limb grip strength assays exist; however, in the present study only forelimb grip strength was measured, as it is more relevant to determining if the mouse's ability to bar press was affected. Grip strength is measured by allowing the animal to grasp a small bar that protrudes from a force meter. The animal is then pulled back until it is no longer able to maintain its grip and the force expended by the animal on the bar is recorded. While grip strength is not directly related to a schizophrenia phenotype, differences between wildtype and VMAT1 knockout mice would potentially confound interpretation from other assays more relevant to schizophrenia, if performance in those assays could be affected by forelimb strength.

Use of Aged Animals. Aging is generally defined as changes in biological function and gene expression that "adversely affect its vitality and function, but most importantly, increase mortality rate as a function of time" (Finch, 1994). Although the exact mechanisms of aging remain unknown, the most common theory is that oxidative stress causes damage to DNA which in turn leads to changes in genetic expression, which result in aging (Johnson, Sinclair, & Guarente, 1999). Patients with schizophrenia do have marked decreases in a number of brain regions, and there is a degenerative effect in cognitive function in patients who develop schizophrenia, but this degeneration seems to occur before clinical onset of the disorder. Aged patients with schizophrenia do show decreased levels of pre-frontal cortex brain-derived

neurotropic factor (BDNF), although the decrease in BDNF does not follow the normal age

related linear decrease seen in healthy controls (Rao, Chiappelli, Kochunov, Regenold, Rapoport,

Hong 2015). However other studies suggest that schizophrenic patients have higher levels of

age-related white matter degradation as compared to healthy controls, suggesting a

neurodegenerative aspect (Kochunov, Glahn, Rowland, Olvera, Winkler, Yang, Sampath,

Carpenter, Duggirala, Curran, Blangero, Hong 2013; Kochunov, Hong, 2014). Still findings are

inconclusive and timing of the study seems to be a larger issue. Longitudinal studies do not find

a poorer cognitive function in the first years after onset, but function may decline after many

years of illness (Rund 2009). Furthermore studies have shown that ventricle size, which is

commonly reported to be increase in schizophrenics, may cycle through increasing in size,

decreasing in size, and then increasing again in a period as short as a few months (DeLisi,

Sakuma, Ge, Kushner 1998).

Rationale

Although schizophrenia is not caused by the presence or absence of alteration in a single

gene, examining the role of these single gene dysfunctions for the different aspects of

schizophrenic-like behavior can help to elucidate how to approach and possibly prevent

behavioral abnormalities seen in the disorder. If a genetic screen of an at-risk person can identify

which specific at-risk polymorphisms an individual has, a specific pharmacological and/or

behavioral therapy could be implemented to potentially forestall on the onset of symptoms or

minimize the loss of quality of life once symptoms begin to present themselves. The discovery of

central nervous system VMAT1 expression, in addition to the neurochemical role of VMAT1,

make it a prime target to investigate how dysfunction of this gene is related to whole organism

behavior. There is a known link between specific non-functioning polymorphism of VMAT1 and

the presence of schizophrenia in multiple ethnic backgrounds. VMAT1 also plays a role in efficient use of dopamine and serotonin, both of which are implicated and/or proposed as neurochemical mechanisms important for schizophrenia and its treatment.

The present study seeks to examine how homozygous VMAT1 knockout mice perform in a number of tasks that have been established to mimic or model specific behavioral deficits seen in or proposed to be related to human schizophrenic patients. To date no one has phenotyped homozygous VMAT1 knockout mice in relation to these tasks, or in relation to such a diverse range of schizophrenic-like behaviors. One study has shown that VMAT1 homozygous mice show a significant reduction in spatial recognition but no change in contextual fear discrimination (Multani et al. 2013). As such, this is an original preclinical study to ascertain the behavioral consequences of VMAT1 dysfunction, as well as how drug response may or may not be changed when this out monoamine transporter is absent.

There are three objectives to this study: first, to examine how VMAT1 dysfunction affects behavioral models of the positive symptoms, negative symptoms, and cognitive deficits related to schizophrenia; second, to determine if dysfunction of VMAT1 changes basic muscle skill and motor memory in rodents.; and third, to determine how the effects of VMAT1 deficiency are modulated, either positively or negatively, with age.

Methods

Age, sample sizes, and weights are provided for each experiment below. Where noted, mice were food-restricted 24 hours before the first testing procedure and maintained at 85-90% of their free feeding body weight, with water available *ad libitum* in the home cage. Mice tested in autoshaping, locomotor activity, Morris water maze, progressive ratio, grip strength, forced swim, and rotarod tasks were individually housed in a vivarium on a 12 h light/dark cycle (lights

on at 600 h). Mice tested in prepulse inhibition were group housed in groups of 2-4 animals based on birth cohorts. Testing occurred between 800 h and 1600 h daily. All behavioral testing procedures were approved by the VCU Institutional Animal Care and Use Committee. All mice were maintained in an animal facility that meets all federal and state requirements and was approved by the American Association for Accreditation of Laboratory Animal Care. For a summary of assays conducted in both young and aged mice refer to table 1.

Assays Conducted in young Animals

Prepulse Inhibition

Apparatus. Testing of sensory motor gating was conducted using 4 startle chambers (San Diego Instruments, San Diego, Calif., USA; Model Number: SR-LAB). Animals were tested in 20 oz paper Dixie cups, as mouse restraints were not available at beginning of testing and this method of animal restraint has been used in previously in this lab to study acoustic startle response and prepulse inhibition. The chamber also contained a high frequency loudspeaker located 24 cm above the animal that produced both a continuous background noise of 65 dB and the various acoustic stimuli. Whole-body startle responses of the mouse caused vibration of the paper cup. These vibrations were converted to analog signals by a piezoelectric accelerometer attached to the underside of the platform, and then digitized and stored by a computer. Data were recorded every ms for 150 ms from the onset of the tone. Peak startle response in this 150 ms period was used as the measure of startle response.

Procedure. 14 Young VMAT1 knockout and 16 young wildtype C57BL/6 mice weighing between 20-25 g were used to test sensory-motor gating in the prepulse inhibition task.

Young Mice Assays	Manipulation	Aged Animal Assay	Manipulation
Prepulse Inhibition	Free Food		
Autoshaping of the Lever Press Response	Food Deprived	Autoshaping of the Lever press Response	Food Deprived
Morris Water Maze	Free Food	Morris Water Maze	Free Food
	Food Deprived		
	Repeated Acquisition		
Locomotor Activity	Free Food	Locomotor Activity	Free Food
	Food Deprived		
	Amphetamine*		
	*Preliminary study		
Progressive Ratio Breakpoint	Food Deprived	Progressive Ratio Breakpoint	Food Deprived
Forced Swim Test	Free Food		
Grip Strength	Free Food	Grip Strength	Free Food
Rotarod	Free Food	Rotarod	Free Food

Table 2: Summary of assays and conditions for young and aged VMAT1 knockout mice and C57BL/6 wildtype mice.

Test sessions began with a 5 minute acclimation period in which animals were exposed to a steady 65 decibel (dB) white noise that served as the background noise; this 65 dB tone was constant throughout the session. After the acclimation period mice were presented with 5 pulse alone trials (120 dB, 40-ms duration). These trials served to habituate the mice to the startle tone and normalize startle response. After these 5 'pulse alone' trials mice were presented with 13 blocks of pseudorandom trials. Each block consisted of 7 different trials: pulse alone, no pulse (65 dB background only), and 5 pulse/prepulse trials with 4, 8, 12, 16, or 20 dB above the background noise. Prepulse tones were presented for 20 ms with 100 ms between the onset of the prepulse and the 120 dB pulse tone. Trial presentation order was randomized across blocks with an inter trial interval average of 15 s (range 10-20 s). After the 13 blocks of trials, 5 pulse alone tones were presented. The test session lasted for a total of 34 minutes and contained 101 trials.

Data Analysis. Startle magnitude was calculated by averaging the response to pulse alone trials within the 13 pseudorandom blocks (first and last 5 pulse alone trials were not included in the measure of startle magnitude). Prepulse startle response was calculated by averaging the whole body startle response for each dB in each of the 13 pseudorandom blocks. PPI was calculated as a percentage using the following formula (100 x [(pulse alone startle - prepulse startle)/ pulse alone startle]). A factorial ANOVA was used to determine the main effects of genotype and prepulse dB on sensorimotor gating. Data analysis was conducted using Prism version 5.0 (GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). All significant differences were at $p < .05$.

Autoshaping of the Lever Press Response:

Apparatus. Autoshaping of the Lever Press Response used six standard computer-interfaced mouse operant condition chambers (Model ENV-307A; Med Associates, St. Albans,

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VT, USA), with two retractable levers positioned in the left and right positions equidistantly (8

cm apart) on the front wall. The levers extended 0.8 cm into the chamber and were positioned 2.5

cm above a grid floor constructed of parallel stainless steel bars, measuring 0.3 cm in diameter.

Between the two levers there was a recessed well where a liquid dipper would deliver 0.02 ml of

sweetened milk (by volume 3 parts sugar, 3 parts powdered non-fat milk, and 10 parts water).

The inner area of the test chamber measured 15 x 11.5 x 17.5 cm and was surrounded by an

aluminum chassis box with 3 Plexiglas walls and a single Plexiglas door. Test chambers were

housed in a sound attenuated cubicle (Med Associates; Model Number: ENV-022).

Experimental events and data collection during these experiments were controlled by Med-PC

for Windows software (Version 4.0 Med Associates).

  Procedure. 13 young VMAT1 knockout and 11 young wildtype C57BL/6 mice,

weighing between 20 and 30 g, were tested for learning and memory of an operant response in

the using autoshaping of the lever press response procedure. Animals were placed in a standard

operant chamber inside the sound attenuating chamber. When the session started, a single lever

was extended into the operant chamber and the house light was turned on. To receive a reinforcer

mice had to press the extended lever during a cued period (6 s signal tone accompanied by a

signal light over the extended lever), which occurred on a variable interval (VI) 45 second

schedule (range 4-132 s). If the mouse pressed the lever during the cued period, the light and

tone terminated and, except during extinction testing, a dipper containing sweetened milk

became available for 4 seconds in the recessed food well. Lever presses made at any time other

than the cued period carried no consequences, but were recorded. While the characteristics of the

cued period did not change throughout the study, consequences to responding or not responding

differed depending on the stage of the study. The first day of the study was a combined

Pavlovian/Instrumental training day, during which the mice received access to a reward if they made a lever press during the cued period or if no response was made during the 6 second cued period, the tone and light terminated and a reinforcer was made available for 4 seconds. Next, the mice underwent 10 days of Instrumental training in which only lever presses made during the 6 second cued period resulted in delivery of the food reward. If no response was made during the 6 second cued period, the light and tone terminated but no reinforcer was delivered. After the 10th day of Instrumental training, the mice underwent 4 days of Extinction testing. During Extinction testing, lever presses made during the cued-period terminated the light and tone, but no reinforcer was made available. Each session lasted for 120 minutes or until 100 reinforced/cued lever presses had been made. Lever position (left versus right) was counterbalanced between groups to control for any possible olfactory cues.

Data Analysis. The number of reinforced lever presses and the total number of lever presses were the primary dependent measures in the autoshaping task. Data were analyzed separately for each experimental condition. An independent samples t-test was conducted to examine differences between genotypes in the Pavlovian/Instrumental condition. A two-way factorial ANOVA was conducted for the main effects of genotype and days 1-10 and the interaction for the Instrumental only condition. To examine the effects of Extinction testing the 10th day of Instrumental only training as well as all 4 days of extinction testing were analyzed using a two-way factorial ANOVA for the main effects of genotype and days, as well as the interaction of the two. Any significant interaction or main effect of days was further examined using a Tukey HSD post-hoc test. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA). All significant differences were p < .05.

Morris Water Maze

Apparatus. Morris Water Maze testing was conducted in a standard water maze pool from Med Associates (Model ENV-594M-W) which is 182.88 cm in diameter and 63.5 cm deep from the floor of the pool to the top edge. The pool was filled with 22 degree Celsius water and non-toxic Colorations Tempera Paint (Colorations Inc, Savanah GA) was added to make the water opaque, obscuring the adjustable platform which sat 2.5 cm below the surface of the water. The pool was filled to approximately 30.5 cm from the top edge, with enough space to place 5 spatial cues along the interior edge of the pool, that were equidistant and approximately 114.3 cm from each other. Three additional cues were placed on the walls around the pool. Cues consisted of simple, unique geometric shapes printed black on white paper and laminated to prevent water damage. A retractable curtain separated the water maze from the computer that controlled the video recording device, to assure that the presence of the experimenter did not affect the animals swim path or search pattern. A video camera (Med Associates, Model Number: VID-CAM-MONO-1) was mounted to the ceiling above the pool and the video was sent to a computer that ran recording and tracking software (v. 1.0.0.426, Med Associates, MED-SYST-VWM). The pool was divided into 4 imaginary quadrants (I, II, III, and IV) using the tracking software. These quadrants were marked on the outside of the pool, but were not used as cues for the animals.

Free Feeding Condition Procedure. 10 young VMAT1 knockout and 10 young wildtype C57BL/6 mice, weighing between 20 and 30 g, were used in this experiment. Mice began each trial in a random pool quadrant (I, II, III, or IV) and were gently lowered into the water while facing the wall of the pool. After the mice were placed in the water, the experimenter would remotely begin the trial. Mice were allowed to freely swim in the pool until either they found the

location of the hidden platform or until 120 seconds had elapsed (platform was in a fixed position for this experiment). If a mouse did not find the platform within 120 seconds, the recording software terminated the trial and the experimenter gently guided the mouse to the platform (animals were not picked up or pushed towards the fixed platform). Once on the platform the mice were allowed to stay on the platform for 20 s. Then the mice were gently hand dried and placed in a warming box with paper towels and a heat lamp 30 cm from one side of the box for 3 minutes before being returned to their home cage. Mice were tested 4 trials per day with approximately 15 minutes between trials, each trial beginning from a different quadrant with the quadrant start order randomized each day. The Morris water maze consisted of 5 days of training and one day of signaled trial testing. On the signaled trial test day the position of the platform was moved to the opposite quadrant of the fixed location the platform had been in for the previous 5 days and a small black film canister was placed on top of the platform, and mice were tested as described above. The signaled trial is important for identifying possible non-cognitive deficits that may affect performance in Morris water maze.

Food Restriction Condition Procedure. 8 young VMAT1 knockout and 8 young wildtype mice, weighing between 20 and 25 g, were used for the food-restricted procedure in the Morris water maze. Twenty-four hours before the first trial, animals were placed on food restriction and maintained at 85-90% of their free feeding body weight for the duration of this procedure. Mice were fed a set amount of standard rodent chow after the completion of all four trials to maintain food-restricted body weights. All other testing procedures were the same as for the free feeding condition.

Repeated Acquisition Learning Procedure. 10 young VMAT1 knockout and 10 young wildtype mice, weighing between 20 and 25 g, were used in this experiment. Repeated

acquisition learning occurred after the free feeding condition using the same cohort of mice. All water maze testing procedures were the same as for the free feeding condition with the exception that on each day the location of the platform was moved to a new quadrant and a different location in that quadrant. The location changed according to a semi-random design using a random number generator (random.org), with each quadrant being selected at least once, but no more than twice and the quadrant could not be the same as the previous day. If the location chosen by the random number generator did not meet both criteria, another number would be generated until both criteria were met.

Data Analysis. Latency to find the platform (seconds) and swim speed (cm/second) were used to analyze differences in learning and memory between VMAT1 knockout and wildtype mice. A factorial ANOVA were used to test for the main effects of genotype and day, as well as the interaction of the two. Significant interaction effects or a main effect of days were further analyzed using a Tukey HSD post-hoc test. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA). All significant differences were at p < .05.

Locomotor Activity

Apparatus. Four standard Locomotor Activity Chambers (Med Associates, ENV-515) were used in this experiment. The chambers measured 43 cm x 43 cm x 30.5 cm, included a ventilated cover, and were housed inside a sound attenuating chamber (Med Associates, ENV-017M). Activity was measured by photocell beam breaks, which was recorded using a series of 3 photocell arrays (16 photocells in each array). Two photocell arrays were used to measure horizontal ambulation for the X & Y dimensions, and one photocell array measured vertical

rearing 4.5 cm above the floor of the chamber. Data were recorded by an Activity Monitor (Med Associates, Model SOF-811).

Free Feeding Condition Procedure. 13 young VMAT1 knockout and 11 young wildtype C57BL/6 mice, weighing between 20 and 25 g, were used in this experiment. Mice were placed in the middle of the locomotor activity chamber and allowed to freely explore for one hour. Data were recorded in 10 minute bins. Test sessions were conducted for six consecutive days. Between test sessions the chambers were cleaned with a 10% EtOH solution.

Food Restricted Condition Procedure. 10 young VMAT1 knockout mice and 10 young wildtype C57BL/6 mice mice, weighing between 20 and 25 g, were used in this experiment. Twenty-four hours before the first test session, animals were placed on food restriction and maintained at 85-90% of their free feeding body weight for the duration of this experiment. Mice were fed a set amount of standard rodent chow at the completion of the one hour locomotor activity session to maintain food-restricted body weights. All other testing procedures were the same as for the free feeding condition.

Amphetamine Condition Procedure. 26 young VMAT1 knockout mice and 30 young wildtype C57BL/6 mice, weighing between 20 and 25 g, were used in this experiment. Mice were placed in the middle of the activity chamber and allowed to freely explore for one hour. Mice were then removed, given an injection of vehicle or 0.5 mg/kg amphetamine and then placed in their home cage for 10 minutes. Fecal boli were collected from the locomotor chamber and the chambers were cleaned with 10% EtOH solution and dried with a paper towel. After 10 minutes the mice were placed back into the locomotor activity chamber for another hour. Data were recorded in 10 minute bins.

Drugs. D-amphetamine (sigma) was dissolved in distilled water and administered at a volume of 10 ml/kg body weight at 10 minute pre-session treatment time. All doses refer to the salt form of the drug (HCl).

Data Analysis. Horizontal ambulation (beam breaks), vertical rearing (beam breaks), and thigmotaxia (defined as the proportion of distance traveled in the outer portion of the chamber) were used as dependent variables. In free feeding and food-restricted conditions a Factorial ANOVA was used to test for main effects of genotype and days and the interaction. If a significant main effect of days or a significant interaction was found, further comparisons with a Tukey HSD post-hoc test was conducted. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). The amphetamine condition data were analyzed using a Factorial ANOVA to test for main effects of genotype, treatment type, and time (across the 10 minute bins). If a significant main effect for time or a significant interaction was found a Tukey HSD post-hoc test was conducted. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). All significant differences were at $p < .05$.

Progressive Ratio

Apparatus. Six standard computer-interfaced mouse operant condition chambers (Model ENV-307A; Med Associates, St. Albans, VT, USA), with two retractable levers positioned in the left and right positions equidistantly (8 cm apart) were used. The levers extended 0.8 cm into the chamber and were positioned 2.5 cm above a grid floor constructed of parallel stainless steel bars, measuring 0.3 cm in diameter. Between the two levers was a recessed well into which a liquid dipper delivered 0.02 ml of sweetened milk (by volume 3 parts sugar, 3 parts powdered non-fat milk, and 10 parts water). The inner area of the test chamber measured 15 x 11.5 x 17.5

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cm and was surrounded by an aluminum chassis box with 3 Plexiglas walls and a single

Plexiglas door. Test chambers were housed in a sound attenuated cubicle (Model ENV-022; Med

Associates).   Experimental events and data collection during these experiments were controlled

by Med-PC for Windows software (Version 4.0 Med Associates).

Procedure. 18 young VMAT1 knockout and 18 young wildtype C57BL/6 mice were

food-restricted 24 hours before the first training session and maintained between 85 and 90% of

their free-feeding body weights for the remainder of the study. Mice were trained on a fixed ratio

1 (FR1) schedule of reinforcement 2 days/week using a 30 minuet training session (Monday and

Thursday). When a lever press was made the levers would retract, the house light would turn off,

the food dipper would rise into the recessed well adjacent to the lever, and a signal light in the

food well would turn on. The purpose behind the signal light was to increase the salience of the

reward presentation. The dipper remained raised and the signal light remained on for 5 seconds

after which the signal light terminated and the food dipper was lowered back into the liquid

reward trough. The house light remained off (following the 5 sec food access) for a total of 10

seconds, functioning as a time out period, after which the house light was turned on and the lever

was extended into the operant chamber to begin the next trial. Mice were tested 2 days/week

(Tuesday and Friday) with a Progressive Ratio schedule of reinforcement. Each progressive ratio

session began with the FR1 schedule and after each FR was completed the FR requirement was

increased according to the formula ([$5e^{(\text{reward number x 0.2})}$]-5) which yields a series of fixed ratios (1,

2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603).

During test sessions both levers were extended; however, the opposite lever was inactive and

responses on the inactive lever were recorded, but had no consequence. If an animal made no

response on either lever for 300 consecutive seconds, the session terminated. Breakpoint was

defined as the current ratio when the session ended either at 60 minutes or at the end of 300 seconds of inactivity.

Data Analysis. Break point was recorded by Med-PC for Windows software (Version 4.0 Med Associates). A Factorial ANOVA was conducted to test for the main effects of genotype and days and the interaction. If a significant main effect of days or a significant interaction was found, further comparisons with a Tukey HSD post-hoc test was conducted. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). All significant differences were at $p < .05$.

Forced Swim Test

Apparatus. The Forced Swim Test was conducted in a clear Plexiglas cylinder that is 45 cm tall, has a 19 cm diameter and is filled with tap water to a depth of 30 cm. Water was kept at 22-23 degrees Celsius with water being changed between each trial. A camera, attached to a tripod, was set approximately 4 ft from the Plexiglas cylinder with the height and tilt of the camera set so that the 30 cm water line was slightly above the midline of the video playback; this was confirmed in the viewfinder before the session begins. This allowed forced swim scorers a full view of the mouse above and below the water line, which was helpful in differentiating mobile and immobile behaviors. The placement of both the camera and the cylinder were marked with tape to ensure that placement of both was consistent for testing each mouse.

Procedure. 12 young VMAT1 knockout and 12 young wildtype C57BL/6 mice were used in this experiment. Each test session began by turning on the camera and recording the animal identifier (a unique animal number that did not identify genotype), date, and experimenter initials. The mouse was removed from its home cage and held by the scruff of its neck and gently placed paws first into the water. The beginning of the test session time on the video recorder was

noted by the experimenter. Each session lasted for a total of 6 minutes. Once the session was over, the mice were placed in a dry box for 4 minutes to warm up and dry off. The water in each tank was changed after each animal to avoid possible confounds due to odor from other mice or fecal matter deposited in the tank.

Data Analysis. The primary dependent variable in this experiment was time spent immobile (in seconds), defined as minimal movement of the fore and hind paws to keep the animal afloat. Test sessions were videotaped to allow decoding of each session by raters blinded to genotype. All behavioral measures obtained from the video recordings were subjected to an analysis of interrater reliability (IRR), a correlation of time spent immobile scores between two individual raters. If IRR did not reach at least 80% scorers were asked to rescore the videos with highest variability and the new immobility score was used. While studies using the task forced swim with rats typically have a habituation trial and then a second day test session, studies have shown that mice require a much shorter habituation period (Castagne, Moser, Roux, Porsolt 2010). Mice are typically tested in single day with a 6 minute test session; however, the first two minutes of the session are used as a habituation period and excluded from the data analysis, so only the last 4 minutes of the test session are used for data analysis. Immobility was scored by measuring the time spent mobile (in seconds), as the presence of this behavior is easier to identify than the absence of it, and that time was subtracted from 240 seconds. Separate independent groups T-tests were conducted between genotypes. All significant differences were at $p < .05$.

Grip Strength

Apparatus. Grip strength was measured using a Digital Force Gauge (Chatillon, DFE2-002) which was mounted on a base that elevates the digital force gauge eighteen inches from the

table where the apparatus is held. The animals were required to grab a triangular metal bar that extends 12 cm from the force gauge with the side of the triangle facing the experimenter measuring 4 cm across. Grip strength was recorded in kilograms force.

Procedure. 14 young VMAT1 knockout and 10 young wildtype C57BL/6 mice at free-feeding body weights were used in this experiment. During testing the mouse was removed from the home cage and weighed. Then it was held by the base of the tail and the scruff of the neck and its two forepaws were placed on the triangle shaped bar. If the animal lost its grip before the experimenter began to pull the mouse back, the mouse was removed completely and the trial started again. The animal was then slowly pulled away from the bar; being held parallel to the table, until it released its grip and the maximal grip force was recorded. Animals were tested for three consecutive trials per day over three days, with one day between each test day. In order to prevent experimenter bias a single blind procedure was used so the researcher testing grip strength did not know the genotype of the mouse that was being testing.

Data Analysis. Force in kilograms was the only dependent measure used in this experiment. Trials were averaged across days, then across animals. A factorial ANOVA was conducted to determine if there were differences between genotypes and days or an interaction. A significant main effect of days or an interaction was further analyzed with a Tukey HSD post hoc test. Data analysis was conducted with Prism version 5.0 (GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). All significant differences were at $p < .05$.

Rotarod

Apparatus. For this procedure the Rotamex-5 (0254-2002L, Columbus Instruments, Columbus, OH) was used. The apparatus consisted of a 3.0 x 50 cm dowel that is partitioned by 5 21 cm diameter grey PVC discs, which created four separate lanes, each 9.5 cm wide, which

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allowed 4 mice to be tested simultaneously. Each lane is monitored by a set of photocell beams, which detects when the mouse falls off of the rotarod (thus ending the trial for that mouse). The dowel is connected to a motor that rotates the rod. The speed of rotation and acceleration of the rod was controlled by a control panel on the front of the rotarod apparatus. The trial ended when the mice fall from the dowel (44.5 cm) to the bottom of the chamber, which was covered in bedding to provide cushioning.

Procedure. 13 young VMAT1 knockout and 11 young wildtype C57BL/6 mice were used for the rotarod procedure. Before the trial began the rotarod was rotating at a speed of 1 revolution per minute (rpm). Once all four lanes were occupied, the trial began and the rotation of the rod accelerated at a speed of 20 rpm/minute until it reached a terminal speed of 60 rpm, or until the animal fell off (whichever came first). Mice were tested for three days with one day between each test session, and each test session consisted of 10 trials. Mice were given 30 seconds after the last mouse fell off the rotarod to rest between trials.

Data Analysis. The primary dependent measure was the latency to fall (measured in seconds). The ten-trials within each session were averaged for each genotype group. A factorial ANOVA was used to test the main effects of genotype and days, and for the interaction. If a significant main effect of days or a significant interaction was found, further comparisons with a Tukey HSD post-hoc test was conducted. Data analysis was conducted with Prism (version 5.0, GraphPad Software; GraphPad Software Inc., La Jolla CA v. 5.0). All significant differences were at $p < .05$.

Assays Conducted in Aged Animals

All apparati, procedures, and statistical analyses for assays conducted in aged mice were the same as those conducted in the young mice except as noted below.

Autoshaping of the Lever Press Response

Procedure. 16 aged VMAT1 knockout and 13 aged wildtype C57BL/6 mice were used in this experiment. All protocols were the same as the autoshaping procedures for the young mice with the exception that the number of reinforcers to end the session was reduced to 50, as previous studies in our lab have shown age dependent effects on lever pressing ability.

Morris Water Maze

Procedure. 12 VMAT1 knockout and 12 aged wildtype mice, weighing between 25 and 30g, were used in this experiment. All protocols for this experiment were the same as young mice except there was no food-restriction or repeated acquisition.

Locomotor Activity

Procedure. 7 aged VMAT1 knockout and 9 aged wildtype C57BL/6 mice weighing between 25 and 30 g, were used in this experiment. All protocols for this experiment were the same as young mice with the exception that there was no food-restricted condition or amphetamine dose curve condition.

Progressive Ratio

Procedure. 12 aged VMAT1 knockout and 12 aged wildtype C57BL/6 mice weighing between 25 and 30 g, were used in this experiment.

Grip Strength

Procedure. 5 aged VMAT1 knockout and 7 aged wildtype C57BL/6 mice weighing between 25-35g were used in this experiment.

Rotarod

Procedure. 6 aged VMAT1 knockout and 13 aged wildtype C57BL/6 mice weighing between 25-35g were used in this experiment.
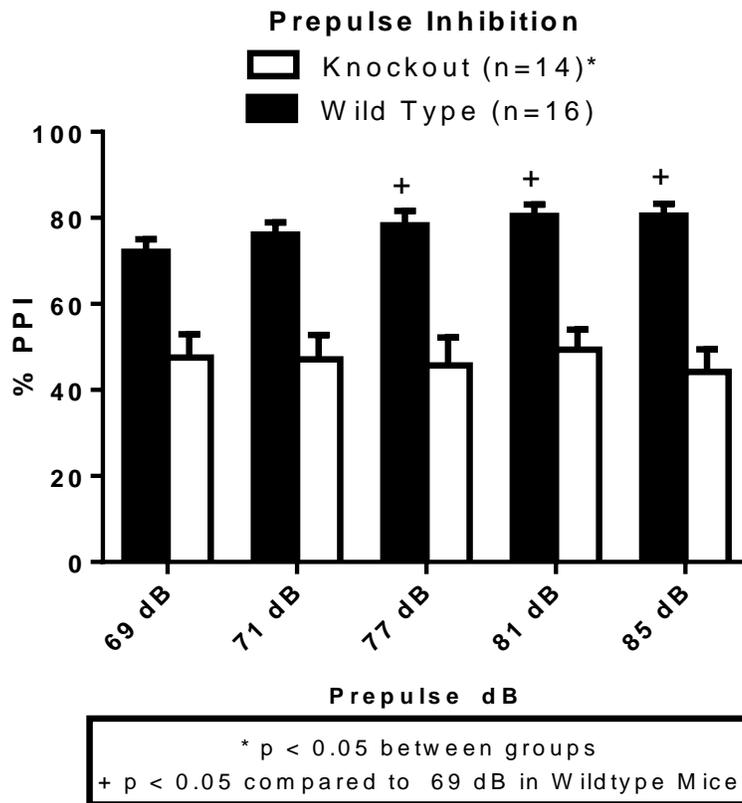
Results

Assays Conducted in Young VMAT1 Knockout and Wildtype Mice

Prepulse Inhibition (Figure 3)

VMAT1 knockout mice displayed a sensorimotor gating deficit with a significant deficit in prepulse inhibition at all decibel levels as compared to wildtype mice ($p < 0.001$). Also, a Newman Keuls post hoc test revealed that wildtype mice showed a significant increase in inhibition of the startle response at 77, 81, and 85 dB compared to 69 dB ($p < 0.05$), while knockout mice failed to show any significant changes in startle across the decibel levels. There was a significant difference in mean startle reactivity of the first and last blocks of five startle alone presentations. Wildtype mice had a mean startle reactivity ($M = 303$, $SEM = 41.92$) higher than VMAT1 knockout mice ($M = 99.1$, $SEM = 14.30$) ($p<.0001$).

Autoshaping of the Lever Press Response (Figure 4)

Reinforced Lever Press Comparison (Figure 4A): Both VMAT1 knockout and wildtype mice learned the task during the instrumental only condition, as there was a significant main effect of days with lever pressing increasing across days ($p < 0.001$). A significant interaction between genotype and days revealed that the VMAT1 knockout mice displayed a significantly slower acquisition of the operant response as evidenced by significantly reduced reinforced lever presses on days 3-6 ($p < 0.05$). The number of responses in VMAT1 knockout mice increased to levels similar to that of the wildtype mice by day 10. Both VMAT1 knockout and wildtype mice exhibited normal extinction learning with significant decreases ($p > 0.05$) in the number of responses over the four days with no significant differences between the two genotypes ($p < 0.05$).

Figure 3: Prepulse Inhibition in young VMAT1 knockout and C57BL/6 wildtype mice. Results for VMAT1 knockout and wildtype mice in prepulse inhibition assay: The percent prepulse inhibition (%PPI) is shown along the Y-axis. The decibel (dB) level of the prepulse is plotted across the x-axis. Data are presented as group means (+/- SEM).

There were significant main effects for genotype $F(1,112) = 28.89$, $p < 0.001$ and decibel level $F(4,112) = 2.86$ $p = 0.027$, and a significant interaction between genotype and decibel level $F(4,112) = 4.08$, $p = 0.004$.
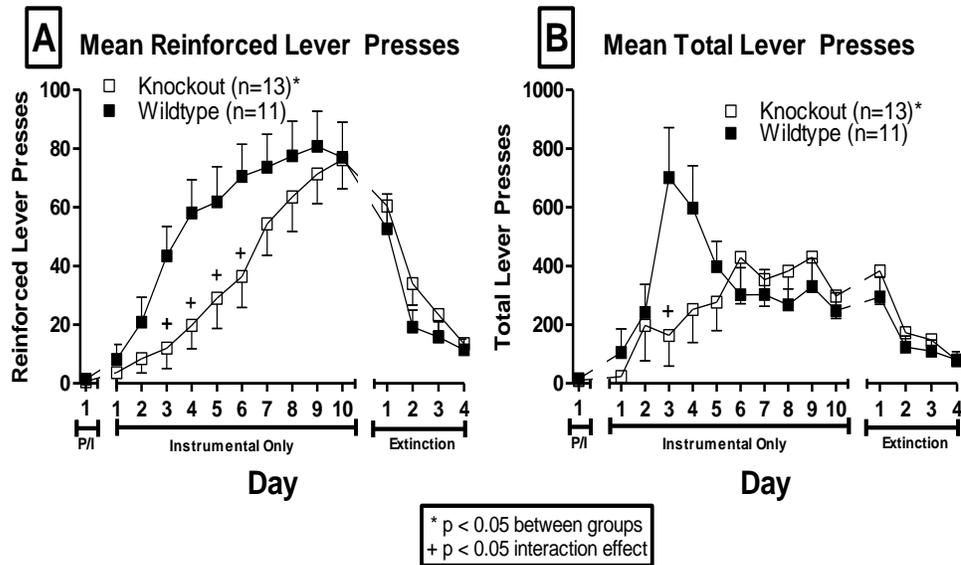
Figure 4: Reinforced & total lever press results for young VMAT1 knockout and C57BL/6 wildtype mice in the autoshaping assay. Reinforced lever presses (mean +/- SEM) is shown in panel A, the total lever presses (mean +/- SEM) is shown in panel B. The condition type and day number is shown on the X-axis (P/I = Pavlovian/Instrumental).

PANEL A: On the P/I day there was no significant difference in reinforced lever presses between VMAT1 knockout and wildtype mice $t(22) = 1.36$, $p = 0.187$. During instrumental only learning, both VMAT1 knockout and wildtype mice learned the task as there was a significant main effect of days $F(9,198) = 33.73$, $p < 0.001$ as shown by the increasing lever presses. While the main effect of genotype was not significant $F(1,198) = 3.15$, $p = 0.090$, the interaction between genotype and day was significant $F(9,198) = 2.40$, $p = 0.013$, and the VMAT1 knockout mice had significantly lower responses on Days 3-6 ($p < 0.05$). During extinction testing there were no significant differences between genotypes $F(1,88) = 0.50$, $p = 0.488.$, but both groups exhibited extinction learning, as there was a significant decrease in reinforced lever pressing $F(4,88) = 52.16$, $p < 0.001$. The interaction between genotype and days in extinction testing $F(4,88) = 0.63$, $p = 0.642$ was not significant.

PANEL B: On the P/I day there was no significant difference in total lever presses between VMAT1 knockout and wildtype mice $t(22) = 0.91$, $p = 0.370$. During the instrumental only condition VMAT1 knockout and wildtype mice did not differ in total lever pressing behavior, as there was no significant main effect of genotype $F(1,198) = 0.91$, $p = 0.352$; however, the interaction between genotype and days was significant $F(9,198) = 2.61$, $p = 0.007$ and a Newman Keuls post hoc test revealed that VMAT1 knockout mice made significantly fewer lever presses on day 3 of instrumental only training. The main effect for days was significant $F(9,198) = 7.62$, $p = 0.007$, with total lever pressing increasing as instrumental only training continued. During extinction testing there was no significant main effect of genotype $F(1,88) = 0.40$, $p = 0.532$. The main effect for days was significant $F(9,88) = 14.34$, $p < .001$. The interaction between genotype and days was not significant $F(9,88) = 0.33$, $p = 0.857$.

Total Lever Pressing Comparison (Figure 4B): The significant interaction between genotype and days ($p < 0.01$) revealed that the VMAT1 knockout mice had a more gradual increase in total lever pressing behavior, than the wildtype mice, and posthoc tests indicated that VMAT1 knockout mice made significantly fewer lever presses on day 3 of instrumental only training. During extinction testing VMAT1 knockout and wildtype mice did not differ significantly ($p > 0.05$) with both groups displaying a significant decrease in total lever pressing during extinction testing ($p < 0.01$).

Locomotor Activity (Figure 5)

Free-Feeding Condition Horizontal Ambulation (Figure 5A). There was no significant difference in horizontal ambulation (beam breaks) between VMAT1 knockout and wildtype mice in the free-feeding condition ($F(1,110) = 0.56$, $p = 0.461$). Both VMAT1 knockout and wildtype mice displayed normal habituation to the locomotor activity chamber as there was a significant decrease in horizontal ambulation over the 6 days of testing (significant main effect of days ($F(5,110) = 7.86$, $p < 0.001$). The interaction of genotype and days was not significant ($F(5,110) = 0.99$, $p = 0.429$).

Free-Feeding Condition Thigmotaxia (Figure 5B). There was no significant difference in thigmotaxia between VMAT1 knockout and wildtype mice in the free-feeding condition $F(1,110) = 0.37$, $p = 0.548$. Both VMAT1 knockout and wildtype mice showed a significant increase in the proportion of time spent around the edge of the locomotor activity chamber (thigmotaxia) over the 6 days of the locomotor activity assay (significant main effect of days $F(5,110) = 36.72$, $p < 0.001$). This increase occurred until day 4, where thigmotaxia leveled off for knockout mice, but not for wildtype mice. The interaction of genotype and days was not significant ($F(5,110) = 0.94$, $p = 0.460$).
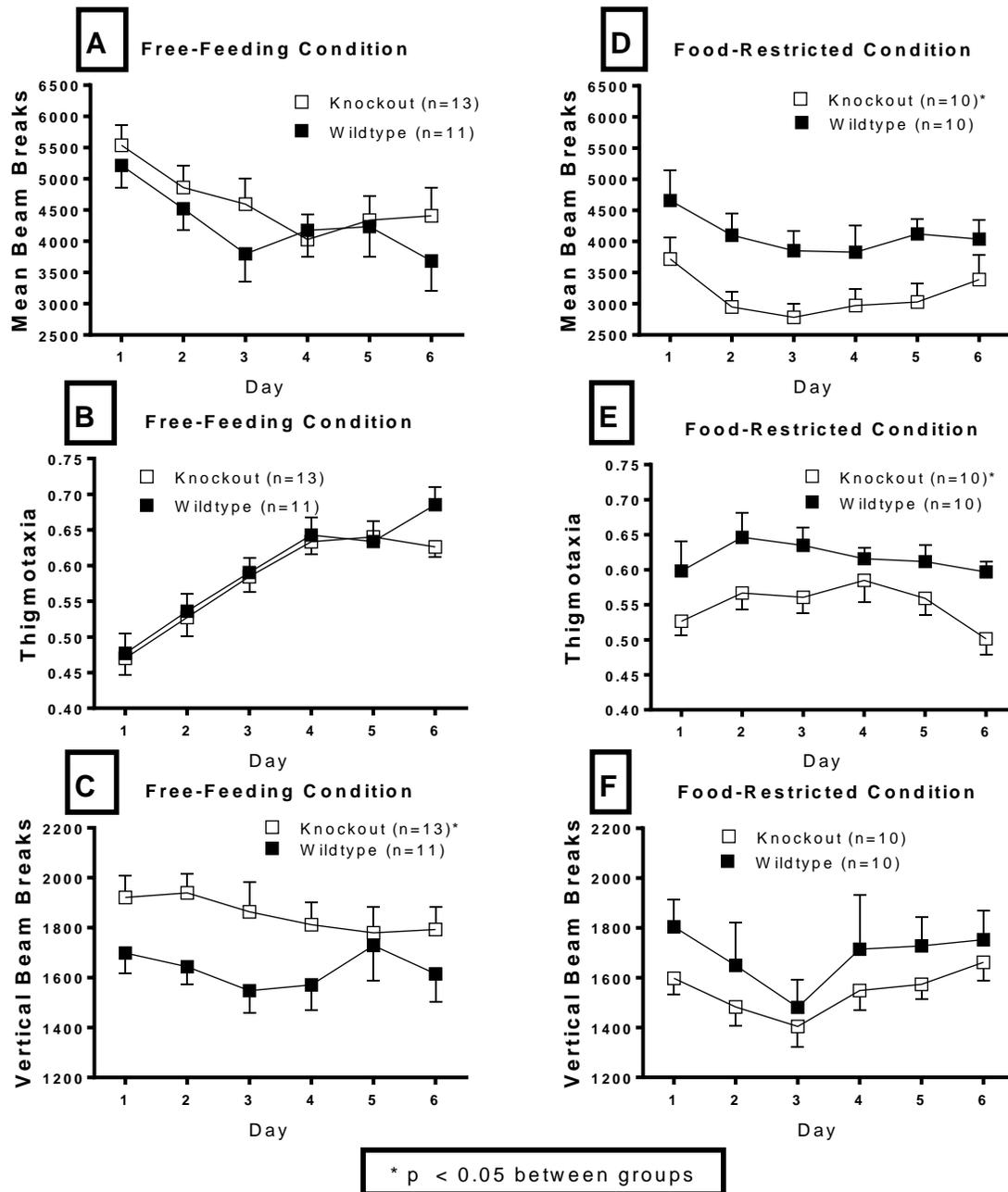
Figure 5: Horizontal ambulation, thigmotaxia, and rearing in young VMAT1 knockout and C57BL/6 wildtype mice in the locomotor activity assay under free-feeding and food-restricted conditions. Panels A, B, & C display data for animals under the free-feeding condition. Panels D, E, & F display data for mice under the food-restricted condition. Mean number of horizontal beam breaks (+/- SEM) is presented in panels A and D. Average proportion of time spent near the perimeter of the activity chamber (+/- SEM) is presented in panels B and E. Average number of vertical beam breaks (+/- SEM) is presented in panels C and F. Days of training are presented across the x-axis.

Free-Feeding Condition Rearing (Figure 5C). There was a significant difference in rearing between VMAT1 knockout and wildtype mice in the free-feeding condition ($F(1,110) = 6.05$, $p = 0.047$) with VMAT1 knockout mice making significantly more vertical beam breaks than wildtype mice across the 6 days of testing . The main effect of days was not significant ($F(5,110) = 0.94$, $p = 0.459$), and the interaction of genotype and days was not significant ($F(5,110) = 0.86$, $p = 0.509$).

Food-restricted Condition Horizontal Ambulation (Figure 5D). In contrast to the free-feeding condition (Figure 5A), the VMAT1 knockout mice made significantly fewer horizontal beam breaks across all test days as compared to the wildtype mice ($F(1,90) = 10.95$, $p = 0.004$). The main effect of days was significant ($F(5,90) = 2.34$, $p = 0.048$) with horizontal beam breaks decreasing as days increased. The interaction of genotype and days was not significant ($F(5,90) = 0.21$, $p = 0.957$).

Food-restricted Condition Thigmotaxia (Figure 5E). The VMAT1 knockout mice showed significantly less thigmotaxia, as they spent a smaller proportion of time along the outer edges of the locomotor activity chamber across all days of the assay ($F(1,90) = 9.65$, $p = 0.006$). The main effect of days was not significant ($F(5,90) = 2.02$, $p = 0.084$). The interaction of genotype and days was not significant($F(5,90) = 0.49$, $p = 0.787$).

Food-restricted Condition Rearing (Figure 5F). In contrast to free-feeding conditions Figure 7C, there was no longer a difference in rearing between VMAT1 knockout and wildtype mice ($F(1,90) = 1.29$, $p = 0.272$) with the VMAT1 mice being consistently lower than the wildtype mice. Rearing in both VMAT1 knockout and wildtype mice decreased until day three, and then increased back to day one levels over the next three days. resulting in a significant main

effect of days (F(5,90) = 3.04, p = 0.014). The interaction of genotype and days was not significant (F(5,90) = 0.19, p = 0.967).

Effects of Food-Restriction on Locomotor Activity (Figure 5). In order to provide a more direct comparison of the effects of food restriction on locomotor activity, the data were reanalyzed comparing the free-feeding condition to the food-restricted condition separately for the VMAT1 knockout mice and the wildtype mice. These results are presented below.

Horizontal Ambulation (Figure 5A Vs Figure 5D). Compared to free-feeding conditions, food-restriction reduced horizontal ambulation (a significant decrease in horizontal beam breaks, F(1,105) = 12.51, p < 0.005) in VMAT1 knockout mice, but not in wildtype animals (F (1,95) = 0.177, p > 0.05). Beam breaks also decreased across days in the VMAT1 knockout animals (significant difference of days F(5,105) = 5.61, p <.001), but there was no significant interaction of the feeding condition and days (F(5,105) = 1.49, p > 0.05). Wildtype mice showed a significant decrease in horizontal beam breaks across days (F(5,95) = 3.59, p < 0.01) and did not show an interaction between feeding condition and days (F(5,95) = 0.60, p > 0.05).

Thigmotaxia (Figure 5B Vs Figure 5E). There was no significant effect of feeding conditions on thigmotaxia in either the VMAT1 knockout mice of the wildtype mice (wildtype F(1,95) = 0.97, p > 0.05; VMAT1 knockout F(1,105) = 1.94, p > 0.05) . Nevertheless, the percent thigmotaxis increased across days in both groups (wildtype F(5,95) = 5.47, p < 0.0005; VMAT1 knockout F(5,105) = 11.38, p < 0.0001).   Also, there was a significant interaction of the feeding conditions and days in both groups. Whereas wildtype mice exhibited less thigmotaxia under the free-feeding conditions on days 1 & 2, (F(5,95) = 6.82, p < 0.0001),

VMAT 1 knockout mice showed more thigmotaxis on day 6 of the free-feeding condition $(F(5,105) = 8.64, p < 0.0001$.

Rearing (Figure 5C Vs Figure 5F). Compared to free-feeding conditions, food restriction significantly reduced vertical rearing in the VMAT1 knockout mice $(F(1,105) = 10.57, p < 0.01)$. There were no significant effects of food restriction in wildtype animals $(F(1,95) = 0.16, p > 0.05)$, and no significant effects of days or interaction of feeding and days in either group.

Morris Water Maze

Stationary Platform Morris Water Maze (Figure 6)

Latency to Platform (Figures 6A and 6C). Results from the Morris water maze under free-feeding (Figure 6A) conditions were nearly identical to those under food-restriction (Figure 6C) conditions. VMAT1 knockout and wildtype mice both learned the Morris Water Maze, as there was a significant decrease in latency to platform as days continued $(p < 0.001)$, but no significant difference between VMAT1 knockout and wildtype mice $(p > 0.05)$.

Swim Speed (Figures 6B and 6D). Under both free-feeding (Figure 6B) and food-restriction conditions (Figure 6D) both genotypes showed a significant increase in swim speed across days $(p < 0.001)$, but again there was no significant difference between the VMAT1 KO mice and wildtype mice $(p > 0.05)$. Although not related to learning, an increase in swim speed could imply acclimation to the environment or change in the salience of the aversive stimuli with repeated exposure.

Repeated Acquisition Condition (Figure 7)

Repeated Acquisition Latency to Platform (Figure 7A). The repeated acquisition task in the Morris water maze was conducted under free-feeding conditions. Both VMAT1 knockout and
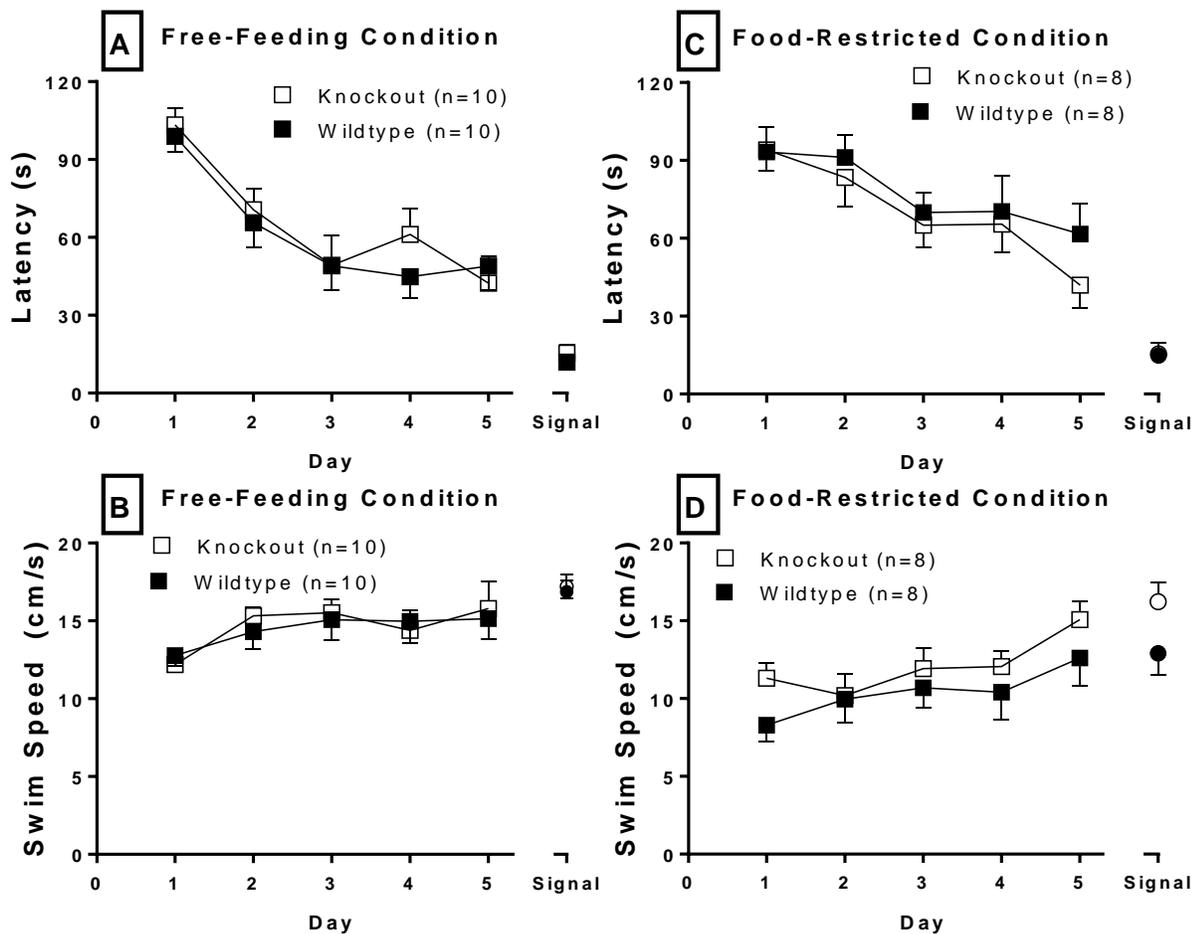
Figure 6: Latency to platform and swim speed for young VMAT1 knockout and C57BL/6 wildtype mice in the Morris water maze under free-feeding and food-restricted condition. Panels A and B display data for mice under the free-feeding condition. Panels C and D display data for mice under the food-restricted condition. Mean latency to platform in seconds (+/- SEM) is presented in panels A and C. Average swim speed (centimeters/second; +/- SEM) is presented in panels B and D. Days of training are presented across the x-axis.

Panel A: There was no significant difference in latency to platform between VMAT1 knockout and wildtype mice in the free-feeding condition $F(1,72) = 0.18$, $p = 0.676$. The main effect of days was significant $F(4,72) = 20.77$, $p < .001$ with latency to platform decreasing as days continued. The interaction of genotype and day was not significant $F(4,72) = 0.69$, $p = 0.598$. There was no significant difference between VMAT1 knockout and wildtype mice in latency to platform on the signal day $t(17) = 0.93$, $p = 0.367$.

Panel B: There was no significant difference in swim speed between VMAT1 knockout and wildtype mice in the free-feeding condition $F(4, 72) = 0.02$, $p = 0.895$. The main effect of days was significant $F(4,72) = 6.56$, $p < 0.001$ with swim speed increasing as days continued. The interaction of genotype and day was not significant $F(4,72) = 0.66$, $p = 0.0653$. There was no significant difference between VMAT1 knockout and wildtype mice in swim speed on the signal day $t(17) = 0.32$, $p = 0.750$.

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Panel C: There was no significant difference in latency to platform between VMAT1 knockout and wildtype mice in the food-restricted condition $F(1,56) = 0.97$, $p = 0.342$. The main effect of days was significant $F(4,56) = 6.10$, $p < 0.001$ with latency to platform decreasing as days continued. The interaction of genotype and day was not significant $F(4,56) = 0.31$, $p = 0.869$. There was no significant difference between VMAT1 knockout and wildtype mice in latency to platform on the signal day $t(14) = 0.11$, $p = 0.916$.

Panel D: There was no significant difference in swim speed between VMAT1 knockout and wildtype mice in the food-restricted condition $F(1,56) = 1.22$, $p = 0.289$. The main effect of days was significant $F(4,56) = 6.74$, $p < .001$ with swim speed increasing as days continued. The interaction of genotype and day was not significant $F(4,56) = 0.78$, p 0.542. There was a significant difference between VMAT1 knockout and wildtype mice in swim speed on the signal day $t(14) = 1.79$, $p = 0.094$ with VMAT1 knockout mice swim faster on average than wildtype mice.
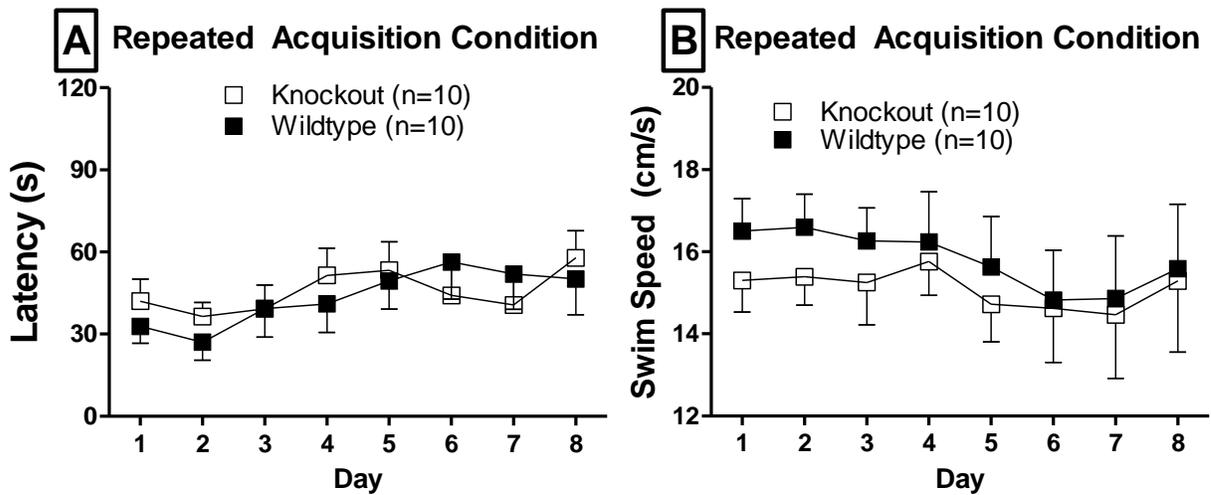
Figure 7: Latency to platform and swim speed for young VMAT1 knockout and C57BL/6 wildtype mice in the Morris water maze under repeated acquisition condition (free-feeding condition).

Mean latency to platform in seconds (+/- SEM) is presented in panel A. Average swim speed (centimeters/second; mean +/- SEM) is presented in panel B. Days of testing are presented across the x-axis.

Panel A: There was no significant difference between VMAT1 knockout and wildtype mice in the repeated acquisition condition $F(1,126) = 0.03$, $p = 0.854$. The main effect of days was significant $F(7,126) = 2.91$, $p = 0.007$ with latency to platform increasing across days. The interaction of genotype and days was not significant $F(7,126) = 1.03$, $p = 0.411$.

Panel B: There was no significant difference between VMAT1 knockout and wildtype mice in the repeated acquisition condition $F(1,126) = 0.24$, $p = 0.630$. The main effect of days was not significant $F(7,126) = 1.67$, $p = 0.124$. The interaction of genotype and days was not significant $F(7,126) = 0.23$, $p = 0.976$.

wildtype mice displayed a small, but significant increased latency to platform across 8 days of testing as shown by a significant main effect of days ($p < 0.01$). The new position for the platform each day affected the search strategies of both genotypes similarly, as there were no significant differences between groups ($p > 0.05$).

Repeated Acquisition Swim Speed (Figure 7B). There were no significant differences in swim speed between VMAT1 knockout and wildtype mice or across days in the repeated acquisition condition ($p > 0.05$).

Progressive Ratio (Figure 8)

There was no significant difference in the number of reinforcers earned between VMAT1 knockout and wildtype mice in the progressive ratio task ($t(20) = 0.55$, $p = 0.588$). This suggests that disruption of VMAT1 did not affect food motivation behaviors in these mice.

Forced Swim Task (Figure 9)

There was no significant difference in immobility time between the VMAT1 knockout and wildtype mice ($t(46) = 0.87$, $p = 0.390$). This suggests that VMAT1 knockout mice did not display any depressant-like behaviors as measured by the forced swim task.

Grip Strength (Figure 10)

Although there was a significant difference in the amount of forelimb grip strength across the days of testing ($F(2,54) = 6.35$, $p = 0.003$), the Newman Keuls post-hoc test failed to reveal any significant differences for individual day comparisons. Visual inspection of the graph does show an increase in grip strength on the second day of testing relative to the first day. There was no significant difference in forelimb grip strength between VMAT1 knockout and wildtype mice ($F(1,54) = 1.29$, $p = 0.313$) and the interaction of genotype and day was not significant $F(2,54) = 0.17$, $p = 0.843$.

Progressive Ratio Rewards
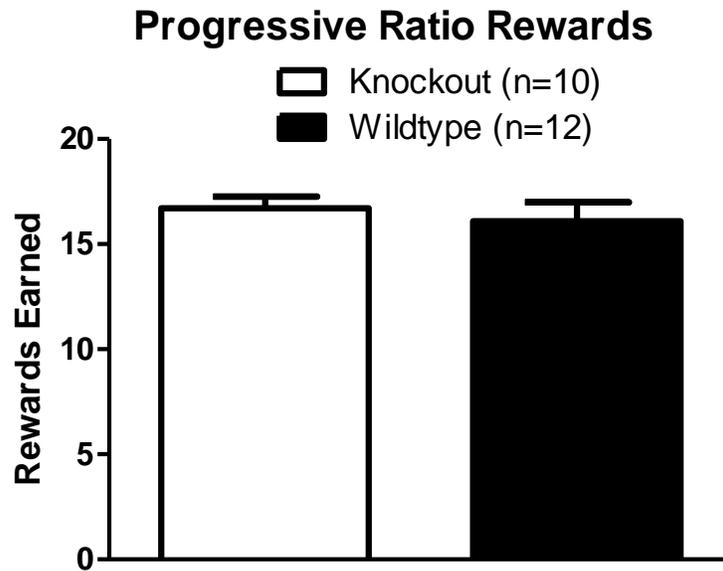
Figure 8: Rewards earned in young VMAT1 knockout and wildtype C57BL/6 mice in the progressive ratio operant assay. Mean number of rewards earned (+/- SEM) is presented.
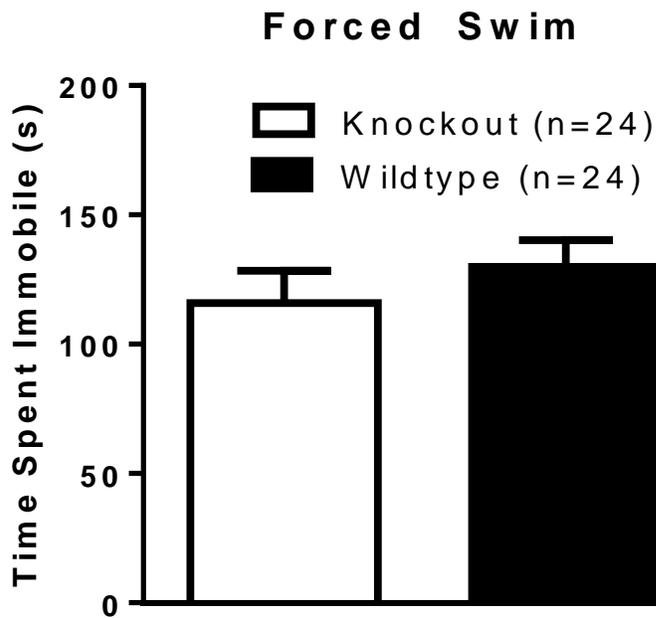
Forced Swim

Figure 9: Time spent immobile in young VMAT1 knockout and C57BL/6 wildtype mice in the forced swim assay. Mean amount of time (in seconds, +/- SEM) spent immobile is presented.
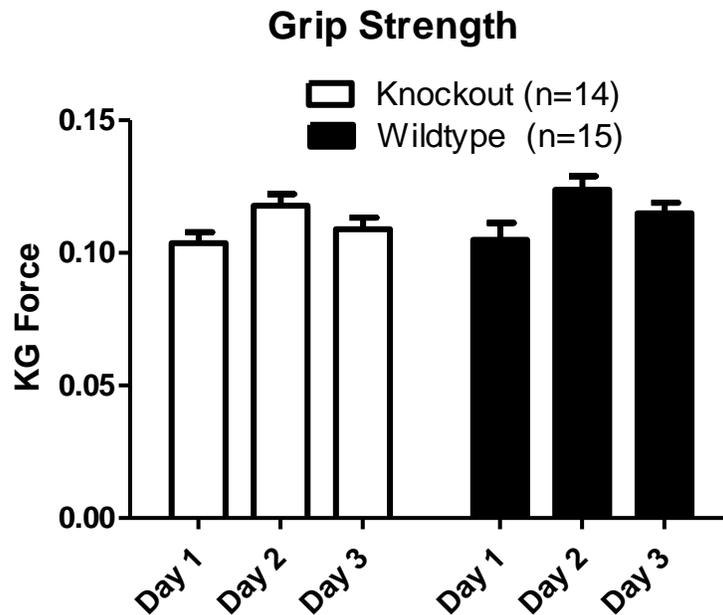
Grip Strength

Figure 10: Kilograms force exerted in young VMAT1 knockout and C57BL/6 wildtype mice in the grip strength assay.
Mean grip strength (kg force exerted; +/- SEM) is presented. Days of training are presented across the x-axis.
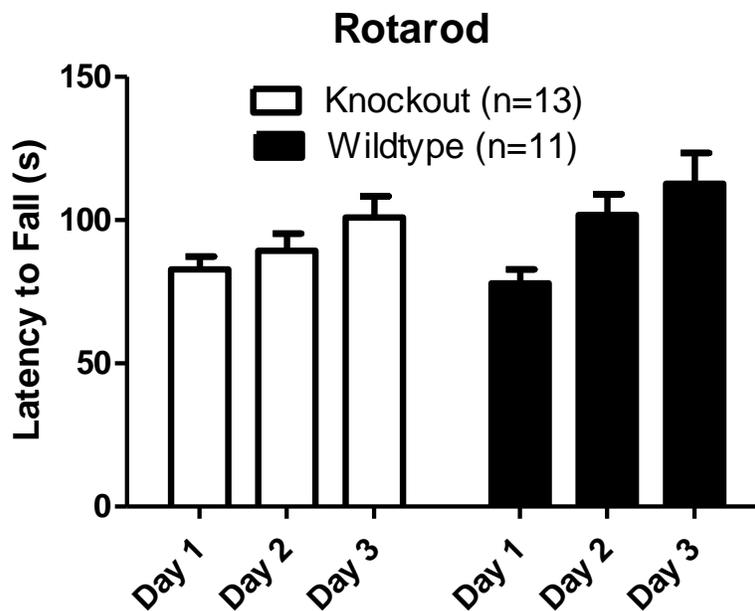
Rotarod

Figure 11: Latency to fall in young VMAT1 knockout and C57BL/6 wildtype mice in the rotarod assay.
Mean latency to fall from the rotarod (in seconds; +/- SEM) is presented. Days of training are presented across the x-axis.

Rotarod (Figure 11)

While there were no significant differences between the VMAT1 knockout and wildtype mice in the latency to fall ($F(1,44) = 0.61$, $p = 0.443$), both genotypes displayed learning as the latency to fall significantly increased across the 3 test days ($F(2,44) = 15.81$, $p < .0001$), demonstrating that the animals improved their motor coordination and balance as they had more practice at the task. The interaction of genotype and days was not significant ($F(2,44) = 2.16$, $p = 0.127$).

## Assays Conducted in Aged VMAT1 Knockout and Wildtype Mice

Autoshaping of the Lever Press Response (Figure 12)

Reinforced Lever Press – All Animals (Figure 12A). Both aged VMAT1 knockout and wildtype mice learned the task during the instrumental only condition, as there was a significant main effect of days with lever pressing increasing across days ($p < 0.001$), although genotypes did not significantly differ ($p > 0.05$). Both genotypes exhibited extinction learning as well, as there was a significant main effect of days during extinction testing ($p < 0.01$). Again, the genotypes did not differ significantly from each other ($p > 0.05$).

Total Lever Press – All Animals (Figure 12B). Total lever pressing during instrumental only training was variable due to high levels of individual differences between aged mice and a high number of non-responders. Although there was no difference in total lever pressing during instrumental only training days ($p > 0.05$), lever pressing behavior did significantly decrease during extinction only training ($p < 0.01$) with Newman Keuls post hoc testing revealing that lever pressing on all four days of extinction testing was lower than the final day of instrumental only training.
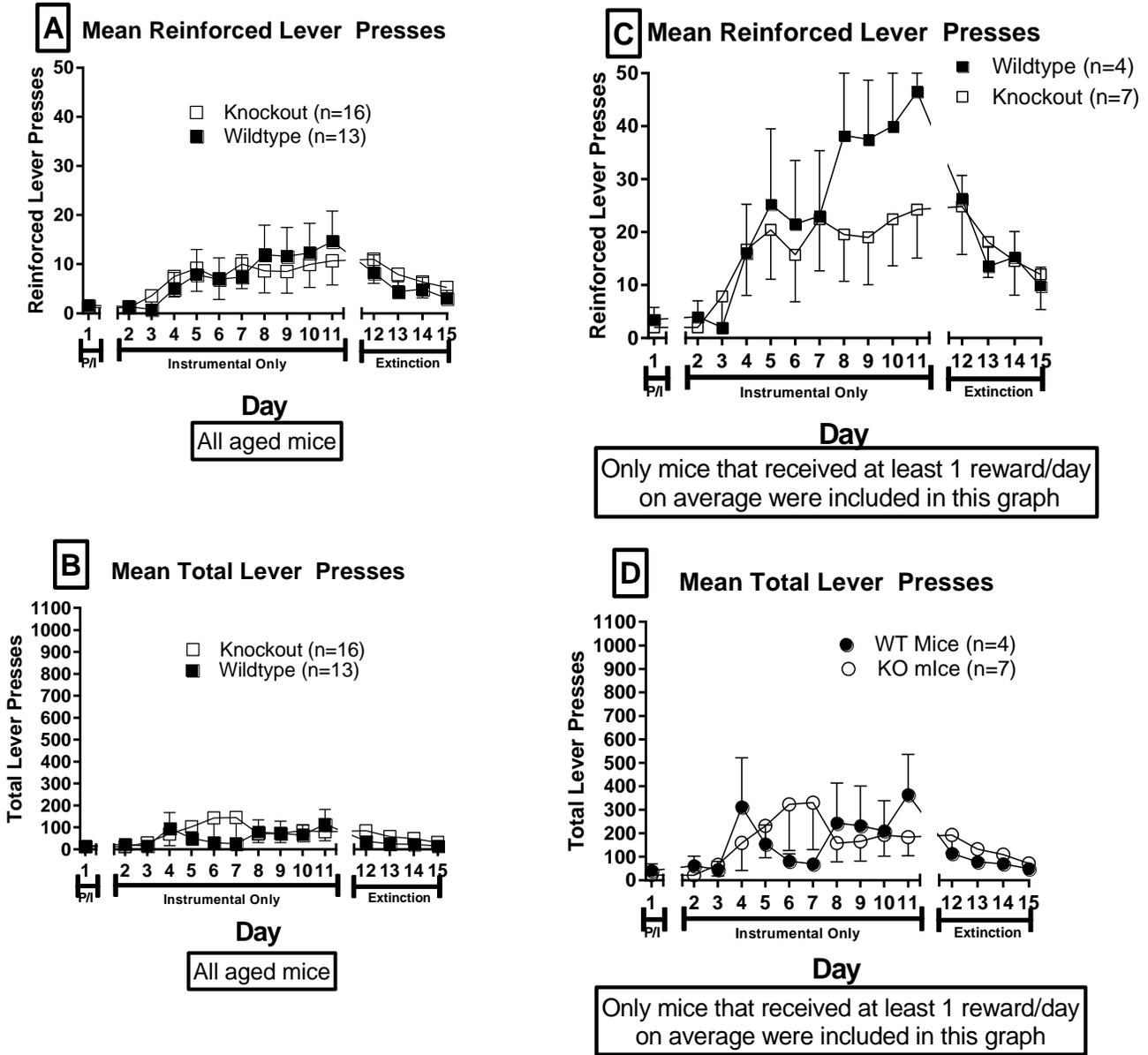
Figure 12: Reinforced & total lever press results for aged VMAT1 knockout and C57BL/6 wildtype mice in the autoshaping assay.
The mean number of reinforced lever presses (+/- SEM) for all mice is shown in panel A and the total number of lever presses (+/- SEM) for all mice is shown in panel B. The mean number of reinforced lever presses (+/- SEM) for animals who learned the task is presented in panel C. The mean number of total lever presses (+/- SEM) for animals who learned the task is presented in panel D. The condition type and day number is shown on the X-axis (P/I = Pavlovian/Instrumental). Significant differences in the interaction as determined by a Newman Keuls post-hoc test are denoted with * if p < 0.05.

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Figure 12 Caption Continued:

Panel A: There was no significant difference between VMAT1 knockout and wildtype mice during the Instrumental/Pavlovian training day $t(27) = 0.83$, $p = 0.429$. During the instrumental onlycondition (trials 2-11) VMAT1 knockout and wildtype mice were not significantly different, as there was no main effect of genotype $F(1,27) = 0.004$, $p = 0.946$. Both VMAT1 knockout and wildtype mice learned the task as there was a significant main effect of days $F(9,27) = 4.71$, $p<.0001$ and a clear upward trend of number of reinforced lever presses as days continued. The interaction of genotype and days was not significant $F(9,27) = 0.59$, $p = 0.802$. During extinction testing genotype did not significantly affect the pattern of reinforced lever pressing as there was no significant main effect of genotype $F(1,27) = 0.06$, $p = 0.665$. All mice did exhibit extinction learning as there was a significant decrease in reinforced lever pressing during extinction testing $F(4,27) = 3.697$, $p = 0.005$. There was not a significant interaction effect between genotype and days in extinction testing $F(4,27) = 1.16$, $p = 0.332$.

Panel B: There was no significant difference between VMAT1 knockout and wildtype mice during the Instrumental/Pavlovian training day $t(27) = 0.11$, $p = 0.916$. During the instrumental onlycondition (trials 2-11) VMAT1 knockout and wildtype mice did not differ significantly $F(1,27) = 0.19$, $p = 0.665$. The main effect of days was not significant $F(9,27) = 1.03$, $p = 0.415$. The interaction of genotype and day was not significant $F(9,27) = 1.05$, $p = 0.399$. During extinction testing VMAT1 knockout and wildtype mice did not differ significantly $F(1,27) = 0.24$, $p = 0.628$. The main effect of days was significant $F(4,27) = 3.97$, $p = 0.005$. The interaction of genotype and day was not significant $F(4,27) = 1.16$, $p = 0.332$.

Panel C: There was no significant difference between VMAT1 knockout and wildtype mice during the Instrumental/Pavlovian training day $t(9) = 0.83$, $p = 0.429$. During the instrumental onlycondition (trials 2-11) VMAT1 knockout and wildtype mice did not differ significantly $F(1,81) = 0.56$, $p = 0.473$. The main effect of days was significant $F(9,81) = 7.37$, $p < .001$ with the number of reinforced lever presses increasing as days continued. The interaction of genotype and days was not significant $F(9,81) = 1.58$, $p = 0.137$. During extinction testing VMAT1 knockout and wildtype mice did not differ significantly $F(1,36) = 0.13$, $p = 0.729$. The main effect of days was significant $F(4,36) = 13.64$, $p < .001$ with the number of reinforce lever presses decreasing as days continued. The interaction of genotype and days was significant $F(4,36) = 4.01$, $p = 0.009$ a Neman-Keuls post hoc test was conducted and suggests that wildtype mice made significantly more reinforced lever pressed on the final day on instrumental onlytraining but were not different during any of the extinction testing days.

Panel D: There was no significant difference between VMAT1 knockout and wildtype mice during the Instrumental/Pavlovian training day $t(9) = 0.88$, $p = 0.403$. During the instrumental onlycondition (trials 2-11) VMAT1 knockout and wildtype mice did not differ significantly $F(1,81) = 0.002$, $p = 0.965$. The main effect of days was not significant $F(9,81) = 1.06$, $p = 0.403$. The interaction of genotype and days was not significant $F(9,81) = 1.10$, $p = 0.374$. During extinction testing VMAT1 knockout and wildtype mice did not differ significantly $F(1,36) = 0.001$, $p = 0.968$. The main effect of days was significant $F(4,36) = 6.12$, $p < .001$ with the number of total lever presses decreasing as days continued. The interaction of genotype and days was not significant $F(4,36) = 2.39$, $p = 0.069$.

Reinforced Lever Presses – Animals Who Learned the Task (Figure 12C). For

the mice that learned the task, there was a significant main effect of days during instrumental

only training (p < 0.001) with Newman Keuls post hoc testing revealing that days 7, 8, 9, & 10

had a higher number of reinforced lever presses than day 1. However, VMAT1 knockout and

wildtype mice did not differ significantly during instrumental only training (p > 0.05). Extinction

testing also saw a significant main effect of days (p < 0.001) as responding declined over the four

days of extinction testing. There was an interaction of genotype and days during extinction

testing (p < 0.01); although, the difference between VMAT1 knockout and wildtype mice was on

the final day of instrumental only training and not during any of the extinction testing Days.

Total Lever Presses – Animals Who Learned the Task (Figure 12D). Although

complete non-responders were removed in this set of analyses, there was still high variability of

onset of responding. This resulted in no significant increases in total number of lever presses

during instrumental only training over the 10 days of testing (p > 0.05), nor was there a

significant difference between genotypes (p > 0.05). However, during extinction testing there

was a significant decrease in the number of total lever presses (p < 0.01), but again no difference

in responding between VMAT1 knockout and wildtype mice (p > 0.05).

Morris Water Maze (Figure 13)

Latency to Platform (Figure 13A). In aged mice both VMAT1 knockout and wildtype

mice learned the task as evidenced by a significant decrease in latency to platform across training

days ($F(4,88) = 10.52$, $p < .001$), although genotypes did not differ significantly from one

another ($F(1,88) = 0.12$, $p = 0.728$). The interaction of genotype and days was not significant

($F(4,88) = 1.55$, $p = 0.194$).
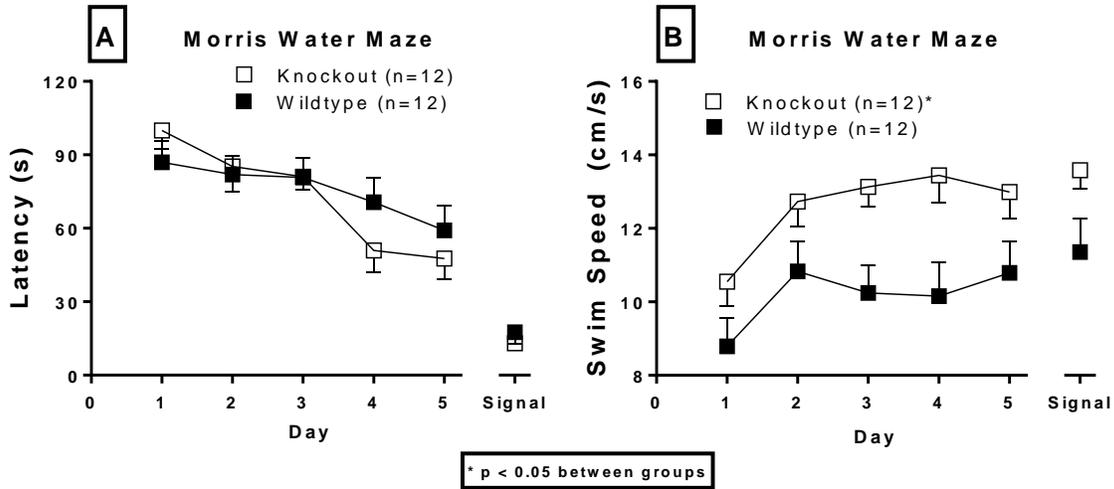
Figure 13: Latency to platform and swim speed for aged VMAT1 knockout and C57BL/6 wildtype mice in the Morris water maze under free-feeding condition. Mean latency to platform in seconds (+/- SEM) is presented in panel A. Average swim speed (centimeters/second; +/- SEM) is presented in panel B. Days of training are presented across the x-axis.

Panel A: VMAT1 knockout and wildtype mice did not differ significantly during the signal day $t(22) = 0.89$, $p = 0.383$.

Panel B: VMAT1 knockout and wildtype mice differed significantly during the signal day $t(22) = 2.13$, $p = 0.045$ with knockout mice swimming faster than wildtype mice.

Swim Speed (Figure 13B). Aged VMAT1 knockout mice swam significantly faster than wildtype mice during the training days ($F(1,88) = 9.07$, $p = 0.006$). There also was a significant increase in swim speed for both genotypes across training days ($F(4,88) = 5.74$, $p < .001$). The interaction of genotype and day was not significant $F(4,88) = 0.68$, $p = 0.606$.

Locomotor Activity (Figure 14)

Horizontal Ambulation (Figure 14A). Although Aged VMAT1 knockout had consistently higher levels of locomotor activity as compared to wildtype mice it failed to reach statistical significance and wildtype mice showed no significant differences in horizontal ambulation ($p = 0.07$). Habituation to the locomotor activity chamber was shown as there was a significant decrease in horizontal beam breaks as testing days continued ($p < 0.001$).

Thigmotaxia (Figure 14B). Aged VMAT1 knockout and wildtype mice did not exhibit any change in thigmotaxia ($p > 0.05$) or differences between the genotypes ($p > 0.05$).

Rearing (Figure 14C). Aged VMAT1 knockout and wildtype mice did not exhibit any change in rearing behavior across days($p > 0.05$) or any differences between the genotypes ($p > 0.05$).

Progressive Ratio (Figure 15)

There were no significant differences for the number of rewards earned between aged VMAT1 knockout and wildtype mice ($t(22) = 1.81$, $p = 0.083$).

Grip Strength (Figure 16)

There were no significant differences between the aged VMAT1 knockout and wildtype mice in grip strength between groups ($F(1,20) = 0.37$, $p = 0.556$) or across days ($F(2,20) = 2.39$, $p = 0.118$). The interaction of genotype and days was not significant ($F(2,20) = 0.17$, $p = 0.848$).

Rotarod (Figure 17)
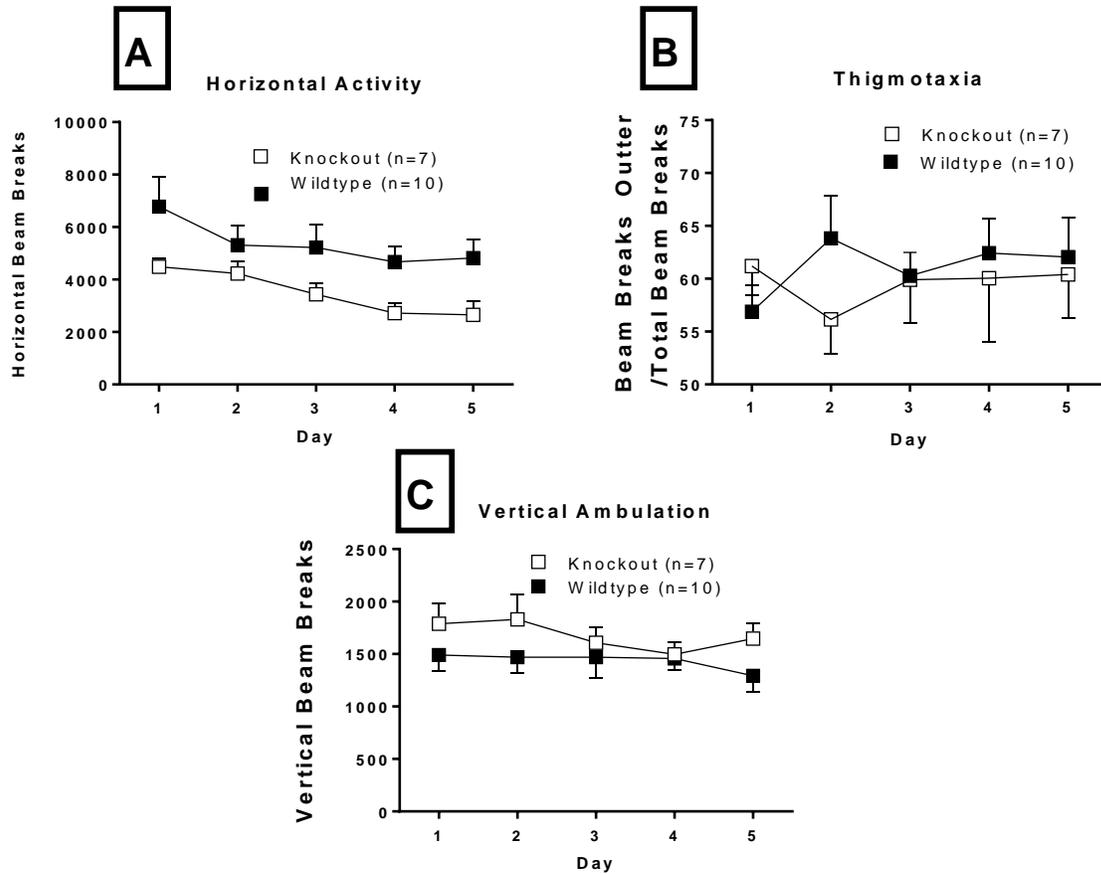
Figure 14: Horizontal ambulation, thigmotaxia, and vertical rearing in aged VMAT1 knockout and C57BL/6 wildtype mice in the locomotor activity assay under free-feeding condition. Mean number of horizontal beam breaks (+/- SEM) is presented in panel A. Average proportion of time spent near the parameter of the activity chamber (+/- SEM) is presented in panel B. Average number of vertical beam breaks (+/- SEM) is presented in panel C. Days of training are presented across the x-axis.

Panel A: There was no significant difference between VMAT1 knockout and wildtype mice in Locomotor Activity $F_{(1,60)} = 3.81$, $p = 0.070$. The main effect of days was significant $F_{(4,60)} = 8.24$, $p < .001$ with horizontal ambulation decreasing as days continued. The interaction of genotype and days was not significant $F_{(4,60)} = 0.72$, $p = 0.579$.
Panel B: There was no significant difference between VMAT1 knockout and wildtype mice in Locomotor Activity $F_{(1,60)} = 0.15$, $p = 0.702$. The main effect of days was not significant $F_{(4,60)} = 0.24$, $p = 0.915$. The interaction of genotype and days was not significant $F_{(4,60)} = 1.28$, $p = 0.288$.
Panel C: There was no significant difference between VMAT1 knockout and wildtype mice in Locomotor Activity $F_{(1,60)} = 1.53$, $p = 0.236$. The main effect of days was not significant $F_{(4,60)} = 1.27$, $p = 0.291$. The interaction of genotype and days was not significant $F_{(4,60)} = 0.88$, $p = 0.482$.

Progressive Ratio Rewards
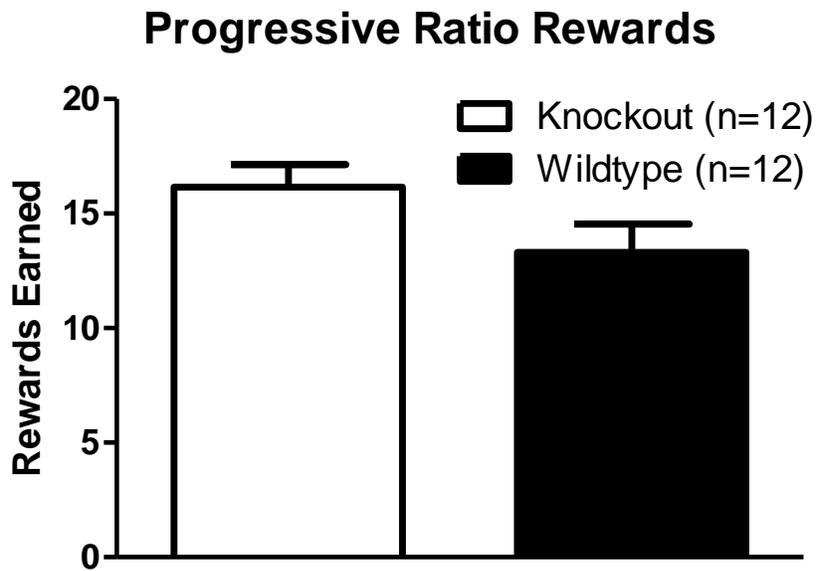
Figure 15: Rewards earned in aged VMAT1 knockout and wildtype C57BL/6 mice in the progressive ratio operant assay. Mean number of rewards earned (+/- SEM) is presented.

Grip Strength

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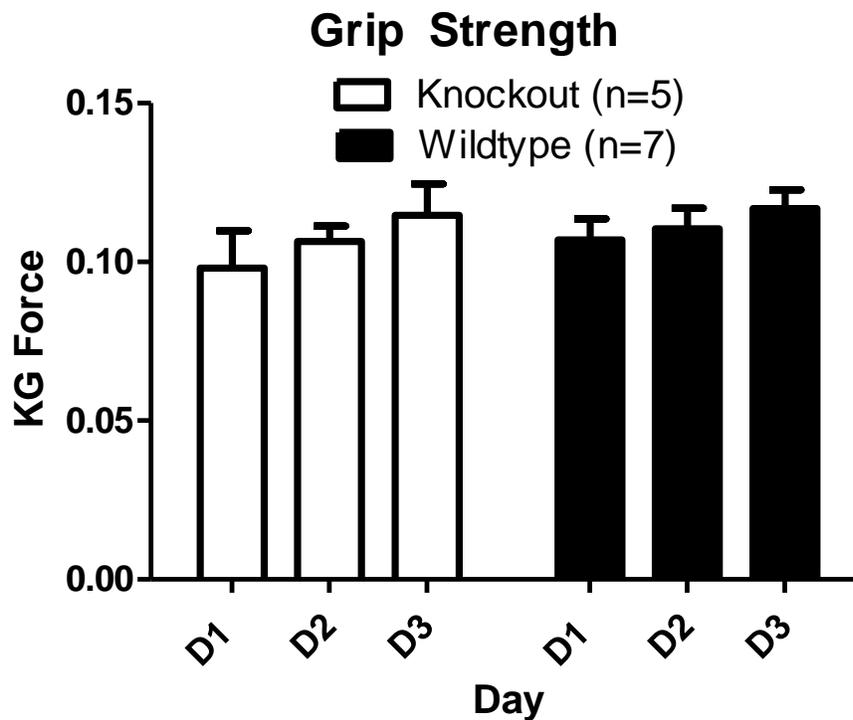
Figure 16: Kilograms force exerted in aged VMAT1 knockout and C57BL/6 wildtype mice in the grip strength assay. Mean grip strength (kg force exerted; +/- SEM) is presented.
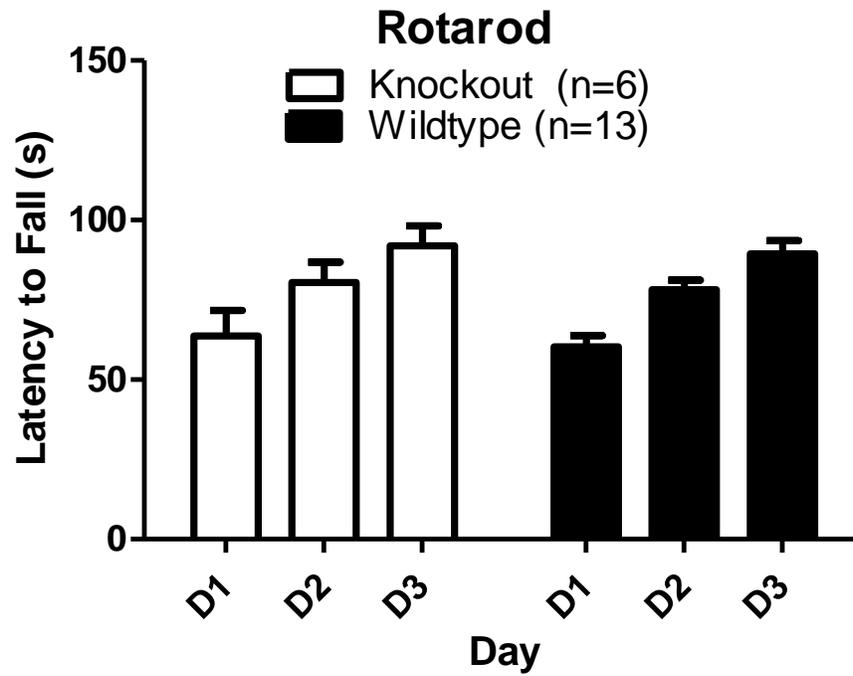
Figure 17: Latency to fall in aged VMAT1 knockout and C57BL/6 wildtype mice in the rotarod assay. Mean latency to fall from the rotarod (in seconds; +/- SEM) is presented. Days of training are presented across the x-axis.

There was a significant increase in latency to fall for aged VMAT1 knockout and wildtype mice in the rotarod task ($F_{(2,34)} = 43.12$, $p < .001$) demonstrating that the animals improved their motor coordination and balance as they had more practice at the task. However, there was no difference between aged genotypes ($F_{(2,34)} = 43.12$, $p < .001$), and the interaction between genotype and days was not significant ($F_{(2,34)} = 0.02$, $p = 0.983$).

Preliminary Studies - Sex Differences

Preliminary Study: Testing Amphetamine in Locomotor Activity. In a study by Wang et al. (1997), it was reported that homozygote VMAT1 knockout mice die within a few days after birth, thus rendering it impossible to study the behavioral consequences of completely removing the VMAT2 gene and VMAT2 transporters. Therefore, they studied VMAT2 heterozygous (+/-) mice to ascertain the role of VMAT2 in the maintenance of presynaptic monoamine functions. One interesting finding from this study was that the VMAT2 (+/-) mice displayed supersensitivity to the psychostimulants cocaine and amphetamine. In order to determine if removal of VMAT1 produced similar effects, a preliminary study was conducted testing the effects of 0.5 mg/kg amphetamine (the same dose used by Wang et al. (1997)) in both male and female young VMAT1 mice.

Young Male 0.5 mg/kg Amphetamine Testing (Figure 18A). Prior to injections male mice showed a significant decrease in distance traveled over the 60 minute period ($F_{(11,264)} = 47.49$, $p < .001$), demonstrating that all animals habituated normally to the activity chamber. There were no significant differences for genotype ($F_{(1,24)} = 0.60$, $p > 0.05$) or for treatment groups ($F_{(1,24)} = 1.47$, $p > 0.05$), and the interaction between genotype and treatment groups also was not significant ($F_{(1,24)} = 0.735$, $p > 0.05$).

Following the injection of either 0.5 mg/kg (s.c.) amphetamine or saline, distance traveled again showed a significant decrease across the 60 minute test session ($F(11,264) = 26.32$, $p < 0.001$). While the main effects for treatment ($F(1,24) = 2.95$, $p > 0.05$) and for genotype ($F(1,24) = 2.71$, $p > 0.05$) were not significant, the interaction between genotype and treatment was significant ($F(1,24) = 4.75$, $p < 0.05$). The Newman Keuls post hoc test revealed a significant increase in locomotor activity for the VMAT1 knockout mice across the entire 60 minute session as compared to the other treatment groups ($p < 0.05$).

Young Female 0.5 mg/kg Amphetamine Testing (Figure 18B). As seen in the male mice, the female mice showed a habituation effect via a significant decrease in distance traveled in the 60 minute period prior to amphetamine or vehicle injection ($F(11,264) = 69.93$, $p < 0.001$). Again, there were no significant differences for genotype ($F(1,24) = 0.00$, $p > 0.05$) or for treatment groups ($F(1,24) = 0.119$, $p > 0.05$), and the interaction between genotype and treatment group was not significant ($F(1,24) = 0.16$, $p > 0.05$).

In contrast to the male mice, there were no significant effects of amphetamine on locomotor activity in the female mice. The main effects for genotype ($F(1,24) = 1.54$, $p > 0.05$) and for treatment groups ($F(1,24) = .61$, $p > 0.05$), and the interaction between genotype and treatment groups ($F(1,24) = .40$, $p > 0.05$) were not significant. There still was a significant decrease in distance traveled following the amphetamine injection ($F(11,264) = 29.76$, $p < .001$).

Preliminary Study – Corticosterone Metabolites in wildtype and VMAT1 Knockout Mice. Measurement of corticosterone metabolites in mouse feces avoids the stress of collecting blood samples and provides a good index of plasma corticosterone levels 12 hours prior to obtaining the fecal sample (Touma, Palme, & Sachser 2004). In this study fecal samples were collected in both young and aged wildtype and VMAT1 knockout mice following 24 h of food

deprivation. Each mouse was placed in a clean empty cage for 1-5 min for collection of 1- 5

fecal boli. Fecal samples were weighed and suspended in a volume of 18% ethanol equaling 10

x the sample weight and shaken overnight. Samples were centrifuged for 15 minutes at 200 x G,

and 0.1 ml of each supernatant was diluted with assay buffer for measurement of corticosterone

metabolite concentrations with a competitive corticosterone ELISA purchased from Enzo Life

Sciences (Plymouth Meeting, PA),

Figure 19A and 19B indicate cortisosterone levels were higher in female than male mice

($p < 0.05$), which is expected in C57/BL6 mice (Verney *et al.* 2002). Furthermore, young

VMAT1 knockout female mice had significantly higher fecal corticosterone levels than young

wildtype females. In aged mice, this difference in corticosterone levels between wildtype and

VMAT1 knockout mice disappeared (Figure 19B). No significant difference between VMAT1

knockout and wildtype corticosterone concentrations was seen in male mice, at either age (Figure

19 A and B).

Figure 20A and 20B show mean body weight of young and aged mice during the

corticosterone collection assay. In young mice only wiltype males had significantly higher body

weight than females. While in aged animals both knockout males and wildtype males each had

significantly higher body weights than wildtype and knockout female mice.
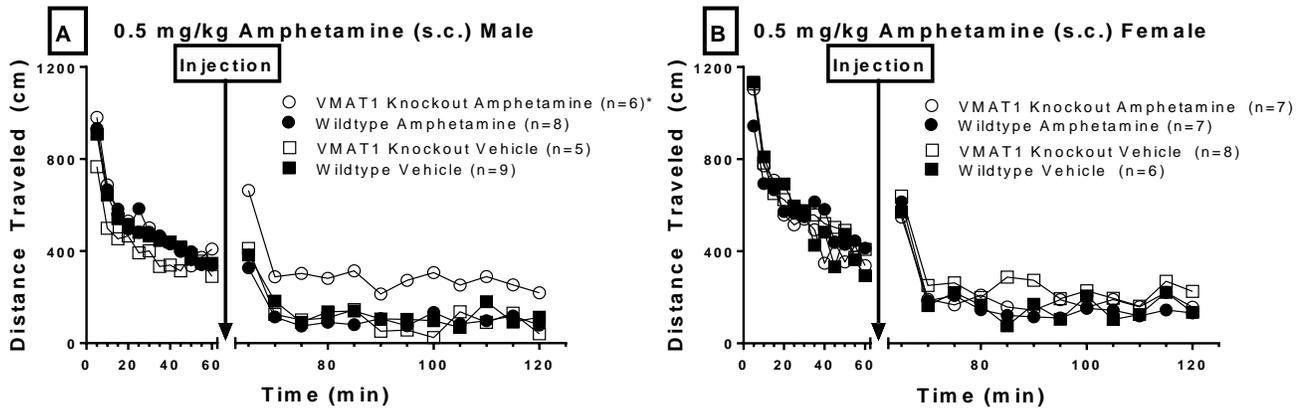
Figure 18: Effects of Amphetamine on Locomotor Activity for both Male and Female Young VMAT1 Knockout and C57BL/6 Wildtype Mice

Results for young male mice are shown in panel A and for young female mice in panel B. The locomotor activity data are plotted in 5 minute bins along the x-axis. Distance traveled (cm) was recorded for 60 minutes prior to the injection of 0.5 mg/kg amphetamine (s.c.). Following a 10 minute delay, the mice were returned to the Locomotor Activity chambers for an additional 60 minutes.

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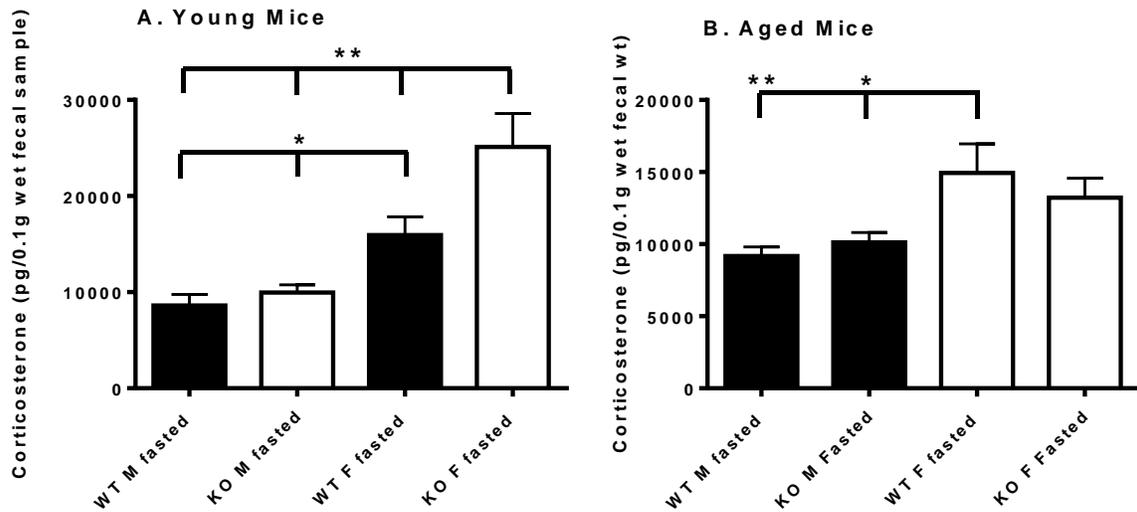
Figure 19. *Concentrations of fecal corticosterone metabolites in VMAT1 knockout and wildtype C57BL/6 mice.* **A**. Fecal corticosterone in fasted young wildtype (WT) and VMAT1 knockout (KO) mice (age 4-5 months). **P<0.001 comparing WT and KO females and *P<0.05 comparing KO female and either KO or WT mice by one-way ANOVA and Turkey's multiple comparison test. **B.** Fecal corticosterone in fasted older mice (age 12-15 months). *P<0.05 comparing WT male and WT female mice by one way ANOVA and Turkey's multiple comparison test.
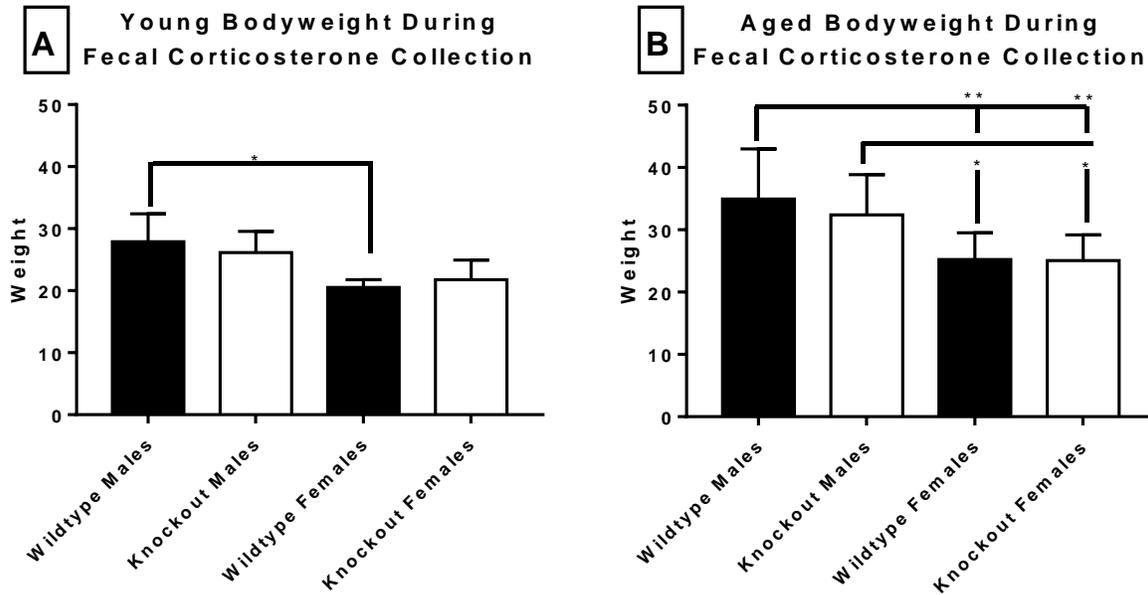
Figure 20. *Bodyweight of young and aged VMAT1 knockout mice and C57BL/6 wildtype controls during corticosterone fecal boli collection assay.* **A**. Body weight in fasted young wildtype (WT) and VMAT1 knockout (KO) mice (age 4-5 months). *P<0.05 comparing WT male and WT female mice by one way ANOVA and Tukey's multiple comparison test. **B.** Body weight in fasted aged mice (age 12-15 months). **P<0.01 comparing WT male and WT female mice & KO females, *P<0.05 comparing KO males and WT females & KO females by one way ANOVA and Turkey's multiple comparison test.

Discussion

The current study represents the first extensive phenotyping of both young and aged mice in which the VMAT1 gene (SLC18A1) has been deleted. The results demonstrated behavioral effects of deleting the VMAT1 gene that may relate to aspects of schizophrenic-like behavioral changes in this model. Specifically, young VMAT1 knockout mice displayed significant deficits in sensorimotor gating in the prepulse inhibition (PPI) task and in the acquisition of operant learning in the autoshaping task. When exposed to a mild stressor (24 hours of food deprivation), young VMAT1 knockout mice displayed a significant reduction in locomotor activity that was not evident under free-feeding conditions. Thus, young VMAT1 knockout mice showed deficits in tasks that model positive symptoms and cognitive deficits seen in schizophrenia; however, they did not display differences in behaviors related to models of the negative symptoms of schizophrenia or deficits in tasks designed to measure motor skills. While less extensive phenotyping was conducted in aged VMAT1 knockout mice, there were no significant deficits evident in any of the assays conducted in older animals. In the sections that follow, the results for each of the assays are discussed, and comparisons between young and aged VMAT1 knockout mice are made when possible.

Sensorimotor Gating (PPI).

Young VMAT1 knockout mice displayed a significant deficit in sensorimotor gating in the prepulse inhibition task (PPI) at all prepulse decibel levels (see Figure 3). In contrast to the wildtype mice, the VMAT1 knockout mice also failed to show an increase in inhibition of the startle response as the decibel level of the prepulse stimulus was increased. This deficit in sensorimotor gating translates well to the deficits that are evident in schizophrenic patients, as deficits in prepulse inhibition are one of the most robust findings in both human schizophrenics

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and animal models of the disorder (Geyer & Ellenbroek 2003; Braff, Geyer, & Swerdlow 2001).

Sensorimotor gating is the ability of an organism to filter out redundant environmental stimuli.

The PPI task uses an unconditioned startle response to a sudden, uncued auditory stimulus. If a

non-startling auditory stimulus is presented shortly before the startling auditory stimulus,

organisms with properly functioning nervous systems are able to inhibit their startle response to

the stimuli, which is reported as a percent reduction of startle response. This is the prepulse

inhibition. Deficits in prepulse inhibition are considered an endophenotype of schizophrenia and

is thought to be a method of modeling the disorganized thoughts and inability to attend to salient

stimuli, a potential neurological correlate to auditory hallucinations in humans. Human

schizophrenics reliably show deficits in prepulse inhibition as compared to healthy controls

(Braff, Geyer, & Swerdlow 2001, Brauer, Strobel, Hensch, Diers, Lesch, & Brocke 2009).

Additionally this effect has been replicated in established models of schizophrenic-like behavior

in animals including post-weaning social isolation (Weiss & Feldon 2001), post-natal

phencyclidine in rats (Gaskins, Alexander, & Fone 2014), and in the *disrupted in schizophrenia-*

1 (DISC1) gene knockout model missense L100P mutation model (Clapcote et al 2007). Thus,

the present findings of a sensorimotor deficit in VMAT1 knockout mice suggest that this gene

deletion may produce a schizophrenia-like behavioral deficit. What remains to be determined is

whether this deficit can be reversed by administration of antipsychotic drugs.

 Autoshaping of the Lever Press Response.

Young VMAT1 knockout mice showed a significant deficit in the acquisition of

reinforced lever presses in the autoshaping task as compared to wildtype mice (see Figure 4).

Although the rates of reinforced lever pressing for the VMAT1 knockout mice eventually

reached wildtype-like responding levels, their inability to differentiate periods when reward was

available suggests that there was a learning deficit in mice without the VMAT1 gene. This suggests that that the young VMAT1 knockout mice initially had greater difficulty learning the association between environmental cues and the response necessary to deliver a reinforcer. Additionally, the wildtype mice displayed a pattern of responding for total lever press responses that peaked on the fourth day of instrumental only, followed by a gradual decrease in total lever pressing, while reinforced lever pressing continued to increase over the 10 days of testing. This increase in 'efficiency' suggests that wildtype mice not only learned the association of external stimuli and reinforcement availability, but also were able to inhibit responding when the stimuli that signaled the availability of reinforcement were not present. Although total lever press responding in VMAT1 knockout mice was similar to wildtype levels of responding near the end of training, the lack of a decrease in total responses may also be indicative of a learning deficit in the acquisition of operant learning.

Serotonin has been shown to play an important role in associative short term and long term memory in invertebrate species (Byrne and Kandel 1996, Cohen et al 2003), as well as in vertebrate mammals (Menes 1999; Buhot et al 2000). Research has shown that learning associated with the autoshaping task causes changes in expression of serotonin (5-HT) receptors and in radioligand binding of serotonin receptors. Rats that underwent Pavlovian autoshaping showed higher levels of ^{3}H-8-OH-DPAT labeled 5-HT$_{1A}$ receptor binding in the prefrontal cortex, septum, and caudate putamen compared to receptor binding in rats that had random, non-paired presentation of conditioned stimulus lever presentations and food reward. The 5-HT$_{1A}$ binding in the dorsal raphe nucleus was *lower* than in the random presentation group, but there was no difference in 5-HT$_{1A}$ receptor binding in other pairing groups. Binding of presynaptic 5-HT$_{2A}$ receptors as indicated by ^{125}I-LSD labeling was found to be higher in three separate areas

of the prefrontal cortex, nucleus accumbens, and caudate putamen (Tomie et al 2003). An

analysis of $5HT_{1A}$ receptors in whole rat brain, showed increases in receptor expression in a

number of brain areas for animals that underwent autoshaping as compared to untrained controls;

however, there was a down regulation (i.e. a decrease) of the $5\text{-}HT_{1A}$ receptors that are thought

to be associated with learning in autoshaping trained animals (Luna-Munguia et al. 2005). The

idea that learning, especially in autoshaping tasks, is related to serotonin activity lends itself well

to the present results, as VMAT1 has been shown to have a higher affinity for serotonin

compared to VMAT2 (Brunk et al, 2006), which is more prevalent in the central nervous system

(Erickson, Eiden, & Hoffman, 1992; Erickson, Schafer, Bonner, Eiden, & Weihe 96). The deficit

seen in acquisition of an operant response by the young VMAT1 knockout mice in the present

study could be related to deficient vesicular transport, which in turn could lead to deficits in

release and usage of serotonin and/or other monoamines in specific brain areas.

Aged VMAT1 knockout mice also were tested in the autoshaping task, but because a

large number of both VMAT1 knockout and wildtype failed to reliably acquire the lever press

response (see Figure 12), comparisons are limited. When only the aged mice that actually

acquired the lever press response were compared (see Figure 12), there appeared to be a learning

deficit in the VMAT1 knockout mice. However, because of the small sample sizes and the very

large variability in responding, this difference was not significant. Additionally C57BL/6, the

background stain used for this knockout, has been known to develop hearing loss much faster

than other mouse strains (Willot 1986), suggesting that the lack of learning may be due, in part,

to the lack of salience through auditory stimulation. Another factor which may play a role in the

deficits in learning seen in aged autoshaping may be that aged mice tended to have a higher basal

body weight than young mice. In essence, fatter animals are less motivated to work for food.

Locomotor Activity

Young VMAT1 knockout mice showed a significant decrease in locomotor activity compared to wildtype mice, when placed under the mild stressor of 24 hour food restriction. When comparing only VMAT1 knockout mice, this significant decrease also was evident. However, there were no significant changes in locomotor activity in the wildtype mice between free-feeding and food-restriction conditions. This suggests that the mild stressor of food restriction was sufficient to cause significant behavioral changes in the VMAT1 knockout mice. These findings may be related to deficient storage and release of adrenal catecholamines in VMAT1 deficient mice. VMAT1 has been shown to have a higher level of expression than VMAT2 in adrenals of adult humans and rodents (Erickson *et al.*1992; Liu *et al.* 1992; Erickson *et al.* 1996). VMAT1 deficiency reduces storage and release of adrenal epinephrine and norepinephrine, which are important in hormonal responses to fasting. When food is not available, these catecholamines promote rapid metabolism of liver glycogen and elevation of plasma glucose. Reduction of the adrenal response permits a more severe hypoglycemia, which stimulates release of other hormones that elevate blood glucose, such as pancreatic glucagon and adrenal corticosterone (review by Goldstein, 2010). Indeed, preliminary results obtained in collaboration with Y. Geng. and K. Stenger (University of Richmond) and J. Stewart (Biology, VCU) showed that fecal corticosterone metabolites were higher in young female VMAT1 knockout mice than in female wild type controls (see Preliminary Results, figure 19), but there were no significant differences in corticosterone in male wild type and knockout animals. Although it is known that C57BL/6 female mice have higher plasma corticosterone and corticosterone metabolites than C57BL/6 males (Verney *et al.* 2002), the exaggerated corticosterone responses in the young female VMAT1 knockout mice may be related to their

lower body weights (lower stored nutrients) making them more susceptible to hypoglycemia. This hypothesis is supported by failure to observe differences in corticosterone in knockout and control females that were older and heavier. Although it is well known that corticosteroids influence depression, anxiety, and cognition, (Derijk & de Kloet, 2005; Fernandez-Guasti et al, 2012), there were no obvious sex differences in the behavioral effects of VMAT1 deletion in the present study, despite differences in corticosterone. Additional research with larger sample sizes are needed to better clarify whether there are differential effects of VMAT1 deletion in male and female animals.

Spatial Learning in the Morris Water Maze

Although VMAT1 knockout mice showed a significant deficit in acquisition of the autoshaping task, there was no differences in spatial learning observed in either the free-feeding condition (both young and aged mice) or the food-restricted condition (young mice only) in the Morris water maze. The VMAT1 knockout and wildtype mice displayed significant levels of learning as the latency to platform decreased over the 5 days of training (see Figures 7 and 14). The differences between the reinforcers in the Morris water maze (i.e. escape from the water; negative reinforcement) and the autoshaping task (i.e. lever pressing to earn food pellets; positive reinforcement) may explain the differences in learning between these two tasks. Water maze tasks use negative reinforcement (i.e. escape from the water to a hidden platform), where the goal behavior is to escape an aversive stimulus, which may cause a different motivational 'state', as opposed to seeking an appetitive reward (i.e. a food pellet). The salience of the aversive stimulus in the Morris water maze may have overridden any deficits in learning that VMAT1 knockout mice expressed in the autoshaping task.

Differences between brain areas involved in spatial learning and operant learning have been suggested, providing another possible explanation for the discordant results between the autoshaping task and the Morris water maze. The hippocampus has been shown to be activated in association with spatial relationships, such as the layout of symbols in relation to a hidden platform, while amygdala activation is important for forming and processing associations between discreet stimuli (McDonald and White, 93, Squire et al 93, Aggleton, 2000). This interpretation is consistent with observations of Multani et al (Multani et al 2013) who reported deficits in spatial object recognition in VMAT1 knockout mice but no effect of the gene deletion on contextual fear conditioning. Multani suggests that the deficit in spatial memory is driven by increase apoptosis in the dente gyrus and decreased cell proliferation in the hippocampus. Cell proliferation in the hippocampus has been implicated to be important for adult memory task performance (Breunig et al 2007), specifically spatial memory tasks (Madsen et al 2003). Multani et al suggested that differences in their results reflect differences in spatial discrimination memory and associative memory. Nevertheless, it is worth noting that both the contextual fear protocol (in the Multani study) and the Morris water maze (in the present study) involve aversive stimuli, which may override learning deficits that would be evident in procedures that do not involve aversive stimuli.

Progressive Ratio and Forced Swim Tasks

The progressive ratio task is used to assess changes in motivation (Uematsu et al 2011) and has been used as a measure of a centrally motivated-motivated state specifically differentiating it from 'sickness' effects, which may decrease eating behavior (Merali, Brennan, Brau & Anisman 2003). Since the number of responses increases with each fixed reward delivery, progressive ratio can be used to measure motivation or lack thereof resembeling an

anhedonic phenotype (Hodos 1961), which is a symptom often seen in both schizophrenic and depressed patients (Carpenter, Heinrichs, & Wagman 1988; van Praag, Uleman, & Spitz 1965). No significant differences in the progressive ratio break point were found between the VMAT1 knock out mice (both young and aged) and the wildtype mice (see Figures 8 and 15). While this finding may seem to be at odds with the results found in the autoshaping task, these two tasks are measuring very different things. First, the autoshaping task really represents a measure of *learning*, i.e. the acquisition of an operant response. In the progressive ratio task, the mice have already learned the operant response, and the work requirement is gradually increased (i.e. the progressive ratio) during the test session until the animal stops lever pressing in order to obtain the food reward. Thus, the progressive ratio task presents a *performance* task. While these results suggest that differences in motivation are not affected by VMAT1 deletion, they do strengthen the claim that the differences between genotypes seen in the autoshaping task were due to differences in the VMAT1 knockout animals' ability to learn - not motivational differences. VMAT1 knockout mice were just as motivated to perform the lever pressing task for a food reinforcer as the wildtype mice were and had similar levels of responding by the end of the 10 days of training. The VMAT1 knockout and wildtype mice also displayed similar decreases in responding during the extinction test conditions of the autoshaping task.

The forced swim task is used both to screen drugs with antidepressant efficacy and also to measure a "depressive-like" state in the animal (Porsolt, Bertin & Jalfre 1977). In the present study, it was used to determine whether or not the VMAT1 knockout mice displayed a "depressive-like" state, as assessed by the forced swim task. No significant differences were found between the young VMAT1 knockout mice and the wildtype mice (see Figure 9),

indicating no evidence of any depressive-like behavior as assessed by this task. Aged VMAT1 knockout mice were not tested in the forced swim task.

Motor Tasks (Grip Strength and Rotarod)

There were no significant differences in forelimb grip strength between VMAT1 knockout and wildtype mice (see Figures 10 and 16). This finding suggests that muscle development was not affected by deletion of the VMAT1 gene. Both young and aged VMAT1 knockout and wildtype displayed similar levels of learning in the rotarod task over the 3 days of testing (see Figures 11 and 17). The significant increase in latency to fall demonstrated that the mice were able to learn this task that required motor coordination to remain on the rotating rod. The results from this task and the grip strength task argue convincingly that deletion of the VMAT1 gene had no effects on motor coordination or on grip strength. Thus, the delayed acquisition of the lever press response in the autoshaping task and the decreased locomotor activity evident under food restriction cannot be attributed to any deficits in motor development.

Preliminary Study – Effects of Amphetamine on Locomotor Activity

The dopamine hypothesis of schizophrenia suggests that there is increased activity of dopamine in the brain (at least in certain areas) (Meltzer & Stahl 1976). Also, to date, there have been no antipsychotic drugs marketed that do not have pharmacological activity at dopamine receptors (mostly antagonism). Thus, monoaminergic systems and the dopamine system in particular, play an important role on the etiology and/or the expression of some symptoms associated with schizophrenia. Hyperactivity to dopamine agonism by drugs like amphetamine has been used both to study antipsychotic drugs and as a phenotype of schizophrenia (Geyer & Ellenbroek 2003). While VMAT2 is more prevalent in brain than VMAT1, deletion of the VMAT2 gene is not a viable phenotype. Wang et al. (1997) studied the effects of the

psychostimulants cocaine and amphetamine in heterozygous (+/-) VMAT2 mice and found that they displayed supersensitivity to dopamine agonism. In the preliminary study, the effects of 0.5 mg/kg amphetamine (the same dose used by Wang et al. (1997)) were tested in both male and female young VMAT1 knockout mice. As can be seen in Figure18, the male VMAT1 knockout mice displayed a significantly greater increase in locomotor activity than wildtype mice following amphetamine, and the response persisted over the 60 minute test session. Interestingly, the female VMAT1 knockout mice were not different from the wildtype mice in their response to amphetamine. These findings replicate those seen in heterozygous VMAT2 knockouts (Wang et al. 1997) and suggest that deletion of the VMAT1 gene may represent a schizophrenic phenotype in male mice. While it is not clear as to why the female VMAT1 knockout mice did not show a response similar to that of male VMAT1 knockout mice, these results point out the importance of examining possible sex differences (and similarities) in future

studies with VMAT1 knockout mice. Finally, it would be important in future studies to determine if this dopamine supersensitivity can be reversed by antipsychotic drugs.

Preliminary Study – Corticosterone Metabolites in wildtype and VMAT1 Knockout Mice

The high corticosterone levels in young VMAT1 KO female mice are interesting because schizophrenia is known to manifest in young adulthood (Pulver *et al.,* 1990), and in the two largest genetic linkage studies of VMAT1 SNPs with behavioral disorders, the associations were stronger in females (Chen et al., 2007;Richards et al., 2006). In addition, VMAT1 polymorphisms have been linked to bipolar disorder (Lohoff et al 2006) and anxiety-related traits in females (Lohoff et al 2008)

Although we have suggested that increased nutrient reserves in animals with higher body weights may explain lower corticosterone responses to fasting, it is conceivable that VMAT2

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expression is greater in the adrenal of animals with lower corticosterone. Tillinger et al. (2010) demonstrated that after rats are subjected to immobilization stress, the adrenal VMAT1 mRNA levels remain constant while adrenal VMAT2 mRNA levels are elevated (Tillinger et al. 2010). While Multani and colleagues observed no significant differences in VMAT2 expression in young VMAT1 knockout and wildtype mice, they did not examine potential sex or age effects on VMAT2 expression (Multani et al 2013). Further investigation is needed to address this issue.

Conclusions

Results from the present study demonstrated that deletion of the VMAT1 gene has behavioral effects that are mediated by changes in brain monoamine function and changes in response to stressors (i.e. food deprivation) that may reflect changes in adrenal gland monoamine function. Specially, VMAT1 knockout mice displayed a significant deficit in sensorimotor gating as measured in the prepulse inhibition task. The knockout mice also displayed a significant impairment in the acquisition of an operant response in the autoshaping task, although it should be noted that they did learn the lever press response and reached performance levels similar to those in wildtype mice. In response to a mild stressor of 24 hours of food deprivation there were significant reductions in locomotor activity in the VMAT1 knockout mice that were most likely mediated by deletion of VMAT1 in the adrenal glands and inability to store and release adrenal catecholamines in response to hypoglycemia. A preliminary study examining the effects of the dopamine agonist amphetamine on locomotor activity revealed supersensitivity in male VMAT1 knockout mice, but not in female VMAT1 knockout mice. These changes in behavioral tasks cannot be attributed to deficits in motor ability, as no motor deficits were evident between VMAT1 knockout and wildtype mice. Additionally, a second preliminary study demonstrated

significantly higher corticosterone levels in young female VMAT1 knockout mice compared to wildtype females. This difference was not found in young male mice or in aged mice.

Whereas the differences between VMAT1 knockout and wildtype mice were limited to specific behavioral deficits, these results support the suggestion that mutation of the VMAT1 gene contributes to the etiology of schizophrenia and bipolar disorder (Lohoff et al 2006; Lohoff et al 2008). Given the large number of genes that have been implicated in the etiology of schizophrenia and bipolar disorder (Purcel et al. 2009; Holzman & Matthysse, 1990; Gonzalez-Mantilla, Moreno-De-Luca, Ledbetter, & Martin 2016; Wilson, Flibotte, Chopra, Melnyk, Honer, & Holt 2005; Caddock & Jones 1999), the impact of a single gene deletion would be expected to be limited to specific aspects of the disorder. There is no single gene that is solely responsible for the development and/or predisposition of schizophrenia and bipolar disorder. Additionally, the development of schizophrenia in humans may require a genetic predisposition followed by some type of an environmental impact (Ellenbroek 2003). Thus, a stressful life event, be it an early life illness (prenatal or postnatal), or later during adolescence, appears to be important in the development of schizophrenia.

Future Studies

Further investigation of the sex differences between genotypes would be a fruitful expansion of this project. As mentioned earlier the human polymorphisms of VMAT1 associated with psychosis are sex dependent (Richards et al 2006, Chen et al 2007). Differences in prevalence, symptomology, prognosis, and psychopharmacology exist between sexes (Saha et al 2005; Aleman et al 2003; Leung et al 2000; Syzmanski et al 1995). Additionally both preliminary sex-separated studies presented in this body of work show important differences between sexes. This suggests that there may be differences in other tasks used here, or that a

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sexual dimorphism in behavior may be masking the power of differences seen between males and females, and further separation of these data may elucidate previously undiscovered differences between sexes.

Schizophrenia is not a purely genetic disorder, this concept is best exemplified by the Genain-Quintuplet case study (Rosenthal 1963). Furthermore locomotor activity data suggests that the absence of VMAT1 may cause knockout mice to be more susceptible to subtle stressors and differences in behavior. Therefore looking at post-natal and adolescent stressors and how that leads to a schizophrenic phenotype is important for phenotyping VMAT1 in relation to psychiatric disorders. Even examining the effect of established environmental models of psychosis (post-weaning social isolation, post-natal phencyclidine administration, chronic mild stress) could be used to examine knockout mice's sensitivity to these stressors. Genotypes that are MORE sensitive to these stressors may more closely mimic humans who develop schizophrenia, bipolar disorder, and depression.

Although this study found effects which seem to suggest a central nervous system activation it is difficult to delineate whether the effects are from losing low, yet salient, levels of VMAT1 in brain, or if behavioral effects seen are a larger downstream effect of the loss of VMAT1 in the periphery, where it is more widely expressed. In fact the differences seen in locomotor activity in relation to food restriction may be explained by adrenal gland hypoglycemia response more so than deficits in VMAT1 mice. Therefore a conditional knockout removing only brain VMAT1 would help to answer the question of where losing VMAT1 is important to the development of these symptoms.

Finally, although this study conducted more extensive phenotyping than Multani 2013 or any other study there are still a number of tasks that can further identify deficits in VMAT1 mice

that are related to psychosis, but not directly addressed here. First delayed-match-to-sample tasks

and novel object recognition could help to assay any potential deficits in working memory.

Indeed if the deficits seen in autoshaping are centrally active then deficits in working memory

are likely to exist as well. Sucrose preference may give a more direct measure of anhedonic-like-

behavior and unconditioned pleasure from naturally rewarding stimuli. Also, as the comparison

of this project and Multani 2013 suggest differential deficits based on how each task is

reinforced further more operant and behavioral tasks using different motivators and different

types of learning/cognition could help to clear just how these differences are effected by removal

of VMAT1 from the animal genome.

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