UNIVERSITY OF CALIFORNIA, SAN DIEGO

If You Give a Mouse a Mutation:
Mouse Models of Mental Illness

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

in

Neurosciences with a Specialization in Computational Neurosciences

by

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The Dissertation of Kerin Kirita Higa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 1, in full, is a reprint of the material as it appears in Ji B, Higa KK, Kim M, Zhou L, Young JW, Geyer MA, Zhou X. *Inhibition of Protein Translation by the DISC1-Boymaw Fusion Gene from a Scottish Family with Major Psychiatric Disorders*. Human Molecular Genetics. 2014 Nov 1;23(21):5683-705. The dissertation author assisted with animal experimentation and data analyses and provided input during manuscript preparation.

Chapter 2, *Mitochondrial Dysfunction in Humanized DISC1-Boymaw Mice*, is being prepared for submission and will include Drs. Baohu Ji, Xianjin Zhou, and Mark A. Geyer as

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Chapter 4, in full, is a reprint of the material as it appears in Young JW, Kamenski ME, Higa KK, Light GA, Geyer MA, Zhou X. *GlyT-1 inhibition attenuates attentional but not learning or motivation deficits of the Sp4 hypomorphic mouse model relevant to psychiatric disorders*. Neuropsychopharmacology. 2015 Nov;40(12):2715-26. The dissertation author assisted with experimentation and data analyses and gave input during manuscript preparation.

Appendix A, in full, is a reprint of the material as it appears in Higa KK, Young JW, Ji B, Nichols DE, Geyer MA, Zhou X. *Striatal dopamine D1 receptor suppression impairs reward-associative learning.* Behavioural Brain Research. 2017 Apr 14;323:100-110. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

If You Give A Mouse a Mutation:
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Serious mental illnesses are debilitating and costly, and the development of effective treatments is imperative. Unfortunately, their heterogeneous and overlapping etiologies and symptomatologies present major obstacles to our understanding of these disorders. Carefully designed animal models and test paradigms with precise cross-species valid outcomes enable us to investigate the mutations, network disruptions, and environmental challenges that

contribute to the development of neuropsychiatric symptoms and may be targeted for treatment. This dissertation presents two mouse models developed to study candidate risk genes associated with neuropsychiatric illnesses, including major depression, bipolar disorder, and schizophrenia. The studies presented herein provide insight into the development and potential treatments for these disorders.

Studies of humanized DISC1-Boymaw mice focus on the effects of a rare translocation associated with psychiatric illnesses in a large Scottish family. Our studies suggest that inhibition of protein synthesis and mitochondrial dysfunction resulting from the fusion of the *DISC1* and *Boymaw* genes may contribute to the pathogenesis of illnesses in this family. The DISC1-Boymaw mice exhibit hypersensitivity to ketamine, a phenotype also observed in patients and in the other rare variant model presented here, the Sp4 hypomorphic (Hyp) mice, which have reduced expression of the transcription factor Sp4. Our investigation of Sp4 Hyp mice suggests that hypersensitivity to ketamine is mediated by inhibitory GABAergic neurons and not excitatory forebrain neurons.

Dysfunction of GABAergic neurons may be associated with cognitive and motivational deficits observed in the Sp4 Hyp mice and in patients. The cognitive deficits associated with psychiatric illnesses are predictive of vocational and social outcomes and should be prioritized in the development of treatments. Our studies suggest that a glycine type-1 transporter (GlyT-1) inhibitor may remediate attention deficits—but not impairments to motivation nor reward learning—in these mice and potentially in patients. It is the author's hope that the findings presented in this dissertation and follow-up studies will contribute to the understanding and development of treatments for neuropsychiatric disorders.

INTRODUCTION

According to the 2016 National Survey on Drug Use and Health, about 1 in 5 adults in America experienced a mental, behavioral, or emotional disorder that met DSM-IV criteria in the past year. Approximately 1 in 25 adults experienced a neuropsychiatric illness that substantially interfered with a major life activity, such as going to school or work (Substance Abuse and Mental Health Services Administration 2017). Serious mental illnesses are among the leading causes of disabilities worldwide (Whiteford et al. 2013) and were estimated to cost $2.5 trillion globally in 2010, primarily due to productivity and income losses, accounting for more economic costs than chronic somatic diseases such as diabetes and cancer (Ota et al. 2014). The economic burden of these illnesses is expected to double by 2030 (Trautmann et al. 2016). Thus, it is imperative that treatments are developed to combat these disorders.

Fortunately, the heritability of neuropsychiatric illnesses—though not neatly Mendelian—is evident. For example, having a parent with the disease increases the chances of developing schizophrenia (~1% in the general population) six times and having a monozygotic twin with the disease increases the chances almost 50-fold (Tsuang 2000; Gottesman and Erlenmeyer-Kimling 2001). With modern genetics techniques, such as genome wide association studies (GWASs), researchers can identify candidate mutations and disrupted networks that might contribute to the development of specific mental illnesses. These genetic mutations and disrupted neural circuitry, as well as environmental stresses, can be modeled in animals, and well-defined biological and behavioral outcomes related to those in patients can be measured, enabling the study of the etiologies and potential treatments of neuropsychiatric illnesses (Young et al. 2010; Razafsha et al. 2013).

Unfortunately, the categorization, or nosology, of psychiatric illnesses is currently undergoing somewhat of a crisis (Young et al. 2010; Kapur et al. 2012; Zachar and Kendler 2017). Disorders, such as major depression (MD), bipolar disorder (BD), and schizophrenia (SZ), are heterogeneous and there is significant symptomatic and genetic overlap between conditions (Craddock and Owen 2010; O'Tuathaigh and Waddington 2015). For example, numerous studies have revealed overlap in genetic susceptibility for BD and SZ (Lichtenstein et al. 2009; Moskvina et al. 2009; Owen and Craddock 2009; Purcell et al. 2009), as well as other neurodevelopmental disorders, including autism (Zhang et al. 2009). The desire to distinguish these conditions, however, may obscure symptomatic overlap. For instance, the negative symptoms of SZ may be characterized as anhedonia in depressive patients or social withdrawal in patients with autism (Goldstein et al. 2002). In light of such findings, it has been suggested that neurodevelopmental disorders, ranging from intellectual disability and autism to SZ, BD, and MD, are not distinct but lie on an etiological and symptomatic gradient (Craddock and Owen 2010).

The problem of nosology, naturally, extends into the indispensable study of animal models of psychiatric illnesses. Given the heterogeneity and polygenic basis of these disorders, a single genetic mutation will not produce a model of any disease in its entirety. The goal of modeling schizophrenia in its entirety, for example, has been repeatedly dismissed (Geyer 2008; Young et al. 2010; Razafsha et al. 2013; Wong and Josselyn 2016). Instead, focus has been placed on the systematic studies of endophenotypes of each disorder. Endophenotypes are intermediate, heritable traits—neurophysiological, biochemical, neuroanatomical, or cognitive, for example—found at higher rates in mutation carriers, regardless of diagnosis, that may contribute to the development of symptoms (Kendler and

Neale 2010). The work included in this dissertation aims to use genetic and pharmacological tools in cell culture and in mouse models to identify and probe endophenotypes that may contribute to the symptomatology of neuropsychiatric illnesses. Through this work, we hope to better understand the molecular and cellular mechanisms that underlie the behavioral symptoms of and guide potential treatments for psychiatric disorders, including SZ, BD, and MD.

Humanized DISC1-Boymaw

Studies in Chapters 1 and 2 of this dissertation examine the *DISC1* mutation, a so-called rare variant that contributes to psychiatric illness susceptibility (Owen 2012). Rather than focusing on common mutations with small effects on disease susceptibility (common-disease/common variant), the common-disease/rare-variant approach focuses on rare point mutations that have large effects on susceptibility (O'Tuathaigh and Waddington 2015). The *DISC1* mutation was discovered in a large Scottish family in which more than 70% of carriers presented with a psychiatric disorder, including SZ, BD, and MD (Millar et al. 2000b; Blackwood et al. 2001). The heterogeneity of diagnoses within the family is in line with the notion that several disorders, as defined in the DSM, may share a common biological basis (Korth 2009). The mutation is a balanced translocation, t(1;11)(q42.1;q14.3), that disrupts the disrupted-in-schizophrenia 1 (*DISC1*) gene on chromosome 1 (Millar et al. 2000a). The *DISC1* gene has been studied extensively and found to play critical roles in neuronal cell proliferation, differentiation, migration, maturation, and synaptic functions (Kamiya et al. 2005; Ishizuka et al. 2006; Duan et al. 2007; Kvajo et al. 2008; Mao et al. 2009; Wang et al.

2011). Although the role of the *DISC1* gene in the pathogenesis of psychiatric illnesses remains unclear and its applicability to the general population has been challenged (Kuroda et al. 2011; Duff et al. 2013; Thomson et al. 2013; Sullivan 2013; Porteous et al. 2014), the mutation has provided a useful entry point to understanding the etiology of various neuropsychological illnesses and may be the most widely studied candidate susceptibility gene (Brandon and Sawa 2011).

Our studies, however, found that the translocation also disrupts a gene on chromosome 11, called *Boymaw* or the *DISC1 fusion protein 1* (*DISC1FP1*) (Zhou et al. 2008). After translocation, two fusion genes, *DISC1-Boymaw* (*DB7*) and *Boymaw-DISC1* (*BD13*) may be generated (Zhou et al. 2010a; Eykelenboom et al. 2012). Chapter 1 explores the effects of these fusion genes *in vitro* and *in vivo* using humanized DISC1-Boymaw mice carrying both human fusion genes. Briefly, we found that the *DB7* fusion gene inhibits intracellular NADH oxidoreductase activities, rRNA synthesis, and protein translation, and the humanized mice display phenotypes associated with SZ and MD. Chapter 2 investigates the hypothesis that the DB7 fusion protein disrupts mitochondrial functioning, potentially contributing to the development of symptoms associated with psychiatric disorders.

Interestingly, our studies of the *DISC1-Boymaw* translocation led us to study the effects of the isolated *Boymaw* gene, the function of which remains unknown. We found that the Boymaw protein alone (not fused to DISC1) localizes to mitochondria and inhibits metabolic activity similarly to the DB7 fusion protein. This led us to investigate the presence of Boymaw in patient samples. Indeed, in postmortem brain tissue, we found that *Boymaw* RNA expression is significantly increased in patients with major psychiatric disorders

compared to healthy controls (Ji et al. 2015). This discovery suggests that overexpression of *Boymaw* may contribute to mitochondrial dysfunction in patients with psychiatric disorders, within and beyond the original Scottish DISC1 family.

Sp4 Hypomorphic Mice

Chapters 3 and 4 explore mutations to the *Sp4* gene that, not unlike *Boymaw* overexpression, was first studied in a mouse model and found to be present in patients with neuropsychiatric illnesses. Originally, mice lacking the *Sp4* gene were studied as a model of cardiac arrhythmia and sudden death but they were found to have behavioral abnormalities (Nguyên-Trân et al. 2000; Zhou et al. 2005). This observation led to the study of the role of Sp4, a transcription factor, in the central nervous system. In order to study the effects of reduced, rather than completely absent, *Sp4* expression, the Sp4 hypomorphic (Hyp) mice were generated. We found that Sp4 Hyp mice displayed several behavioral phenotypes relevant to psychiatric disorders, including deficits in prepulse inhibition and hypersensitivity to ketamine (Zhou et al. 2005, 2007, 2010b).

Our studies of Sp4 Hyp mice led to the discovery of several SNPs in the *SP4* gene associated with MD, BD, and SZ (Zhou et al. 2009, 2010b; Tam et al. 2010; Pinacho et al. 2011; Shi et al. 2011; Greenwood et al. 2013). The *SP4* gene has been found to be spontaneously deleted in patients with SZ (Tam et al. 2010; Zhou et al. 2010b), SP4 protein levels are reduced in peripheral blood mononuclear cells in first-episode SZ patients (Fusté et al. 2013), and the expression of the SP4 protein is reduced in postmortem brain tissue of BD

patients (Pinacho et al. 2011). These studies support the Sp4 Hyp mice as a mouse model of psychiatric illnesses.

Chapter 3 investigates a particular SZ-related endophenotype, hypersensitivity to ketamine, which we also observed in the DISC1-Boymaw mice in Chapter 1. Administration of non-competitive N-methyl-D-aspartate receptor (NMDAR) antagonists, such as ketamine, induces behaviors that resemble aspects of psychiatric disorders in healthy people (Krystal et al. 1994; Xu et al. 2015). Furthermore, prolonged effects and the exacerbation of symptoms have been reported in SZ patients after the administration of ketamine (Lahti et al. 1995; Malhotra et al. 1997; Holcomb et al. 2005). These findings, as well as prefrontal cognitive deficits in patients, have implicated impaired NMDAR neurotransmission in SZ (Lewis et al. 2005; Kantrowitz and Javitt 2010). In fact, decreased *NMDAR1* subunit expression has been reported in postmortem brain tissue of SZ patients (Weickert et al. 2012), and anti-NMDAR encephalitis results in schizophrenia-like symptoms and/or loss of memory (Dalmau et al. 2008; T. Iizuka et al. 2008). Consistent with these findings, expression of NMDAR subunit *Nmdar1* is reduced to 40-50% in the hippocampus and cortex of Sp4 Hyp mice (Zhou et al. 2010b). We wished to investigate the mechanism underlying NMDAR antagonist sensitivity in the Sp4 Hyp mice. Cortical GABAergic inhibitory neurons have been proposed as the primary targets of NMDAR antagonists, so we hypothesized that ketamine sensitivity in the Sp4 Hyp mice is regulated by Sp4 expression in GABAergic neurons.

The construction of the Sp4 Hyp mice allows for the restoration of *Sp4* expression using Cre-Lox recombination (Zhou et al. 2005). Through crosses with specific Cre-expressing mouse lines, we found that restoration of *Sp4* expression in GABAergic inhibitory,

but not forebrain excitatory, neurons reduced hypersensitivity to ketamine in Sp4 Hyp mice. These findings, consistent with the work of other groups (Belforte et al. 2010; Rompala et al. 2013), support the hypothesis that GABAergic neurons dampen locomotor responses to ketamine and that Sp4 is essential for this process. Therefore, *Sp4* mutations may confer abnormal function of GABAergic neurons, providing a pathway that may be targeted in some patients with impaired GABAergic functioning.

Dysfunction of GABAergic neurons may also contribute to the cognitive and negative symptoms of psychiatric disorders, which may be more clinically relevant than hypersensitivity to ketamine—which is associated with the positive symptoms and/or reduced habituation to novel environments in patients with SZ (Perry et al. 2009). Indeed, the negative and cognitive symptoms of SZ are thought to form the core of the disorder (Geyer 2008; Geyer et al. 2012) and are predictive of poor social and vocational outcomes (Green 2006; Bowie et al. 2008; Tsang et al. 2010). Chapter 4 aims to assess the behaviors of Sp4 Hyp mice relevant to the negative cognitive symptoms of SZ using cross-species relevant paradigms (Cuthbert 2014) (Young et al. 2009; Young and Geyer 2015).

We measured cognitive control using the 5-Choice Continuous Performance Test (5C-CPT) (Young et al. 2009, 2013; Lustig et al. 2013), approach motivation using a probabilistic learning task (Bari et al. 2010; Amitai et al. 2014), and effort valuation using the progressive ratio breakpoint paradigm (Bensadoun et al. 2004; Young and Geyer 2010). Patients with SZ exhibit deficits in all three domains in comparable tests designed for humans (Ellenbroek et al. 2000; Gold et al. 2008; Waltz et al. 2011; Armstrong et al. 2012; Ragland et al. 2012; Young et al. 2013; Wolf et al. 2014).

Using other mouse models, author of this dissertation has utilized these paradigms to investigate the contributions of striatal dopamine D1 receptors to motivation and reward- and punishment-associated learning and of α7 nicotinic acetylcholine receptors to nicotine withdrawal-induced inattention. These studies, found in Appendices A and B, respectively, demonstrate how the carefully designed paradigms used in Chapter 4 enable the parsing of precise aspects of cognition (e.g., inattention v. response disinhibition) that can be associated with specific biological impairments (e.g., lack of α7 nicotinic acetylcholine receptors).

In Chapter 4, we found that the Sp4 Hyp mice exhibited impaired attention, reduced motivation to work for a reward, and impaired probabilistic learning, consistent with findings in patients. Based upon findings in patients, we also tested whether the Sp4 Hyp mice are more sensitive to distraction (flashing lights) during the 5C-CPT challenge (Demeter et al. 2013; Lustig et al. 2013). In fact, the Sp4 Hyp mice were impaired similarly to WT mice, perhaps suggesting that the overall attention deficit—and not specifically attentional control during distraction—should be prioritized in the development of treatments.

Given the reduced NMDAR function in the Sp4 Hyp mice, we tested whether an inhibitor of the NMDAR co-agonist glycine transporter type-1 (GlyT-1), which should potentiate NMDAR function, could remediate the deficits observed. Indeed, GlyT-1 inhibition attenuated the attention deficit of the Hyp mice in the 5C-CPT. Interestingly, neither the motivation nor the probabilistic learning deficit was attenuated by GlyT-1 treatment, suggesting that the mechanisms underlying the attentional deficit and these reward-related deficits involved distinct neural networks. Our studies indicate that GlyT-1 inhibition may treat attentional deficits in patients with low Sp4 levels.

All together, the studies collected within this dissertation serve to illustrate the ways in which mouse models can be used to probe the etiology and develop treatments for neuropsychiatric illnesses. Future studies investigating the overlapping endophenotypes between models, the influence of additional genetic and environmental insults, and responses to proposed treatments are warranted.

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Chapter 1:

Inhibition of protein translation by the DISC1-Boymaw fusion gene from a Scottish family with major psychiatric disorders

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Inhibition of protein translation by the DISC1-Boymaw fusion gene from a Scottish family with major psychiatric disorders

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The t(1; 11) translocation appears to be the causal genetic lesion with 70% penetrance for schizophrenia, major depression and other psychiatric disorders in a Scottish family. Molecular studies identified the disruption of the disrupted-in-schizophrenia 1 (DISC1) gene by chromosome translocation at chromosome 1q42. Our previous studies, however, revealed that the translocation also disrupted another gene, Boymaw (also termed DISC1FP1), on chromosome 11. After translocation, two fusion genes [the DISC1-Boymaw (DB7) and the Boymaw-DISC1 (BD13)] are generated between the DISC1 and Boymaw genes. In the present study, we report that expression of the DB7 fusion gene inhibits both intracellular NADH oxidoreductase activities and protein translation. We generated humanized DISC1-Boymaw mice with gene targeting to examine the *in vivo* functions of the fusion genes. Consistent with the *in vitro* studies on the DB7 fusion gene, protein translation activity is decreased in the hippocampus and in cultured primary neurons from the brains of the humanized mice. Expression of Gad67, Nmdar1 and Psd95 proteins are also reduced. The humanized mice display prolonged and increased responses to the NMDA receptor antagonist, ketamine, on various mouse genetic backgrounds. Abnormal information processing of acoustic startle and depressive-like behaviors are also observed. In addition, the humanized mice display abnormal erythropoiesis, which was reported to associate with depression in humans. Expression of the DB7 fusion gene may reduce protein translation to impair brain functions and thereby contribute to the pathogenesis of major psychiatric disorders.

INTRODUCTION

Over 20 years ago, St Clair *et al*. (1) reported a large Scottish family with major psychiatric disorders. In this family, a balanced chromosome translocation t(1;11) co-segregates with schizophrenia, major depression and other psychiatric disorders, suggesting the translocation as a causal genetic mutation (2). The disrupted-in-schizophrenia 1 (DISC1) gene was subsequently identified from the breakpoint of the translocation on chromosome 1 (3). Since then, the DISC1 gene has been extensively studied and proposed to play important roles in neuronal cell proliferation, differentiation, migration, maturation and synaptic functions (4–8). In a comprehensive summary of DISC1 studies, Brandon and Sawa (9) provided a recent review on the potential roles of DISC1 in the pathogenesis of psychiatric disorders.

Our previous studies, however, found that the translocation also disrupted another gene, Boymaw (also termed DISC1 fusion partner 1, DISC1FP1), on chromosome 11 in the Scottish family (10). After translocation, the DISC1-Boymaw (DB7) and the Boymaw-DISC1 (BD13) fusion genes are generated. The DB7 fusion protein is insoluble (11). Insoluble DISC1 proteins were reported in the postmortem brains of sporadically collected patients with schizophrenia, major depression and bipolar disorder (12). These findings prompted us to study the functions of insoluble DB7 fusion protein in hopes of elucidating molecular mechanisms underlying the major psychiatric disorders.

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In this study, we investigated the effects of full-length DISC1 (DISC1-FL), truncated DISC1, DISC1-Boymaw (DB7) and Boymaw-DISC1 (BD13) protein expression in different cell lines. We found that expression of the DB7 fusion gene inhibits oxidoreductase activities, rRNA synthesis and protein translation. Humanized DISC1-Boymaw mice were generated via gene targeting to examine the *in vivo* functions of the fusion genes. We confirmed inhibition of protein translation by the DISC1-Boymaw fusion gene in the humanized mice. Molecular and behavioral characterizations of the humanized mice were also conducted.

RESULTS

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction and DB7 fusion gene expression

The DISC1 gene is mostly expressed in neuronal cells (13,14). To examine potential functions of the DB7 fusion gene, we conducted our studies in HEK293T cells that share 90% of expressed genes with human brain (15, 16). The cDNA genes encoding DISC1-FL, truncated DISC1 (1–597 amino acids), DISC1-Boymaw (DB7) and Boymaw-DISC1 (BD13) are tagged with an HA epitope at their C-terminal (Fig. 1A). Full-length DISC1-FL, truncated DISC1 and BD13 fusion proteins were abundantly expressed in contrast to low levels of DB7 fusion proteins exclusively found in the pellet fraction (Fig. 1A, overexposure in Supplementary Material, Fig. S1A). Similar expression patterns were also observed in transfected COS-7 cells (Supplementary Material, Fig. S1B and C). Since we do not know what exact functions the DB7 protein may serve, we first chose an assay to measure the broad cellular activity of the cells transfected with these genes. MTT reduction assays were therefore conducted (17–20). Surprisingly, only the DB7 fusion gene expression significantly decreased MTT reduction while MTT reduction did not differ between cells expressing DISC1-FL, truncated DISC1, BD13 and green fluorescent protein (GFP) control genes (Fig. 1B). Decreased MTT reduction by the DB7 fusion gene expression was also observed in both COS-7 and neuroblastoma SH-SY5Y cells (Supplementary Material, Fig. S1D and E). We were perplexed by the low expression of the DB7 fusion proteins because all expression constructs contain the same promoter and transcription terminator. One possible explanation could be that the DB7 mRNA is less stable than the mRNA transcripts of the other three genes. To examine such a possibility, bi-cistronic gene constructs were generated (Fig. 1C). If the DB7 mRNA were unstable, we would expect little expression of the fluorescence marker gene downstream of the DB7 open reading frame (ORF). Surprisingly, expression of the fluorescence marker gene was not different between cells expressing any of the four bi-cistronic genes (Fig. 1C), indicating no difference in their mRNA abundance. To examine whether low level expression of the DB7 fusion proteins may be caused by rapid protein degradation, we constructed fusion genes in which each gene was fused with a fluorescence timer (FT) E5 (Fig. 1D) (21). The FT E5 proteins change color from green to red, 9 h after translation. All fusion genes, except the DB7-FT, produced strong co-localized green and red fluorescence (Fig. 1D). However, we could only detect weak green fluorescence in the absence of any red fluorescence in cells expressing the DB7-FT proteins, suggesting possible rapid protein degradation. In further support of this conclusion, addition of proteasome inhibitor *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) to HEK293T cells transfected with the DB7 fusion gene remarkably increased the level of DB7 protein expression (Supplementary Material, Fig. S1F). To provide direct evidence for rapid protein degradation, we performed a proteomic analysis of cells expressing the DB7 fusion gene using the isobaric tags for relative and absolute quantification (iTRAQ) (Supplementary Material, Fig. S2A, B and C). Total proteins from the DISC1-FL and the DB7 over-expression cells were labeled with 114, 115, 116 and 117 Da mass tags, respectively. Hundreds of unique DISC1 peptides were identified in cells expressing the DB7 fusion gene, and their average abundance was ~70% of that in cells expressing the DISC1-FL gene. The abundance of a representative DISC1 peptide is shown between cells expressing full-length DISC1 and the DB7 fusion genes (Fig. 1E). We conclude that these abundant DISC1 peptides from cells expressing the DB7 fusion gene are likely peptide fragments generated by rapid protein degradation within the cell.

Characterization of the Boymaw gene

Since the truncated DISC1 proteins did not decrease MTT reduction, we wondered whether the Boymaw, rather than the DISC1, was responsible for both decreased MTT reduction and rapid protein turnover of the DB7 fusion proteins. Boymaw was therefore in-frame fused at the C-terminal of a randomly selected gene (the FT E5 proteins, FT-Boymaw) to follow the same fusion pattern of the DISC1-Boymaw (DB7) gene (Fig. 2A). In contrast to strong green and red fluorescence in cells expressing the control construct FT, weak green fluorescence was detected in the absence of red fluorescence in cells expressing the FT-Boymaw fusion gene (Fig. 2B). Consistent with the fluorescence images, western blot analyses detected abundant control FT proteins with little FT-Boymaw fusion proteins (Fig. 2C). A significant decrease of MTT reduction was also observed in cells expressing the FT-Boymaw fusion gene in comparison with the controls (Fig. 2D). These data indicate that the Boymaw cause decreased MTT reduction as well as rapid protein turnover of both the FT-Boymaw and the DISC1-Boymaw (DB7) fusion proteins. To investigate whether the inhibition of MTT reduction causes rapid protein turnover or vice versa, a series of C-terminal deletions of the FT-Boymaw gene were generated (Fig. 2E). The C4-del was as effective as the FT-Boymaw in decreasing MTT reduction (Fig. 2F). However, deletion of a short C-terminal sequence in the C4-del dramatically improved protein stability in contrast to barely detectable FT-Boymaw (FT-B) fusion proteins (Fig. 2E). The C2-del had a comparable MTT reduction to the controls (FT) (Fig. 2F), but was less abundant (Fig. 2E). These data indicate that different, although overlapping, regions of the Boymaw gene contribute to the regulation of rapid protein turnover and inhibition of MTT reduction.

Reduced NADH oxidoreductase activities

Our studies found that decreased MTT reduction is not caused by reduced proliferation of the cells expressing the DISC1-Boymaw (DB7) fusion gene (Supplementary Material, Fig. S3A). To rule out potential differences in MTT uptake in living cells,

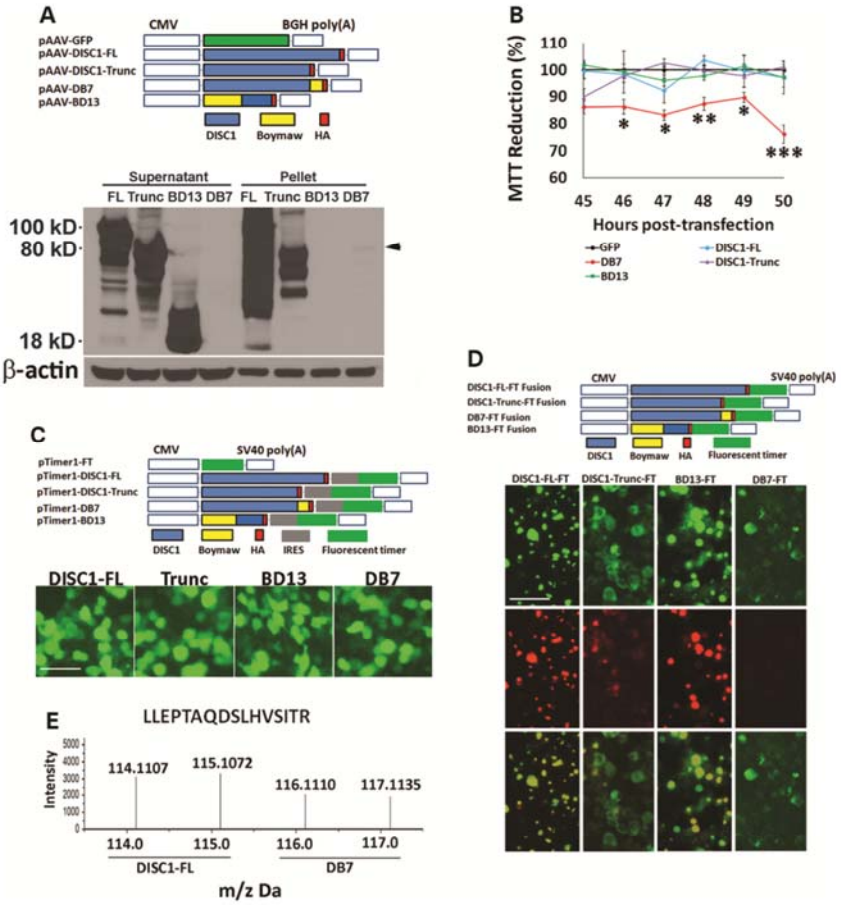

Figure 1. MTT reduction and rapid degradation of DB7 fusion protein. (**A**) pAAV plasmid constructs. Each expression construct was tagged with HA epitope. Western blot analyses revealed abundant expression of full-length DISC1 (FL), truncated DISC1 (Trunc) and BD13 (Boymaw-DISC1). The expression of the DB7 (DISC1-Boymaw) was very low (black arrowhead), and restricted to the pellet fraction. Equal amounts of proteins were loaded for the western blot (see β-actin control). **(B)** MTT reduction assays at multiple time points. There were 4–6 replica wells per construct per time point. The mean value of the MTT reduction in cells transfected with GFP was used as the control (100%) to calculate relative MTT reduction for all other constructs at each time point. ANOVA analysis revealed a significant gene effect [$F(4,126) = 28.09$, $P < 0.0001$]. *Post hoc* analyses (Tukey studentized range test) revealed that cells transfected with the DISC1-Boymaw (DB7) construct displayed a significant decrease in MTT reduction at all time points except 45 h post-transfection. Error bar: SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**C**) Bi-cistronic constructs in pTimer1 plasmid vector. Each gene was linked with a fluorescence marker (timer) gene via an IRES site. Green fluorescence was examined 48 h after transfection of HEK293T cells. There was no difference between any of the four gene constructs. Scale bar: 25 μm. (**D**) The FT E5 was in-frame fused with DISC1-FL, truncated DISC1, DB7 and BD13 in pTimer plasmid vector. Expression of the fusion proteins was examined 48 h after transfection of HEK293T cells. Both green and red fluorescence were readily detected and co-localized in DISC1-FL-FT, DISC1-trunc-FT and BD13-FT. Weak green, and no red, fluorescence was detected in comparable number of cells expressing DB7-FT only after overexposure. Scale bar: 25 μm. (**E**) In iTRAQ experiments, proteins from cells expressing the full-length DISC1 gene were labeled with 114 and 115 Da mass tags; proteins from cells expressing the DB7 gene were labeled with 116 and 117 Da mass tags. Abundant human DISC1 peptides were also detected in cells expressing the DB7 gene.

the transfected cells were partially lysed for the MTT assay. DB7 cell lysate display a similar magnitude of MTT reduction deficit to the living cells (Fig. 3A). These data therefore indicate that intracellular MTT reduction activities are decreased in DB7 expressing cells. NADH oxidoreductases are major intracellular electron donors in the reduction of MTT (20). We therefore first examined whether NADH concentration was decreased in cells expressing the DB7 gene. No significant difference in intracellular NADH was found between cells expressing DISC1-FL and DB7 genes (Supplementary Material, Fig. S3B). Cytosolic

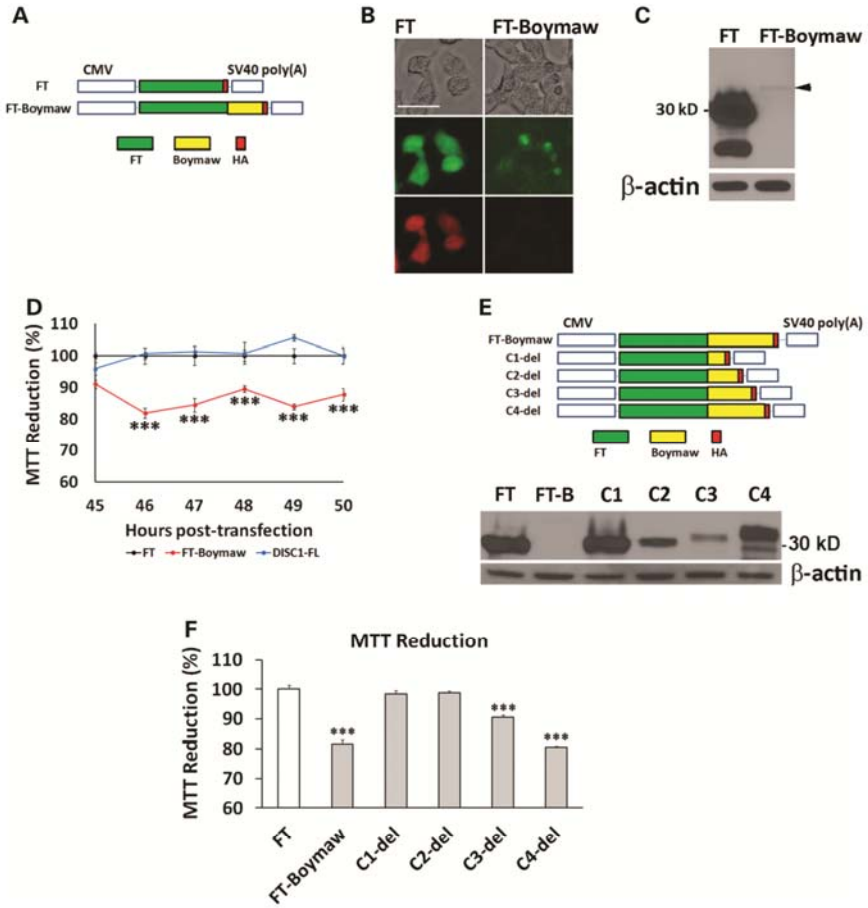

Figure 2. Characterization of the Boymaw gene. (**A**) Boymaw was fused with a FT tagged with HA in pTimer plasmid vector. The pTimer-HA plasmids (FT) were used as controls. (**B**) Expression of the FT-Boymaw fusion proteins was examined 48 h after transfection of HEK293T cells. While green and red fluorescence of the control FT proteins were very strong, little green and no red fluorescence of FT-Boymaw was detected. Scale bar: 15 μm. (**C**) Western blot analysis barely detected expression of the FT-Boymaw fusion proteins (black arrowhead) in comparison with abundant control FT proteins. (**D**) MTT assays revealed decreased MTT reduction by expression of the FT-Boymaw fusion gene in comparison with the controls and DISC1-FL [$F(2,66) = 145.12$, $P < 0.0001$]. *Post hoc* analyses (Tukey studentized range test) revealed that cells transfected with the FT-Boymaw construct displayed a significant decrease in MTT reduction at all time points except 45 h post-transfection. There were 4–6 replica wells per construct per time point. The mean value of the MTT reduction in cells transfected with FT was used as the control (100%) to calculate relative MTT reduction for the other two constructs at each time point. (**E**) A series of C-terminal deletion of FT-Boymaw fusion gene. Western blot analysis revealed expression of each deletion construct. (**F**) MTT assays were conducted 48 h after transfection of each deletion construct. There was a significant group effect on MTT reduction [$F(8, 27) = 92.14$, $P < 0.000001$]. *Post hoc* analyses (Tukey studentized range test) revealed significant MTT reduction in cells transfected with FT-Boymaw, C3-del and C4-del. There were four replica wells per construct. The mean value of the MTT reduction in cells transfected with FT was used as the control (100%) to calculate relative MTT reduction for all other constructs. Error bar: SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

NADH concentration was also examined with NADH fluorescence sensor Peredox (22), and no difference was found either (Supplementary Material, Fig. S3C). To further examine whether NADH oxidoreductase activities were reduced in DB7 expressing cells, recombinant *Escherichia coli* NADH alcohol dehydrogenase (ADH), which catalyzes MTT reduction in the presence of NADH, was added to the DISC1-FL and DB7 cell lysates, respectively. Addition of the ADH dramatically increased MTT reduction in the DB7 cell lysate, but not in the DISC1-FL cell lysate (Fig. 3B). These data indicate that the DB7 cell lysate have decreased activities of NADH oxidoreductases. To localize the NADH oxidoreductases, we conducted

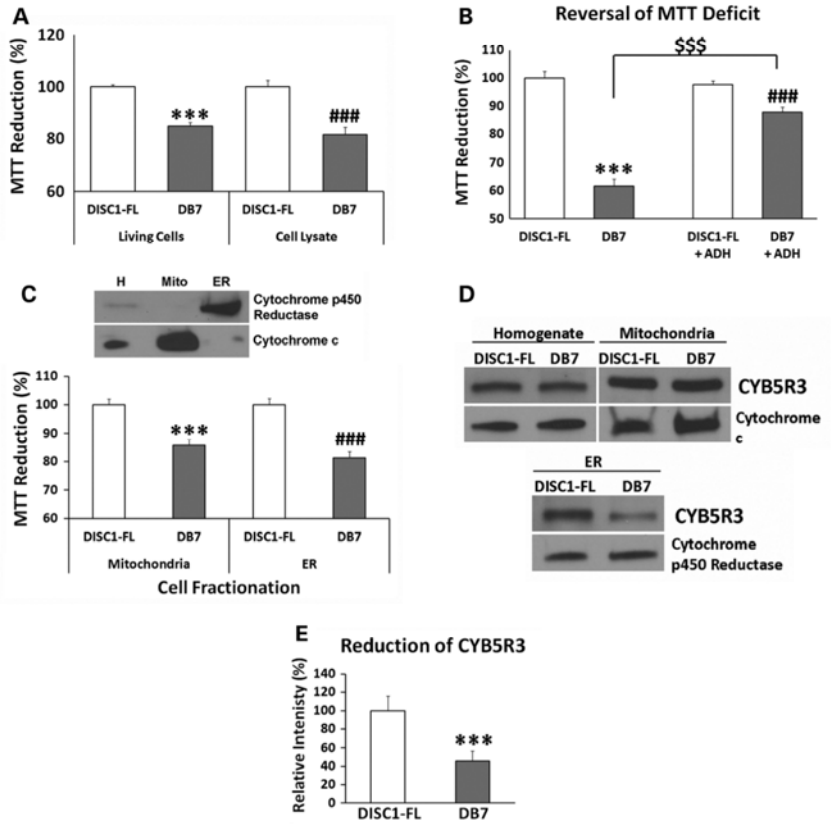

Figure 3. Reduced NADH oxidoreductase activities. (**A**) HEK293T cells were transfected with DISC1-FL and DB7, respectively. Two days after transfection, MTT reduction was performed in both living cells and cell lysate, respectively. Student's *t*-tests (unpaired, two-tailed) were used to compare MTT reduction between DISC1-FL and DB7 in living cells and cell lysate, respectively. The mean value of the MTT reduction in cells transfected with DISC1-FL was used as the control (100%) to calculate relative MTT reduction for DB7 in each group. Significantly decreased MTT reduction in DB7 transfection was observed in living cells ($n = 3$) [$t(4) = 10.74$, $P < 0.001$].and cell lysate ($n = 6$) [$t(10) = 4.92$, $^{\#\#\#}P < 0.001$]. (**B**) Reversal of MTT reduction deficit in cell lysate. There were 12 replica wells for each gene and treatment. Unpaired student's *t*-test (two-tailed) was used for statistical analysis. Significantly decreased MTT reduction was observed in HEK293T cell lysate from DB7 transfection in comparison with DISC1-FL [$t(22) = 11.50$, $P < 0.001$]. *E. coli* ADH can catalyze MTT reduction in the presence of NADH. Addition of the ADH did not completely reverse the deficit of MTT reduction in DB7 lysate in comparison with DISC1-FL lysate [$t(22) = 4.26$, $^{\#\#\#}P < 0.001$]. However, addition of recombinant *E. coli* ADH significantly improved MTT reduction in DB7 cell lysate [$t(22) = 8.66$, $^{\$\$\$}P < 0.001$ (comparison between DB7 lysate with and without ADH)]. No difference was found in MTT reduction of the DISC1-FL cell lysate with or without ADH. The mean value of the MTT reduction of DISC1-FL samples without ADH was used as the control (100%) to calculate relative MTT reduction for DB7, DISC1-FL + ADH and DB7 + ADH. (**C**) Purification of mitochondria and endoplasmic reticulum (ER) from transfected HEK293T cells using density-gradient ultracentrifugation. Large amount of cells was transfected for each purification experiment. Cytochrome c and cytochrome p450 reductase were used as marker proteins for mitochondria and ER respectively. MTT reduction assays were conducted in purified mitochondria and ER from HEK293T cells transfected with DISC1-FL and DB7 constructs. The value of MTT reduction was further normalized with total amount of proteins in mitochondria and ER, respectively. Significantly reduced MTT reduction was observed in both mitochondria ($n = 6$)[$t(10) = 5.53$, two-tailed, $P < 0.001$) and ER ($n = 6$)[$t(10) = 6.59$, two-tailed, $^{\#\#\#}P < 0.001$] isolated from cells transfected with DB7 expression construct. (**D**) The level of CYB5R3 protein expression was examined with western blot analysis, and normalized with the expression of ER marker cytochrome p450 reductase (CYPOR). CYB5R3 expression was reduced in the ER of the cells expressing the DB7 fusion gene. No significant difference in CYB5R3 expression was observed between DISC1-FL and DB7 in either cell homogenate or mitochondria after normalization with mitochondrial marker cytochrome c. (**E**) CYB5R3 expression was quantified in ER preparations of each construct ($n = 4$). Unpaired student's *t*-test (two-tailed) analysis revealed a significant reduction of CYB5R3 in DB7 [$t(6) = 18.84$, $P < 0.001$] Error bar: SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

density-gradient ultracentrifugation to isolate both mitochondria and microsomes (fragmented endoplasmic reticulum). Western blot analyses demonstrated enriched expression of cytochrome c in the mitochondrial fraction and cytochrome p450 reductase in the microsomal fraction (Fig. 3C). A significant decrease of MTT reduction was observed in both

mitochondrial and microsomal fractions from the cells expressing the DB7 gene. Further analysis identified a significant reduction of cytochrome b5 reductase 3 (CYB5R3) proteins in the microsomal fraction of the cells expressing DB7 (Fig. 3D and E). No differential CYB5R3 expression was detected between DISC1-FL and DB7 in either cell homogenate or mitochondria.

To fully characterize the effects of the DB7 fusion gene expression, we conducted proteomic analysis of cells expressing the DB7 fusion gene. We did not find additional candidate NADH oxidoreductases whose expression was significantly decreased (Supplementary Material, Fig. S2A, S2B, S2C, and Supplementary Material, Table S1). Several reasons could account for the results. First, proteomic analysis can only survey a portion of total cellular proteins. Second, we found that expression of several NADH oxidoreductases was decreased, but did not reach statistical significance because of a limited number of identified peptides. Third, NADH oxidoreductase activities are modulated not only by protein expression but also by protein modifications. The molecular nature of the reduced activities of NADH oxidoreductases in cells expressing the DB7 fusion gene remains to be fully investigated. Among the top 18 differentially expressed proteins ranked according to their p values, however, seven were directly involved in the regulation of protein translation (Supplementary Material, Fig. S2C).

Inhibition of rRNA expression and protein translation *in vitro*

To investigate whether expression of the DISC1-Boymaw (DB7) fusion gene alters protein translation, we first examined rRNA expression in cells expressing the DISC1-FL, truncated DISC1, DB7 and BD13 genes, respectively. Interestingly, we found a significant reduction of total RNA in cells expressing the DB7 fusion gene (Fig. 4A). Reduction of the total RNA was not caused by differential cell proliferation (Supplementary Material, Fig. S3D). Since rRNA makes up more than 80% of total RNA, we quantified the expression of both 28S and 18S rRNAs. Reduction of both 28S and 18S rRNAs was found only in cells expressing the DB7, not the other three genes. Consistent with the effects of the DB7 fusion gene expression, expression of the FT-Boymaw fusion gene also decreased total RNA and rRNA expression (Fig. 4B). To provide direct evidence for inhibition of protein translation, we conducted SUnSET (surface sensing of translation) experiments to measure protein translation in the transfected cells (23). In the presence of cycloheximide, an inhibitor of protein translation, we detected little puromycin incorporation in newly synthesized proteins in western blot analysis (Fig. 4C). In pulse-labeling experiments, we found significant reduction of puromycin labeling from cells expressing the DB7 fusion gene, suggesting decreased protein translation activities (Fig. 4D and E). There was no significant difference in puromycin labeling between cells expressing the other three genes. We confirmed that expression of the FT-Boymaw fusion gene also inhibits protein translation similar to the DB7 fusion gene (Fig. 4F and G). All together, it appears that it is the Boymaw gene, rather than the DISC1 gene, that contributes to the inhibition of oxidoreductase activities, rRNA expression and protein translation in both the DISC1-Boymaw and FT-Boymaw fusion genes. We did not observe any changes of these cellular activities in cells expressing the full-length DISC1, truncated DISC1 or the Boymaw-DISC1 fusion genes.

Generation of humanized DISC1-Boymaw mice

To investigate the *in vivo* functions of the DB7 fusion gene, we generated humanized DISC1-Boymaw mice. Human DISC1-Boymaw fusion gene (DB7) and Boymaw-DISC1 fusion gene (BD13) were knocked-in to replace mouse endogenous *disc1* gene (Fig. 5A). A bi-cistronic gene, consisting of the two fusion genes connected with an internal ribosome entry site (IRES), was designed to splice with exon 1 of human DISC1 gene. The bi-cistronic cassette was also flanked with two *loxP* sites upstream of a human DISC1 gene for future genetic rescue experiments. Germ-line transmission of the bi-cistronic gene was obtained. To examine whether the bi-cistronic gene is expressed and correctly spliced with exon 1, we conducted reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of total RNA extracted from the brains of humanized DISC1-Boymaw mice ($DISC1^{H}$) (Fig. 5B). A 150 bp DNA fragment was specifically amplified. Further sequencing confirmed the correct splicing (data not shown). Before examination of the expression of the DB7 proteins of the bi-cistronic gene in the humanized mice, we first evaluated eight different anti-DISC1 antibodies using our DISC1 over-expressing cell lysates (Supplementary Material, Fig. S4A). Five antibodies (ab55808, ab62069, ab59017, 3D4 and 14F2) with the highest affinities to human DISC1 proteins were selected for western blot detection of the DB7 fusion protein. Unfortunately, there were extensive non-specific cross-reactions of the antibodies with proteins from mouse brain homogenate in western blot analysis (data not shown), preventing the detection of the fusion proteins. However, the antibodies have less background in western blot analyses of mouse primary neuron culture (H. Song, personal communication). We therefore performed our analyses in cultured primary neurons from neonatal mice. The antibody against the N-terminal residues of human DISC1 protein recognized a 75 kDa protein band in western blot analysis of the primary neurons from the humanized DISC1-Boymaw mice, but not wild-type mice (Fig. 5C). Two reasons support this band as the DB7 fusion protein. First, the protein band is about the calculated size of the DB7 fusion protein (73 kDa). Second, the absence of this band in wild-type mice rules it out as a non-specific cross-reaction of the antibody. To detect the expression of the BD13 proteins of the bi-cistronic gene, we further screened the DISC1 antibodies in our BD13 over-expressing cell lysate. Two antibodies recognizing the C-terminal residues of human DISC1 proteins displayed some, but not high (compared with anti-HA antibody), affinity to the BD13 proteins (Supplementary Material, Fig. S4B). Due to the low affinity of the two antibodies, we could not detect the expression of the BD13 proteins expected to be ~17 kDa (Supplementary Material, Fig. S4C, S4D). To further confirm the expression of the DB7 fusion proteins, immunocytochemical analyses were conducted. Co-localization of the DB7 proteins with neuronal cell marker MAP2 was observed in the primary neurons from the heterozygous DISC1-Boymaw mice (Fig. 5D). Expression of the DB7 protein did not cause gross anatomical abnormalities in the brain of the heterozygous DISC1-Boymaw mice (Supplementary Material, Fig. S5). To

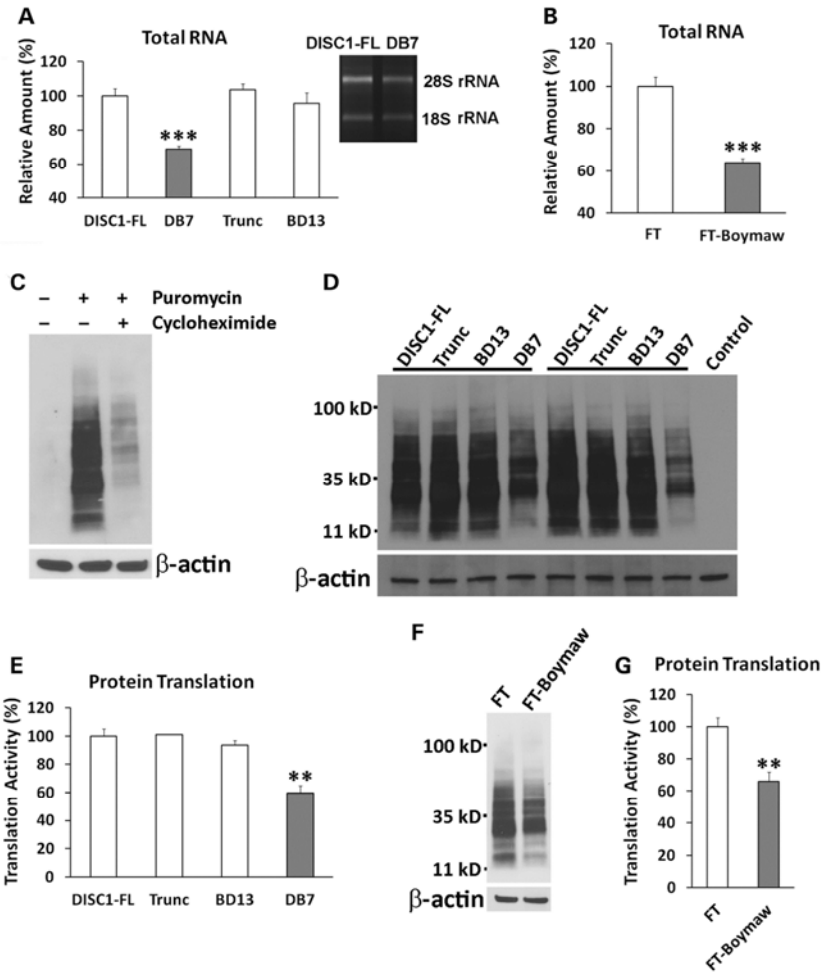

Figure 4. Inhibition of rRNA expression and protein translation. (**A**) Reduction of total RNA in cells expressing the DB7 gene [unpaired, two-tailed student's *t*-test, $t(10) = 9.84$, $P < 0.001$]. There were three to six replica wells for each gene construct. Significant reduction of rRNA expression was observed between DB7 and DISC1-FL. The same amount of rRNA reduction was further confirmed in later transfection experiments. Expression of total RNA and rRNA was normalized against the number of cells. The level of total RNA and rRNA expression of the DISC1-FL was used as the reference (100%) to calculate relative expression of the other three groups. (**B**) Reduction of total RNA and rRNA in cells expressing FT-Boymaw fusion gene compared with the control FT gene [unpaired, two-tailed student's *t*-test, $t(8) = 7.6$, $P < 0.001$). There were five replica wells for each construct. Significant reduction of rRNA was observed in cells expressing the FT-Boymaw fusion gene. (**C**) Pulse-labeling of new protein synthesis via incorporation of puromycin. Anti-puromycin antibody has no cross-reactions with HEK293T cellular proteins. A 10 min pulse-labeling generated massive incorporation of puromycin in western blot analysis. However, the presence of cycloheximide, an inhibitor of protein translation, dramatically reduced the puromycin labeling. (**D**) Inhibition of protein translation. Protein translation activities were measured with SUnSET in cells at 48 h post-transfection. Remarkable reduction of puromycin labeling was observed in cells expressing the DB7 construct in two representative sets of transfection experiments. (**E**) Quantification of reduction of puromycin incorporation. There were four replica dishes for each construct. There was a significant reduction of protein translation in cells expressing the DB7 construct in comparison with the DISC1-FL construct [unpaired, two-tailed student's *t*-test, $t(6) = 5.03$, $P < 0.01$]. The mean value of the incorporation of puromycin in DISC1-FL samples was used as the control (100%) to calculate relative intensity of puromycin incorporation in the other three genes. No difference was found in protein translation activity between the other three constructs. (**F**) There was a reduction of protein translation in cells expressing the FT-Boymaw fusion gene compared with the control FT gene in SUnSET. (**G**) There were six replica dishes for each construct. Significant reduction of puromycin incorporation was observed in cells expressing the FT-Boymaw fusion gene [unpaired, two-tailed student's *t*-test, $t(10) = 4.48$, $P < 0.01$]. Error bar: SEM. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

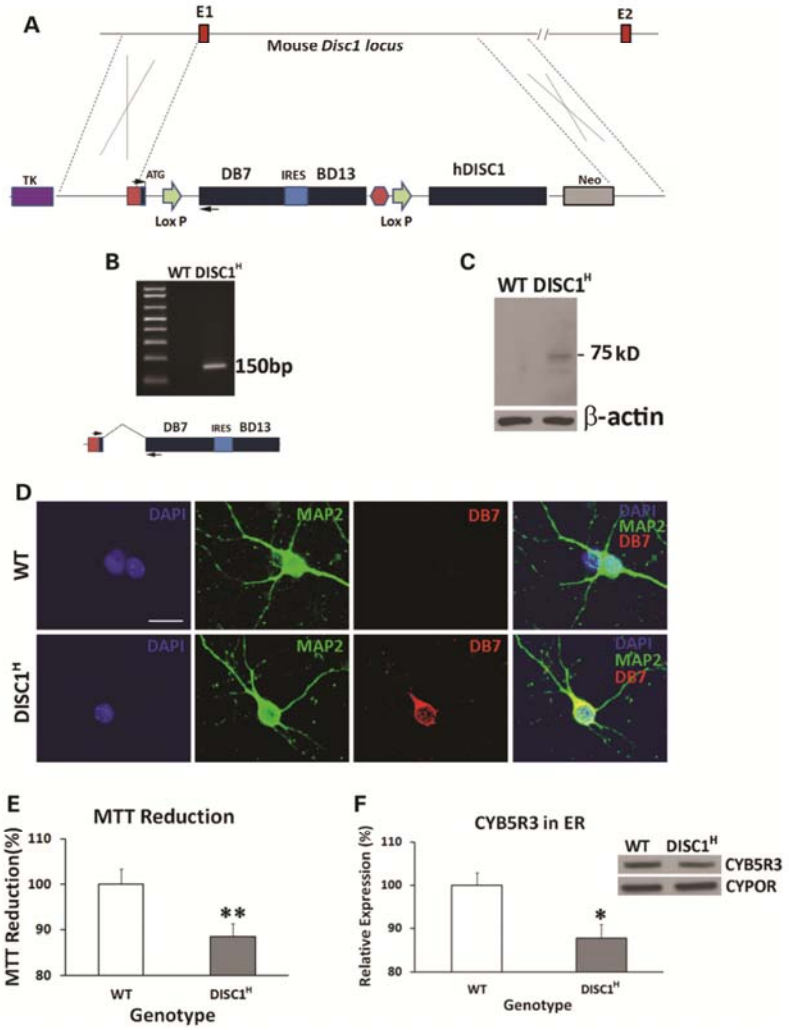

Figure 5. Generation of humanized DISC1-Boymaw Mice. (**A**) Both the DISC1-Boymaw and the Boymaw-DISC1 fusion genes were knocked-in to replace mouse endogenous *disc1* gene. E1 and E2 are exon 1 and 2 of mouse *disc1* gene. (**B**) RT-PCR analysis of the expression of the DB7 fusion genes in humanized DISC1-Boymaw mice ($DISC1^{H}$). The two PCR primers were localized in exon 1 and DB7, respectively (arrows). The expression of the bi-cistronic gene generated a 150 bp cDNA fragment after RT-PCR. Correct splicing was confirmed after sequencing. (**C**) Western blot analysis of the DB7 fusion protein in cultured primary neurons. Expression of the DISC1-Boymaw (DB7) fusion proteins was detected from the primary neurons isolated from the humanized DISC1-Boymaw mice. The 75 kDa protein band is very close to predicted molecular size (~73 kDa). (**D**) Immunocytochemical staining of the DB7 fusion proteins in the primary neurons. Primary neurons were isolated from postnatal Day 1 mice, and cultured 4 days *in vitro* before immunostaining. Co-localization between MAP2 and DB7 proteins was observed. Scale bar: 20 μm. (**E**) Decreased MTT reduction in brain homogenate of neonatal heterozygous mice ($DISC1^{H}$) ($n = 14$) in comparison with wild-type control littermates ($n = 12$) after normalization with total amount of proteins ($t(24) = 2.984$, $P < 0.01$, unpaired, two-tailed student's *t*-test). (**F**) Reduced expression of Cyb5r3 in the ER isolated from the brains of neonatal DISC1-Boymaw mice ($n = 16$) and wild-type littermates ($n = 18$) after normalization with ER marker Cypor (unpaired, two-tailed student's *t*-test, $t(32) = 2.58$, $P < 0.05$). Error bar: SEM. ($^{*}P < 0.05$, $^{**}P < 0.01$).

examine whether expression of the DB7 fusion protein decreases MTT reduction *in vivo*, we performed MTT reduction assays in the brain homogenate of neonatal heterozygous DISC1-Boymaw and wild-type control mice. A significantly decreased MTT reduction was observed in the heterozygous DISC1-Boymaw mice (Fig. 5E). Western blot analysis also confirmed a significant decrease of Cyb5r3 proteins in ER isolated from the brains of the heterozygous DISC1-Boymaw mice (Fig. 5F).

Inhibition of rRNA expression and protein translation *in vivo*

We were particularly interested in the inhibition of both rRNA expression and protein translation in HEK293T cells expressing the DB7 fusion gene. To investigate whether expression of the DB7 fusion gene also decreases rRNA expression *in vivo*, we extracted total RNA from mouse hippocampus and cortex. Heterozygous DISC1-Boymaw mice had significantly less hippocampal rRNA than their wild-type siblings (Fig. 6A). No difference in hippocampal DNA was found between the two genotypes. Reduction of rRNA expression was also observed in the cortex of the heterozygous DISC1-Boymaw mice (Supplementary Material, Fig. S6A). To confirm whether rRNA expression is reduced in neuronal cells, we conducted non-radioactive chromogenic RNA *in situ* hybridization to visualize 18S rRNA expression (Fig. 6B) (Supplementary Material, Fig. S6B). Reduced expression of 18S rRNA was observed across mouse brain including both hippocampal and cortical neuronal cells in the heterozygous DISC1-Boymaw mice. Consistent with reduction of rRNA expression, we found significant reduction of protein translation in the hippocampus of the heterozygous DISC1-Boymaw mice after intracerebroventricular (ICV) injection of puromycin in *in vivo* SUnSET experiments (Fig. 6C and D) (24). To investigate whether the inhibition of protein translation is independent of potential differential brain activities, we examined protein translation activities in cultured primary neurons isolated from postnatal Day 1 mice. Protein translation activity was significantly lower in the primary neurons isolated from the heterozygous DISC1-Boymaw mice than their wild-type siblings (Fig. 6Eand F).

Decreased Gad67, Nmdar1 and Psd95 expression

We next examined whether general inhibition of protein translation in the humanized mice reduces protein expression of genes critical for pathophysiology of schizophrenia and other psychiatric disorders. Genes encoding Gad67, Nmdar1 and Psd95 proteins were examined because of their central roles in GABAergic and glutamatergic neurotransmission as well as synaptogenesis. There are also many reports on their down-regulation in psychiatric disorders (25–32), and degradation of PSD95 is proposed to play a key role in autism (33). We first conducted real-time RT-PCR quantification of their RNA expression, and did not find significant reduction in the humanized mice (Fig. 7A). To examine their protein expression, we conducted western blot analyses. Significant reduction of Gad67, Nmdar1 and Psd95 was observed in the heterozygous DISC1-Boymaw mice (Fig. 7B). No significant difference was found in synaptophysin. It has been reported that neural circuitry activity can regulate Gad67 gene expression at the system level (34). To rule out any potential effects from differential neural circuitry activity of individual mice, we examined Gad67 expression in primary neuron culture. Reduction of Gad67 proteins was remarkable in the primary neurons isolated from both E18.5 embryos and postnatal Day 1 (P1) DISC1-Boymaw mice (Fig. 7C). Moderate reduction of Psd95 was also observed. Expression of Nmdar1 was very low and inconclusive. Immunohistochemical analyses were also conducted to examine expression pattern of these proteins in adult mouse brains (Fig. 7D). Gad67 and Nmdar1 proteins displayed similar expression patterns between the heterozygous DISC1-Boymaw mice and wild-type siblings. In the heterozygous DISC1-Boymaw mouse brains, there is no obvious loss of Gad67-positive cortical GABAergic interneurons (data not shown). Anti-Psd95 antibody did not work on mouse paraffin sections. We also included anti-parvalbumin antibody in the analysis to examine whether this major subgroup of cortical GABAergic interneurons may be abnormal in the heterozygous DISC1-Boymaw mice. We did not find differences in either the number of positive neurons or intensity of parvalbumin expression between the two genotypes.

Behavioral analyses of the humanized DISC1-Boymaw mice

Since the DB7 fusion gene is a gain-of-function mutation in inhibiting protein translation, the function of the fusion gene was therefore studied in the heterozygous DISC1-Boymaw mice. Mice on the C57 background carry wild-type *Disc1* genes, but 129S mice are natural null mutants of the full length *disc1* gene (6, 35) (Supplementary Material, Fig. S6C and D). By following the recommendation of using F1 genetic background by Banbury Conference on Genetic Background in Mice (36), we generated heterozygous DISC1-Boymaw mice on F1 129S/C57 genetic background for behavioral studies (Fig. 8A). In the F1 generation, both wild-type and the DISC1-Boymaw heterozygous mice carry one allele of mouse wild-type *Disc1* gene. The DISC1-Boymaw heterozygous mice were healthy and indistinguishable from their wild-type sibling mice. Prepulse inhibition (PPI) and habituation of acoustic startle were examined because they are both deficient in patients with schizophrenia and/or other major psychiatric disorders (37, 38). No differences in PPI (100 ms interstimulus interval; ISI), startle, or startle habituation were found between the genotypes (Fig. 8B and C). However, we noticed that there was a large startle variation between individuals with a significant number of mice with low startle (<50) (Fig. 8D), which could complicate the measurement of PPI. The source of such a large startle variation remains unknown since there is no genetic segregation in F1 generation. Schizophrenia patients are reported to have high sensitivity and prolonged responses to ketamine, an NMDA receptor antagonist (39–41). Cortical GABAergic inhibitory interneurons have been proposed to be the primary targets of NMDAR antagonists (42, 43). In light of our findings of reduced Nmdar1 and Gad67 proteins, we examined responses of the heterozygous DISC1-Boymaw male mice to ketamine in the mouse behavioral pattern monitor (BPM) (44). A significant gene by ketamine interaction was observed [$F(1,27) = 4.30$, $P < 0.05$]. *Post hoc* analyses revealed that the heterozygous DISC1-Boymaw male mice display a larger increase in total distance traveled (Fig. 8E), counts (Supplementary Material,

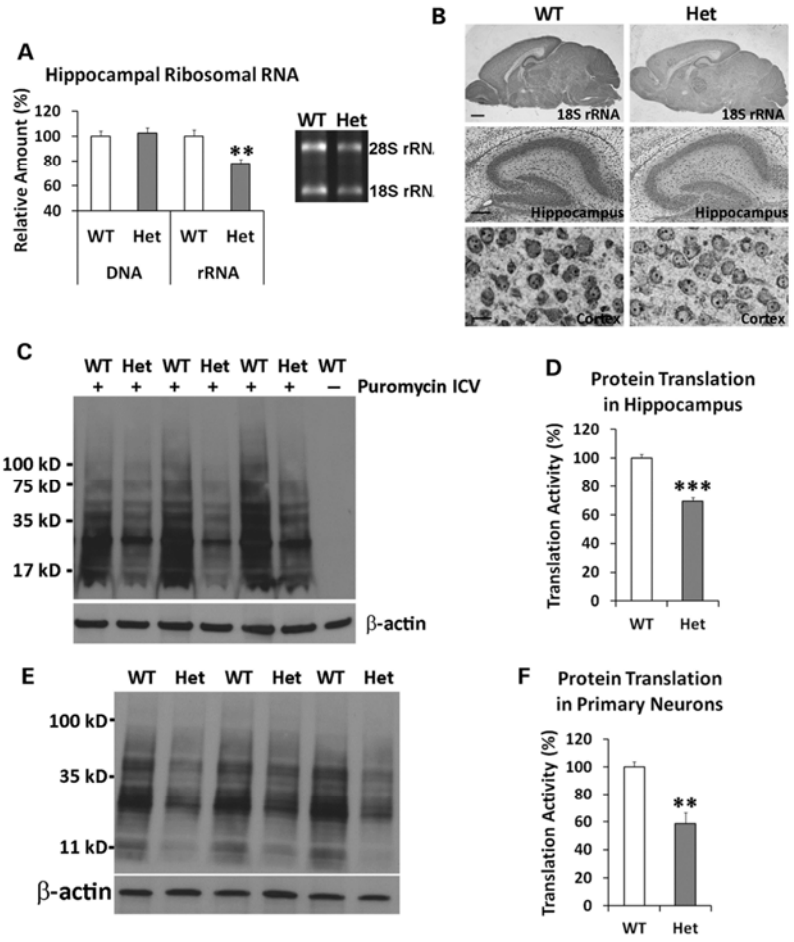

Figure 6. Decreased rRNA expression and protein translation. (**A**) Hippocampal DNA and RNA were isolated from 3-week old heterozygous DISC1-Boymaw ($n = 8$) and wild-type control mice ($n = 12$) respectively. No difference in total amount of DNA was observed between the two genotypes. However, significant reduction of total RNA was observed [unpaired, two-tailed student's *t*-test, $t(18) = 3.33$, $P < 0.01$]. After gel electrophoresis, 28S and 18S rRNA displayed significant reduction in the hippocampus of the heterozygous DISC1-Boymaw mice. (**B**) Chromogenic RNA *in situ* hybridization of 18S rRNA was conducted in the mouse brains at postnatal Day 7 (scale bar: 400 μm). Reduction of 18S rRNA was observed in both hippocampal neuronal cells (scale bar: 100 μm) as well as cortical neuronal cells (scale bar: 10 μm). Nuclear rRNA was readily detected in the nucleolus of the cortical neurons. (**C**) Protein translation activities in hippocampus. Intracerebroventricular injection of puromycin generated massive labeling of new protein synthesis in mouse hippocampus. Decreased puromycin labeling can be readily observed in the heterozygous mice. (**D**) Significant reduction of protein synthesis activities was found in the hippocampus of the heterozygous DISC1-Boymaw mice in comparison with wild-type sibling mice [unpaired, two-tailed student's *t*-test, $t(4) = 9.28$, $P < 0.001$]. (**E**) Primary neurons were isolated from postnatal Day 1 mice and cultured at 4 days *in vitro* before *SUnSET* experiments. After pulse-labeling, western blot revealed less puromycin labeling in the primary neurons from the heterozygous DISC1-Boymaw mice than their wild-type siblings. (**F**) Significant reduction of protein synthesis was detected [unpaired, two-tailed student's *t*-test, $t(4) = 4.74$, $P < 0.01$]. Error bar: SEM. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Fig. S7A) and transitions (Supplementary Material, Fig. S7B) than sibling wild-type mice in locomotion after ketamine injection. It is unlikely that less locomotion of the wild-type mice results from ketamine overdose, since we did not observe initial suppression and later increase of locomotion by ketamine overdosing in the wild-type mice. However, multiple dosages of ketamine are needed to fully address the ketamine sensitivity of the heterozygous DISC1-Boymaw mice in the future.

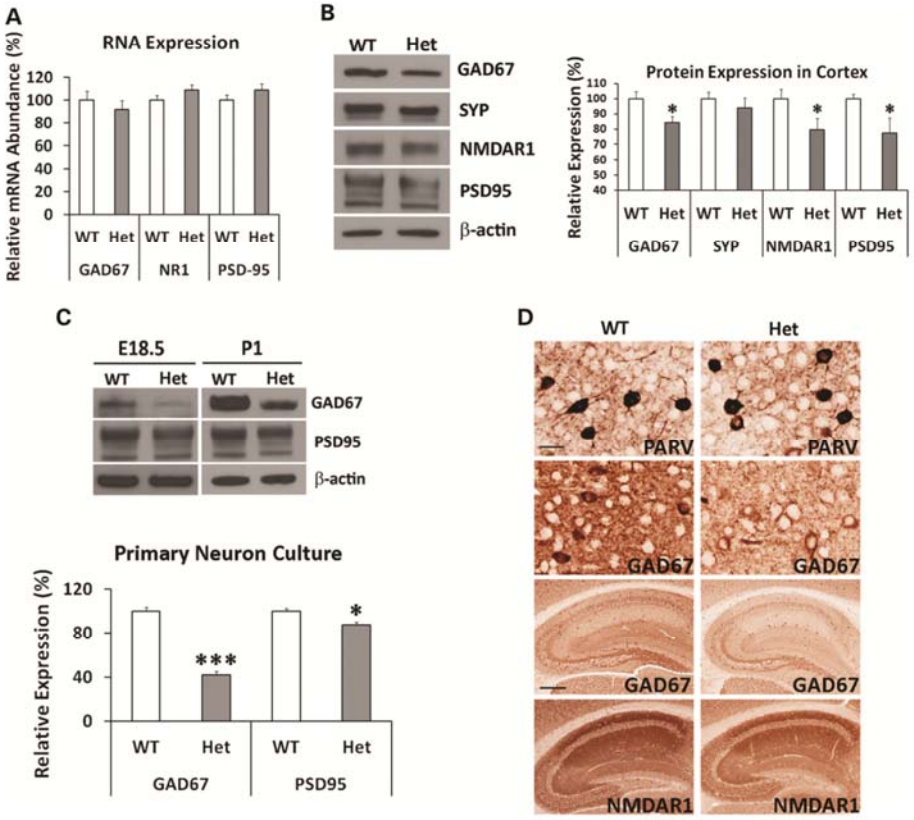

Figure 7. Reduction of Gad67, Nmdar1 and Psd95 Proteins. (**A**) Q-PCR analysis of mRNA transcripts of *Gad67*, *Nmdar1* and *Psd95* genes in adult mouse brain. There was no significant difference between wild-type and the heterozygous DISC1-Boymaw mice in *Gad67* [unpaired, two-tailed student's *t*-test, $t(19) = 0.73$, n.s.], *Nmdar1* [unpaired, two-tailed student's *t*-test, $t(19) = -1.44$, n.s.] and *Psd95* [unpaired, two-tailed student's *t*-test, $t(19) = -1.17$, n.s.] expression. (**B**) Western blot analysis and quantification of Gad67, synaptophysin (Syp), Nmdar1 and Psd95 in the brains of the adult heterozygous DISC1-Boymaw ($n = 9$) and wild-type ($n = 9$) mice. Unpaired two-tailed student's *t*-test was used for statistical analysis. No significant reduction of synaptophysin was observed between the two genotypes. Significant reduction of Gad67 [$t(16) = 2.37$, $P < 0.05$], Nmdar1 [$t(16) = 2.55$, $P < 0.05$] and Psd95 [$t(16) = 2.58$, $P < 0.05$] proteins was observed after normalization with either total amount of loading protein or β-actin expression. (**C**) In primary neurons isolated from cortex and striatum of postnatal Day 1 mice, significant reduction of both Gad67 ($t(4) = 11.92$, $P < 0.001$) and Psd95 [$t(4) = 3.30$, $P < 0.05$) proteins was observed in cultured primary neurons at 4 days *in vitro* from the DISC1-Boymaw mice ($n = 3$) in comparison with wild-type controls ($n = 3$) (unpaired, two-tailed student's *t*-test). Reduction of both proteins was further confirmed in the primary neuron culture (isolated from cortex and striatum) at 4 days *in vitro* from E18.5 embryos. (**D**) Immunohistochemical analysis of parvalbumin, Gad67, and Nmdar1 proteins in the heterozygous DISC1-Boymaw mice (anti-Psd95 antibodies did not work on paraffin sections). Reduction of Gad67 and Nmdar1 was observed in both hippocampus (scale bar: 100 μm) and cortex (scale bar: 20 μm). No difference was detected in parvalbumin expression. White 'spots' were nuclei not stained by antibodies. Error bar: SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

We next examined the behavioral phenotypes of the humanized mice in the absence of full-length wild-type *Disc1* gene on 129S genetic background (Fig. 9A). Because wild-type 129S mice carry mutant *disc1* genes, we define that 'wild-type mice' in our studies refer to mice that do not carry the knocked-in DISC1-Boymaw fusion gene, rather than refer to the status of mouse endogenous *disc1* gene. Both wild-type and heterozygous mice were healthy; however, male heterozygous mice were slightly, but significantly, smaller than their wild-type littermates (Fig. 9B). No significant body weight difference was observed in females between the two genotypes. In contrast to mice on mixed 129S/C57 genetic background, mice on 129S pure genetic background have higher startle with less variation between individuals (Supplementary Material, Fig. S8A), which makes the 129S genetic background more suitable for PPI studies. To extend the PPI studies with ISI at 100 ms in the F1 mice, we performed PPI tests with various ISIs in this cohort. When PPI was conducted with a short ISI at 25 ms,

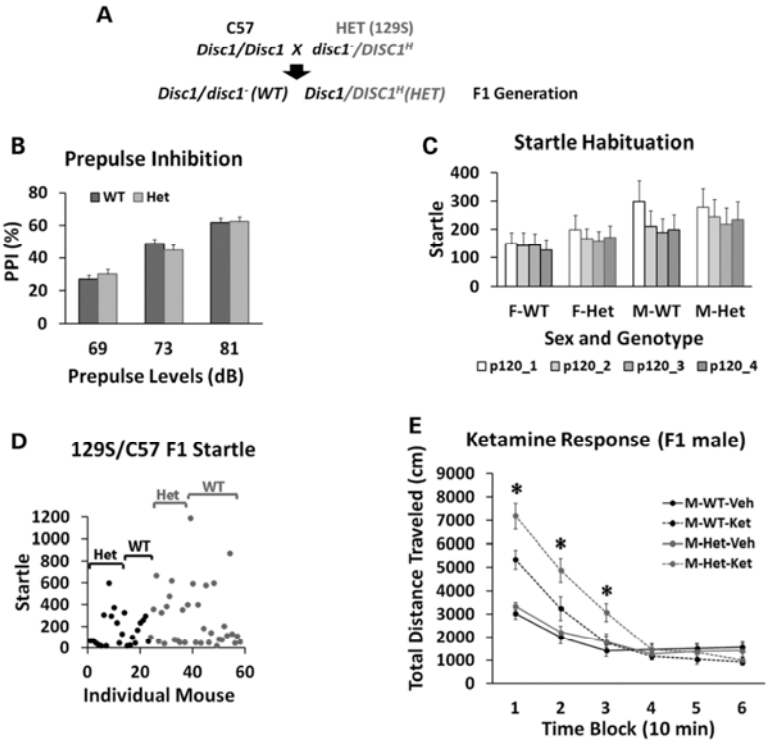

Figure 8. Behavioral analyses of the humanized DISC1-Boymaw mice on F1 129S/C57 genetic background. (**A**) The breeding scheme of mice on F1 129S/C57 background. In the F1 generation, there is no genetic segregation between individual mice. Both wild-type and the heterozygous DISC1-Boymaw mice carry one wild-type copy of mouse *Disc1* gene. (**B**) The cohort of F1 mice consists of 28 wild-type and 26 heterozygous DISC1-Boymaw mice. There was no sex effect or gene effect on PPI [$F(1,52) = 0.00$, n.s.]. (**C**) There was no gene effect on startle habituation [$F(1,50) = 0.21$, n.s.]. (**D**) A large startle variation was observed between individual mice despite the fact that they are genetically identical. Dots represent individual mice (black = females, pale = males). (**E**) Males were tested for their responses to ketamine in the BPM. There were 15 wild-type and 14 heterozygous age-matched males. A significant gene by ketamine interaction was observed [$F(1,27) = 4.30$, $P < 0.05$]. *Post hoc* analyses (Tukey studentized range test) revealed that the heterozygous mice displayed significantly increased responses to ketamine in comparison with wild-type male controls in the first three blocks. Error bar: SEM. #$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

however, enhanced PPI, or impaired prepulse facilitation, was observed in the heterozygous DISC1-Boymaw mice, suggesting abnormal information processing (Fig. 9C). After ketamine administration in the BPM, significant gene [$F(1,52) = 11.42$, $P < 0.01$], ketamine [$F(1,52) = 24.06$, $P < 0.0001$] and gene X ketamine X sex interaction [$F(1,52) = 5.40$, $P < 0.05$] effects were observed in total distance traveled. In male mice, a significant gene X ketamine interaction [$F(1,26) = 8.61$, $P < 0.01$] was observed in the distance traveled (Fig. 9D) and other measures of locomotion activity (Supplementary Material, Fig. S8B, S8C, S8D). *Post hoc* analyses revealed significantly increased locomotion in the heterozygous DISC1-Boymaw mice injected with ketamine in all blocks except the first and last, suggesting increased and prolonged responses to ketamine. In female mice, a significant gene effect [$F(1,26) = 5.63$, $P < 0.05$] was detected without gene X ketamine interaction (Supplementary Material, Fig. S8E). To examine whether the heterozygous mice display behavioral phenotypes related to depression, a tail suspension test was conducted to assess mouse behavioral despair (45, 46). The heterozygous DISC1-Boymaw mice displayed significantly more immobility time than wild-type sibling controls without sex effects ($P < 0.05$) (Fig. 9E). A saccharin preference test was also conducted to examine anhedonia-like behavior, as seen in depression in humans, in the heterozygous mice. Wild-type female mice displayed significantly higher preference for saccharin than the heterozygous female mice (Fig. 9F). Because wild-type male mice had no significant preference for saccharin, the heterozygous DISC1-Boymaw male mice cannot be assessed for depression-like behavior using this behavioral paradigm (Supplementary Material, Fig. S8F). It has been reported that some rodent strains display a sex difference in saccharin preference in which females have strong preference while males have little preference or even some avoidance (47). In general, 129S mice are reported to have only slight saccharin preference

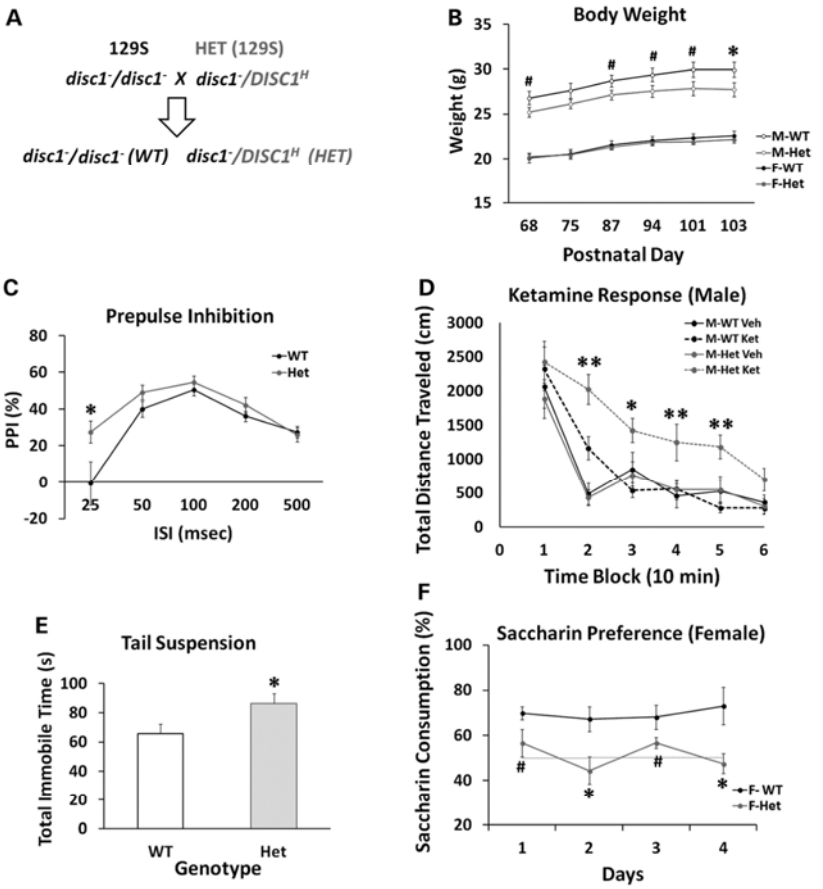

Figure 9. Behavioral characterization of the humanized DISC1-Boymaw mice on 129S background. (**A**) Breeding of wild-type and the heterozygous DISC1-Boymaw mice on 129S pure genetic background. 129S mice carry a frame-shift mutation in exon 6 of the *disc1* gene. (**B**) The cohort consisted of 14 wild-type females, 14 heterozygous females, 13 wild-type males and 15 heterozygous males. The age range of the birth date between individual mice was less than a week. Body weight was compared between the wild-type and the heterozygous mice. Heterozygous males displayed slightly, but significantly, reduced body weight at postnatal Day 103 [unpaired, two-tailed student's *t*-test, $t(26) = 2.132$, $P < 0.05$]. No difference was found in body weight between female wild-type and heterozygous mice. (**C**) There was a significant gene effect [$F(1,54) = 4.41$, $P < 0.05$] in PPI at 69 dB prepulse. A significant gene X ISI interaction was also observed [$F(4,216) = 2.90$, $P < 0.05$]. *Post hoc* analysis revealed that the heterozygous DISC1-Boymaw mice displayed increased PPI (or impaired prepulse facilitation) with ISI at 25 ms. No sex difference was observed. (**D**) The male heterozygous DISC1-Boymaw mice display a significant gene effect [$F(1,26) = 6.26$, $P < 0.05$] as well as a gene X ketamine interaction [$F(1,26) = 8.61$, $P < 0.01$]. *Post hoc* analyses (Tukey studentized range test) revealed that the male heterozygous mice displayed significantly increased and prolonged responses to ketamine in comparison with wild-type male controls in all time blocks except the first and last one. (**E**) Total immobility time (seconds) was recorded during a 6 min suspension test. Five wild-type male mice were lost because of fighting in cages after previous tests. The heterozygous DISC1-Boymaw mice ($n = 29$) displayed significantly more immobility time than the wild-type control mice ($n = 22$) [unpaired, two-tailed student's *t*-test, $t(49) = 2.22$, $P < 0.05$). (**F**) Two or three mice were grouped in a single cage according to genotype. Saccharin preference test was conducted for four consecutive days. Consumption of saccharin was measured each day, and compared between the wild-type and the heterozygous mice in each sex. Significant gene effect was detected [$F(1,11) = 17.85$, $P < 0.01$] between female wild-type ($n = 7$) and the DISC1-Boymaw heterozygous mouse cages ($n = 6$). *Post hoc* analysis revealed that the female heterozygous mice displayed significantly less saccharin consumption than female wild-type controls mice at Day 2 and Day 4. Error bar: SEM. #$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

(http://www.criver.com/SiteCollectionDocuments/129.pdf). These findings may explain why wild-type male mice on the 129S background did not display saccharin preference. Taken together, the humanized DISC1-Boymaw male mice consistently displayed a prolonged response to ketamine on different genetic backgrounds, mimicking phenotypes of schizophrenia and potentially depression. Replication of this phenotype in different cohorts of mice helped rule out type I error by multiple comparisons.

Abnormal information processing of startle and depressive-related behaviors has also been suggested, and further confirmation is needed in future.

Abnormal erythropoiesis in humanized DISC1-Boymaw mice

Mutations of ribosomal protein genes, which impair protein translation, have been demonstrated to cause Diamond–Blackfan anemia (48). Since the human DISC1 gene is also expressed in lymphoblastoid cells, we conducted hematological analysis of the heterozygous DISC1-Boymaw mice to investigate whether inhibition of protein translation by the DISC1-Boymaw fusion gene may cause anemia (Fig. 10A). Interestingly, 2 out of 10 heterozygous DISC1-Boymaw male mice displayed lower concentrations of hemoglobin (Fig. 10B) (Supplementary Material, Table S2), indicating different degrees of anemia (49). A significant difference between the two genotypes is an increase of red blood cell distribution width (RDW), which measures the variation of red blood cell volume, in the heterozygous DISC1-Boymaw mice (Fig. 10C). We re-examined RDW in some of these mice 1 week later to investigate RDW stability. RDW displayed little change in wild-type mice, but higher RDW fluctuation was observed in their sibling heterozygous DISC1-Boymaw mice (Fig. 10D). These data indicated that the DISC1-Boymaw fusion gene impairs the stability of erythropoiesis in the heterozygous DISC1-Boymaw mice.

DISCUSSION

The t(1;11) chromosome translocation in the Scottish family co-segregates with schizophrenia and major depression with 70% penetrance (1–3). Despite extensive studies on the DISC1 gene, the role of the disruption of the DISC1 gene alone remains unclear in the pathogenesis of major psychiatric disorders (35,50,51). Our previous studies revealed that the DISC1 gene disruption is accompanied by disruption of the Boymaw gene and generation of the DISC1-Boymaw and Boymaw-DISC1 fusion genes (10,11). In the present study, we found that expression of the DB7 fusion gene decreases NADH oxidoreductase activities, rRNA synthesis and protein translation in both *in vitro* cell studies and *in vivo* humanized DISC1-Boymaw mice. Despite inhibition of protein translation by the DB7 fusion gene, however, we did not find gross anatomical abnormalities in the brain of the heterozygous DISC1-Boymaw mice. Inhibition of protein translation does not always cause obvious developmental defects. For example, reduced protein translation by silencing translational initiation factor eIF4E isoform (IFE-2) has no effects on *Caenorhabditis elegans* development and body size at 20°C (52). Even though we did not find obvious developmental abnormalities, reduced expression of Gad67, Nmdar1 and Psd95 proteins was observed in the brain of the heterozygous DISC1-Boymaw mice. Behavioral studies showed that the humanized mice display phenotypes related to both schizophrenia and depression. Our studies therefore suggest that the DB7 fusion gene could be one of the causal gene mutation(s) in the pathogenesis of major psychiatric disorders in the Scottish family. It should be kept in mind, however, that the humanized mice also harbor the BD13 fusion gene in addition to the DB7 fusion gene. Reduction of protein translation in the brains of the humanized mice is likely caused by the expression of the DB7 fusion gene, according to our *in vitro* cell transfection studies. However, we cannot rule out a potential contribution from the BD13 fusion gene to the behavioral phenotypes, although we did not find any cellular phenotypes in HEK293T cells expressing the BD13 fusion gene.

Our *in vitro* studies revealed that the DB7 fusion protein is unstable and its expression decreases intracellular oxidoreductase activities, rRNA synthesis and protein translation. These phenotypes are unlikely caused by the insolubility of the DB7 proteins *per se*, since the much more abundant insoluble DISC1 and truncated DISC1 proteins did not generate any of these cellular phenotypes in the over-expressing HEK293T cells. However, the insoluble DISC1 proteins in HEK293T cells may be structurally different from the insoluble proteins found in postmortem human brains (12). Surprisingly, fusion of the Boymaw gene to a randomly selected gene (fluorescence marker) generated the same cellular phenotypes as the DB7 fusion gene. It will be interesting to know whether the human Boymaw gene encodes any protein or peptide. Regardless of the Boymaw gene being a coding or non-coding RNA the DB7 fusion gene appears to be a gain-of-function mutation. The role of the Boymaw gene merits further studies. Unfortunately, there is no human Boymaw gene orthologue in mouse genome.

Eykelenboom *et al* (53) identified three different fusion transcripts from the patient lymphoblastoid cells from the Scottish family. The DB7 fusion gene used in our studies encodes exactly the same fusion protein as their CP60 transcript. The second CP69 fusion transcript is generated by alternative splicing 12 nucleotides upstream of the stop codon of the CP60 transcript. Our deletion analysis demonstrated that the last few amino acid residues of Boymaw are not required for inhibition of MTT reduction. Therefore, it is likely that the CP69 fusion protein is also capable of inhibiting MTT reduction. The third CP1 fusion transcript encodes a protein with just one more amino acid addition to the C-terminus of the truncated DISC1 protein that has been used in the generation of several mouse DISC1 models (54,55). The CP1 protein does not display any detectable toxicity to mitochondria, which is consistent with our observation that over-expression of truncated DISC1 proteins has no effect on MTT reduction, rRNA expression and protein translation. Our immunocytochemical analysis confirmed localization of the DB7 fusion protein (but not DISC1-FL, truncated DISC1 or BD13) in mitochondria (Supplementary Material, Fig. S9). Interestingly, MTT reduction was also reduced in mitochondria of cells expressing the DB7 fusion gene (Fig. 3C). Decreased activities of mitochondrial NADH oxidoreductases may relate to loss of mitochondrial membrane potential in cells expressing the DB7 fusion protein (53). We therefore hypothesize that reduction of oxidoreductase activity may decrease cell metabolism to down-regulate rRNA expression and protein translation. One potential pathway could be that inhibition of the mitochondrial oxidoreductase activities reduces adenosine triphosphate (ATP) production (Fig. 11), which is supported by the finding of loss of mitochondrial membrane potential in cells expressing the DB7 fusion protein (53). Reduction of ATP can trigger activation of the AMP-activated protein kinase (AMPK), a sensor of cell energy status (56). The AMPK pathway is a well-established pathway to regulate protein translation. In this pathway, the activated AMPK phosphorylates TSC1/TSC2 to

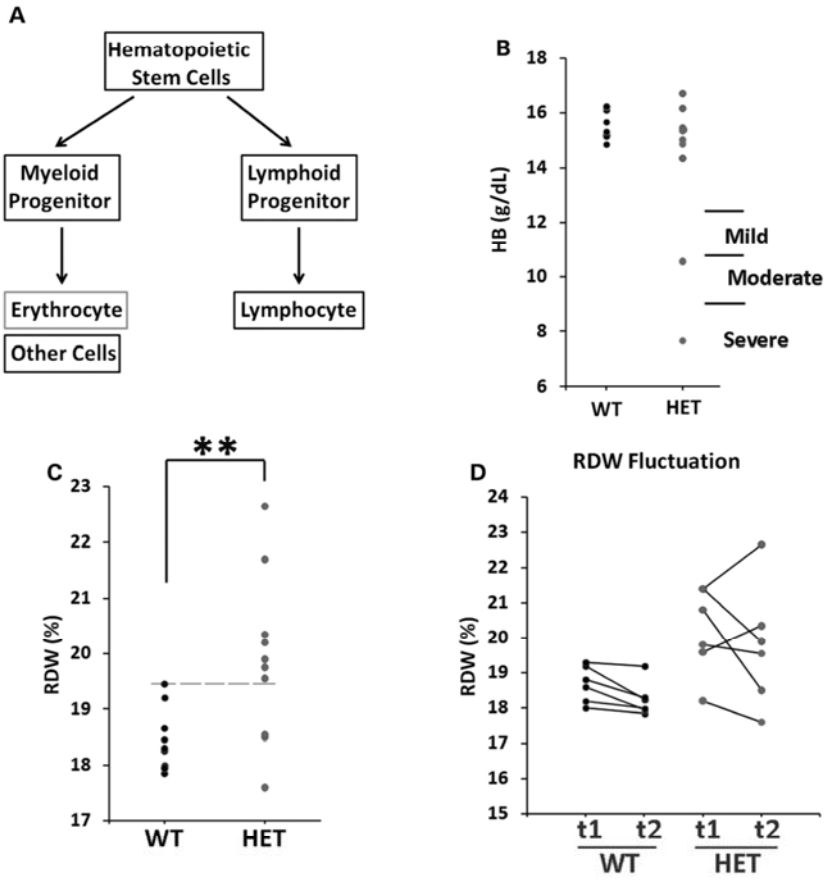

Figure 10. Increased RDW in the humanized DISC1-Boymaw mice. **(A)** Schematic pathway of erythropoiesis. **(B)** Concentration of hemoglobin (HB) was measured in the blood of individual male mice. Severe, moderate and mild anemia was diagnosed according to hemoglobin concentrations (49). Two heterozygous DISC1-Boymaw mice suffered from severe and moderate anemia. All wild-type mice were normal. Each dot represents an individual mouse. **(C)** Homogeneity of variances of RDW data was first assessed with Levene's test ($P = 0.057$). Significantly higher RDW was found in the heterozygous DISC1-Boymaw mice [$F(1,19) = 8.451$, $^{**}P < 0.01$]. Most of these mice have RDW above the upper limit of the normal RDW range of their wild-type sibling mice (dashed line). **(D)** Six wild-type and six heterozygous DISC1-Boymaw male mice were re-examined 1 week later (t2) for their RDW stability, and compared with their initial ($t1$) RDW measurement. There was little change in RDW of the wild-type mice during this period. However, high magnitude of RDW fluctuation was observed in the heterozygous DISC1-Boymaw mice, indicating unstable erythropoiesis.

inhibit mammalian target of rapamycin (mTOR). mTOR kinase plays a central role to stimulate protein translation either directly via activation of translational initiation factors (57) or indirectly via phosphorylating transcription initiation factor TIF-1A to promote rRNA transcription (58). Mutation of TSC1/TSC2 leads to autism (59), which shares many common psychopathological features with major psychiatric disorders. Another potential pathway linking cellular metabolism to rRNA transcription could be initiated through the Sirtuin family of proteins, which are NAD^+-dependent deacetylase to sense cellular NAD^+ changes. SIRT1 (Sirtuin 1) has been demonstrated to epigenetically silence rDNA chromatin (60, 61). However, it is also reported that nucleolar SIRT7 (Sirtuin 7) functions as an activator for rRNA transcription (62). It is clear that the potential underlying mechanisms are complex and much more work is needed.

One of the key questions is how expression of the DB7 fusion protein reduces oxidoreductase activity. Identification of these NADH oxidoreductases in mitochondrion and cytoplasm is critical to unravel the signaling pathways from cellular metabolic activity to protein translation. In contrast to inhibition of protein translation by the DB7 fusion gene, exaggerated protein translation has been suggested as the core pathophysiology of Fragile

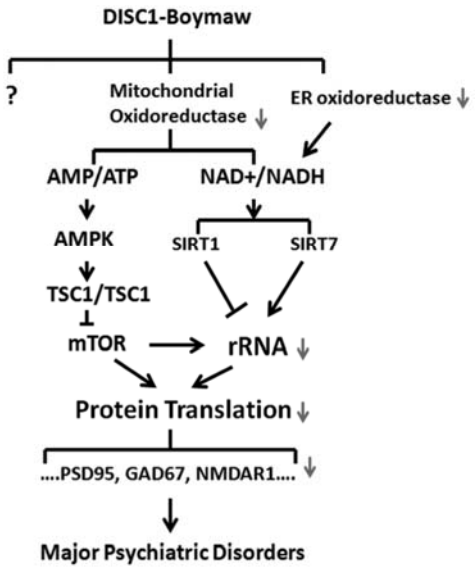

Figure 11. Hypothetical pathways of the DB7 fusion gene. Cell metabolism is tightly coupled with protein synthesis. Reduction of NADH oxidoreductase activity may alter production of ATP and NAD^+ which can trigger at least two known pathways (AMPK and Sirtuin) to down-regulate rRNA transcription and protein translation. AMPK, AMP-activated protein kinase; TSC1 and TSC2, tuberous sclerosis complex 1 and 2; mTOR, mammalian target of rapamycin.

X Syndrome (63) and other autism genes (24). It is possible that an optimal protein translation is necessary for neural plasticity (64), and excessive or less protein translation are harmful to brain functions. Translational control of protein synthesis may play important roles in many other psychiatric disorders. Indeed, it was reported that hypermethylation of ribosomal RNA gene promoter was associated with suicide (65), mild cognitive impairment and Alzheimer's disease (66), as well as Huntington's diseases (67). Considering that rRNA synthesis is a rate-limiting step of ribosome biogenesis (68), reduction of rRNA will likely result in a decrease of protein translation. Our SUnSET experiments clearly demonstrated inhibition of protein translation in both the cells transiently expressing the DB7 fusion gene and the heterozygous DISC1-Boymaw mice. Since we did not find significant reduction of mRNA transcripts of *Gad67*, *Nmdar1* and *Psd95* genes, the reduction of general protein translation therefore could be responsible for the decrease of Gad67, Nmdar1 and Psd95 protein expression in the heterozygous DISC1-Boymaw mice. Indeed, it was reported that cellular abundance of proteins is predominantly controlled at the level of protein translation (69,70). However, it is also possible that protein turnover may be altered and contributes to the reduction of these proteins in the humanized mice. The effects of the expression of the DB7 gene may not be restricted to the inhibition of protein translation. For example, we observed reduced expression of Cyb5r3 reductase in endoplasmic reticulum (ER), where the enzyme plays an important role in lipid biosynthesis and has been demonstrated to be essential for brain development (71–73).

Impaired NMDAR-mediated neurotransmission has been well established in the pathophysiology of schizophrenia. Prolonged ketamine effects and exacerbation of symptoms are observed in schizophrenia patients (39–41) and our mouse genetic model (74). Consistent with the findings in human patients, we found reduction of both Nmdar1 and Gad67 proteins in heterozygous DISC1-Boymaw mice which display prolonged responses to the non-competitive NMDAR antagonist, ketamine. This phenotype has been replicated in different cohorts of mice on various genetic backgrounds regardless of the presence or absence of wild-type *Disc1* gene. It is unknown why the female humanized mice do not show prolonged responses to ketamine. The female humanized mice are not symptom-free, since they display abnormal phenotypes in other behavioral paradigms. We found increased PPI in the heterozygous DISC1-Boymaw mice at a short ISI (25 ms) in both sexes. In mice, PPI diminishes with decreasing ISI starting from 100 ms, and eventually transforms into prepulse facilitation. Abnormally enhanced PPI at short ISI is indicative of altered information processing in the heterozygous DISC1-Boymaw mice. Although this alteration in PPI differs from that seen in patients with schizophrenia (75), enhanced PPI at short ISI has been reported in rats treated with dopamine receptor agonists (76, 77).

Inhibition of protein translation by the fusion gene impacts not only the brain but also erythropoiesis of the heterozygous DISC1-Boymaw mice. We found an incomplete penetrance of the DISC1-Boymaw fusion gene to anemia, consistent with the role of impaired protein translation demonstrated by ribosomal protein gene mutations in Diamond–Blackfan anemia (48). Interestingly, the heterozygous DISC1-Boymaw mice also display increased RDW. In human, over 2 million red blood cells are generated every second with small variation in cell volume (human RDW: 11.5–14.5%). It is possible that such an extremely active and precise biological process in mouse is highly susceptible to the DISC1-Boymaw mutation which confers even mild inhibition of protein translation. Recently, an association between increased RDW and depression was reported from a multi-year large scale study in humans (78). The study found a gradient positive correlation between increased RDW and the risk of developing depression in thousands of patients. It will be interesting to know whether patients with major depression in the Scottish family may display increased RDW.

It should be kept in mind that our studies are focused on the investigation of the biological functions of the DISC1-Boymaw fusion genes. To that end, the 129S genetic background, which contains a frame-shift mutation in endogenous *disc1* gene, provides a unique advantage for our studies to avoid *Disc1* gene dosage difference between wild-type and the heterozygous DISC1-Boymaw mice. However, the chromosome translocation in the Scottish family generates not only the fusion genes but also loss of one allele of the *DISC1* gene. Therefore, our humanized DISC1-Boymaw mice on 129S background should be viewed as a genetic model for the DISC1-Boymaw fusion genes rather than the chromosome translocation in the Scottish family. In the future, we could generate mice carrying full-length human *DISC1* genes on mouse *Disc1* locus using the *Cre* recombinase. After breeding these *DISC1* mice with the heterozygous DISC1-Boymaw mice, we could generate wild-type mice carrying two human *DISC1* (*DISC1/DISC1*) genes and heterozygous

mice carrying one human *DISC1* and one DISC1-Boymaw fusion gene (*DISC1/DISC1^{H}*). Comparisons between wild-type (*DISC1/DISC1*) and the heterozygous (*DISC1/DISC1^{H}*) will provide a more complete model for the chromosome translocation in the Scottish family.

MATERIALS AND METHODS

DNA constructs

Full length DISC1, truncated DISC1, DB7 and BD13 fusion genes were generated through PCR amplification and ligation. All expression constructs were tagged with HA epitopes as described (11). The adeno-associated virus (AAV) shuttle and pTimer plasmid vectors were used to generate expression constructs, bi-cistronic constructs connected with the IRES of encephalomyocarditis virus (EMCV) virus (79), and FT fusion protein constructs, respectively. Boymaw was also fused with FT in pTimer plasmid vector. All constructs were confirmed with complete sequencing. A series of FT-Boymaw C-terminal deletion were generated by using PCR amplification and subsequent In-Fusion ligation (Clontech). The following pairs of PCR primers were used: C1-del: Boymaw-C1-del-f: 5′CCTTGAGTAC CA TACGACG TTCCAGACT3′; Boymaw-C1-del-r: 5′TATGGGT ACT CAAGGCTTCT ATCTGTGGT3′; C2-del: Boymaw-C 2-del-f: 5′TCTACCATAC CCATACGACG TTCCAGACT3′; Boymaw-C2-del-r: 5′TATGGGTATG GTAGAAGCCT GCAG ACAAAG3′; Boymaw-C3-del-f: 5′GAAGTCATAC CCATA CGACG TTCCAGACT3′; Boymaw-C3-del-r: 5′TATGGGTA TG ACTTCAGGTA ACTGAAACC3′; Boymaw-C4-del-f: 5′GA GACATTAC CCATACGACG TTCCAGACT3′; Boymaw-C4-del-r: 5′TATGGGTAAT GTCTCTTATT CCAGTCTGT3′.

In vitro cell culture

HEK293T, COS-7 and SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin (Life Technologies, CA) at 37°C in a humidified atmosphere containing 5% CO_2. HEK293T, COS-7 cell transfections were performed using Mirus TransIT® reagent (Mirus, WI), according to manufacturer's instructions. SH-SY5Y cells were transduced with recombinant AAV virus. Primary culture of mouse neuronal cells was established from both wild-type and heterozygous DISC1-Boymaw embryos at E18.5 and P1 as described in our previous studies (80). The mouse cerebrum were dissected and cut into small pieces in Neurobasal A medium on ice. The tissue was triturated 10 times with a fire polished 9-in. Pasteur pipette and allowed to settle on ice for 1 min. The supernatant was then transferred to a new tube and centrifuged gently at 600–700 rpm for 5 min to pellet the cells. The cells were resuspended in B27/Neurobasal medium (B27/Neurobasal with 0.5 mM glutamine, no glutamate, 5 ng/ml FGF2).

MTT reduction assays

HEK293T cells and COS-7 cells were seeded at cell density of 1×10^5 cells per well in 24-well plates, 24 h prior to transfection. Cell proliferation was measured by counting cell numbers with 0.04% Trypan blue 48 h after transfection. MTT reduction assay was modified from Mosmann T (81). MTT (Sigma, MO) was dissolved in 1× PBS at the concentration of 5 mg/ml, and filtered through 0.2 μm membrane. The stock solution was stored at −20°C. For MTT reduction in living cells, MTT stock solution was added at the final concentration of 0.5 mg/ml, and the cells were incubated at 37°C for 1.25 h. For MTT assay in cell lysate, transfected cells were first lysed in lysis buffer (18) (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.01%Triton X-100, protease inhibitors cocktail) at 4°C for 30 min with gentle shaking. MTT was added to the lysate at the final concentration of 0.5 mg/ml, and the reaction was incubated at 37°C for 1 h. For MTT assay in purified ER and mitochondria, NADH (Sigma, MO) was added in the assay at the final concentration of 0.5 mM, and the reactions were incubated at 37°C for 10 min for mitochondria, and 30 min for ER, respectively. For the reversal of deficient MTT reduction, 2.5 Unit of *E. coli* ADH (Cat# 49641, Sigma, MO) was added into the DISC1-FL and DB7 cell lysate, respectively, and further incubated at 37°C for 2 h. MTT crystal were collected and dissolved in DMSO. Absorbance at 540 nm was measured with SpectraMax M5 Multi-Mode Microplate Readers (Molecular Devices, PA). Neonatal mouse brains were dissected on postnatal Day 1. After washing three times with pre-chilled PBS, brains were cut into small pieces in 1.5 ml isotonic extraction buffer (IEB) (10 mM HEPES, pH 7.8 with 0.25 M sucrose, 1 mM EGTA, and 25 mM KCl) containing protease inhibitor cocktail (Sigma, MO), and further homogenized with Dounce homogenizer on ice. Equal amounts of protein homogenate were used for MTT reduction assays.

Western blot analysis

Supernatant soluble proteins were extracted from HEK293T cells and COS-7 cells in Passive Lysis Buffer (PLB) (Promega, WI) containing 0.2% Sarkosyl and 1× protease inhibitor cocktail (Sigma, MO) at room temperature for 15 min on a shaker. Total proteins from cell pellets, endoplasmic reticulum, mitochondria and brain homogenate of neonatal mice were solubilized with sonication in PLB containing 1× protease inhibitor cocktail. Protein concentration was measured with Bradford (Abs. 595 nm) method using Coomassie Plus Protein Assay (Thermo Scientific, IL). After electrophoresis, proteins were transferred onto PVDF membranes which were subsequently blocked with 5% non-fat dry milk in TBST buffer (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. The membranes were incubated with the following primary antibodies at 4°C overnight: mouse monoclonal anti-cytochrome C (1:5000), ab110325 (Abcam, MA); rabbit polyclonal anti-Cyb5r3 (1:600), sc-67284; mouse monoclonal anti-Cypor (1:400); sc-25270; mouse monoclonal anti-β-actin (1:5000); sc-47778 (Santa Cruz Biotechnology, CA). After washing three times, the membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5000, Cell signaling, MA), anti-rabbit IgG (1:5000, Santa cruz, CA) for 1.5 h at room temperature. Rat monoclonal anti-HA antibody (1:500) conjugated with peroxidase (3F10) (Roche, CA) was used to directly detect HA epitope. Quantification of protein expression was conducted with Image J.

Proteomic analysis

HEK293T cells were transfected with DISC1-FL and DB7 genes, respectively. Two days later, cells were harvested and sent to UCSD Mass Spectrometry Facility Core for iTRAQ analysis. The samples were labeled with 114, 115, 116 and 117 Da mass tags, respectively. Protein identification and quantitation were performed using the Paragon™ Algorithm in ProteinPilot™ Software, searching against the NCBInr database.

NADH concentration

Intracellular NADH extraction was conducted as described in Cyclex NAD+/NADH colorimetric Assay Kit (CycLex, Japan). In brief, 2×10^5 cells were seeded in each well of a12-well plate one day before transfection. Cells were collected 48 h post-transfection. NADH extraction buffer [50 mM NaOH and 1 mM ethylenediaminetetraacetic acid (EDTA)] was added to cell pellets and gently vortexed 2–3 times. To reduce viscosity, samples were incubated at 60°C for 30 min. Equal volume of neutralization buffer (0.3 M potassium phosphate buffer, pH 7.4) was added and incubated at least 5 min on ice. NADH was extracted from the supernatant solution after spinning down the neutralized samples at 15 000 rpm for 5 min at 4°C. The cell pellets were solubilized to measure protein concentration for the normalization of NADH concentration. *E. coli* ADH (Sigma) was used to measure NADH concentration in MTT reduction assays.

Density-gradient ultracentrifugation

Mitochondria and ER were purified from HEK293T cells and neonatal mouse brains via density-gradient ultracentrifugation as described in Endoplasmic Reticulum Isolation Kit (Sigma, MO). In brief, HEK293 T cells ($\sim 5 \times 10^7$) were incubated in hypotonic buffer (10 mM HEPES pH7.8, 1 mM EGTA and 25 mM KCl) for 20 min at 4°C. The cells were pelleted with 600 × g for 5 min, and resuspended with appropriate volume of the IEB (10 mM HEPES, pH 7.8, with 0.25 M sucrose, 1 mM EGTA and 25 mM KCl). For mouse brains, dissected brains were cut into small pieces in the IEB. The cells and the tissue were further homogenized with Dounce homogenizer on ice with 2 ml of IEB. Large cellular debris and nuclei were pelleted after centrifuging at 1000 × g for 10 min at 4°C. The supernatant was transferred into a new centrifuge tube and further centrifuged at 12 000 × g for 15 min at 4°C. Crude mitochondria were pelleted, and the supernatant fraction containing ER was transferred to a new ultracentrifuge tube and centrifuged at 150 000 × g in a Beckman Optima™ LE-80K (Beckman Coulter, CA) at 4°C for 1 h to purify ER. ER pellets were resuspended in IEB containing protease inhibitors. Crude mitochondrion pellet was further purified as described (82). In brief, mitochondrion pellet was resuspended in 1 ml of mitochondrion isolation buffer (MIB) (70 mM sucrose, 210 mM D-mannitol, 1 mM EDTA-K_2, 0.23 mM PMSF, 5 mM HEPES–Tris, pH 7.4, and protease inhibitors cocktail) and layered onto 1.5 ml of 7.5% (w/v) Ficoll-sucrose medium (7.5% Ficoll-MIB) on top of 1 ml of 10% (w/v) Ficoll-sucrose medium (10% Ficoll-MIB) and centrifuged in a Beckman Optima™ LE-80K (Beckman Coulter, CA) for 1.5 h at 100 000 × g at 4°C. Mitochondria were pelleted at the bottom of the ultracentrifuge tube. The pellets were suspended in 1 ml of MIB and then centrifuged for 5 min at 15 000 × g at 4°C. The mitochondrion pellets were washed once in MIB and finally resuspended in appropriate volume of MIB. The purified mitochondria were either used for MTT reduction or stored at −80°C for subsequent analyses.

SUnSET

HEK293 cells seeded at cell density of 1×10^5 cells per well in 24-well plates, 24 h prior to transfection. After transfection, cells were pulse-labeled with puromycin at the concentration of 10 μg/ml for 10 min in the presence or absence of cycloheximide at final concentration of 25 μM. Total proteins were extracted and load on polyacrylamide electrophoresis gel electrophoresis (PAGE) gel (10 μg per lane) for western blot analysis. Anti-puromycin antibody (1:10 000) (12D10, Millipore) was used to detect incorporation of puromycin in newly synthesized proteins. Blots were stripped later and re-probed with anti-β-actin antibody. Image J was used to quantify the incorporation of puromycin.

Generation of humanized DISC1-Boymaw mice

Both DB7 and BD13 fusion genes were connected with IRES of EMCV virus (11, 79). The bi-cistronic gene was terminated with SV40 poly (A). A splicing acceptor was added to generate in-frame splicing of the bi-cistron with the first exon of human DISC1 gene. The bi-cistron gene cassette was flanked with two *loxP* sites. Downstream of the bi-cistron, human DISC1 cDNA was capped with a splicing acceptor. Such a design aimed for conditional restoration of DISC1 gene expression in genetic rescue experiments in the future. Two homology arms with respective sizes of 4 and 3 kb were amplified from the mouse disc1 gene of 129S ES cells (129S2/SvPasCrl) to assemble the final gene targeting construct. The final gene targeting construct was electroporated into mouse embryonic stem (ES) cells. After positive and negative selections with G418 and ganciclovir, ∼1500 ES cell colonies were picked for further screening. Two homologous recombinant ES cell colonies were identified. After blastocyst injection, chimeric mice were obtained and bred with 129S females for germline transmission of the targeted DISC1 allele.

Mouse breeding

Heterozygous DISC1-Boymaw mice were bred with wild-type mice to generate wild-type and heterozygous mice on 129S genetic background (129S2/SvPasCrl, Charles River) for all molecular studies. Different cohorts of mice were generated on the F1 hybrid (129S/C57) and 129S genetic backgrounds for behavioral studies. PCR was used for DISC1-Boymaw mouse genotyping (DISC1-Common-R: 5′TAACAACAGCCAGTGTGC AAG 3′; DISC1-Neo-F: 5′GGTGGGCTCTATGGCTTCTG A3′; DISC1-F: 5′TGCATTCACATGTGTTGCCTT TG 3′). Mice were housed in groups (two to four mice per cage) in a climate-controlled animal colony with a reversed day/night cycle. Behavioral testing occurred during the dark cycle. Food (Harlan Teklab, Madison, WI) and water were available *ad*

libitum, except during behavioral testing. All behavioral testing procedures were approved by the UCSD Institutional Animal Care and Use Committee (permit number: A3033–01) prior to the onset of the experiments. Mice were maintained in American Association for Accreditation of Laboratory Animal Care (AALAC) approved animal facilities. For behavioral studies, all mice were 2- to 6-month old siblings, and the age range of the birth date between all mice was less than a week.

Hematological analysis

A cohort of wild-type and heterozygous DISC1-Boymaw mice was generated on 129S background. Male mice were anesthetized with isofluorane before blood collection. Submandibular venous lancets (Goldenrod 5 mm) were used to collect ~100 μL into EDTA anticoagulated 0.5 ml Microtainer tubes (Becton-Dickinson). Hematological analysis was conducted using the Hemavet 950 FS at the UC San Diego Hematology Core Laboratory. Six adult wild-type male mice and six heterozygous male DISC1-Boymaw siblings were first used for hematological analysis. To replicate the findings, another independent group of wild-type mice and their heterozygous DISC1-Boymaw siblings ($N = 4$, for each genotype) was used.

RNA, DNA extraction and RT-PCR analysis

DNA and RNA were extracted from cells and mouse brains using Trizol method. RNA expression of the DB7 fusion gene was examined with RT-PCR using the primers: human DISC1 e1-f: 5′GCGGCGTGAGCCACCGCGCAG3′ human DISC1 e2-r: 5′GAACCGGAACAGTGTGCCCAC3′. Mouse endogenous *disc1* RNA expression was examined for alternative splicing of exon 6 using primers:

mouse Disc1 exon2f: 5′CCATGCTGCAGGACTACCTA3′
mouse Disc1 exon3f: 5′GAAAAAGTGGTCGAGGATGG3′
mouse Disc1 exon4f: 5′GCAGCTTCTTGGGTTACCTG3′
mouse Disc1 exon5f: 5′ACCAGGAGGGACTGGCTTAT3′
mouse Disc1 exon6f: 5′GCTGTGACCTGATGGCACT3′
mouse Disc1 exon10r: 5′ACCTTCCAACACTTCCATGC3′

For the real-time RT-PCR quantification of mRNA transcripts of *Gad67*, *Nmdar1* and *Psd95* genes, cDNA was first synthesized with random hexamers using the Superscript III First-Strand Synthesis Kit (Invitrogen). A comparative Ct method was used for quantification with SYBR Green, according to the manufacturer's protocol (Bio-Rad CFX384). Beta-actin was used as a reference control for normalization. The following primers were used in Q-PCR:

Mouse *Gad67* f: 5′TCACCCTCGATTTTTCAACC3′
Mouse *Gad67* r: 5′TTGACCATCCAACGATCTCTC3′
Mouse *Nmdar1* f: 5′GGCAGTAAACCAGGCCAATA3′
Mouse *Nmdar1* r: 5′GTGGGAGTGAAGTGGTCGTT3′
Mouse *Psd95* f: 5′AAGTCAGAGCCCCCTACTCG3′
Mouse *Psd95* r: 5′AGGGTGTCCGTGTTGACAAT3′
Mouse β-Actin f: 5′TTCTACAATGAGCTGCGTGTG3′
Mouse β-Actin r: 5′GGGGTGTTGAAGGTCTCAAA3′

Immunohistochemical and immunocytochemical staining and western blot

Immunohistochemical staining of parvalbumin, Gad67, Nmdar1 were conducted in mouse brain paraffin sections as described in our previous publication (83). For immunocytochemical staining, primary neurons were fixed in 3.7% formalin/PBS at room temperature for 10 min. After 1 h of blocking with 5% goat serum containing 0.2% Triton X-100 at room temperature, the slides were incubated with mouse monoclonal anti-DISC1 (3D4) (1:1000 dilution, gift from Dr Carsten Korth) and rabbit polyclonal anti-MAP2 (1:2,000 dilution, PRB-547C, Covance) antibodies at 4°C overnight. After rinsing three times with PBST, the slides were incubated with Alexa Fluor® 568 goat anti-mouse IgG (1:500 dilution, Invitrogen) and Alexa Fluor® 488 goat anti-rabbit IgG (1:500 dilution, Invitrogen) for 1 h at room temperature. The slides were washed three times with PBST each for 5 min, and mounted with VECTASHIELD® Hard Set™ Mounting Medium with DAPI (Vector, CA). The DB7 fusion proteins were detected in western blot analysis of primary neuron culture using rabbit polyclonal anti-DISC1 antibody (1:500 dilution, ab55808, Abcam) recognizing N-terminal residues of human DISC1proteins. Expression of Gad67, Nmdar1, Psd95, synaptophysin and β-actin in mouse brain and primary culture of neuronal cells was examined using the antibodies: mouse monoclonal anti-Gad67 (1:10 000 dilution, G5419, sigma); mouse monoclonal anti-Nmdar1 (1:5000 dilution, 556308, BD Biosciences); mouse monoclonal anti-Psd95 (1:3000 dilution, Ab99009, Abcam); rabbit polyclonal anti-synaptophysin (1:3000 dilution, 5461S, Cell signaling); mouse monoclonal anti-β-actin (1:5000 dilution, sc-47778, Santa Cruz Biotechnology).

SUnSET in mice

The *in vivo* SUnSET experiments were conducted as described (24). Sex-, and age-matched 2-month old sibling wild-type and heterozygous mice were anesthetized. Unilateral ICV injection of puromycin (25 μg/2.5 μl) was performed with coordinates: −0.22 mm anterioposterior, −1.0 mm mediolateral, and −2.4 mm dorsoventral. Mice were sacrificed by cervical dislocation 1 h after the injection. The hippocampus was dissected for total protein extraction. Equal amounts of protein (70 μg) were loaded on PAGE gel for western blot analysis. Anti-puromycin antibody (1:1000)(12D10, Millipore) was used to detect incorporation of puromycin in newly synthesized proteins. Blots were then stripped and re-probed with ant-β-actin antibody. Image J was used to quantify the incorporation of puromycin.

RNA *in situ* hybridization

We developed a modified protocol for chromogenic RNA *in situ* hybridization according to branched DNA amplification assays (84, 85). Oligonucleotide adaptors were added to the 5′ end of three 18S rRNA probes (5′TCACTGTACC GGCCGTGCGT3′; 5′CCTAGCTGCGGTATCCAGGC3′; 5′GCGATCGGCCCG AGGTTATC3′). In brief, paraffin sections were dewaxed, rehydrated and partially digested with pepsin (4 mg/ml) at 37°C for 10 min. After PBS washing, sections were pre-hybridized with Quick-hyb Solution (Stratagene, 201220) with 0.1 M DTT, and 50 μg/ml denatured ssDNA. Pre-hybridization was conducted

at 40°C for 15 min in pre-heated wet plastic chamber. After pre-hybridization, probes were added to the final concentration of 200 ng/ml. Hybridization was conducted at 40°C overnight in pre-heated wet pyrex chamber. After hybridization, sections were washed, and hybridized with oligonucleotide amplifier labeled with biotin. Horseradish peroxidase-conjugated avidin (Thermo Scientific, 21123) was used to bind biotin at final concentration of 5.5 μg/ml. Chromogenic reaction was performed using DAB Peroxidase Substrate Kit (Catalog# SK-4100, Vector Labs).

Acoustic startle

Startle reactivity and PPI were measured with startle chambers (SR-LAB, San Diego Instruments, San Diego, CA) using 65 dB background noise, three types of prepulse-pulse trials (69, 73 and 81 dB) and pulse-alone (120 dB) (86). The session started with five pulse-alone trials at 120 dB intensity to stabilize startle. The prepulse testing block contained three prepulse trial types in which the 69 dB prepulse onset preceded the 120 dB pulse by 25, 50, 100, 200 and 500 ms. Prepulse trials and pulse alone trials were presented in a pseudorandom order. The session ended with five pulse-alone trials. There was an average of 15 s (range: 12–30 s) between trials. Mice were assigned in a pseudo-random order, and placed in the startle chambers, where a 65-dB background noise level was presented for a 10-min acclimation period and continued throughout the test session.

Tail suspension

The tail suspension test was conducted as described previously with some modifications (45, 46). In brief, mice were suspended by the tail with adhesive tape placed 1–2 cm from the end of the tail. The mice were visually isolated at the center of a white box. Mice were suspended for 6 min, and the duration of immobility—during which the mice did not produce any apparent voluntary movements—was recorded by an observer blind to genotype.

Saccharin preference

The saccharin preference test was conducted as described with some modifications (87). In brief, mice were separated and housed, two or three per cage, by genotype. Saccharin sodium salt hydrate (Sigma) was dissolved in tap water to 1.0%. Each cage was supplied with one bottle of saccharin solution and one bottle of normal drinking water. The bottles were weighed before placement in each cage and 24, 48, 72 and 96 h thereafter. Preference for the saccharin solution was calculated as the percentage of saccharin solution consumed out of the total liquid consumption each day. Food was available *ad libitum* during the course of the experiment.

Behavioral pattern monitor

Spontaneous behavioral data were recorded using the mouse BPM (San Diego Instruments, San Diego, CA), as described previously (44, 88). In brief, a single chamber consists of a 30.5 × 61 × 38-cm area, with a Plexiglas hole board floor that was equipped with floor holes in the front, middle, and rear parts of the floor and eight wall holes (three along each side of the long walls and two holes in the front and back walls). Mice were tested during the dark phase of their light cycle. During testing, a white noise generator produced background noise at 65 dB. The measurement of transitions, center time and spatial coefficient of variation were based on the nine divided regions of the chambers. The status of the photobeams was sampled every 0.1 s. The session lasted 60 min. Raw data were transformed into the location of the animal (in $X-Y$ coordinates), whether holepoking or rearing occurred (events), and the duration of each event (time). The chambers were cleaned thoroughly between testing sessions.

Ketamine treatment

Ketamine was dissolved in saline, and administered i.p. at a volume of 5 ml/kg with a dosage of 60 mg/kg immediately prior to the start of the BPM session. A within-subjects design was used for the ketamine studies. After administration of ketamine, mouse locomotor activities were recorded for 60 min in BPM chambers.

Statistical analysis

All data were first tested for normal distribution using the Kolmogorov–Smirnov test before calculation of differences. For statistical analyses, repeated-measures analysis of variance (ANOVA) with genotypes as a between-subjects factor and drug treatment, block and prepulse intensity as within-subjects factors were performed on the %PPI data and locomotor activity (e.g., total distance). *Post hoc* analyses were carried out using Newman–Keuls or Tukey's test. Alpha level was set to 0.05. All statistical analyses were carried out using the BMDP statistical software (Statistical Solutions Inc., Saugus, MA).

AUTHORS' CONTRIBUTIONS

X.Z. conceived, performed, analyzed and wrote the studies. B.J., K.H., M.K., L.Z. and J.Y. performed and analyzed the research. M.G. provided critical discussion and comments on the studies.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Chapter 1, in full, is a reprint of the material as it appears in Ji B, Higa KK, Kim M, Zhou L, Young JW, Geyer MA, Zhou X. *Inhibition of Protein Translation by the DISC1-Boymaw Fusion Gene from a Scottish Family with Major Psychiatric Disorders*. Human Molecular Genetics. 2014 Nov 1;23(21):5683-705. The dissertation author assisted with animal experimentation and data analyses and provided input during manuscript preparation.

Chapter 2:

Mitochondrial dysfunction in humanized DISC1-Boymaw mice

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Abstract

Mitochondrial dysfunction has been implicated in the pathogenesis of psychiatric disorders. In particular, disruption to the Disrupted-in-Schizophrenia 1 (*DISC1*) gene may alter mitochondrial function and transport, contributing to abnormal neuronal development and function in patients with depression, bipolar disorder, and schizophrenia. We have found that fusion of *DISC1* with *Boymaw* may also contribute to mitochondrial and metabolic dysfunction in mice carrying the human *DISC1-Boymaw* fusion genes and potentially in human carriers of the *DISC1* translocation. Although there is evidence for impaired mitochondrial functioning at the cellular level in our humanized mice, interactions with additional genetic and environmental insults may be necessary to observe behavioral and metabolic dysfunction on a global level.

Introduction

Mitochondria serve essential functions, including energy production and calcium buffering, that are necessary for healthy neuronal development and communication (Nemoto et al. 2000; Mattson et al. 2008; Rizzuto et al. 2012; Khacho and Slack 2017). Disturbances to mitochondrial function can lead to neuronal degeneration and death (Di Carlo et al. 2012). Therefore, it is not surprising that mitochondrial dysfunction has been implicated in the pathogenesis of neurological disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Lin and Beal 2006; Carvalho et al. 2015). More recently, mitochondrial dysfunction has been associated with psychiatric disorders, including major depression, bipolar disorder, and schizophrenia (Kung and Roberts 1999; Rezin et al. 2009; Manji et al. 2012; Flippo and Strack 2017).

For example, patients with bipolar disorder and schizophrenia have impaired ATP production (Deicken et al. 1995; Gardner et al. 2003; Clay et al. 2011) and Ca^{2+} homeostasis (Yoon et al. 2001; Bojarski et al. 2010; Giegling et al. 2010), both of which rely on intact mitochondrial functioning. Genome-wide association studies have identified numerous single-nucleotide polymorphisms in mitochondrial DNA associated with major psychiatric disorders in the general population (Rollins et al. 2009; Hudson et al. 2014). Furthermore, patients with mitochondrial disorders often present with psychiatric disorders (Fattal et al. 2006; Inczedy-Farkas et al. 2012), and the prevalence of type 2 diabetes mellitus is increased in patients with severe mental illness (Holt 2006; Meetoo 2013; Vancampfort et al. 2016). The underlying mechanisms linking mitochondrial dysfunction and psychiatric disorders remain poorly understood, but recent studies on animal models of mitochondrial and

psychiatric disorders have begun to bridge that gap (Kasahara et al. 2006; Gong et al. 2011; Manji et al. 2012).

In particular, *Disrupted-in-Schizophrenia* (*DISC1*) has been found to play a role in mitochondrial function and distribution, and disruption to this gene may contribute to the pathogenesis of psychiatric disorders (Park and Park 2012; Norkett et al. 2016). A balanced translocation in *DISC1* was found to co-segregate with major psychiatric disorders across five generations in a Scottish family (St. Clair et al. 1990; Blackwood et al. 2001). The high penetrance of the *DISC1* translocation for psychiatric disorders has led to discoveries of its role in neuronal development, signaling, and synapse regulation (Brandon and Sawa 2011; Niwa et al. 2016; Tomoda et al. 2016).

In vivo and *in vitro* studies have found that DISC1 proteins localize to mitochondria and interact with mitochondrial proteins, including mitofilin and CHCHD6 (Park et al. 2010; Norkett et al. 2016; Piñero-Martos et al. 2016). Mutations to *DISC1* may increase localization of DISC1 to mitochondria, leading to aberrant mitochondrial trafficking and function (Ogawa et al. 2014; Norkett et al. 2017). Both knockdown of *DISC1* and expression of truncated *DISC1* (amino acids 1-597) resulted in reduced NADH oxidoreductase activity and ATP reduction (Park et al. 2010).

Our previous studies found that the chromosome translocation in the Scottish *DISC1* family also disrupts another gene, *Boymaw*, on chromosome 11 (Zhou et al. 2008). After translocation, two fusion genes (*DISC1-Boymaw* and *Boymaw-DISC1*) may be generated between the *DISC1* and *Boymaw* genes (Zhou et al. 2008, 2010; Eykelenboom et al. 2012). We generated mice carrying the human fusion genes, *DISC1-Boymaw* (*DB7*) and *Boymaw-*

DISC1 (*BD13*). These mice exhibit heightened sensitivity to ketamine, as well as some depressive-like symptoms (Ji et al. 2014). We and others found that the DB7 protein localizes to mitochondria more than wild-type DISC1 and truncated DISC1 in COS7 cells and primary neurons (Eykelenboom et al. 2012; Ji et al. 2014; Norkett et al. 2016). Interestingly, we found that the Boymaw protein alone specifically localizes to mitochondria, and both *DB7* and *Boymaw* inhibit NADH oxidoreductase activity (Ji et al. 2014, 2015). We also found in postmortem samples that *Boymaw* RNA expression is significantly increased in the brains of patients with major psychiatric disorders compared to healthy controls (Ji et al. 2015). These findings suggest that Boymaw and the DB7 fusion protein may contribute to mitochondrial dysfunction in patients with psychiatric disorders, within and beyond the original Scottish *DISC1* family.

Mitochondria are vital for ATP production. Some ATP can be produced from glucose via glycolysis and anaerobic respiration, producing lactate, but most of the energy derived from carbohydrates and fatty acids is produced via aerobic respiration, primarily through oxidative phosphorylation (OXPHOS). Without an electrochemical gradient across the mitochondrial membrane, OXPHOS cannot occur. Therefore, mitochondrial dysfunction can lead to energy shortage and a shift toward glycolysis as a means of energy production. Impaired glucose regulation has long been associated with schizophrenia (Cramond 1987; Stone et al. 2004), and this is consistent with findings that patients with bipolar disorder and schizophrenia have elevated CSF lactate concentrations (Dager et al. 2004; Regenold et al. 2009).

In the current study, we hypothesized that ATP production would be impaired in the brains of heterozygous (HET) mice carrying one copy of the *DB7* fusion gene. AMP-

activated kinase (AMPK) is an energy sensor that is activated by a high AMP/ATP ratio (i.e., low energy) (Hardie 2004). AMPK activation can be measured via phosphorylation of its α subunit at threonine 172 (Thr172). To approximate rates of ATP production, we measured AMPK phosphorylation, hypothesizing that cells expressing DB7 would have elevated AMPK activation relative to controls. Because *DISC1* is expressed in many adult tissues, including muscle, pancreas, and liver (Millar et al. 2000; Ozeki et al. 2003), we hypothesized that the metabolic effects of *DB7* expression would be observable beyond the brain. To assess global metabolic functioning, we calculated the respiratory exchange ratio (RER) of mice. RER = VCO_2/VO_2, where VCO_2 is the volume of CO_2 produced and VO_2 is the volume of O_2 consumed. When a mouse metabolizes fat for energy via beta-oxidation, it utilizes more O_2 than CO_2 produced, and its RER is close to 0.7. When a mouse metabolizes carbohydrates for energy via glycolysis, its RER is 1.0. Therefore, predicting a shift to glycolysis and decreased reliance on OXPHOS, we hypothesized that DISC1-Boymaw mice would have an elevated RER compared to wild-type littermates. Such a finding would support mitochondrial dysfunction as a primary underlying cause for co-morbid metabolic disorders and psychiatric symptoms (Jurczyk et al. 2016).

Materials and methods

DNA constructs

Full-length *DISC1* and *DB7* adeno-associated virus (AAV) plasmid constructs were generated through PCR amplification and ligation, as previously described (Ji et al. 2014). Constructs were driven by a CMV promoter, tagged with HA epitopes, and followed by a bGH Poly(A) tail. Constructs were confirmed with complete sequencing.

Cell culture

HEK 293T/17 and COS-7 cells (American Type Culture Collection [ATCC], Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA) at 37˚C in a 5% CO2 humidified incubator. Cells were transfected with either HA-tagged full-length *DISC1* or *DB7* fusion genes with TransIT1-COS Transfection reagent and COS Boss Reagent (TransIT®-COS Transfection Kit, Mirus, Madison, WI).

Mice

Humanized *DISC1-Boymaw* on a 129S background were generated in-house, as described (Ji et al. 2014). Male and female wild-type (WT, N=21), heterozygous (HET, N=22), and homozygous (HOMO, N=8) littermates were bred and tested at 3-7 months old (20-35 g). Mice were group-housed (3-4 per cage) in climate-controlled animal colonies on a 12 h light-dark cycle in facilities that are approved by the American Association for Accreditation of Laboratory Animal Care and meet all federal and state requirements for care and treatment of laboratory animals. Food (Harlan Teklad, Madison, WI) and water were

available ad libitum. All procedures were approved by the University of California San Diego (UCSD) Animal Care and Use Committee.

MitoTracker

Two days after transfection of COS7 cells with full-length *DISC1* or *DB7*, MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA) was added to culture medium at a concentration of 50 nM and incubated at 37˚C for 15 minutes. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature followed by permeabilization with methanol at -20˚C for 5 minutes. Fixed cells were blocked with 2% goat serum/PBS containing 0.2% Triton-X100 at room temperature for 1 h, and further incubated with 1:250 dilution of rabbit anti-HA antibody (H6908, Sigma-Aldrich, St. Louis, MO) overnight. After rinsing with PBST, the cells were incubated with Alexa Fluor1488 goat anti-rabbit IgG (1:500, Invitrogen) for 1 h at room temperature. After washing with PBST, the cells were mounted with VECTASHIELD HardSet Mounting Medium containing DAPI (Vector, CA). Confocal images were acquired with an Olympus FluoViewTM FV1000 confocal microscope.

Western blot

Two days after transfection with full-length *DISC1* or *DB7*, HEK293T cells were harvested for Western blot analysis. Adult mouse brains were dissected and minced on ice. Cell pellet and brain homogenate were solubilized with sonication in PLB containing 1x protease inhibitor cocktail (Sigma). Protein concentration was measured by absorbance at 595 nm using the Bradford method (Coomassie Plus Protein Assay, Thermo Scientific, Rockford, IL). After electrophoresis, proteins were transferred onto PVDF membranes which

were subsequently blocked with 5% non-fat dry milk in TBST buffer (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. HA-tagged recombinant proteins were detected directly with a high affinity rat monoclonal anti-HA antibody conjugated with peroxidase (1:500, Roche, CA). The same membrane was stripped and re-probed as described (Kim et al. 2012) with the following primary antibodies at 48˚C overnight: rabbit monoclonal anti-phospho-AMPKα antibody (1:10k; Cell Signaling, Danvers, MA); rabbit monoclonal anti-AMPKα (1:10k, Cell Signaling); mouse monoclonal anti-b-actin (1:5000, Santa Cruz Biotechnology, CA). After washing, the membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5,000, Cell Signaling, MA) or anti-rabbit IgG (1:5000, Santa Cruz, CA) for 1.5 h at room temperature. Quantification of protein expression was conducted with Image J.

Comprehensive Metabolic Evaluation

The Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) measured locomotor activity (X-axis beam breaks, "XTOT"), oxygen consumption (VO_2), carbon dioxide production (VCO_2), and food intake ("feed"). The respiratory exchange ratio (RER), the ratio of VCO_2/VO_2, is used to estimate the relative proportions of fat and carbohydrate utilized by each mouse. Heat is an estimate of energy expenditure for each mouse, based on VCO_2 and VO_2. Measurements were made every 13 minutes for approximately 72 h. Mice were placed in the CLAMS chambers around 14:00 h. After an approximately 5-hour acclimation period in the light, data were averaged for every 12-hour period beginning in the first full dark period (D1) and ending in the final full dark period (D3) (See **Fig. 2.3** for timeline). CLAMS was conducted by the UCSD Animal Care Program (ACP) Animal Phenotyping Core.

Statistics

Protein levels were tested for normal distribution using the Kolmogorov–Smirnov test before calculation of differences and analyzed using one-way analysis of variance (ANOVA) with gene (*DB7* v. full-length *DISC1*) or genotype (WT v. HET) as a between-subjects factor. CLAMS data were analyzed using repeated measures with period as a within-subjects factor and genotype as a between-subjects factor. Tukey *post hoc* analyses were conducted on all significant main effects and interactions. The alpha level was set to 0.05. All statistics were performed using SPSS (24.0, Chicago, IL, USA).

Results

MitoTracker

The MitoTracker dye emits bright red fluorescence dependent on mitochondrial membrane potential; a weaker signal indicating lower potential. As reported previously (Eykelenboom et al. 2012), we found that cells transfected with *DB7* but not with full-length *DISC1* exhibited a reduced MitoTracker signal (**Fig. 2.1**), suggesting that expression of *DB7* abolishes mitochondrial membrane potential.

AMPK phosphorylation

We transfected cells with *DB7*, full-length *DISC1*, or a control plasmid vector and compared levels of phosphorylated AMPKα (p-AMPKα) at 24, 32, and 48 h after transfection. There was no difference in total AMPKα protein (relative to β-actin expression) between transfection groups (for all time points: $F<1$, *ns*). We found, however, that there

were significant group differences in p-AMPKα at 32 and 48 h after transfection, measured in relation to total AMPKα (**Fig. 2.2A-2.2B**; 32 h: $F(2,9)=7.2$, $p<0.05$; 48 h: $F(2,9)=13.3$, $p<0.005$) or β-actin (32 h: $F(2,9)=9.3$, $p<0.01$; 48 h: $F(2,9)=67.7$, $p<0.001$). *Post hoc* analyses revealed that this group effect was carried by significant differences in p-AMPKα in cells transfected with *DB7* (relative to total AMPKα cf. Controls at 32 h: $t(6)=2.9$, $p<0.05$, 48 h: $t(6)=4.2$, $p<0.01$; relative to β-actin cf. Controls at 32 h: $t(6)=3.9$, $p<0.01$, 48 h: $t(6)=9.1$, $p<0.001$). There was no difference in p-AMPKα 24 h after transfection relative to total AMPKα or β-actin ($F<1$, ns).

Consistent with our hypothesis and our *in vitro* transfection studies, we found that both HET and HOMO DISC1-Boymaw mice display significantly higher levels of p-AMPKα in frontal cortex than their WT littermates (**Fig. 2.2C-2.2D**). We did not observe differential expression of total AMPKα protein ($F<1$, ns). One-way ANOVA demonstrated a trend toward an effect of genotype ($F(2,24)=3.2$, $p<0.06$). Based on our *a priori* hypothesis that p-AMPKα would be greater in both HET and HOMO mice, we performed *post hoc* analysis, which confirmed significantly higher levels of p-AMPKα in the brains of HET ($t(17)=2.8$, $p=0.03$) and HOMO ($t(15)=2.4$, $p=0.03$) mice, compared to their WT littermates.

CLAMS

CLAMS Experiment 1:

There was no main effect of Genotype on any measures but a main effect of 12-h Period on all measures and significant Period × Genotype interactions on VCO_2 and RER (**Table 2.1**). *Post hoc* analyses revealed significantly higher RER for HET mice during the second dark period (D2) and third light period (L3) (**Fig. 2.4A**). Because RER fluctuates

during the day, we assessed light and dark periods separately. Across light periods (L2 and L3, not including D1-D3), there was a significant Period × Genotype interaction ($F(1,10)=7.5$, $p=0.02$). There was no effect of Period in WT mice ($F(1,5)=2.1$, ns) and a trend toward an effect of Period in HET mice ($F(1,5)=5.4$, $p=0.07$), with increased RER during L3 compared to L2. Across dark periods (D1-D3, not including L2 and L3), there was a significant Period × Genotype interaction ($F(2,20)=3.6$, $p<0.05$) and a trend toward an effect of Genotype ($F(1,10)=3.6$, $p=0.09$). When analyzed separately, there was no effect of Period in the WT mice ($F<1$, ns) but an effect of Period in the HET mice ($F(2,10)=4.0$, $p=0.05$), driven by a significant difference between RER in D1 versus D2 ($t(5)=3.2$, $p=0.03$).

RER closer to 1.0 may suggest carbohydrate metabolism, rather than fat metabolism, for which RER is closer to 0.7. RER can also be increased with greater activity, so *post hoc* analyses were conducted on activity to determine if increased RER was due to greater activity of the HET mice during D2 and L3. In fact, HET mice were significantly less active than WT littermates during D3 (**Fig. 2.4B**). This profile suggests a bioenergetic shift from mitochondrial oxidative phosphorylation to cytoplasmic glycolysis, as predicted.

CLAMS Experiment 2 and 3:

We hoped to replicate the findings from CLAMS Experiment 1 in a second cohort. In Experiments 2 and 3, we tested female and male HET and WT DISC1-Boymaw mice at 3 mo. In both experiments, there was a main effect of Period but no main effect of Genotype nor significant Period × Genotype interaction (**Table 2.2**). Despite evidence for decreasing fatty acid oxidation, aging mice rely increasingly on fat for energy production, lowering RER (Houtkooper et al. 2011). If this was the case for WT mice, elevated RER in HET mice may

be more evident in aging mice. Furthermore, since mitochondrial function deteriorates with age, the effects of mitochondrial dysfunction might be more profound in older mice. Thus, we hypothesized that the lack of effect in the 3-mo mice may have been due to their relative youth compared to the mice in Experiment 1.

CLAMS Experiments 4 and 5:

We tested the same mice from Experiments 2 and 3 at 7 mo for Experiments 4 and 5. We expected results similar to those in Experiment 1 for the older mice, but again we observed a main effect of Period but no main effect of Genotype nor significant Period × Genotype interaction (**Table 2.2**).

Discussion

Research suggests that mitochondrial dysfunction may contribute to the development of psychiatric illnesses (Manji et al. 2012; Park and Park 2012). Impaired energy metabolism and ATP production may contribute to sleep disturbances, as well as lack of motivation, concentration, and working memory, associated with these illnesses (Picard and McEwen 2014). The exact relationship between impaired mitochondrial and psychiatric symptoms, however, is not understood.

We have studied how the *DISC1-Boymaw* (*DB7*) fusion gene, generated in the Scottish *DISC1* family, may contribute to pathogenesis of psychiatric disorders (Ji et al. 2014). The humanized DISC1-Boymaw mice expressing the human DB7 fusion gene displayed behavioral abnormalities that may be the result of mitochondrial dysfunction and

inhibition of protein synthesis. In this study, we investigated whether the co-localization of the DB7 gene with mitochondria influences mitochondrial functioning.

Consistent with previous findings (Eykelenboom et al. 2012),we confirmed that the DB7 protein (but not full-length DISC1) predominantly localizes in mitochondria and reduces mitochondrial membrane potential (**Fig. 2.1**). Mitochondrial membrane potential is essential for the generation of ATP. Loss of mitochondrial membrane potential indicates reduction of ATP in cells expressing the DISC1-Boymaw fusion protein. Reduction of ATP can trigger activation of the AMP-activated protein kinase (AMPK) by phosphorylation at Thr 172 of its α subunit (p-AMPKα), a sensor of cell energy status (Hardie 2004). AMPK coordinates metabolic pathways to balance nutrient supply with energy demand. For example, AMPK mediates the stimulation of glucose intake and shift to glycolysis. We can measure levels of p-AMPK relative to total AMPK to evaluate the relative AMP/ATP ratio in a sample. We found that cells transfected with DB7 in vitro exhibited increased levels of p-AMPKα (**Fig. 2.2A-2.2B**). Similarly, p-AMPKα is significantly increased in the brains of HET and HOMO DISC1-Boymaw mice (**Fig. 2.2C-2D**).

AMPK activity, notably in the hypothalamus, regulates whole-body metabolic changes—including thermogenesis and food intake (Hardie 2015; López et al. 2016). Therefore, elevated AMPK phosphorylation in tissue sample may indicate metabolic changes not limited to ATP production. Our findings in transfected cells, however, suggest that expression of the *DB7* gene can confer a targeted disadvantage to mitochondria and metabolic processes on a cellular level. Direct targeting of the ATP synthesis pathway is also supported reduced oxidoreductase activity (measured via MTT reduction) in the brain tissue of mice carrying the *DB7* fusion gene (Ji et al. 2014).

DISC1 is expressed in tissues throughout the body (Ozeki et al. 2003; Millar et al. 2005), and we expect that the DB7 protein is expressed in those same tissues in the DISC1-Boymaw mice. We suspect that, in patients and in our model, a single mutation or set of mutations affecting mitochondria throughout the body could lead to behavioral abnormalities, as well as global metabolic disorder (Clay et al. 2011; Flippo and Strack 2017).

We utilized the Comprehensive Lab Animal Monitoring System (CLAMS) to measure the activity, food intake, O_2 consumption, and CO_2 production of a small cohort of female DISC1-Boymaw mice. In these mice, compared with their WT female littermates, we observed an increased respiratory exchange ratio (RER, **Table 2.1**). RER can be used to estimate the source of energy production, and increased RER supports the idea of a shift to glycolysis, consistent with increased p-AMPKα. Interestingly, the WT mice had relatively stable RER across dark periods and light periods, with lower RER during the inactive light periods compared to the active dark periods, consistent with previous studies (Eckel-Mahan et al. 2012; Wang et al. 2013). The HET mice, on the other hand, exhibited increased RER during the second dark period (D2) and third light period (L3). It is possible that the HET mice are less able to stabilize their RER after a stressful event, such as being transferred into the CLAMS environment. Unfortunately, due to the short testing period, we are unable to determine if the heightened RER of the HET mice would return to a baseline similar to the WT mice or stabilize at a higher rate.

We hoped to explore this in our future cohort. However, when we repeated the experiment in a second, younger cohort including males and females, we were unable to replicate our findings. We hypothesized that because mitochondrial functioning declines with age, we might see the detrimental effects of DB7 expression later in life. Therefore, we tested

the same mice at 7 mo, comparable to our first cohort. Again, we were unable to replicate the results of Experiment 1.

There are several potential reasons why we did not observe increased RER in the second cohort. Of course, it is possible that differences between WT and HET groups in the first cohort occurred by chance or were unrelated to genotype. In fact, it was surprising that we observed a phenotype in the first cohort at all. In our studies, published and unpublished, the humanized DISC1-Boymaw mice have exhibited relatively mild phenotypes. Given the incomplete penetrance and variable manifestation of the mutation in humans, we cannot necessarily expect a strong phenotype in our mouse model. In fact, mouse models of severe mitochondrial dysfunction, such as *Ndufs4* knockout mice that model Leigh syndrome, can develop normally, compensating for loss of function with other proteins (Kruse et al. 2008; Quintana et al. 2010; Farrar et al. 2013). Even xenomitochondrial cybrids—carrying mitochondria from evolutionarily divergent species—do not display obvious phenotypic abnormalities due to compensatory mechanisms (Cannon et al. 2011; Dunn et al. 2012).

It is possible that multiple genes are necessary to cause problems on an observable scale. For example, in the Scottish *DISC1* family, *DISC1* haploinsufficiency—which inhibits mitochondrial trafficking—may interact with the *DISC1-Boymaw* fusion gene to exacerbate mitochondrial dysfunction (Atkin et al. 2011; Ogawa et al. 2014; Norkett et al. 2017). This could explain the failure to find *DISC1* alone as a risk gene for psychiatric illnesses in the general population (Huang et al. 2010; The Network and Pathway Analysis Subgroup of the Psychiatric Genomics Consortium 2015; Tomoda et al. 2016). Future studies incorporating compounded mitochondrial mutations in DISC1-Boymaw mice may reveal the impact of *DISC1-Boymaw* expression on mitochondrial function and behavior.

The notion that the genetic environment influences susceptibility to a particular mutation reminds us that an individual's physical environment and experiences can also greatly affect outcome. Our cohorts of mice were born at different times and despite our greatest efforts may have experienced different stressors before CLAMS testing. Notably, the second cohort experienced CLAMS testing at 3 mo, and the exposure to the testing procedure in their relative youth may have affected retesting at 7 mo. Ideally, the experiment would have been re-run in a third cohort that, like the first, only experienced CLAMS testing at 7 mo.

As has been observed in the xenomitochondrial cybrids, the lack of observable phenotype in our animal model does not invalidate the effects at the cellular level that still may contribute to the development of psychiatric illnesses in humans. It is still of interest to investigate the effects of DB7 on mitochondrial more closely.

Future studies will be necessary to determine exactly how DB7 aggregates and affects mitochondrial function. For example, we wish to determine which mitochondrial oxidoreductases are inhibited by DB7. It will be crucial to determine DB7 interaction partners to investigate whether the effects of DB7 can be prevented or treated. We have proposed two mechanisms by which the DB7 gene may contribute to the development of psychiatric illnesses.

First, we proposed that the AMPK-mTOR pathway may link impaired mitochondrial function to inhibition of protein translation activity (Ji et al. 2014). The AMPK-mTOR pathway has been implicated in the pathogenesis of neurological disorders, including autism spectrum and cognitive disorders (Ehninger et al. 2009; Ehninger and Silva 2011). The

AMPK-mTOR pathway is a critical regulator of protein translation. An optimal level of protein synthesis is necessary for neural plasticity (Bhakar et al. 2012), and the excess or deficiency of protein translation may harm brain development and function. Overactive AMPK activity may lead to inhibited protein translation, which could impair neural plasticity and contribute to the pathogenesis of major psychiatric disorders. Although DB7 is only present in the Scottish family, overexpression of *Boymaw* in the general population of patients with schizophrenia seems to affect protein translation in the same way (Ji et al. 2015). Therefore, studies on the AMPK-mTOR pathway in the DISC1-Boymaw mice may be applicable to the general population of psychiatric patients, and targeting the pathway (with L-Leucine, for example) may have therapeutic effects.

In addition to the developmental effects of reduced protein translation, impaired ATP production might directly target specific, vulnerable neuronal populations. We hypothesize that mitochondrial dysfunction in energetically-demanding neurons might cause behavioral abnormalities. For example, fast-spiking, parvalbumin-expressing (PV^+) cortical GABAergic interneurons may consume more ATP than other neurons because of their high-frequency firing (Hasenstaub et al. 2010). If so, cortical PV^+ interneurons may be particularly vulnerable to DB7-induced mitochondrial dysfunction. These abnormalities could be particularly relevant to the pathogenesis of schizophrenia and other major psychiatric disorders in which impaired cortical GABAergic interneurons are consistently observed (Benes and Berretta 2001; Hashimoto et al. 2003; Costa et al. 2003; Curley and Lewis 2012). Electrophysiological and imaging experiments will be crucial to identifying which neuron populations and circuits are particularly vulnerable to the DB7 protein.

Although we were unable to replicate our findings of global metabolic impairments in DISC1-Boymaw mice, we have confirmed that expression of the *DB7* gene impairs mitochondrial function at the cellular level. This impaired mitochondrial function may contribute to the development of psychiatric symptoms in the DISC1 family, as well as in the general population. Additional genetic and environmental insults may be necessary for the development of more profound metabolic dysfunction observed in patients with psychiatric illnesses.

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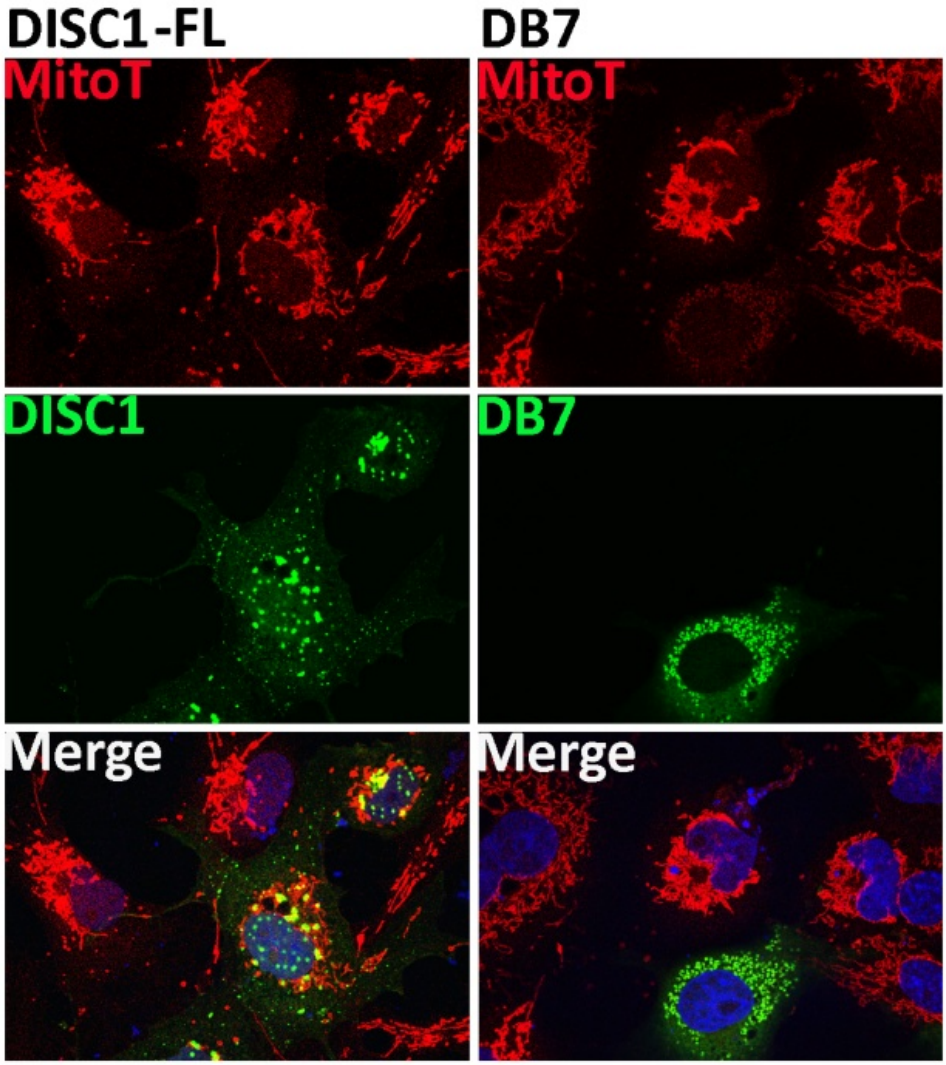

Figure 2.1: DB7 expression reduces mitochondrial membrane potential. MitoTracker ("MitoT," red) was used to illuminate mitochondrial membrane potential in live COS7 cells transfected with either full-length DISC1 (DISC1-FL) or DISC1-Boymaw (DB7) in green. Cells expressing DB7, but not DISC1-FL, displayed remarkable reduction of mitochondrial membrane potential. Nuclei were stained with DAPI (blue).

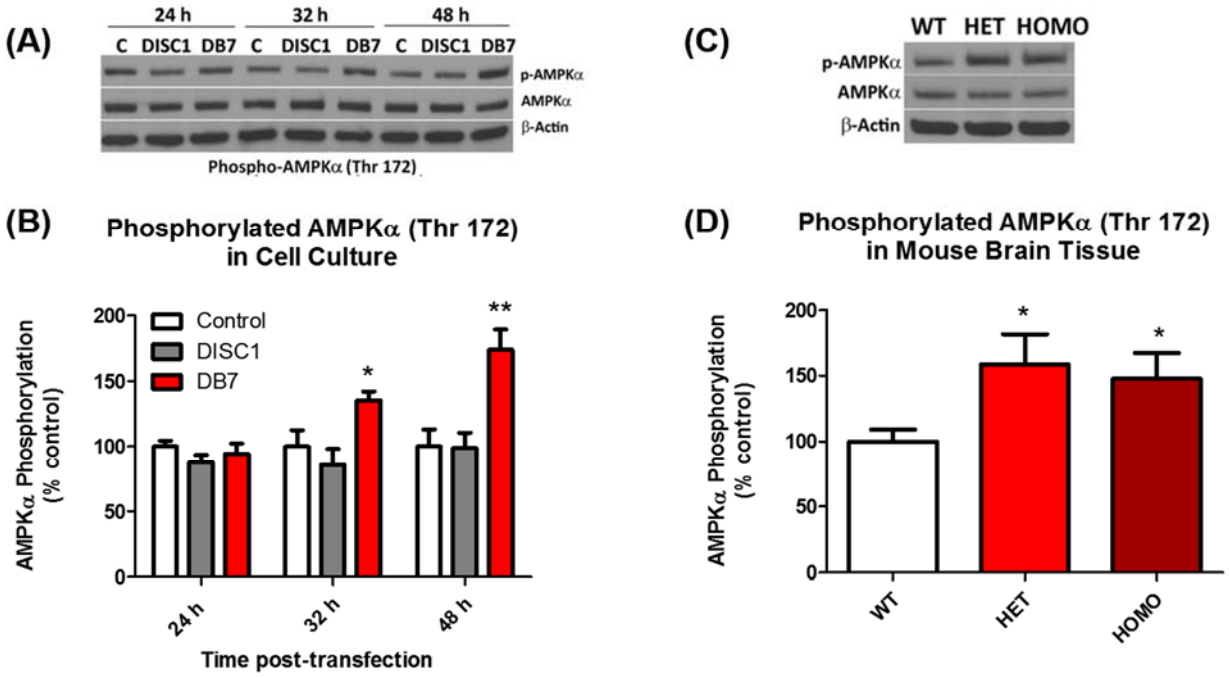

Figure 2.2: DISC1-Boymaw expression increases AMPKα phosphorylation. (A) Human HEK293 cells were transfected with the control plasmid vector (C), DISC1-FL, or DB7. Phosphorylated AMPKα (p-AMPKα) was assessed 24, 32, and 48 h after transfection. Western blot revealed increased p-AMPKα antibody in cells transfected with the DB7 gene, but not DISC1-FL or control plasmids. (B) Relative p-AMPKα was quantified against the total amount of AMPKα protein. There was significantly more relative p-AMPKα in cells transfected with DB7 at 32 and 48 h. (C) Both HET and HOMO DISC1-Boymaw mice had higher levels of p-AMPKα in frontal cortex. (D) There was significantly more relative p-AMPKα in the frontal cortex of HET and HOMO mice, compared with WT littermates. *$p<0.05$

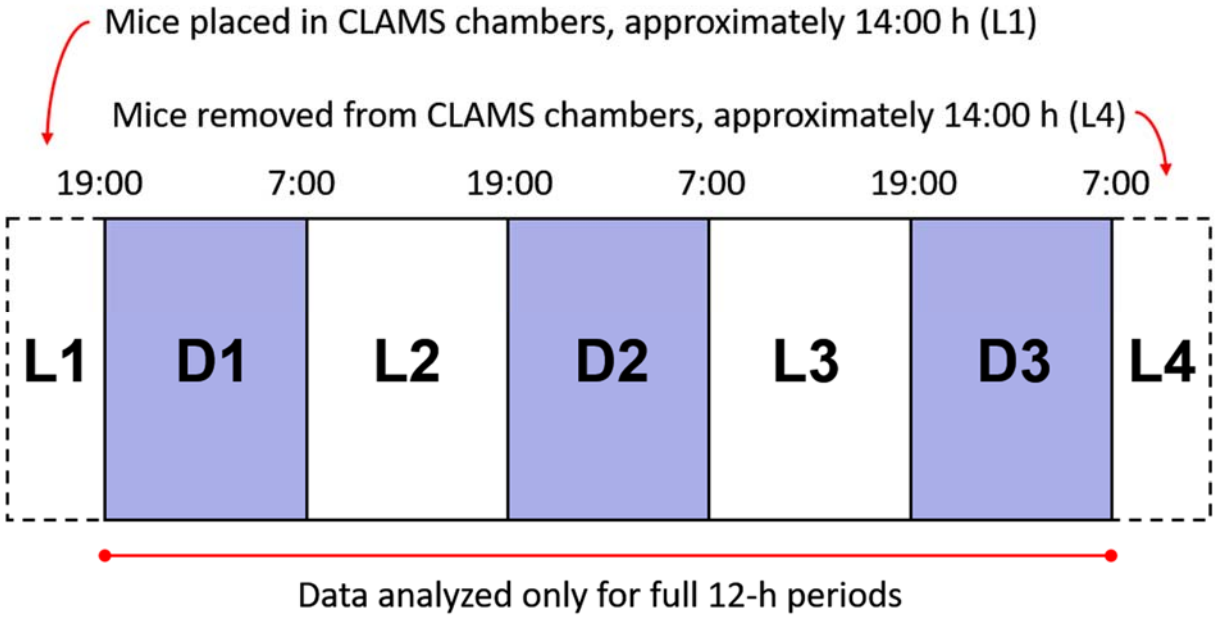

Figure 2.3: Timeline for CLAMS Experiments 1-5. Light periods (L1-4) began at 7:00 h, and dark periods (D1-3) began at 19:00 h. Data were analyzed only for full 12-h periods: D1, L2, D2, L3, and D3.

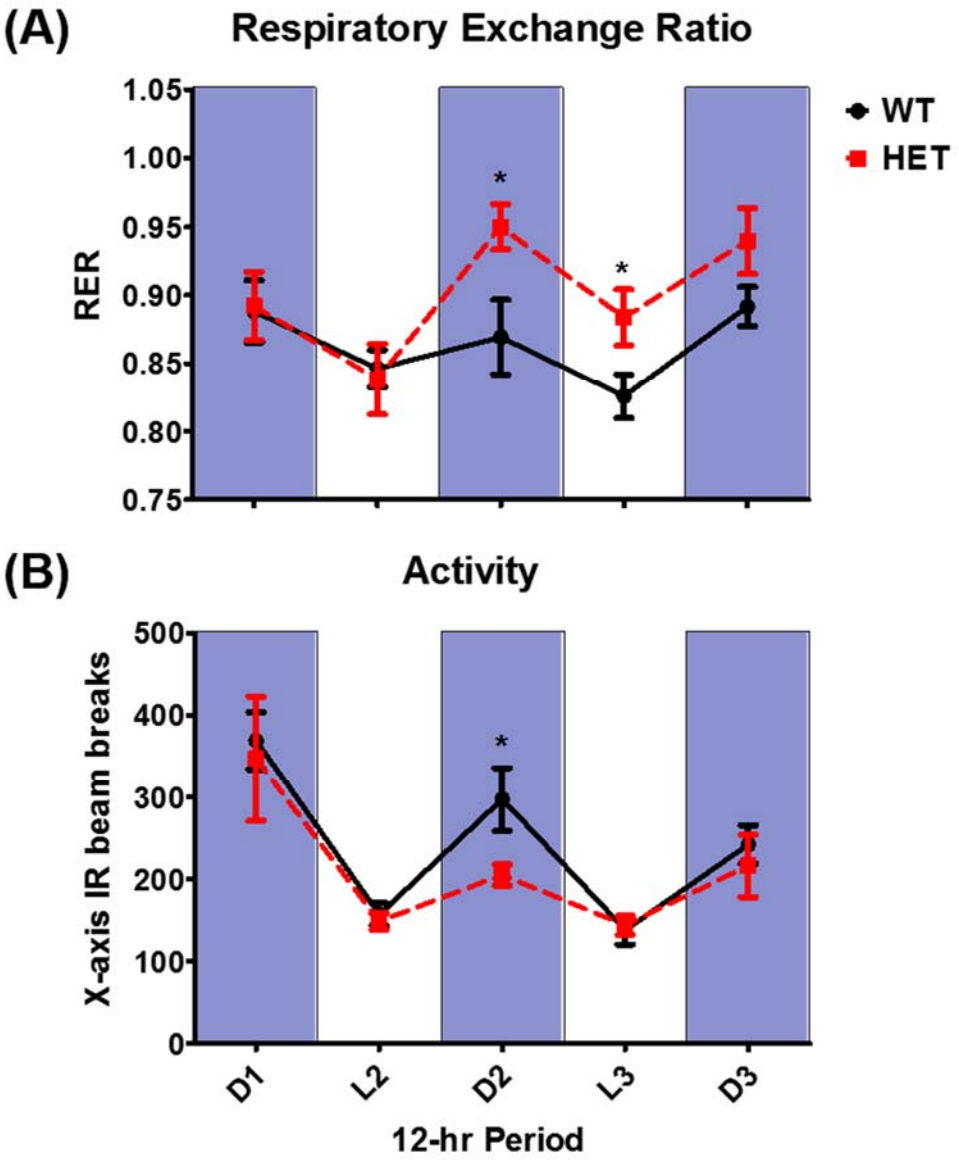

Figure 2.4: CLAMS analysis revealed altered respiratory exchange ratio (RER, A) and activity patterns (B) in 7-mo female HET DISC1-Boymaw mice. *Post hoc* analyses revealed significantly higher RER for HET mice during D2 and L3 and lower activity during D2. *$p<0.05$

Table 2.1: CLAMS results for 7-mo female DISC1 HET mice and WT littermates. **Bolded** indicates significant effects and interactions. *Post hoc* analyses were performed to assess differences between genotypes during isolated dark (D) and light (L) periods according to *a priori* hypotheses or Period × Genotype interactions.

CLAMS Experiment 1
Female, 7 mo

Measure	Period	Genotype	Period × Genotype
VO_2	**F(4,40)=10.4, *p*<0.001**	F(1,10)<1, ns	F(4,40)=2.3, *p*=0.074
VCO_2	**F(4,40)=17.9, *p*<0.001**	F(1,10)<1, ns *Post hoc* L3 F(1,10)=4.1, *p*=.070	**F(4,40)=3.7, *p*=0.012**
Feed	**F(4,40)=10.0, *p*<0.001**	F(1,10)=1.5, ns	F<1, ns
Activity	**F(4,40)=22.8, *p*<0.001**	F(1,10)=1.0, ns *Post hoc* **D2 F(1,10)=6.2, *p*=0.032**	F(4,40)=1.1, ns
RER	**F(4,40)=9.7, *p*<0.001**	F(1,10)=3.4, *p*=0.097 *Post hoc* **D2 F(1,10)=7.6, *p*=0.02** **L3 F(1,10)=5.9, *p*=0.035** D3 F(1,10)=3.5, *p*=0.091	**F(4,40)=3.2, *p*=0.024**

Table 2.2: CLAMS results for female and male DISC1 HET mice and WT littermates at 3 and 7 mo. **Bolded** indicates significant effects and interactions. Post hoc analyses were performed to assess differences between genotypes during isolated dark (D) and light (L) periods according to a priori hypotheses or Period × Genotype interactions.

	CLAMS Experiment 2 Female, 3 mo			**CLAMS Experiment 3** Male, 3 mo		
Measure	Period	Genotype	Period × Genotype	Period	Genotype	Period × Genotype
VO_2	**F(4,40)=8.2, p<0.001**	F<1, ns	F<1, ns	**F(4,40)=9.8, p<0.001**	F<1, ns	F(4,40)=2.4, p=0.069
VCO_2	**F(4,40)=34.7, p<0.001**	F<1, ns	F<1, ns	**F(4,40)=54.7, p<0.001**	F<1, ns	F(4,40)=1.0, ns
Feed	**F(4,40)=53.1, p<0.001**	F<1, ns	F(4,40)=1.2, ns	**F(4,40)=17.9, p<0.001**	F<1, ns	F(4,40)=1.2, ns
XTOT	**F(4,40)=7.6, p<0.001**	F<1, ns	F(4,40)=1.7, ns	**F(4,40)=4.4, p=0.005**	F<1, ns	F<1, ns
RER	**F(4,40)=22.2, p<0.001**	F<1, ns	F<1, ns	**F(4,40)=40.0, p<0.001**	F<1, ns *Post hoc* D3 F(1,10)=3.4, p=0.093	F(4,40)=1.6, ns

	CLAMS Experiment 4 Female, 7 mo			**CLAMS Experiment 5** Male, 7 mo		
	Period	Genotype	Period × Genotype	Period	Genotype	Period × Genotype
VO_2	**F(4,40)=12.0, p<0.001**	F(1,10)=1.6, ns	F<1, ns	**F(4,40)=49.0, p<0.001**	F<1, ns	F(4,40)=1.0, ns
VCO_2	**F(4,40)=24.5, p<0.001**	F(1,10)=1.7, ns	F<1, ns	**F(4,40)=69.9, p<0.001**	F<1, ns	F(4,40)=1.4, ns
Feed	**F(4,40)=38.1, p<0.001**	F<1, ns	F<1, ns	**F(4,40)=13.6, p<0.001**	F(1,10)=1.2, ns	F<1, ns
XTOT	**F(4,40)=15.7, p<0.001**	F<1, ns	F<1, ns	**F(4,40)=8.8, p<0.001**	F<1, ns	F<1, ns
RER	**F(4,40)=21.2, p<0.001**	F<1, ns	F(1,10)=1.3, ns	**F(4,40)=19.6, p<0.001**	F<1, ns	F(4,40)=1.3, ns

Chapter 2, *Mitochondrial Dysfunction in Humanized DISC1-Boymaw Mice*, is being prepared for submission and will include Drs. Baohu Ji, Mark A. Geyer, and Xianjin Zhou, as co-authors. This work was supported by the Veterans Affairs VISN 22 Mental Illness Research, Education, and Clinical Center and NIH grants R01-MH073991, R21-MH086075, and F31-MH109218. The authors thank the UCSD ACP Animal Phenotyping Core, Dr. Chuck Heyser for help with data analysis and Marilyn Hardee for conducting the CLAMS experiment. The dissertation author was the primary investigator and author of this paper.

Chapter 3:

Restoration of Sp4 in forebrain GABAergic neurons rescues hypersensitivity to ketamine in Sp4 hypomorphic mice

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RESEARCH ARTICLE

Restoration of *Sp4* in Forebrain GABAergic Neurons Rescues Hypersensitivity to Ketamine in *Sp4* Hypomorphic Mice

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Abstract

Background: Ketamine produces schizophrenia-like behavioral phenotypes in healthy people. Prolonged ketamine effects and exacerbation of symptoms after the administration of ketamine have been observed in patients with schizophrenia. More recently, ketamine has been used as a potent antidepressant to treat patients with major depression. The genes and neurons that regulate behavioral responses to ketamine, however, remain poorly understood. Sp4 is a transcription factor for which gene expression is restricted to neuronal cells in the brain. Our previous studies demonstrated that *Sp4* hypomorphic mice display several behavioral phenotypes relevant to psychiatric disorders, consistent with human *SP4* gene associations with schizophrenia, bipolar disorder, and major depression. Among those behavioral phenotypes, hypersensitivity to ketamine-induced hyperlocomotion has been observed in *Sp4* hypomorphic mice.
Methods: In the present study, we used the *Cre-LoxP* system to restore *Sp4* gene expression, specifically in either forebrain excitatory or GABAergic inhibitory neurons in *Sp4* hypomorphic mice. Mouse behavioral phenotypes related to psychiatric disorders were examined in these distinct rescue mice.
Results: Restoration of *Sp4* in forebrain excitatory neurons did not rescue deficient sensorimotor gating nor ketamine-induced hyperlocomotion. Restoration of *Sp4* in forebrain GABAergic neurons, however, rescued ketamine-induced hyperlocomotion, but did not rescue deficient sensorimotor gating.
Conclusions: Our studies suggest that the *Sp4* gene in forebrain GABAergic neurons regulates ketamine-induced hyperlocomotion.

Keywords: GABAergic, genetic rescue, ketamine, locomotor activity, Sp4

Introduction

Sp4 is a transcription factor that recognizes GC-rich sequences in the "CpG islands" around the promoters of many genes (Heisler et al., 2005). In contrast to the *Sp1* gene, *Sp4* gene expression is restricted to neuronal cells in the brain (Supp et al., 1996; Zhou et al., 2005). Our previous studies found that *Sp4* hypomorphic mice displayed several behavioral phenotypes

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relevant to psychiatric disorders, including deficits in prepulse inhibition and hypersensitivity to ketamine (Zhou et al., 2005, 2007, 2010). In humans, the SP4 gene was reported to be sporadically deleted in patients with schizophrenia (Tam et al., 2010; Zhou et al., 2010), and the SP4 protein is reduced in the post-mortem brains of bipolar patients (Pinacho et al., 2011). Additionally, human genetic studies reported the association of the SP4 gene with bipolar disorder, schizophrenia, and major depression (Shyn et al., 2009; Zhou et al., 2009; Shi et al., 2010; Greenwood et al., 2011). These studies suggest the *Sp4* hypomorphic mice as a promising mouse genetic model for human psychiatric disorders.

Administration of ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptors antagonist, induces behaviors that resemble several aspects of psychiatric disorders in healthy people (Krystal et al., 1994; Xu et al., 2015). Prolonged ketamine effects and exacerbation of symptoms were reported in schizophrenia patients after administration of ketamine (Lahti et al., 1995; Malhotra et al., 1997; Holcomb et al., 2005). On the other hand, ketamine was recently found to act as a potent antidepressant that provides rapid relief to patients who are resistant to treatment with classical antidepressants (Zarate et al., 2006; Price et al., 2009; aan het Rot et al., 2010; Diazgranados et al., 2010; Li et al., 2010). In rodents, ketamine also disrupts prepulse inhibition of startle (PPI), a form of sensorimotor gating that is deficient in patients with either schizophrenia or bipolar disorder, but not in patients with depression (Braff et al., 2001; Geyer, 2006). It is unclear however, what genes and neurons are the key regulators of such responses to ketamine.

Sp4 hypomorphic mice have robust behavioral phenotypes such as deficient PPI and hypersensitivity to ketamine-induced hyperlocomotion (Ji et al., 2013). By taking advantage of a genetic rescue strategy used in the generation of *Sp4* hypomorphic mice (Zhou et al., 2005), we restored *Sp4* expression in forebrain excitatory and GABAergic inhibitory neurons, respectively. Deficient PPI and ketamine-induced hyperlocomotion were examined in the two different neuron-specific *Sp4* rescue mouse lines.

Materials and Methods

Mouse Strains and Breeding

Following the recommendation of the Banbury Conference on Genetic Background in Mice (Banbury, 1997) to use the F1 genetic background, we generated *Sp4* hypomorphic mice on an F1 129S/Black Swiss genetic background for behavioral studies, as previously described (Ji et al., 2013). The *Emx1-Cre* mouse line (stock number: 005628) and *Dlx6a-Cre* mouse line (stock number: 008199) were purchased from Jackson laboratory. Both lines were backcrossed with Black Swiss mice for more than six generations. Each *Cre* line was then bred with *Sp4* heterozygous mice on a Black Swiss background to obtain double heterozygous mice. The double heterozygous mice were finally bred with *Sp4* heterozygous mice on a 129S background to obtain F1 generation mice (Figure 1S). All F1 *Sp4* heterozygous mice were sacrificed, and the remaining four genotypes were used for molecular and behavioral analyses (Supplemental Table I). Mice were housed in a climate-controlled animal colony with a reversed day/night cycle. Food (Harlan Teklabl) and water were available *ad libitum*, except during behavioral testing. Behavioral testing began when mice were 3 months old with PPI and ketamine-induced locomotor activity tests separated by at least 2 weeks. All testing procedures were approved by the UCSD Animal Care and Use Committee (permit number: A3033-01) prior to the onset of the experiments. Mice were maintained in American Association for Accreditation of Laboratory Animal Care–approved animal facilities at UCSD and the local Veteran's Administration Hospital. These facilities meet all Federal and State requirements for animal care.

LacZ Staining

20 μm thick cryostat sections were cut from fresh adult mouse brains. The sections were fixed, permeabilized, and stained as previously described (Zhou et al., 2005).

Western Blot

Total protein was extracted from mouse cortices and striata and Western blot analyses were subsequently performed as described (Ji et al., 2014). Anti-Sp4 antibody was purchased from Santa Cruz Biotechnology (sc-13019, sc-645).

Immunohistochemical Analysis

Adult mice were anesthetized with carbon dioxide, and perfused transcardially with 2% phosphate buffered saline paraformaldehyde. Mouse brains were dissected out and further fixed in 4% paraformaldehyde solution at 4°C for 24 hrs. Paraffin sections were generated. Sequential chromogenic immunohistochemistry was conducted to examine the co-localization of Sp4 protein with Gad67 protein, a marker for GABAergic neurons in mouse cortex, as described by Kim et al. (2012).

Prepulse Inhibition

Startle reactivity and prepulse inhibition (PPI) were measured with startle chambers (SR-LAB, San Diego Instruments) as described by Ji et al. (2013). The background noise level was 65 dB, and the pulse was 120 dB for 40 ms. The prepulse intensities were 69, 73, and 81 dB (4, 8, and 16 dB above the 65-dB background noise, respectively), delivered 80 ms before the pulse. The test session began and ended with five presentations of the "pulse-alone" trial. In between, each acoustic or "no stimulus" (65 dB background noise only) trial type was presented ten times in a pseudo-random order. The amount of PPI was calculated as a percentage score for each acoustic prepulse trial: %PPI = 100% × {1 - [(Startle response for "prepulse + pulse")/(Startle response for "pulse-alone")]}.

Video-Tracking Locomotion Tests

Locomotor activity was measured using the Video-Tracker (VT) system as previously described (Ji et al., 2013). Mice were tested during their dark period with lights on for the entire duration of the test. They were first acclimated to the testing room for 60 min. Then, they were placed into white plastic enclosures (41 × 41 × 34 cm^3), surrounded by an opaque plastic curtain. A video camera, mounted 158 cm above the enclosures, generated the signal for the Polytrack digitizer (San Diego Instruments). The position of each animal (x, y; in pixels) was sampled with a frequency of 18.18 Hz, which was used to generate a coordinate file (x, y, t) consisting of the x-location, y-location, and the duration (time t) spent in that location. After acclimation, mice were placed in their enclosures for 30 min of habituation. They then received ketamine (described below) and were placed back in their enclosures for another 60 min.

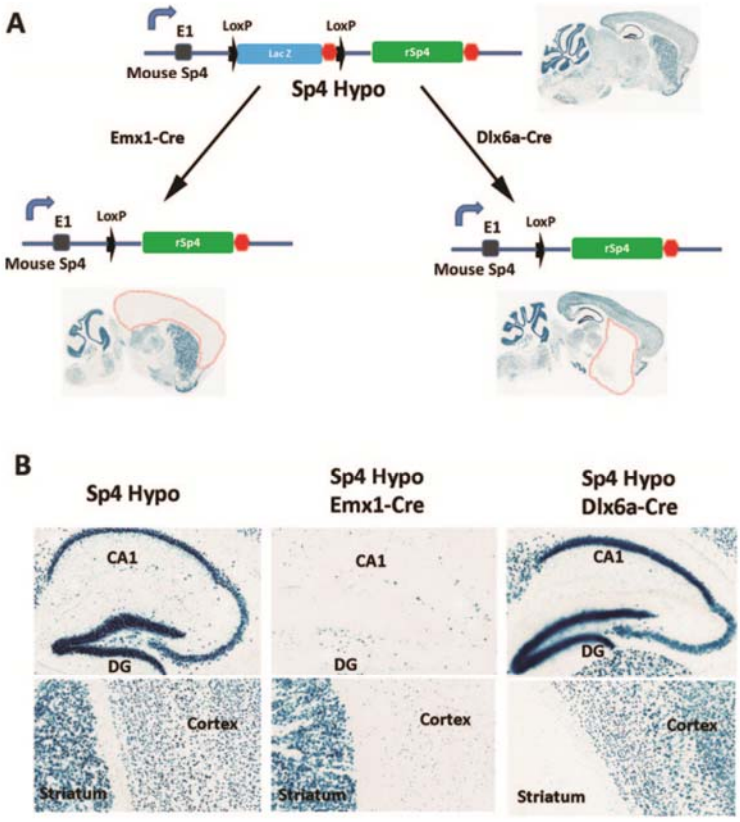

Figure 1. Restoration of rat *Sp4* gene in forebrain excitatory neurons and GABAergic neurons, respectively. (A) A nuclear *LacZ* expression cassette was capped with a splicing acceptor and further flanked by two Lox P sites. The Lox P flanked *LacZ* was inserted in the first intron of mouse *Sp4* gene. Expression of *LacZ* gene revealed that the *Sp4* gene is specifically expressed in neuronal cells in mouse brain. After breeding with *Emx1-Cre* and *Dlx6a-Cre* mouse lines, the *LacZ* gene cassette was excised in forebrain excitatory neurons and GABAergic neurons, respectively. The removal of the *LacZ* cassette allows expression of the downstream rat *Sp4* gene that was also capped with a splicing acceptor to splice with mouse exon 1 to generate a functional full-length *Sp4* gene. The absence of blue staining in neuronal cells indicates restoration of the *Sp4* gene. Because mouse cortices predominantly consist of excitatory neurons, *LacZ* blue staining almost completely disappeared in the cortices of *Sp4* hypomorphic mice carrying the *Emx1-Cre* gene. In contrast, mouse striata predominantly contains GABAergic neurons, the *LacZ* blue staining almost completely disappeared in the striata of *Sp4* hypomorphic mice carrying *Dlx6a-Cre* gene. (B) Under higher magnification, neurons that were not derived from the *Emx1* lineage remain blue in the cortex of *Sp4* hypomorphic mice carrying *Emx1-Cre* gene. Many of them are GABAergic neurons.

Ketamine

Ketamine was dissolved in saline and administered i.p. at a volume of 5 ml/kg after 30 min habituation in the VT arena (Brody et al., 2003). The doses of ketamine were determined from the same F1 genetic background mouse in previous studies (Ji et al., 2013). A within-subjects crossover design was used for drug studies, with 2 weeks between drug treatments.

Statistical Analysis

Repeated measures analysis of variance with genotype as a between-subjects factor and drug treatment, block, and prepulse intensity as within-subjects factors were performed on the %PPI data and total distance traveled. Post hoc analyses were carried out using Newman-Keuls or Tukey's test. Alpha level was set to 0.05. No outliers were detected or removed from the analysis. All statistical analyses were carried out using the BMDP statistical software (Statistical Solutions Inc.).

Results

Generation of Neuron-Specific *Sp4* Rescue Mice

Mouse *Emx1* (Briata et al., 1996) and *Dlx5/6* (Robledo et al., 2002) expression are first detected at embryonic day 9.5 (E9.5) and E8.5, respectively. Forebrain excitatory neurons originate from the *Emx1* cell lineage (Gorski et al., 2002), while forebrain GABAergic neurons are derived from the *Dlx5/6* cell lineage (Monory et al., 2006). Since mouse *Sp4* expression starts around E9.5 (Supp et al., 1996), the *Cre* driven by *Emx1* and *Dlx5/6* genes will restore the *Sp4* gene during the entire development of forebrain excitatory

and GABAergic neurons, respectively. In F1 *Sp4* homozygous mice carrying either the *Emx1-Cre* or *Dlx6a-Cre* (*Dlx5/6-Cre*) gene (Figure S1), the nuclear *LacZ* cassette was excised to restore *Sp4* expression in each cell lineage (Figure 1). Consistent with previous reports on *Emx1-Cre* expression (Gorski et al., 2002), introduction of *Emx1-Cre* into *Sp4* heterozygous mice abolished *LacZ* staining in both cortical and hippocampal excitatory neurons (Figure 1A). Under higher magnification, many *LacZ* blue spots remained in both the cortex and hippocampus. They presumably consisted of GABAergic and other types of neurons (Figure 1B). Introduction of *Dlx6a-Cre* into *Sp4* heterozygous mice abolished *LacZ* staining in striatal GABAergic neurons. Because cortical GABAergic neurons are a minority group of neurons scattered across the cortex, the disappearance of *LacZ* staining in these GABAergic neurons cannot be directly visualized in the cortices of *Sp4* heterozygous mice carrying a *Dlx6a-Cre* transgene. However, *Dlx6a-Cre* expression in cortical GABAergic neurons was readily detected in the same *Dlx6a-Cre* mouse line when breeding with *ROSALacZ* (*Gtrosa26*^{tm1Sor}) reporter mice (http://www.informatics.jax.org/recombinase/specificity?id=MGI:3758328&systemKey=4856356).

Western blot provided validation of restoration of the Sp4 transcription factor in the cortex of the [*Sp4* $^{Hypo/Hypo}$; *Emx1*$^{+/+(Cre)}$] rescue mice (Figure 2A). Restoration of the Sp4 transcription factor was also confirmed in the striata of [*Sp4* $^{Hypo/Hypo}$; *Tg(Dlx6a-Cre)*] rescue mice (Figure 2B). An increased level of Sp4 protein was observed in the cortices of GABAergic *Sp4* rescue mice [*Sp4* $^{Hypo/Hypo}$; *Tg(Dlx6a-Cre)*]. Nevertheless, this increase was inconclusive because there are fewer GABAergic neurons than excitatory neurons in mouse cortices. To examine whether Sp4 expression is specifically restored in the cortical GABAergic neurons of *Sp4* rescue mice [*Sp4* $^{Hypo/Hypo}$;

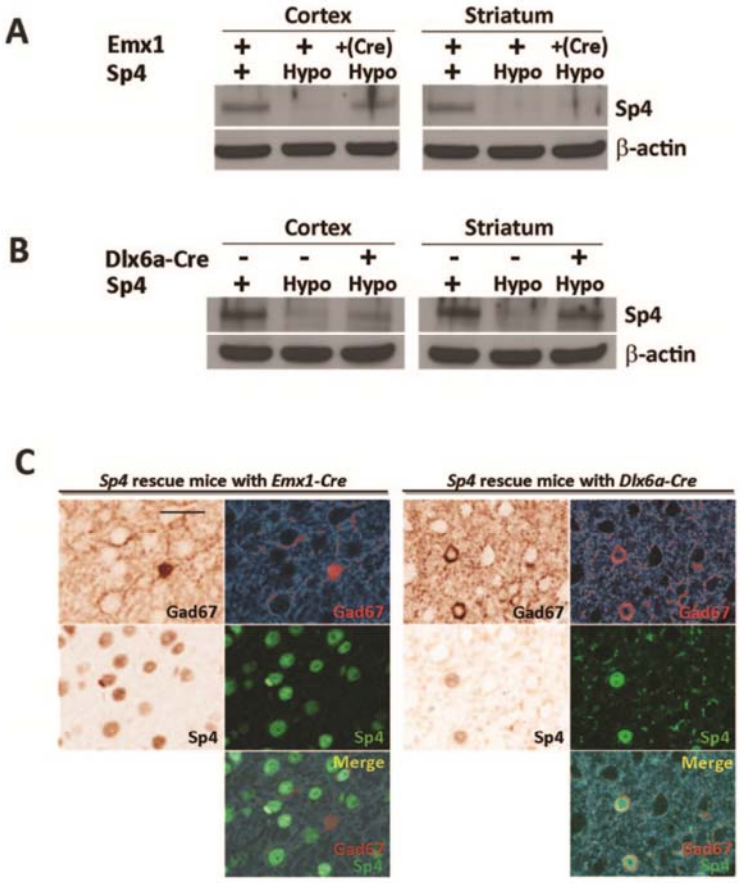

Figure 2. Sp4 expression in neuron-specific *Sp4* rescue mice. (A) Western blot confirmed expression of Sp4 protein in the cortices of rescue mice [*Sp4Hypo/Hypo; Emx1+/+(Cre)*]. (B) Western blot confirmed expression of Sp4 protein in the striata of rescue mice [*Sp4Hypo/Hypo; Tg(Dlx6a-Cre)*]. A low level of Sp4 protein in the cortices of the rescue mice likely comes from restored Sp4 expression in cortical GABAergic neurons. (C) Co-localization between Sp4 and Gad67 proteins was analyzed using sequential chromogenic immunohistochemistry in the cortices of *Sp4* rescue mice with either the *Emx1-Cre* or the *Dlx6a-Cre* gene. In *Sp4* hypomorphic mice carrying the *Emx1-Cre* gene, Sp4 protein expression was restored only in cortical excitatory neurons, but not in Gad67-positive cortical GABAergic inhibitory neurons. In contrast, Sp4 protein expression was only detected in Gad67-positive cortical GABAergic neurons in *Sp4* hypomorphic mice carrying the *Dlx6a-Cre* gene. Expression of Sp4 and Gad67 proteins was pseudocolored into green and red, respectively, and superimposed for their co-localization (Kim et al., 2012). Scale bar: 25 μm.

Tg(Dlx6a-Cre)], we conducted sequential immunohistochemical analyses of Sp4 and Gad67 proteins (Kim et al., 2012). Sp4 protein was detected only in the Gad67-positive GABAergic interneurons in the Sp4 rescue mice carrying the *Dlx6a-Cre* gene (Figure 2C). There is no detectable Sp4 protein in the surrounding Gad67-negative neurons, which are mostly cortical excitatory neurons. In contrast, most cortical neurons, except Gad67-positive interneurons, express Sp4 protein in the *Sp4* rescue mice carrying the *Emx1-Cre* gene.

Behavioral Characterization of the *Emx1-Cre* Rescue Mice

Deficits in PPI and ketamine-induced hyperlocomotion were examined in the two different neuron-specific *Sp4* rescue mice. In the *Emx1-Cre* rescue cohort, startle reactivity (Supplemental Table II) and PPI (Supplemental Table III and IV) were investigated. *Sp4* hypomorphic mice showed significantly reduced PPI [$F(1,80)=18.23$, $p < 0.0001$; Figure 3A], which was not rescued by restoration of the *Sp4* gene in forebrain excitatory neurons in [*Sp4* $^{Hypo/Hypo}$; *Emx1* $^{+/+(Cre)}$] rescue mice [*Sp4* X *Emx1-Cre* interaction, $F(1,80) < 1$, ns]. No sex effects were observed. We examined the locomotor response of these mice to ketamine (50 mg/kg) using video-tracking (VT) equipment. Before ketamine treatment, there was no significant *Sp4* gene effect on distance traveled during habituation. After ketamine injection, *Sp4* hypomorphic mice exhibited significantly more ketamine-induced hyperactivity compared to wild-type controls {Figure 3B; *Sp4* X ketamine interaction [$F(1,79)= 12.97$, $p = 0.0006$] on distance traveled}. No *Sp4* X *Emx1-Cre* X ketamine interaction was detected [$F(1,79) < 1$, ns].

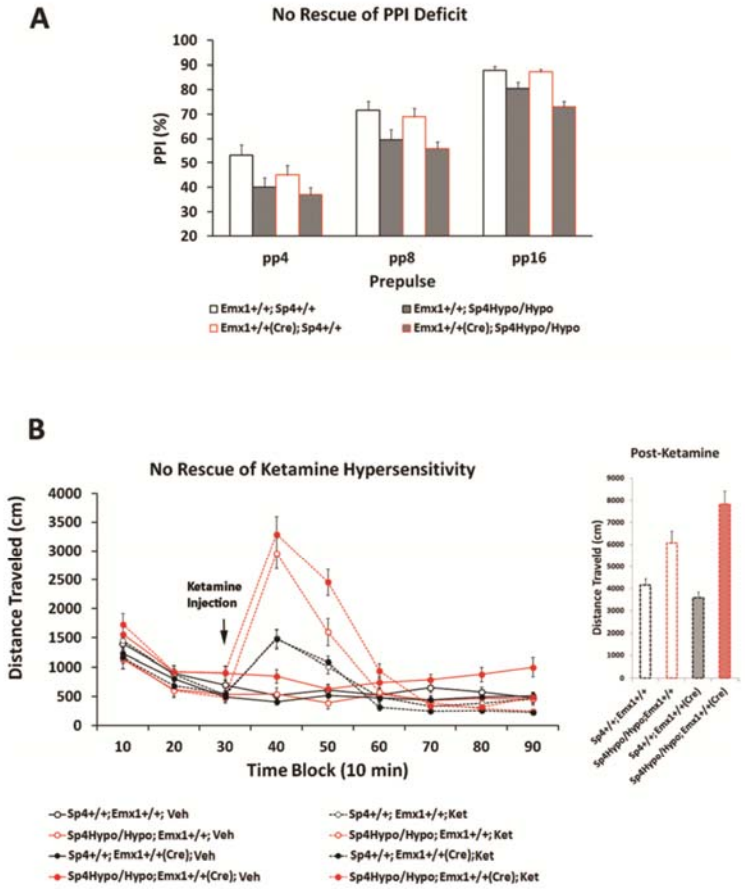

Figure 3. Characterization of rescue mice with restoration of Sp4 gene only in forebrain excitatory neurons. Male and female mice were balanced in each genotype. There were 18 wild-type (*Sp4+/+; Emx1+/+*), 20 Sp4 hypomorphic (*Sp4Hypo/Hypo; Emx1+/+*), 19 wild-type carrying *Emx1-Cre* (*Sp4+/+; Emx1+/+(Cre)*), and 27 rescue mice (*Sp4Hypo/Hypo; Emx1+/+(Cre)*) for behavioral analyses. (A) Prepulse inhibition was conducted in these mice. No sex effect was found. *Sp4* hypomorphic mice displayed prepulse inhibition of startle (PPI) deficits across three different prepulse intensities compared to wild-type mice, regardless of the presence or absence of the *Emx1-Cre* gene. (B) All mice were habituated for 30 min before injection of ketamine (50 mg/kg). Injection of ketamine significantly increased locomotor activity in all four genotypes. A significantly larger increase of locomotor activity was observed in *Sp4* hypomorphic mice, with or without the *Emx1-Cre* gene, than in wild-type mice {*Sp4* X ketamine interaction [$F(1,79)= 12.97$, $p = 0.0006$] on distance traveled}. However, there was no *Sp4* X *Emx1-Cre* X ketamine interaction. Error bar: standard error of the mean.

The rescue mice [*Sp4* $^{Hypo/Hypo}$; *Emx1* $^{+/+(Cre)}$] displayed similar ketamine-induced hyperlocomotion as the (*Sp4* $^{Hypo/Hypo}$; *Emx1* $^{+/+}$) *Sp4* hypomorphic mice after ketamine injection, suggesting that there was no rescue of ketamine hypersensitivity.

Behavioral Characterization of the *Dlx6a-Cre* Rescue Mice

In the *Dlx6a-Cre* rescue cohort, startle reactivity (Supplemental Table V) and PPI (Supplemental Table VI and VII) were investigated. *Sp4* hypomorphic mice exhibited reduced PPI [$F(1,85) = 9.73$, $p = 0.002$; Figure 4A]. However, no *Sp4* X *Dlx6a-Cre* interaction was detected [$F(1,85) = 1.38$, ns], with the [*Sp4* $^{Hypo/Hypo}$; *Tg(Dlx6a-Cre)*] rescue mice exhibiting similar PPI deficits to *Sp4* hypomorphic mice. The response to ketamine was subsequently examined with the VT locomotion test. No significant *Sp4* effect was observed during habituation. After ketamine injection, a significant *Sp4* X ketamine interaction [$F(1,85) = 14.54$, $p < 0.001$] was observed (Figure 4B). Interestingly, there was a significant *Sp4* X *Dlx6a-Cre* X ketamine interaction [$F(1,85)=7.44$, $p = 0.008$].

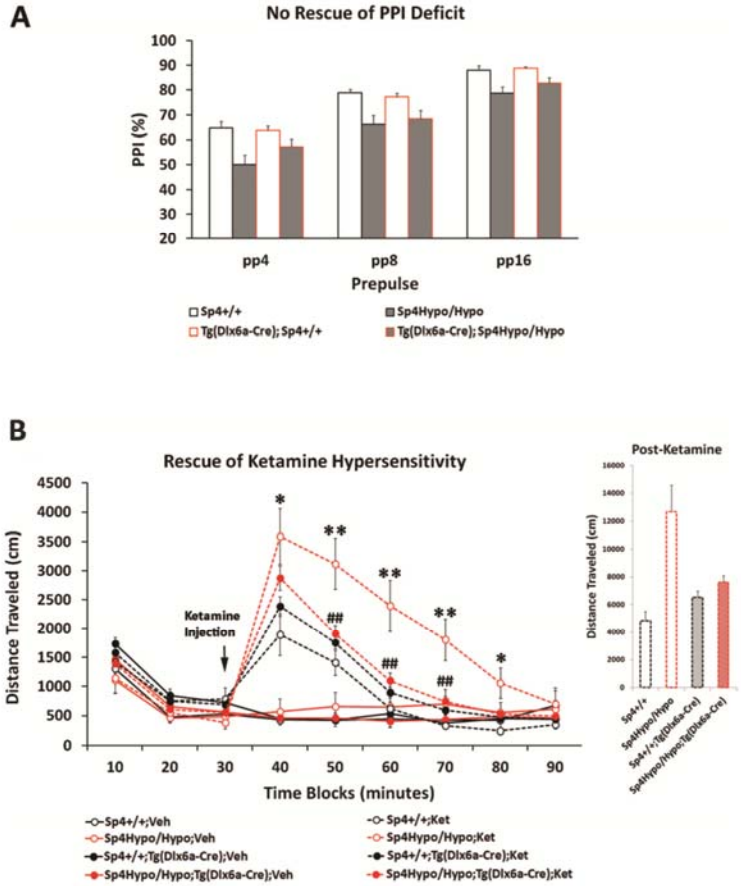

Figure 4. Prevention of ketamine-induced hyperlocomotion in rescue mice with restoration of *Sp4* gene only in forebrain GABAergic neurons. Male and female mice were balanced in each genotype. There were 9 wild-type (*Sp4+/+*), 13 *Sp4* hypomorphic (*Sp4Hypo/Hypo*), 46 wild-type carrying *Dlx6a-Cre* [*Sp4+/+; Tg(Dlx6a-Cre)*], and 21 rescue mice [*Sp4Hypo/Hypo; Tg(Dlx6a-Cre)*] for behavioral analyses. Our breeding generated fewer wild-type and *Sp4* hypomorphic mice. More rescue mice and the control wild-type mice carrying the *Dlx6a-Cre* transgene were produced. (A) Prepulse inhibition was examined in all four genotypes of mice. There was no sex effect on prepulse inhibition of startle (PPI). *Sp4* hypomorphic mice displayed PPI deficits across three different prepulse intensities compared to wild-type mice. No *Sp4* X *Dlx6a-Cre* interaction was observed. (B) After 30 min habituation in VT chamber, mice were injected with ketamine (50 mg/kg). As expected, *Sp4* hypomorphic mice displayed significantly more locomotor activity after ketamine injection [$F(1,85)=14.54$, $p < 0.001$]. *Post hoc* analysis revealed more distance traveled in the *Sp4* hypomorphic mice than wild-type mice at 10 ($^*p < 0.05$), 20 ($^{**}p < 0.01$), 30 ($^{**}p < 0.01$), 40 ($^{**}p < 0.01$), and 50 min ($^*p < 0.05$) post-ketamine injection. A significant *Sp4* X *Dlx6a-Cre* X ketamine interaction was found [$F(1,85)=7.44$, $p = 0.008$]. Post hoc analysis revealed significant differences in the distance traveled between the *Sp4* hypomorphic mice in the absence or presence (rescue) of the *Dlx6a-Cre* transgene at 20 ($^*p < 0.01$), 30 ($^*p < 0.01$), and 40 ($^*p < 0.01$) min post-ketamine injection. The rescue mice [*Sp4 Hypo/Hypo; Tg(Dlx6a-Cre)*] displayed no difference in locomotor activity from the wild-type mice carrying the *Dlx6a-Cre* gene, suggesting an almost complete rescue of the excessive response to ketamine. Error bar: standard error of the mean.

The rescue mice [*Sp4* $^{Hypo/Hypo}$; *Tg(Dlx6a-Cre)*] displayed the same time-course pattern of locomotive hyperactivity as the control mice [*Sp4* $^{+/+}$; *Tg(Dlx6a-Cre)*] after ketamine injection, suggesting that restoration of *Sp4* in forebrain GABAergic neurons is sufficient to rescue ketamine-induced hyperlocomotion in *Sp4* hypomorphic mice.

Discussion

Genetic manipulations are powerful tools for disentangling complex phenotypic profiles. We previously demonstrated that a full restoration of the *Sp4* gene in the whole animal by crossing with a protamine-cre mouse line rescued the complete phenotypic profile seen in *Sp4* hypomorphic mice (Zhou et al., 2005). Conventional and conditional gene-knockout strategies can determine whether a gene is necessary for a particular phenotype in a specific type of cells. Conditional rescue of a phenotype in knockout mice via restoration of the gene in only one specific cell type can determine whether the gene in that specific cell type is necessary and sufficient for controlling the phenotype. Many behaviors are complex, and likely modulated by interactions between different types of neurons from different brain regions. Given that different types of neurons are involved in the modulation of behavior, it is not surprising that *Sp4* gene restoration in only one specific type of neuron may not be sufficient to rescue the full behavioral profile. Indeed, we failed to rescue deficient PPI in *Sp4* hypomorphic mice regardless of whether the *Sp4* gene was restored in forebrain excitatory neurons or GABAergic neurons, although PPI was rescued previously when the *Sp4* gene was rescued in the whole animal (Zhou et al., 2005). Importantly, however, we found that restoration of the *Sp4* gene in GABAergic neurons was sufficient to rescue ketamine-induced hyperlocomotion in the *Sp4* hypomorphic mice.

Sp4 hypomorphic mice (*Sp4* $^{Hypo/Hypo}$) from the *Dlx6a-Cre* rescue cohort displayed a prolonged response to ketamine relative to the response in wild-type control mice. However, *Sp4* hypomorphic mice (*Sp4* $^{Hypo/Hypo}$; *Emx1*$^{+/+}$) from the *Emx1-Cre* rescue cohort had a similar time-course response to ketamine with their sibling wild-type mice. The prolonged response to ketamine in the *Sp4* hypomorphic mice from the *Dlx6a-Cre* rescue cohort is a more typical response of *Sp4* hypomorphic mice according to our previous studies (Ji et al., 2013). Why did the same dosage of ketamine not elicit a prolonged response to ketamine in the *Sp4* hypomorphic mice from the *Emx1-Cre* rescue cohort? We speculate that there may be still subtle differences between the genetic backgrounds of the *Emx1-Cre* and *Dlx6a-Cre* mouse lines despite the fact that both *Emx1-Cre* and *Dlx6a-Cre* mouse lines were backcrossed for more than six generations. The genetic background of the *Emx1-Cre* rescue cohort may have been overall less sensitive to ketamine than that of the *Dlx6a-Cre* rescue cohort. We consider that different time-course responses to ketamine may be generated by differential ketamine sensitivities between mouse cohorts or different dosages of ketamine. Indeed, increasing dosages of ketamine produced more locomotor activity and prolonged responses (Irifune et al., 1991). This observation may explain why the same dosage of ketamine generated different time-courses in the locomotor responses between the two different mouse cohorts.

Ketamine acts primarily, although not exclusively (Kapur and Seeman, 2002), as a noncompetitive antagonist of NMDA receptors that are present in both excitatory and GABAergic neurons in multiple brain regions. Cortical GABAergic inhibitory neurons have been suggested to be the primary targets of NMDA receptor antagonists (Moghaddam et al., 1997; Homayoun and Moghaddam, 2007). Our studies suggest that the mouse *Sp4* gene is essential for forebrain GABAergic neurons to dampen locomotor responses to ketamine. Reduced *Sp4* expression may impair the ability of GABAergic neurons to control locomotor hyperactivity in *Sp4* hypomorphic mice after ketamine injection (Ji et al., 2013). In contrast, the *Sp4* gene expression appears dispensable in excitatory neurons in controlling the locomotor response to ketamine. Since the genetic restoration of the *Sp4* gene in *Dlx6a-Cre* lineage cells starts from early embryogenesis, our studies cannot differentiate whether altered development and/or function of forebrain GABAergic neurons are responsible for ketamine hypersensitivity in *Sp4* hypomorphic mice. Although we did not find gross abnormalities in the brains of *Sp4* hypomorphic mice (Zhou et al., 2005; Ji et al., 2013), we cannot rule out subtle structural alterations in GABAergic neurons. As for molecular mechanisms, the NMDA receptor has been proposed as the primary target of ketamine. The protein, but not mRNA, of Nmdar1, the obligatory subunit of NMDA receptors, was down-regulated in the brains of *Sp4* hypomorphic mice (Zhou et al., 2010; Sun et al., 2015). Those findings, however, came from mouse brain tissue that contained different types of neurons and glial cells, and hence do not necessarily exclude the *Nmdar1* gene as a direct target of the Sp4 transcription factor in a small group of specific neurons. There is still a possibility that the Sp4 transcription factor may directly regulate mRNA expression of the *Nmdar1* gene in some forebrain GABAergic neurons (Krainc et al., 1998). Forebrain GABAergic neurons consist of striatal neurons and different cortical GABAergic neurons. It remains to be investigated which subset of GABAergic neurons is responsible for the rescue effects observed here. Investigation of molecular mechanisms can eventually be conducted after identification of the critical GABAergic neurons. It should be kept in mind that other receptors and channels, in addition to NMDA receptors, may also mediate the effects of ketamine in *Sp4* hypomorphic mice (Jamie Sleigha et al., 2014). In the future, restoration of the *Sp4* gene in forebrain GABAergic neurons via temporal and region-specific (e.g. cortex or striatum) control of Cre-mediated DNA recombination may clarify the role of development of GABAergic neurons and functions of regional GABAergic neurons in controlling locomotor responses to ketamine.

Cortical GABAergic inhibitory interneurons have been proposed to be the primary targets of NMDA receptor antagonists (Moghaddam et al., 1997; Homayoun and Moghaddam, 2007) and contribute to the pathogenesis of schizophrenia (Lewis et al., 2005; Lewis and Moghaddam, 2006). Abnormalities in GABA receptors have been reported in the brains of patients with schizophrenia, bipolar disorder, or major depression (Fatemi et al., 2013). Ketamine produces schizophrenia-like behavioral phenotypes in healthy people and exacerbates symptoms in schizophrenia patients. Our studies suggest that SP4, deleted in some patients with schizophrenia, could be a missing piece that links a genetic susceptibility gene with ketamine hypersensitivity and abnormal function of GABAergic neurons in schizophrenia. The influence of the *Sp4*-GABAergic-ketamine pathway may not be limited to the regulation of locomotor activity in response to ketamine. Given that ketamine functions as a potent antidepressant, it will be interesting to investigate whether the *Sp4*-GABAergic-NMDA pathway may also be involved in the regulation of depressive behaviors. Indeed, our most recent studies suggested that *Sp4* hypomorphic mice displayed depressive-like behaviors (Young et al., 2015). In the future, such depressive-like phenotypes will be examined after ketamine treatment in *Sp4* hypomorphic mice with restoration of the *Sp4* gene in GABAergic neurons.

Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

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Statement of Interest

In the past three years, Dr Geyer has received consulting compensation from Abbott, Dart, Lundbeck, Neurocrine, Omeros, Otsuka, and Sunovion, and holds an equity interest in San Diego Instruments. Dr Young has received consulting compensation from Cerca Insights, Lundbeck Ltd, Omeros, and Amgen. Dr Powell held service contracts with Servier and ACADIA pharmaceuticals. Dr Risbrough has received research funding from Johnson and Johnson, Omeros, and Sunovion.

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Chapter 3, in full, is a reprint of the material as it appears in Higa KK, Ji B, Buell MR, Risbrough VB, Powell SB, Young JW, Geyer MA, Zhou X. *Restoration of Sp4 in forebrain GABAergic neurons rescues hypersensitivity to ketamine in Sp4 hypomorphic mice.* International Journal of Neuropsychopharmacology. 2015 Jun 2;18(11):pyv063. The dissertation author conducted animal experimentation and data analyses and assisted with manuscript preparation.

Chapter 4:

GlyT-1 inhibition attenuates attentional but not learning or motivation deficits of the Sp4 hypomorphic mouse model relevant to psychiatric disorders

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GlyT-1 Inhibition Attenuates Attentional But Not Learning or Motivational Deficits of the Sp4 Hypomorphic Mouse Model Relevant to Psychiatric Disorders

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Serious mental illness occurs in 25% of the general population, with many disorders being neurodevelopmental, lifelong, and debilitating. The wide variation and overlap in symptoms across disorders increases the difficulty of research and treatment development. The NIMH Research Domain of Criteria initiative aims to improve our understanding of the molecular and behavioral consequences of specific neurodevelopmental mechanisms across disorders, enabling targeted treatment development. The transcription factor Specificity Protein 4 (SP4) is important for neurodevelopment and is genetically associated with both schizophrenia and bipolar disorder. Reduced Sp4 expression in mice (hypomorphic) reproduces several characteristics of psychiatric disorders. We further tested the utility of Sp4 hypomorphic mice as a model organism relevant to psychiatric disorders by assessing cognitive control plus effort and decision-making aspects of approach motivation using cross-species-relevant tests. Sp4 hypomorphic mice exhibited impaired attention as measured by the 5-Choice Continuous Performance Test, an effect that was attenuated by glycine type-1 transporter (GlyT-1) inhibition. Hypomorphic mice also exhibited reduced motivation to work for a reward and impaired probabilistic learning. These deficits may stem from affected anticipatory reward, analogous to anhedonia in patients with schizophrenia and other psychiatric disorders. Neither positive valence deficit was attenuated by GlyT-1 treatment, suggesting that these and the attentional deficits stem from different underlying mechanisms. Given the association of *SP4* gene with schizophrenia and bipolar disorder, the present studies provide support that personalized GlyT-1 inhibition may treat attentional deficits in neuropsychiatric patients with low SP4 levels.

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INTRODUCTION

Mental health disorders affect approximately one-quarter of the world's population and are commonly lifelong and debilitating. The sufferers of such disorders exhibit a myriad of distinct yet overlapping symptoms. This overlap is consistent with evidence that many illnesses share numerous associated genetic polymorphisms. Recognizing these overlapping genes and symptoms, the National Institutes of Mental Health began the Research Domain Criteria (RDoC) initiative to provide a new way of classifying psychopathology based on dimensions of functioning (Cuthbert, 2014). By investigating genetic constructs associated with numerous psychiatric disorders—particularly those relevant to neurodevelopment (Insel and Wang, 2010)—and testing behavioral domains affected across those disorders, more circuit-specific targeted treatments may be developed.

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Specificity protein 4 (*SP4*) gene is a member of the SP1 family of transcription factors and is important for neurodevelopment. The *SP4* gene recognizes GC-rich sequences of 'CpG islands' around the promoters of a variety of genes and is neuronally localized (Supp *et al*, 1996; Zhou *et al*, 2005). SP4 is therefore highly expressed during neuronal differentiation (Sun *et al*, 2014; Zhou *et al*, 2007), and regulates dendritic patterning and neural development during maturation (Ramos *et al*, 2007; Zhou *et al*, 2007). Importantly, the *SP4* gene was deleted in some patients with schizophrenia (Tam *et al*, 2010; Zhou *et al*, 2010), whereas single-nucleotide polymorphisms of SP4 have also been associated with schizophrenia (Zhou *et al*, 2009), bipolar disorder, and major depressive disorder (Pinacho *et al*, 2011; Shi *et al*, 2011; Zhou *et al*, 2009). Such associations are likely functionally significant given that SP4 protein levels are reduced in peripheral blood mononuclear cells in first-episode schizophrenia patients (Fuste *et al*, 2013). Reducing Sp4 expression (hypomorphic (Hyp)) in mice results in abnormalities relevant to schizophrenia and bipolar patients, eg, reduced sensorimotor gating (Zhou *et al*, 2005) and reduced *N*-methyl-D-aspartate receptor (NMDAR) protein expression (Zhou *et al*, 2010). These mice are also hypersensitive to the

NMDAR noncompetitive antagonist ketamine and competitive NMDAR antagonist SDZ 220–581, resulting in reduced habituation to a novel environment (Ji *et al*, 2013). Interestingly, acutely ill schizophrenia patients exhibit reduced habituation to a novel environment (Perry *et al*, 2009). Moreover, patients with schizophrenia are hypersensitive to phencyclidine (Luby *et al*, 1959), another NMDAR antagonist. Hence, Sp4 Hyp mice—that are genetically relevant to psychiatric patients—recreate some behavioral abnormalities seen in such patients.

The behavior of Sp4 Hyp mice has yet to be assessed in cross-species cognitive/behavioral paradigms that are RDoC relevant however (tasks such as those reviewed in Young and Geyer, 2015). RDoC domains such as cognitive control can be measured using the 5-Choice Continuous Performance Test (5C-CPT) (Lustig *et al*, 2013; Young *et al*, 2009, 2013a), in which patients with schizophrenia (Young *et al*, 2013a) and bipolar mania (Young, Geyer, Minassian and Perry; unpublished observations) exhibit deficits. Positive valence, specifically the subconstruct of preference-based decision making of approach motivation, can be measured using a probabilistic learning task (Amitai *et al*, 2013; Bari *et al*, 2010), in which schizophrenia patients exhibit deficits (Armstrong *et al*, 2012; Gold *et al*, 2008; Ragland *et al*, 2012a; Waltz *et al*, 2011). The subconstruct of effort valuation can be measured using the progressive ratio breakpoint paradigm (PRBP; Bensadoun *et al*, 2004; Young and Geyer, 2010,) that is also impaired in schizophrenia patients (Ellenbroek and Cools, 2000; Wolf *et al*, 2014). Hence, in the current studies, we examined the cognitive control and aspects of approach motivation of Sp4 Hyp mice and their wild-type (WT) littermates in these paradigms. Given the reduced NMDAR function in these mice, we also tested whether a glycine type-1 transporter (GlyT-1) inhibitor could remediate any deficits observed, as glycine is a NMDAR co-agonist (Javitt, 2012; Javitt *et al*, 2001).

MATERIALS AND METHODS

Animals

For all behavioral experiments, the same cohort of male Sp4 WT ($n=17$) and Hyp ($n=11$) mice were generated from heterozygous breeding pairs. The Sp4 mice were generated as previously described (Zhou *et al*, 2005) and maintained as Sp4 heterozygous in 129S mice and Black Swiss backgrounds. In brief, a nuclear *LacZ* expression cassette was capped with a splicing acceptor and further flanked by two *loxP* sites. The floxed LacZ cassette was inserted into the first intron of mouse *Sp4* gene, and followed by a rat *Sp4* cDNA gene fused in frame into the second exon of mouse *Sp4* gene (Zhou *et al*, 2005). Mouse endogenous *Sp4* gene was replaced by the targeting cassette via homologous recombination. Expression of the rat *Sp4* gene was blocked by the upstream *LacZ* gene tagged with three transcription terminators. Because of leakage of transcription termination *in vivo*, there is 2–5% of the level of Sp4 expression in Sp4 homozygous mice compared with WT mice. These Sp4 mutant mice were therefore termed Sp4 Hyp mice. After breeding the Sp4 Hyp mice with Cre lines, the *LacZ* gene can be deleted by the Cre to restore Sp4 gene expression. All test mice were the F1 generation mice with the same genetic background (Ji *et al*, 2013; Zhou *et al*, 2010), were 3 months old at the time of testing, and weighed between 21 and 28 g. All animals were group housed (maximum four/cage) and maintained in a temperature-controlled vivarium (21 ± 1 °C) with a reversed 12 h day/night cycle (lights off at 0700 h and on at 1900 h). During training, mice were food restricted to maintain weight at 85% of their free-feeding weight, as is commonly used during reward-learning training (Young *et al*, 2011). For quantification of GlyT-1 levels, 8 WT and 7 Hyp mice at 5 months were used (see Supplementary Methods). Water was available *ad libitum* except during training and testing that occurred during the dark phase of the day/night cycle between 0800 and 1200 h. All behavioral testing procedures were approved by the UCSD Animal Care and Use Committee before initiation of experiments. The UCSD animal facility meets all federal and state requirements for animal care.

Drugs

Org 24598 lithium salt and d-amphetamine were purchased from Sigma Aldrich (St Louis, MO) and dissolved in saline. Based on previous reports (Achat-Mendes *et al*, 2012; Lido *et al*, 2012), Org 24598 (0.3, 1, and 3 mg/kg) was injected intraperitoneally with a volume of 5 ml/kg, 30 min before testing. d-amphetamine (1 mg/kg) was injected intraperitoneally with a volume of 5 ml/kg, 5 min before testing, based on evidence of improvement in probabilistic learning (Young, Khan and Powell; unpublished observations). Free-base drug weight was used in all drug calculations.

Apparatus

5-Choice chambers. Training and testing took place in four 5-hole operant chambers ($25\times25\times25$ cm; Med Associates, St Albans, VT) consisting of an array of five square holes ($2.5\times2.5\times2.5$ cm) arranged horizontally on a curved wall 2.5 cm above the grid floor (Figure 1a) as described elsewhere (van Enkhuizen *et al*, 2013). This array was opposite a food delivery magazine (Lafayette Instruments, Lafayette, IN) located at floor level with a house light located near the ceiling. The chamber was located in a sound-attenuating box, ventilated by a fan that also provided a low level of background noise. An infrared camera installed in each chamber enabled the monitoring of performance during training and testing. Mice were trained to respond with a nose-poke to an illuminated LED recessed into the holes. Responses were detected by infrared beams mounted vertically and located 3 mm from the opening of the hole. Liquid reinforcement in the form of strawberry milkshake (Nesquik plus nonfat milk, 30 µl) was delivered by peristaltic pump (Lafayette Instruments) to a well located in the magazine opposite the 5-hole wall. Magazine entries were monitored using an infrared beam mounted horizontally 5 mm from the floor and recessed 6 mm into the magazine. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates) using custom programming (Amitai *et al*, 2013; Young *et al*, 2012, 2013c).

Progressive ratio breakpoint testing. Mice were trained in the 5-choice serial reaction-time task daily, 5 days per week,

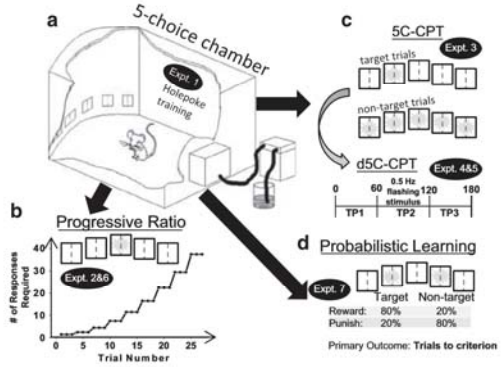

Figure 1 Behavioral challenges on Sp4 hypomorphic and wild-type mice. (a) Sp4 mutant mice were trained to respond to lit holes in 5-choice chambers whereby a response in any one of the five recessed apertures (detected by infrared beams, vertical dashed line) resulted in strawberry milkshake reward delivery. (b) After initial training mice were tested in the progressive ratio breakpoint schedule of reinforcement. Because the number of responses required in the central aperture for a single reward kept increasing after three3 responses/rewards at that level, this challenge established the motivation of mice to work for a single reward. The 'breakpoint' of the mice indicated their willingness to work for a single reward. (c) Mice were then trained on the 5-choice continuous performance test (5C-CPT) that required mice to differentiate between target and nontarget trials, requiring responding in the singly lit aperture or inhibiting when all five apertures were lit respectively. Mice were then challenged in the distracting (d)5C-CPT where during trial periods 1 and 3 (trials 1–60 and 121–180) the task was consistent with the standard 5C-CPT, but during trial period 2 (trials 61–120) a 0.5 Hz flashing light continuously occurred. (d) After these studies, mice were challenged in the probabilistic learning paradigm, wherein mice were presented with a right or left stimulus. One stimulus was assigned as target and the other as nontarget, with responses to the former rewarded (milkshake) 80% and punished (4 s illuminated house light) 20% of the time and vice-versa for the latter.

as described previously (Young and Geyer, 2010,). Each session lasted 30 min or 120 trials, whichever was completed first. Training began by associating the magazine delivering reward in conjunction with its illumination (Hab1). Mice were then trained to respond into any one of the five apertures for a single reward (Hab2). Once responding reliably (>70 responses in 30 min for 2 consecutive days), mice were challenged in the breakpoint study. The number of nose-pokes required to gain a reward increased according to the following progression: 1, 2, 4, 7, 11, 16, 22, 29, 37, 46, 56, and 67. To maintain responding, each ratio had three steps before moving to the next ratio (Figure 1b). Importantly, for each completed step, mice only received one reward. The session continued for 60 min or until 5 min had passed without a nose poke. The *breakpoint* was defined as the last ratio to be completed before the session ended. Mean reward latencies (MRLs) and mean response latencies were also calculated. The progressive ratio challenge was conducted on Tuesday with normal shaping on the previous day.

5-Choice continuous performance test training. After PRBP testing, mice were retrained in Hab2 requiring responses in all apertures (Wednesday–Friday). The mice were then moved onto 5C-CPT training as previously described (Young *et al*, 2009, 2013c). Each trial was initiated by the mouse nose-poking and then removing its nose from the magazine. After a 5-s ITI, a light stimulus appeared in one of the five apertures located opposite the magazine. A nose-poke in the lit aperture during the stimulus duration (SD) plus a 2-s limited hold period resulted in a *correct* (Hit) response being registered and a reward being delivered in the magazine. A nose-poke in any other aperture over this period was registered as an *incorrect* response and resulted in a 4-s time-out (TO). Failure to respond in any aperture during the SD+limited hold was registered as an *omission* (omission+incorrect = Miss) and also resulted in a TO. Response in any aperture during the ITI registered a *premature* response and triggered a TO. The next trial began when the mouse entered and then exited the magazine. The SD started at 20 s and was reduced to 10, 8, and 4 s after the attainment of each criterion (a mean correct latency less than half the current SD for 2 consecutive days) across sessions. At this point, mice were transferred to a variable ITI (3–7 s). Once performance stabilized (~1 week), the mice were transferred to the 5C-CPT. For the 5C-CPT, 100 trials were target trials, identical to trials described in the 5-choice serial reaction-time task where a cue stimulus could appear in any 1 of the 5 apertures, 20 trials were nontarget trials, unique to the 5C-CPT in which all 5 apertures were illuminated, and the mouse was required to inhibit responding (Figure 1c). Training took ~4 months. Consistent with human CPTs (Riccio *et al*, 2002), successful inhibition of a response in a nontarget trial resulted in a *correct rejection* (*CR*) being recorded and reward delivered. Responding in a nontarget trial, however, resulted in a *false alarm* (*FA*) being registered and a TO occurring. These nontarget trials were interspersed pseudorandomly within the 100 target trials (maximum of 3 sequential no-go trials). For all 3 tasks, the *mean correct latency* (*MCL*) was calculated along with the following parameters:

Measures resulting from target trials only

$$accuracy = \frac{Hit}{Hit + Incorrect} \quad \%Omissions = \left(\frac{omissions}{TotalTrials}\right) \times 100$$

$$p(HR) = \frac{Hit}{Hit + Miss}$$

Measures resulting from target and nontarget trials

$$p(FA) = \frac{FA}{FA + CR}$$

Based upon these basic parameters, signal detection indices (Green and Swets, 1966; McNicol, 1972) were calculated to assess both sensitivity index (SI) and responsivity index bias (RI). The SI was calculated using the following formula:

$$SI = \frac{p(HR) - p(FA)}{2[p(HR) + p(FA)] - [p(HR) + p(FA)]^2}$$

SI provides a nonparametric assessment of sensitivity (Frey and Colliver, 1973). Values for SI vary from −1 to +1, with

+1 indicating that all target events were responded to, whereas all nontarget events were inhibited from responding to, and 0 indicating chance levels of distinguishing between signal and nonsignal events. SI was also the basis by which McGaughy and Sarter (1995) developed their vigilance index measure and hence would produce comparable results for mice to those seen in rats performing their vigilance paradigm. To mirror the use of SI, the nonparametric response bias measure RI (Frey and Colliver, 1973) was chosen to provide a measure of the 'tendency to respond' (Frey and Colliver, 1973; Marston, 1996; Sahgal, 1987).

$$RI = \frac{p(HR) + p(FA) - 1}{1 - [p(FA) - p(HR)]^2}$$

Both SI and RI are based on the same geometric logic and are both appropriate for use with single choice procedures (respond or not; Marston, 1996).

Probabilistic learning assessment. This task was based on a similar task developed by Bari *et al* (2010) and previously described (Amitai *et al*, 2013). Briefly, the task presented two stimuli (holes 2 and 4) wherein responses by mice in one aperture (target) were reward 80% and punished 20% of the time, whereas the contingencies were reversed for responses in the other aperture (nontarget; Figure 1d). This session lasted 1 h, and learning criterion performance was defined as eight consecutive nose-poke responses (rewarded or unrewarded) into the target location. The apertures were illuminated until a response was made. The primary outcome measure was *trials to criterion*. Secondary measures included a measurements of motor impulsivity—*premature responses*—and strategy formation analysis—including *target win-stay ratio* (no. of target responses after rewarded target response/ no. of total responses after rewarded target response), *nontarget win-stay ratio* (no. of nontarget responses after rewarded nontarget response/no. of total responses after rewarded nontarget response), *target lose-shift ratio* (no. of nontarget responses after punished target response/no. of total responses after punished target response), and *nontarget lose-shift ratio* (no. of target responses after punished nontarget response/no. of total responses after punished nontarget response).

Experimental Challenges

Once trained to respond in lit apertures (experiment 1), the Sp4 mice were tested in the progressive ratio breakpoint procedure (experiment 2) in order to gauge the motivation of mice to work for a single reward (Barnes *et al*, 2014; Markou *et al*, 2013), as described previously (Bensadoun *et al*, 2004; Young and Geyer, 2010,). The mice were then trained in the 5C-CPT and stable baseline performance recorded (experiment 3). The cognitive control of mice was then challenged on a single test day (Wednesday) between normal training sessions (Monday, Tuesday, Thursday, and Friday) using the distracting (d)5C-CPT (Figure 1c). This challenge mirrored work by Sarter and colleagues (Demeter *et al*, 2013; Howe *et al*, 2010), designed to further measure control of attention (Lustig *et al*, 2013). The test session was split into 3 blocks of 60 trials wherein the second (middle) block included a flashing light during trials, located 2 cm above the array and flashing at 0.5 Hz (experiment 4). This challenge was then used to test the effects of GlyT-1 inhibition on performance. Mice were treated with Org 24598 before testing in the d5C-CPT (experiment 5). Mice were then retested in the progressive ratio breakpoint challenge with Org 24598 treatment at 1 mg/kg before testing (experiment 6). The mice were then tested in the within-session probabilistic learning paradigm to assess the effects of Org 24598 or amphetamine on the learning of these mice (experiment 7). Finally, assessment of GlyT-1 levels in a new cohort of mice was established (experiment 8).

Statistics

Acquisition of hole-poking in the 5-choice chambers was analyzed by comparing the number of days to criterion for Hab1 (>30 reward associations for 2 consecutive days) and Hab2 (>70 responses for 2 consecutive days) between the two genotypes using independent samples *t*-tests. Baseline 5C-CPT data were analyzed using a two-way repeated measures ANOVA with day as a within-subjects factor and genotype as the between-subjects factor. The distraction challenge data were analyzed using a two-way repeated measures ANOVA with trial period as a within-subjects factor and genotype as a between-subjects factor. When tested with the GlyT-1 inhibitor, drug dose was also included as a within-subjects factor. To assess motivation, mice were tested in the progressive ratio breakpoint study using an independent *t*-test to compare measures between genotypes. When tested with the GlyT-1 inhibitor, data were analyzed using two-way ANOVAs with genotype as a between-subjects factor and drug as a within-subjects factor. For the probabilistic learning study, data were analyzed using two separate two-way ANOVAs with the GlyT-1 inhibition and amphetamine data were analyzed separately as within-subject factors and genotype as the between-subjects factor. GlyT-1 levels were compared across genotypes using a two-tailed *t*-test. All data were analyzed using SPSS (20.0, Chicago, IL).

RESULTS

Experiment 1: Response Acquisition Training

Sp4 Hyp mice took longer to acquire both the association between food delivery and illumination ($t_{(26)} = -5.7$, $p < 0.0001$) and hole response for reward ($t_{(26)} = -4.1$, $p < 0.0001$; see Table 1). When trained to hole-poke in a single lit cue for a reward, Sp4 Hyp took significantly longer to acquire the task than WT littermates ($t_{(26)} = -2.4$, $p < 0.05$).

Experiment 2: Sp4 Mutant Effects on Progressive Ratio Breakpoint Schedule of Reinforcement

Once responding stably, the mice were challenged with a PRBP to assess motivation. Overall, Sp4 Hyp mice tended to have a lower breakpoint ($F_{(1,25)} = 3.6$, $p < 0.1$; Supplementary Figure 1A) and exhibited a significantly slower mean response time ($F_{(1,25)} = 12.8$, $p < 0.005$; Supplementary Figure 1B) compared with WT mice. However, latencies to

Table 1 Comparison of Operant Training between Sp4 Wild-Type (WT) and Hypomorphic (Hyp) Mice

Sp4	Magazine illumination/ reward delivery acquisition (HAB1)	5-Hole array responding (HAB2)	Single lit hole choice acquisition
WT	3.1 (±0.1)	6.5 (±0.4)	18.0 (±2.1)
Hyp	5.1 (±0.4)*	20.9 (±4.3)*	25.8 (±4.1)*

Data presented as mean ± SEM.
*$P<0.05$ cf. WT.

collect rewards did not differ between the two genotypes ($F<1$, NS; Supplementary Figure 1C).

Experiment 3: 5C-CPT: Baseline Performance

Once trained, the stability of 5C-CPT performance of Sp4 mutant mice was assessed across 4 days. Sp4 Hyp mice exhibited poorer vigilance compared with WT mice ($F_{(1,22)}=3.8$, $p<0.05$; Supplementary Figure 2A). In one-way *t*-test analyses, WT mice exhibited higher vigilance than chance responding ($t_{(15)}=3.5$, $p<0.005$), whereas Sp4 mice did not ($t_{(13)}=1.0$, NS). This deficit was driven by a lower Hit Rate ($F_{(1,22)}=12.5$, $p<0.001$; Supplementary Figure 2B) compared with WT mice, as the False Alarm Rate was unchanged ($F<1$, NS; Supplementary Figure 2C). Sp4 Hyp mice were also less responsive than WT mice ($F_{(1,22)}=4.5$, $p<0.05$; Supplementary Figure 2D), as measured using the RI. However, no genotype effect was observed in the total number of trials ($F<1$, NS). Sp4 Hyp mice were also less accurate in responding than WT mice ($F_{(1,22)}=6.3$, $p<0.05$; Supplementary Figure 2E). The lower Hit Rate of Sp4 Hyp mice was driven by a higher % omissions than WT mice ($F_{(1,22)}=10.7$, $p<0.001$; Supplementary Figure 2F). The Sp4 Hyp mice were also slower to correctly respond compared with WT mice ($F_{(1,22)}=16.7$, $p<0.005$; Supplementary Figure 2G). No genotypic difference in premature responses was observed ($F<1$, NS; Supplementary Figure 2G). Stability of performance was demonstrated by a lack of improvement in any of these measures over days as well as a lack of interaction with genotype ($F<1.6$, NS; Supplementary Figure 2H).

Experiment 4: 5C-CPT: Effects of Within-Session Distraction

After identifying poor vigilance of Sp4 mutant mice, we then assessed their control of attention using a visual distracting paradigm. During trials 41–80, a distracting visual stimulus located above the stimulus array flashed at 0.5 Hz (Figure 2i). A main trial-period effect ($F_{(2,50)}=5.9$, $p<0.001$; Figure 2a) revealed that vigilance performance was worst during the distracting trial period compared with the first and third trial periods ($p<0.05$). No genotype × trial-period interaction was observed ($F<1$, NS), revealing that Sp4 Hyp mice tended to perform worse ($F_{(2,32)}=3.7$, $p=0.067$) than WT mice throughout the session.

Overall, no effect of Trial Period, genotype, or their interaction was observed for Hit Rate ($F<2.4$, NS; Figure 2b) or False Alarm Rate ($F<1$, NS; Figure 2c). However, Trial Period tended to affect response bias as measured by RI ($F_{(2,50)}=3.1$, $p=0.063$; Figure 2d), indicating that mice in trial period 3 (after distraction) were more responsive compared with the other two trial periods ($p<0.05$). No effect of Trial Period, genotype, or their interaction was observed for Accuracy ($F<2.0$, NS; Figure 2e). The % omissions of mice were lower in trial period 3 after distraction ($F_{(2,50)}=6.6$, $p<0.005$; Figure 2f) compared with trial periods 1 and 2 ($p<0.05$). No effect of genotype or interaction with Trial Period was observed ($F<2.5$, NS). A main effect of Trial Period was observed in premature responses ($F_{(2,50)}=12.3$, $p<0.001$; Figure 2g), driven by higher levels during the distracting period compared with the other two trial periods ($p<0.001$). No effect of genotype or its interaction with Trial Period was observed ($F<1$, NS). Finally, mice exhibited faster reaction times ($F_{(2,50)}=4.2$, $p<0.05$; Figure 2h) in trial periods 2 and 3 (during and after distraction) compared with the first trial period ($p<0.05$).

Experiment 5: GlyT-1 Inhibitory Effects on 5C-CPT of SP4 Mutant Mice during Distraction

Unlike baseline performance, Sp4 mice did not exhibit lower d' levels overall ($F_{(1,16)}=2.0$, NS), likely as a result of genotype × GlyT-1 inhibition interaction ($F_{(2,32)}=4.9$, $p<0.05$; Figure 3). The *post hoc* ANOVA analyses revealed that GlyT-1 inhibition tended to lower d' in WT mice ($F_{(2,22)}=2.7$, $p=0.092$), but significantly increased it in Sp4 Hyp mice ($F_{(2,10)}=3.2$, $p<0.05$). These effects were irrespective of trial period and hence were unlikely to be attributable to treatment effects on distraction. Although no overall effect of trial period was observed ($F_{(2,32)}=2.1$, NS), performance was significantly lower during distraction compared with trial period 3 ($p<0.05$).

During this challenge, Sp4 Hyp mice had a comparable Hit Rate with WT mice ($F<1$, NS). Moreover, genotype did not interact with trial period, drug treatment, or both ($F<1.6$, NS). However, a trial period × drug treatment interaction was observed ($F_{(4,64)}=5.0$, $p<0.005$). The *post hoc* analyses revealed that Hit Rate during the distracting trial period was significantly lower ($F_{(2,32)}=3.6$, $p<0.05$) than the other two periods ($p<0.05$) in mice treated with saline. Interestingly, a main trial period effect was also seen in mice treated with 0.3 mg/kg of the GlyT-1 inhibitor ($F_{(2,32)}=6.5$, $p<0.005$) but this effect was driven by poor performance in the final trial period *after* the distracting trial period. No main effect of trial period was seen in mice treated with 1 mg/kg of the GlyT-1 inhibitor ($F_{(2,32)}=2.2$, NS), although performance was worst during the distracting trial period. In terms of response disinhibition measured by false alarms, no effect of any factor or their interaction was observed ($F<1.1$, NS). Neither genotype nor Trial Period nor GlyT-1 inhibition

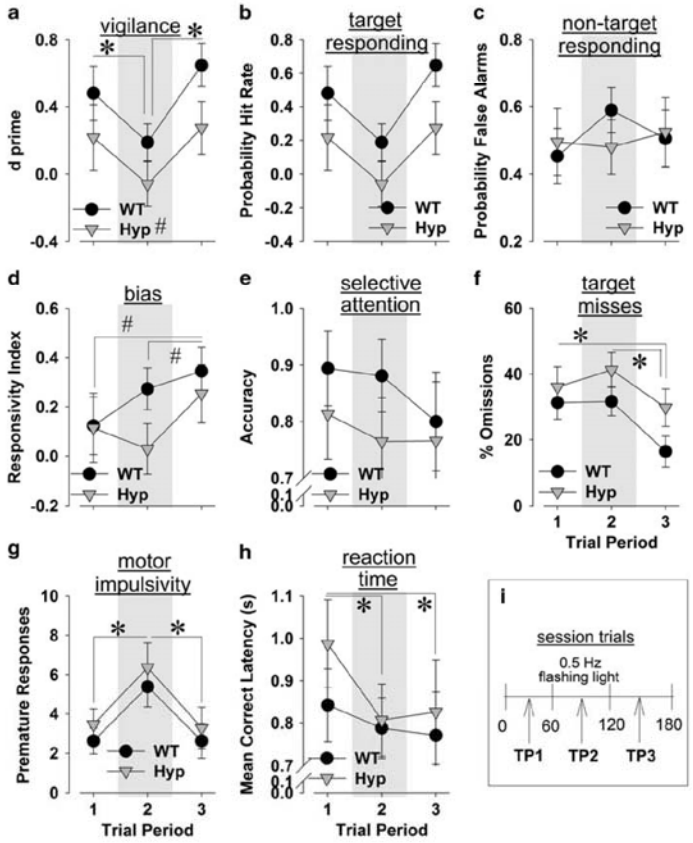

Figure 2 Effect of distraction on the control of attention of Sp4 hypomorphic (Hyp) and wild-type (WT) littermate mice. After baseline assessment, the control of attention of Sp4 Hyp and WT littermate mice was assessed by flashing 0.5 Hz light stimulus during the middle trial period of testing (shaded section). The vigilance performance of both WT and Hyp mice was deleteriously affected by distraction, with Sp4 Hyp tending to exhibit poorer performance irrespective of Trial Period (a). Responses to targets were primarily, though nonsignificantly, affected by the distracting stimulus (b) as response inhibition was unaffected (c). Interestingly, mice were responding more liberally after the distraction irrespective of genotype (d). The selective attention (accuracy) of mice was unaffected by the distracting stimulus (e), whereas target misses were reduced after distraction (f). Mice were more motorically impulsive during the distracting Trial Period (g), whereas reaction times (latency to respond) were also sped during and after distraction irrespective of genotypes (h). The breakdown of distracting *vs* nondistracting trial period is depicted (i). Data presented as mean ± SEM, $^{*}P<0.05$, $^{\#}p<0.1$ compared with indicated direction.

affected or interacted together to affect response bias measured using the RI ($F<2.1$, NS).

Sp4 Hyp mice did not differ in accuracy ($F<1.9$, NS) compared with WT mice during this challenge. No effect of Trial Period, drug, or the interaction with any of these factors was observed ($F<2.0$, NS). GlyT-1 inhibition affected % omissions ($F_{(2, 32)}=3.4$, $p<0.05$), although this effect was driven by differences in mice receiving 1.0 *vs* 0.3 mg/kg ($p<0.05$). No main effect or interaction with any other factor was observed ($F<2.0$, NS). The main effects of trial period ($F_{(2, 32)}=9.3$, $p<0.005$) and a GlyT-1 × trial period interaction ($F_{(4, 64)}=4.1$, $p<0.01$) were observed for motor impulsivity measured by premature responses. No genotype or any other interaction was observed ($F<1.8$, NS). During distraction, there was a significant increase in premature responses compared with the other trial periods ($p<0.05$). Higher motor impulsivity during distraction was seen in mice treated with saline ($F_{(2, 32)}=9.5$, $p<0.001$) and 0.3 mg/kg GlyT-1 inhibitor ($F_{(2, 32)}=8.1$, $p<0.001$), but not at 1.0 mg/kg ($F<1$, NS), compared with the other two trial periods ($p<0.05$). Higher premature responses in mice receiving 1 mg/kg of the GlyT-1 inhibitor ($F_{(2, 32)}=3.7$, $p<0.05$) compared with saline ($p<0.05$) were observed during trial period 3 but not at any other trial period. GlyT-1 inhibition affected mean reaction time ($F_{(2, 32)}=3.8$, $p<0.05$) without interacting or main effect of any other factor ($F<1.2$, NS). Finally, the reaction time of mice given 1 mg/kg GlyT-1 inhibitor was slower than those given 0.3 mg/kg

($p<0.05$) and tended to be slower than those receiving saline ($p<0.1$).

Experiment 6: GlyT-1 Inhibitory Effects on Breakpoint in Sp4 Mutant Mice

Sp4 Hyp mice exhibited a lower breakpoint than WT mice ($F_{(1, 16)}=9.0$, $p<0.05$; Figure 4a). GlyT-1 inhibition did not affect the breakpoint of mice or interact with genotype ($F<1$, NS). Sp4 Hyp mice collected rewards as fast as WT mice ($F_{(1, 16)}=2.0$, NS; Figure 4b), which was slowed by GlyT-1 inhibition in both genotypes ($F_{(1, 16)}=10.5$, $p<0.01$), as exhibited by a lack of interaction of the two factors ($F<1$, NS). Interestingly, GlyT-1 inhibition did not affect response latency, nor was there an effect of, or interaction with, genotype ($F<1.4$, NS; Figure 4c).

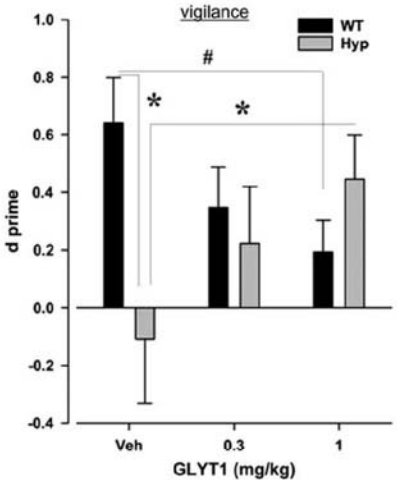

Figure 3 GlyT-1 inhibition-induced changes in vigilance of Sp4 hypomorphic (Hyp) and wild-type (WT) littermate mice. GlyT-1 inhibition using Org 24598 significantly improved the vigilance of Sp4 Hyp mice as measured by *d'* measured over the entire session. However, GLYT1 inhibition tended to deleteriously affect the vigilance of WT littermate mice. Data presented as mean+SEM, $^{*}P<0.05$, $^{\#}p<0.1$ compared with indicated control.

Experiment 7: GlyT-1 Inhibitory Effects on Probabilistic Learning in Sp4 Mutant Mice

Sp4 Hyp mice required more trials to attain criterion compared with WT mice ($F_{(1, 14)}=7.6$, $p<0.05$; Figure 5a). However, GLYT-1 inhibition did not affect this learning ($F_{(3, 42)}=1.9$, NS), neither in WT nor Hyp mice as exhibited by a lack of drug and genotype interaction ($F<1$, NS). Importantly, the positive control (amphetamine treatment) tended to improve trials to criterion performance compared with saline treatment ($F_{(1, 14)}=3.6$, $p=0.077$) irrespective of genotype ($F_{(1, 14)}=2.4$, NS). This trend for an amphetamine-induced improvement is consistent with previous findings that were significant with a larger sample size (data not shown).

GlyT-1 inhibition, amphetamine, genotype, or their interactions did not affect premature responding ($F<1$, NS; Figure 5b). GlyT-1 inhibition ($F_{(3, 42)}=3.4$, $p<0.05$) and genotype ($F_{(1, 14)}=11.2$, $p<0.01$) affected target win-stay behavior without interacting ($F<1$, NS; Figure 5c). The *post hoc* analyses revealed that the middle dose increased target win-stay choices compared with saline ($p<0.05$), whereas Sp4 Hyp mice had lower overall target win-stay choices than WT mice ($p<0.05$). Interestingly, the middle dose resulted in the lowest trials to criterion, although not significantly different from vehicle (see above). In terms of shifting after punishment, GlyT-1 inhibition did not affect, nor interact with genotype, target lose-shift behavior ($F<1.2$, NS), although Sp4 Hyp mice tended to have lower shift levels after punishment from the target than WT mice ($F_{(1, 14)}=4.2$,

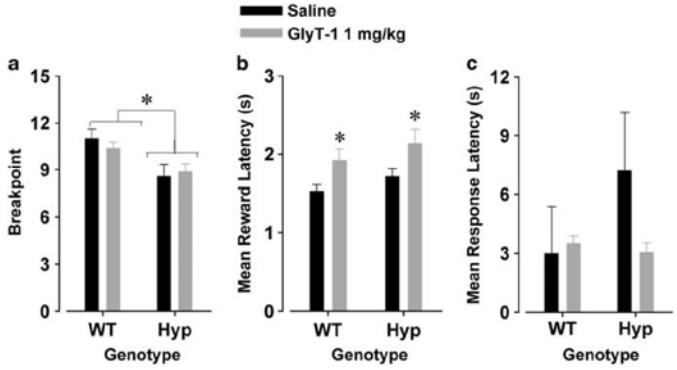

Figure 4 GlyT-1 inhibition did not alter poor motivation of Sp4 hypomorphic (Hyp) or wild-type (WT) littermate mice. Consistent with baseline testing, Sp4 Hyp mice exhibited a reduced breakpoint compared with WT littermates (a). During this testing period, Hyp mice also exhibited a slower latency to collect rewards (b), but no changes in latency to respond (c). None of these behaviors were affected by GlyT-1 inhibition via acute Org 24598 treatment. Data presented as mean+SEM, $^{*}P<0.05$ compared with indicated group or saline.

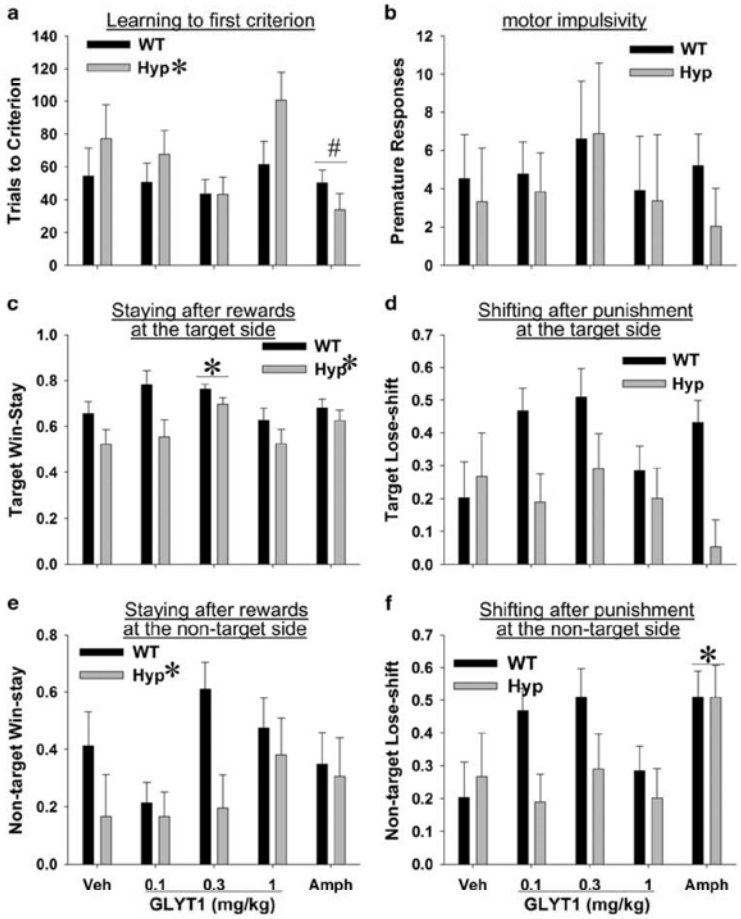

Figure 5 GlyT-1 inhibition did not alter poor reward-associative learning of Sp4 hypomorphic (Hyp) or wild-type (WT) littermate mice. Sp4 Hyp mice exhibited poorer probabilistic reward-associative learning compared with WT littermate mice as measured by trials to criterion (a). Although partial remediation was seen at 0.3 mg/kg of GlyT-1 inhibition treatment by Org 24598, this effect did not reach statistical significant. Amphetamine treatment tended to improve learning of both Sp4 WT and Hyp mice. Motor impulsivity as measured by premature responses was not affected by genotype, GlyT-1 inhibition, or amphetamine treatment (b). Impaired probabilistic learning of Sp4 Hyp mice was likely driven by lower target win-stay, reflecting that WT mice were more likely to stay at the target side compared with Hyp mice (c). GlyT-1 inhibition at 0.3 mg/kg improved target win-stay in both genotypes. Shifting after a punishment on the target side was unaffected by any treatment (d). Sp4 Hyp mice were also less likely to stay at the nontarget side after being rewarded, with neither GlyT1 inhibition nor amphetamine treatment affecting this behavior (e). Amphetamine treatment improved probabilistic learning primarily by increasing the likelihood of the animal to shift after being punished on the nontarget stimulus (f). Data presented as mean+SEM, $^{*}P<0.05$ compared with indicated control (genotype or saline), $^{\#}p<0.1$ compared with saline.

$p=0.061$; Figure 5d). GlyT-1 inhibition did not significantly affect nontarget win-stay ($F_{(3,42)}=2.2$, NS; Figure 5e), whereas Sp4 mice exhibited lower nontarget win-stay behavior than WT ($F_{(1,14)}=5.2$, $p<0.05$), again with no interaction between these two factors. Finally, neither GlyT-1 inhibition ($F<1$, NS) nor genotype ($F_{(1,14)}=2.4$, NS; Figure 5f) nor their interaction ($F<1.4$, NS) affected nontarget lose-shifting behavior. Amphetamine did not exert any main effects on any of these measures ($F<1.6$, NS) except for nontarget lose-shift ($F_{(3,42)}=5.7$, $p<0.05$), wherein it elevated lose-shift in WT and Hyp mice compared with saline ($p<0.05$).

Experiment 8: Comparable GlyT-1 Levels in Sp4 WT and Hyp Mice

The GlyT-1 levels of Sp4 WT and Hyp mice were measured and compared. GlyT-1 levels did not differ by genotype

($t_{(13)} = 1.3$, $p = 0.215$). Hence, the differential effect of GlyT-1 inhibition was likely a result of altered downstream mechanism(s) from this inhibition (Supplementary Figure 3).

DISCUSSION

Sp4 Hyp mice exhibited impaired cognitive control, poor reward-associative learning, reduced effort to gain rewards, and low positive valence. This pattern of results is highly consistent with profiles of deficits observed in schizophrenia patients using similar tasks. Hence, reduced Sp4 expression during neurodevelopment results in a behavioral profile that is consistent with cognitive and negative symptoms of schizophrenia. Moreover, by using this model organism relevant to schizophrenia, we demonstrated that GlyT-1 inhibition (Org 24598) significantly reversed the attentional but not learning or motivational deficits of these mice. Interestingly, GlyT-1 inhibition impaired the attention of WT littermate mice without affecting their learning or motivation (summarized in Table 2). These data support GlyT-1 inhibition as a potential treatment for attentional but not positive valence deficiencies (negative symptoms) related to schizophrenia as well as a U-shape dose response of optimal synaptic glycine levels for attentional performance.

Sp4 Hyp mice exhibit impaired attentional performance at baseline in the 5C-CPT (Supplementary Figure 2A and Figure 2a) consistent with the impaired attention of chronic sufferers with schizophrenia in the human 5C-CPT (Young *et al*, 2013a). However, some subtle differences between Sp4 mice and patients exist in specific 5C-CPT performance measures, including impaired accuracy and a significantly more conservative response bias in these mice (Supplementary Figure 2D and E), although the direction of effect for these measures was the same in patients as in Sp4 Hyp mice (Young *et al*, 2013a). Another notable difference is that Sp4 mice exhibited only chance levels of responding (Supplementary Figure 2A), suggesting they may exhibit difficulties in differentiating between target and nontarget stimuli. However, when treated with the GlyT-1 inhibitor, performance increased significantly above chance levels (Figure 3). Combined with unaltered GlyT-1 levels in Sp4 Hyp mice (Supplementary Figure 3), these findings support a putative NMDAR mediation of attentional deficits in Hyp mice related to their reduced NMDAR protein expression (Zhou *et al*, 2010). Importantly, increasing glycine levels demonstrated that the Sp4 Hyp mice knew how to perform the task, enabling them to perform at similar levels to WT mice. In contrast however, GlyT-1 inhibition worsened the 5C-CPT performance of WT mice at the same doses. These data support recent findings of a U-shaped optimal glycine level required for attentional maintenance during a delay-dependent memory task in non-human primates (Castner *et al*, 2014). Hence, optimizing glycine levels using a GlyT-1 inhibitor may be a useful treatment for patients with schizophrenia with impaired attentional functioning as measured by the 5C-CPT.

Although GlyT-1 inhibition clearly affected overall attentional functioning in a manner consistent with previous findings and hypothesized models, it did not interact with attentional performance while under distraction using the 5C-CPT challenge. This challenge was based on work by Sarter and colleagues (Demeter *et al*, 2013; Howe *et al*, 2010) demonstrating that competing flashing stimuli during attentional performance deleteriously affected attentional performance. As reported for humans and rats (Demeter *et al*, 2013; Howe *et al*, 2010), we demonstrated that the attentional performance of mice was deleteriously affected by distracting stimuli (trial period 2, cf. trial periods 1 and 3; Figure 2a and i). Distraction did not affect simple accuracy of responding but increased premature responding (Figure 2e and g). Interestingly, Sp4 Hyp mice were as distracted as WT mice as measured by d'. This finding is consistent with the report that healthy subjects and patients with schizophrenia exhibit similar reductions in performance during distraction in the dSAT (Demeter *et al*, 2013). Thus, despite the reported importance of deficits in attentional control (in response to distraction) in schizophrenia patients (Luck *et al*, 2011; Lustig *et al*, 2013), perhaps general attention/vigilance remains primarily important to improve outcome for patients with schizophrenia. The Sp4 Hyp mice may prove a valid model organism—and GlyT-1 inhibition a viable therapeutic target—for impaired attention seen in patients with schizophrenia (Javitt, 2012).

In addition to impaired attention, Sp4 Hyp mice tended to exhibit a reduced breakpoint in the progressive ratio task (Supplementary Figure 1A) that was confirmed as significant upon retesting (Figure 4a). Reduced breakpoint in a progressive ratio setting has often been suggested as a measure of reduced motivation in mice that is relevant to negative symptoms in schizophrenia (Barnes *et al*, 2014; Ellenbroek and Cools, 2000; Markou *et al*, 2013; Young *et al*, 2010). Support for these assertions comes from recent evidence of reduced breakpoint in patients with schizophrenia that negatively correlated with negative symptom scale ratings (Wolf *et al*, 2014). One could theorize that this reduced breakpoint stems from lower NMDAR expression in these mice, but this deficit was not remediated by GlyT-1 inhibition (Figure 4a), unlike that seen for attention (Figure 3). Considering the importance of Sp4 expression during neurodevelopment (Ramos *et al*, 2007; Zhou *et al*, 2007),

Table 2 Summary of Cognitive/Motivational Profile of Sp4 Hypomorphic (Hyp) Compared with Wild-Type (WT) Mice

Paradigm	Domain measured	Sp4 Hyp effect?	GlyT1 inhibition effect?
5-Choice continuous performance test	Control of attention, inhibition	*Impaired*: driven by inattention	Attenuated deficit, impaired WT mice
Probabilistic learning	Feedback-driven learning/decision making	*Impaired*: driven by poor reward learning	No effect, but improved with amphetamine
Progressive ratio Breakpoint study	Effortful motivation	*Impaired*: driven by reduced effort	No effect

the mechanism(s) underlying this amotivation of Sp4 Hyp mice remain unclear.

Consistent with evidence of reduced motivation to work for a reward, the Sp4 Hyp mice also exhibited impaired probabilistic learning compared with their WT littermates (Figure 5a). This deficit was driven by a reduced likelihood of staying at the target side after being rewarded at that side—hence reduced reward sensitivity (Figure 5c). These findings support the premise that these mice exhibit impaired anticipation of reward. Such impaired probabilistic (reward-associative) learning has also been linked to anhedonia and negative symptoms in schizophrenia (Dowd and Barch, 2010; Waltz and Gold, 2007), via impaired reward anticipation (Acheson *et al*, 2013; Gold *et al*, 2008). However, these deficits could still be linked to impaired cognition, given that probabilistic learning was suggested by the CNTRICS initiative to measure long-term relational memory (Armstrong *et al*, 2012; Ragland *et al*, 2012b). As with the progressive ratio challenge, GlyT-1 inhibition did not ameliorate the probabilistic learning deficits of Sp4 Hyp mice, although modest improvements were seen at 0.3 mg/kg. The LTP deficits of Sp4 Hyp mice may underlie their associative learning deficits. As GlyT-1 inhibition did not remediate their learning deficits but instead improved their attention, it is unlikely it would remediate their LTP deficits. However, improved probabilistic learning was observed in both WT and Sp4 mice treated with amphetamine (Figure 5). Hence, it appears that these negative symptom-relevant behavioral deficits can be attenuated by elevating dopamine and norepinephrine activity, but not glycine levels. Because amphetamine can enhance LTP (Xu *et al*, 2010), it is possible that it would also improve the LTP of Sp4 Hyp mice, as will be assessed in future studies. Considering however that probabilistic learning of both Sp4 WT and Hyp mice was improved by amphetamine treatment, this finding may not specifically reveal the mechanism(s) underlying the deficit of Sp4 Hyp mice.

Taken together, the inability of GlyT1-inhibition to remediate behaviors relevant to negative symptoms is surprising given that: (1) lower plasma and cerebrospinal fluid glycine level of patients with schizophrenia is linked to negative symptoms (Hashimoto *et al*, 2003); and (2) glycine treatment (although chronic) modestly lowers negative symptom ratings (Heresco-Levy *et al*, 1999; Javitt *et al*, 1994). However, such positive findings have not always been reproduced (Buchanan *et al*, 2007). In fact, Roche recently stopped trials testing a GlyT-1 inhibitor for the improvement of negative symptoms in schizophrenia, perhaps due in part to the sole reliance on clinical rating scales rather than objective translational laboratory tests as primary outcome measures. Testing negative symptoms using laboratory-based measures with relevance to those presented here may provide more relevant cross-species findings (Barnes *et al*, 2014; Der-Avakian *et al*, 2013; Young *et al*, 2013b) and greater sensitivity to the effects of GlyT-1 inhibitors. Constitutively reducing Sp4 expression in mice resulted in impaired attention that was remediated by GlyT-1 inhibition. The finding that this treatment did not remediate motivational deficits suggests that the attentional deficits of Sp4 mice are unlikely a result of altered motivation or learning. The lack of effect on motivation could be because of a requirement of longer treatment duration, although it improved attention acutely. Alternatively, more direct NMDAR1 activation may be required. Furthermore, the mechanism(s) underlying impaired motivation and learning resulting from reduced Sp4 expression have yet to be delineated. As SP4 regulates the transcription of NMDA receptor subunits GluN1, GluN2A, and GluN2B (Priya *et al*, 2014; Priya *et al*, 2013), further investigation of other mechanisms using these mice is warranted.

In conclusion, reduced Sp4 expression in mice largely recreates the attentional deficits observed in patients with schizophrenia as measured by the 5C-CPT (Young *et al*, 2013a). These data support pairing attentional assessment with evidence of reduced SP4 levels in peripheral blood mononuclear cells, as seen in first-episode patients with schizophrenia (Fuste *et al*, 2013). Such an approach might provide a useful personalized biomarker for predicting whether GlyT-1 inhibition may remediate attentional deficits in individual patients. However, such treatment would unlikely treat impaired positive valence related to reward anticipation. SP4 rare copy number variations and reduced protein levels are linked to several psychiatric disorders that exhibit attentional deficits including schizophrenia, bipolar disorder, and major depressive disorder (Pinacho *et al*, 2011; Shi *et al*, 2011; Tam *et al*, 2010; Zhou *et al*, 2009). Hence, identifying the mechanism(s) of how reducing Sp4 levels negatively affect attention and the neurobiology underlying GlyT-1 inhibition-induced reversal of these effects will prove vital. Importantly though, the present work using this model organism provides opportunities for personalized medicine for the treatment of attentional deficits in neuropsychiatric patients having low SP4 levels (McMahon and Insel, 2012).

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Chapter 4, in full, is a reprint of the material as it appears in Young JW, Kamenski ME, Higa KK, Light GA, Geyer MA, Zhou X. *GlyT-1 inhibition attenuates attentional but not learning or motivation deficits of the Sp4 hypomorphic mouse model relevant to psychiatric disorders*. Neuropsychopharmacology. 2015 Nov;40(12):2715-26. The dissertation author assisted with experimentation and data analyses and gave input during manuscript preparation.

DISCUSSION

This dissertation investigated two mouse lines modeling mutations to candidate risk genes, *DISC1/Boymaw* and *Sp4*, which have been associated with the development of multiple mental illnesses, including major depression (MD), bipolar disorder (BD), and schizophrenia (SZ). The heterogeneous diagnostic outcomes associated with these candidate genes in patients reflect the complex nature of psychiatric illnesses: a single mutation may lead to the development of multiple illnesses with a wide range of symptoms. Within a single illness, like BD for example, outcomes vary depending on a patient's physical and genetic environment. In animal models, too, behavioral outcomes are difficult to predict.

A major challenge in the development and behavioral analysis of animal models is that researchers must select both the manipulations and the measures by which to define and validate the model. Even across mouse models of *DISC1* mutations (modeling simple disruption to the *DISC1* gene, not fusion with *Boymaw* studied here), no consistent behavioral phenotypes have been found (O'Tuathaigh and Waddington 2015; Wong and Josselyn 2016). The presence or absence of anxiety- and depressive-like phenotypes varies across models, for example. These inconsistencies across models may be due to differences in the genetic manipulation, extent of dysfunction, functional diversity of this gene, or environmental effects. Even so, much can be gained from determining the disrupted brain circuitry and associated behavioral changes within each model. Consideration of multiple models can also lead to the discovery of shared behavioral endophenotypes that may share a common underlying mechanism.

Phenotypic convergence across models

After characterizing mutant DISC1-Boymaw and Sp4 hypomorphic mice in a wide battery of tests, we observed convergence on a behavioral phenotype relevant to schizophrenia: hypersensitivity to ketamine in the behavioral pattern monitor. Furthermore, DISC1-Boymaw and Sp4 Hyp mice exhibited low expression of the NMDA receptor subunit 1 *(NMDAR1*) and GABA-synthesizing enzyme glutamic acid decarboxylase 67 (*GAD67)*, which is also observed in patients with schizophrenia. Although the defining mutations for these models are seemingly unrelated, and the mice do not share a complete phenotypic profile, the overlap in these phenotypes may inform us of the shared mechanisms that underlie the development of related symptoms in patients with psychiatric illnesses affected by a variety of genetic, epigenetic, and environmental insults.

In Chapter 3, we showed that dysfunction of GABAergic neurons is necessary to produce ketamine hypersensitivity in Sp4 Hyp mice. This finding is consistent with the suggestion that ketamine acts primarily on GABAergic NMDARs (Homayoun and Moghaddam 2007). Although several GABAergic neurons may be affected by the lack of Sp4, PV^+ interneurons are the most abundant cortical interneuron type and may be particularly susceptible to genetic, epigenetic, and environmental insults related to SZ (Jiang et al. 2013). Furthermore, gamma oscillations—the generation of which relies on NMDARs in PV^+ interneurons (Gonzalez-Burgos and Lewis 2012; Nakazawa et al. 2012; Carlén et al. 2012)—are abnormal in patients with SZ (Uhlhaas and Singer 2010), as well as our Sp4 Hyp

mice (Ji et al. 2013). Therefore, we hypothesize that NMDAR function in PV^+ interneurons is likely disrupted in the Sp4 Hyp mice, contributing to their hypersensitivity to ketamine.

It has been suggested that fast-spiking PV^+ cortical GABAergic interneurons consume more ATP than other types of neurons because of their high frequency firing (Hasenstaub et al. 2010). Therefore, PV^+ interneurons may be particularly vulnerable to reduced ATP production in the DISC1-Boymaw mice due to DB7 aggregation in mitochondria. Thus, we hypothesize that PV^+ interneuron dysfunction is responsible for ketamine hypersensitivity observed in the DISC1-Boymaw mice, as well. Because the majority of cortical PV^+ neurons lose their synaptic NMDAR component by adulthood (Wang and Gao 2009, 2010; Rotaru et al. 2011), the role of NMDAR dysfunction in PV^+ neurons has been challenged. Deficits to PV^+ neurons during development, however, may have long-lasting effects on cortical synchronization and inhibitory/excitatory balance that remain evident in adulthood (Nakazawa et al. 2012, 2017).

Interestingly, PV expression is not reduced in either mouse line. PV is a calcium (Ca^{2+}) buffer that regulates Ca^{2+}-dependent GABA release, so reduction in PV expression may be a consequence of low GABA release (Nakazawa et al. 2012; Berridge 2014). Therefore, although we did not observe low PV expression in naïve mice, stressful environmental conditions and/or normal aging might eventually lead to decreased PV expression in these mice. Therefore, unaltered PV expression in either mouse model is not inconsistent with findings that PV expression (but not PV^+ interneuron density) is decreased in patients (Lewis et al. 2012).

Of course, we cannot rule out subtle structural alterations in GABAergic neurons, as well as compensatory changes in PV^+ and other interneuron types to maintain a stable excitation/inhibition balance during development. These developmental changes may result in the acute effects of ketamine. Future studies will require greater temporal and regional restoration of Sp4 expression in more specific GABAergic populations to further elucidate the ketamine hypersensitivity phenotype. It is possible that the phenotypic overlap does not reside primarily in PV^+ interneurons but in other interneuron types. Furthermore, decreased Ca^{2+} signaling via PV, NMDAR, and/or mitochondrial dysfunction, may play a greater role than discussed here (Berridge 2014). We do not have reason to expect that the Sp4 Hyp mice will exhibit a mitochondrial deficit like that observed in the DISC1-Boymaw mice, but the metabolic profile of the Sp4 Hyp mice is worth investigation and a deficit would support mitochondrial deficits as a primary cause of SZ in some patient populations.

Thus, although the overlapping phenotypes between DISC1-Boymaw and Sp4 Hyp mice have not been investigated within this dissertation, the results presented here point toward a potentially fruitful line of investigation that is worth pursuing in these and other mouse models.

Cognitive deficits in schizophrenia

While the studies described above focus on ketamine sensitivity, continued investigation of GABAergic interneuron dysfunction in the context of cognition is also important. Although much emphasis has been placed on treating the positive symptoms of schizophrenia, the recent prioritization of the negative and cognitive symptoms of the disease

is now widely accepted. Efforts such as the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) program developed by the National Institute of Mental Health (NIMH) aim to clarify the specific domains of cognition that are impaired in schizophrenia (Marder and Fenton 2004; Marder 2006). These investigations will benefit greatly from the use of cross-species valid tasks that can assess comparable processes in patients and in rodents (Young and Geyer 2015). Examples of cross-species valid paradigms used to assess mouse cognition in 5-choice operant chambers can be found in Appendices A and B, written primarily by the author of this dissertation.

In Chapter 4, we assessed cognition in the Sp4 Hyp mice and observed impaired attention, reduced motivation, and impaired probabilistic learning, consistent with patient profiles. We found that a glycine transporter 1 (GlyT-1) inhibitor, which should increase NDMAR function, remediated the attention deficits but not impairments to motivation nor reward learning in these mice. This was surprising given that low glycine levels in plasma and cerebrospinal fluid have been associated with negative symptoms of SZ and glycine treatment has been shown to lower negative symptom ratings (Javitt et al. 1994; Heresco-Levy et al. 1999; Javitt 2012). However, these results indicate that the attention deficits are not due to motivation or learning deficits. It is possible that chronic, rather than acute, treatment with glycine or GlyT-1 inhibitors may be necessary, and alternative targets should also be studied. Nevertheless, these studies illustrate the potential use of the Sp4 Hyp mice for preclinical evaluation of treatments for the cognitive deficits associated with SZ and other psychiatric illnesses. Expanding such tests to additional models, such as the DISC1-Boymaw mice, would aid in the prediction of clinical efficacy or perhaps indicate which subpopulations of patients could be best treated.

Challenges and limitations

For some genetic mouse models, such as the α7 nicotinic acetylcholine receptor knock-out (α7 nAChR KO) mice presented in Appendix B, compensatory changes for the mutation during development may present a confounding factor. For example, based on our studies of the α7 nAChR KO mice alone, we cannot conclude with certainty that the lack of nicotine withdrawal-induced inattention in these mice is due specifically to the lack of α7 nAChRs or to other chemical and anatomical changes resulting from development without α7 nAChRs. In the DISC1-Boymaw and Sp4 models, however, our hope is that the developmental changes associated with each mutation are conserved from mice to humans and may be informative as to which circuits are disrupted during development and, in a patient's adulthood, might be targeted for treatment.

It is inevitable, however, that some compensatory mechanisms—and even the deficits that are compensated for—will differ across species, leading to unexpected and potentially uninformative outcomes. Because, as stated above, the investigators have chosen the manipulation and must decide on relevant outcomes, it is impossible to know if an observed phenotype (or lack thereof) is due to the desired manipulation, developmental changes, or unidentified environmental factors. Therefore, studies on consistent findings across distinct models—such as those which may link impaired GABAergic functioning and ketamine hypersensitivity in DISC1-Boymaw and Sp4 Hyp mice, described above—may be particularly informative and compelling.

Future directions

The experiments in Chapter 3 illustrate the ability to restore *Sp4* expression in Sp4 Hyp mice using Cre-mediated recombination. We were unable to rescue the prepulse-inhibition (PPI) deficit in Sp4 Hyp mice regardless of restoration of *Sp4* expression in forebrain excitatory neurons or GABAergic interneurons, although PPI was rescued when *Sp4* expression was restored in the whole animal (Zhou et al. 2005). Since PPI deficits are robust and consistent with sensorimotor gating deficits in SZ patients, it would be worthwhile to pinpoint the neuronal population in which *Sp4* restoration could rescue the PPI deficit. As discussed above, it will also be important to restore *Sp4* with greater temporal, regional, and cell-type specificity to clarify to role of GABAergic neurons in controlling the locomotor response to ketamine. Considering the association of the GABAergic neuron dysfunction with cognitive impairment, similar studies should be performed to determine the role of GABAergic neurons in the cognitive deficits in Sp4 Hyp mice observed in Chapter 4.

The DISC1-Boymaw mice are also equipped with the potential to express the full human *DISC1* (*hDISC1*) gene using Cre recombination. A criticism of the DISC1-Boymaw mouse on the 129S background is that 129S mice have a naturally occurring *Disc1* mutation (Koike et al. 2006; Clapcote and Roder 2006; Juan et al. 2014). Therefore, the DISC1-Boymaw mice have a gain-of-function mutation. Because we aimed to study the outcomes of the gained fusion *DB7* and *BD13* genes, we were less concerned with *Disc1* haploinsufficiency and modeling the genotype of the Scottish family exactly. Had we conducted our studies in a different strain with a functional mouse *Disc1*, in fact, it would have been difficult to parse the effects from the gain of *DB7* and *BD13* versus the loss of

Disc1. We hoped to address the issue of background strain in Chapter 1 by testing DISC1-Boymaw mice on a mixed 129S/C57 background. Importantly, the 129S/C57 F1 generation also exhibited heightened sensitivity to ketamine. Given the possibility of *hDISC1* expression, however, future studies should compare mice expressing two copies of *hDISC1* (comparable to healthy human subjects) with mice expressing one copy of *hDISC1* and one copy of the DISC1-Boymaw fusion genes (mimicking human carriers of the *DISC1* mutation).

The experiments highlighted in this dissertation focused primarily on the behavioral outcomes in the DISC1-Boymaw and Sp4 Hyp mice. Therefore, the effects of the respective mutations on neuronal function are lacking, and electrophysiological characterization and functional imaging of the neurons affected by DISC1-Boymaw and Sp4 Hyp mice are critical. For example, we do not know if the mitochondrial deficits proposed in Chapter 2 indeed alter neuronal function, so electrophysiological and imaging experiments would be essential before we can make any conclusions regarding the effects of mitochondrial deficits on behavior. In addition to these and pharmacological experiments on mouse behavior, the search for targets for treatments would be aided by the investigation of the interaction partners of *Sp4* and *Boymaw* and the downstream effects of the mutations. A closer look into the behavior of DB7 within mitochondrial would also be informative.

It is evident that there are complex genetic interactions that increase the risk of developing psychiatric illnesses (Purcell et al. 2009; Rudd et al. 2014; Power et al. 2015). Developmental and dynamic environmental influences can also greatly affect neuronal functioning and behavioral outcomes (van Os et al. 2010; Brown 2011; Meyer-Lindenberg and Tost 2012; Kannan et al. 2013). Both the Boymaw-DISC1 and Sp4 Hyp models would

be useful for multiple-hit hypotheses testing gene × gene and gene × environment interactions. We have proposed, for example, that the AMPK-mTOR protein synthesis pathway is disrupted in DISC1-Boymaw mice. This pathway may also be disrupted by sleep deprivation, leading to cognitive dysfunction and other behavioral abnormalities (Gessa et al. 1995; McDermott et al. 2003; Banks and Dinges 2007). Therefore, DISC1-Boymaw mice may be particularly vulnerable to the effects of sleep deprivation, which has been shown to result in deficits in fear conditioning associated with psychiatric disorders (Holt et al. 2009; Maren et al. 2013). Measuring contextual fear conditioning in the DISC1-Boymaw mice following sleep deprivation could demonstrate one way in which environmental and genetic factors converge to exacerbate symptoms.

Multiple-hit studies could also be used to address the lack of robust behavioral and metabolic phenotypes in the DISC1-Boymaw mice. Studies on mice with much greater mitochondrial deficits—including mice with mitochondria from evolutionarily divergent species—do not consistently reveal obvious phenotypic abnormalities unless triggered by stress or infection (Cannon et al. 2011; Dunn et al. 2012). Therefore, additional developmental perturbations via genetic mutation or environmental stresses may be necessary to better understand the contribution of the DISC1-Boymaw genes to the development of psychiatric disorders. Such investigations may help to explain the range of diagnoses observed in the Scottish DISC1 family.

The studies presented in this dissertation illustrate just some of the ways mouse models can be used in the study of psychiatric disorders. The possibilities for future experiments using the DISC1-Boymaw and Sp4 Hyp mice are endless. The author of this

dissertation hopes that the studies presented here and to follow using these mice will be informative for ongoing efforts to better understand and develop treatments for neuropsychiatric disorders.

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Appendix A:

Striatal dopamine D1 receptor suppression impairs reward-associative learning

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Research report

Striatal dopamine D1 receptor suppression impairs reward-associative learning

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ABSTRACT

Dopamine (DA) is required for reinforcement learning. Hence, disruptions in DA signaling may contribute to the learning deficits associated with psychiatric disorders. The DA D1 receptor (D1R) has been linked to learning and is a target for cognitive/motivational enhancement in patients with schizophrenia. Separating the striatal D1R contribution to learning vs. motivation, however, has been challenging.

We suppressed striatal D1R expression in mice using a D1R-targeting short hairpin RNA (shRNA), delivered locally to the striatum via an adeno-associated virus (AAV). We then assessed reward- and punishment-associative learning using a probabilistic learning task and motivation using a progressive-ratio breakpoint procedure.

We confirmed suppression of striatal D1Rs immunohistochemically and by testing locomotor activity after the administration of (+)-doxanthrine, a full D1R agonist, in control mice and those treated with the D1RshRNA. D1RshRNA-treated mice exhibited impaired reward-associative learning, while punishment-associative learning was spared. This deficit was unrelated to general learning impairments or amotivation, because the D1shRNA-treated mice exhibited normal Barnes maze learning and normal motivation in the progressive-ratio breakpoint procedure.

Suppression of striatal D1Rs selectively impaired reward-associative learning whereas punishment-associative learning, aversion-motivated learning, and appetitive motivation were spared. Because patients with schizophrenia exhibit similar reward-associative learning deficits, D1R-targeted treatments should be investigated to improve reward learning in these patients.

1. Introduction

Neuropsychiatric disorders are life-long and debilitating. Although cognitive deficits associated with such disorders predict functional outcomes, no pharmacological treatments currently exist that target/treat these symptoms. Behavioral therapy using positive feedback-associated learning can help [1,2], but not in all patients [3,4], possibly hampered by poor reward-associative learning [5,6]. Dopamine (DA) is the major neurotransmitter required for reinforcement learning. According to the DA reward prediction error hypothesis, unexpected rewards strongly activate midbrain DA neurons, and this phasic activity drives reinforcement learning [7–9]. DA is also important for motivation and reward-seeking behavior required for learning [10]. Disruptions in DA signaling may therefore contribute to learning deficits associated with psychiatric disorders [11–14].

The precise mechanism(s) by which DA signaling affects specific aspects of learning remain unclear. Operant learning can be affected by decreased liking or wanting of the reward or by impaired aversion to or avoidance of the punishment used to shape learning [15]. Specific impairments in reward-associative versus punishment-associative learning have been reported for patient groups with various psychiatric disorders [11,13,14,16]. For example, Brambilla et al. [12] reported that patients with schizophrenia exhibit poorer overall learning whereas patients with bipolar disorder exhibit impaired learning due to heightened reward sensitivity. Furthermore, the relationship between motivation and reinforcement learning in health and disease is poorly understood [17]. The relative impairments to motivation and to reward- and punishment-associative learning in the context of

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0166-4328/

psychiatric disorders are only recently being investigated, in part because the physiological mechanisms accompanying these distinct aspects of learning are complex, even in health.

The "direct" and "indirect" pathways of the basal ganglia are thought to mediate reward- and punishment-associative learning, respectively. DA medium spiny neurons (MSNs) projecting to the substantia nigra express DA D1 receptors (D1Rs) or DA D2 receptors (D2Rs). D1Rs are expressed in MSNs in the direct pathway of the basal ganglia, whereas D2Rs are expressed in MSNs in the indirect pathway [18–20]. Previous studies in rodents have shown that the direct pathway and D1Rs are associated with reward valuation and habit formation, whereas D2Rs are associated with behavioral flexibility and punishment-associative learning [21–26]. Kravitz et al. [27] used optogenetic techniques to stimulate striatal D1R- or D2R-expressing neurons during a simple reinforcement learning task. They found that D1R-expressing MSNs in the direct pathway mediate persistent reinforcement, whereas D2R-expressing MSNs in the indirect pathway mediate transient punishment. Beyond the striatum, D1Rs and D2Rs in the prefrontal cortex (PFC) also play distinct roles in reward- and punishment-associated learning and decision-making. For example, pharmacological blockade of PFC D1Rs may increase sensitivity to punishment and behavioral flexibility, whereas blockade of D2Rs may increase sensitivity to reward [28,29]. While optogenetic studies are invaluable for determining the major roles of each neuron type in each pathway, optogenetic stimulation does not necessarily induce natural activity of the neurons. Furthermore, this technique does not enable the identification of the specific receptor types responsible for reinforcement learning. Similarly, the commonly used D1R antagonist, SCH23390, blocks the entire D1R family, including D5Rs.

In this study, we developed a D1R-selective suppression technique and used a cross-species relevant reinforcement learning tasks to disentangle some of the molecular and behavioral mechanisms contributing to the learning deficits associated with psychiatric illnesses. To address the issue of receptor specificity in the investigation of reinforcement learning, we used RNA interference to suppress D1Rs in the striatum [30,31]. We designed a short hairpin RNA (shRNA) sequence targeting the D1R gene (D1R-shRNA, Fig. 1a). Self-complementary recombinant adeno-associated viruses (scAAVs) were generated to deliver the D1R-shRNA expression construct (Fig. 1b). After delivery and expression of the D1RshRNA, we assessed the effects of striatal D1R suppression on reward- and punishment-associative learning (using a probabilistic learning paradigm consistent with clinical studies [4,11]), motivation for reward [using the progressive ratio breakpoint procedure (PRBP), also seen as deficient in schizophrenia [32], and aversively motivated learning [using the Barnes Maze (BM)], comparing their effects to control mice treated with green fluorescent protein (GFP). We hypothesized that reducing striatal D1R expression would: 1) impair reward- but not punishment-related learning; and 2) not affect motivation or aversively motivated behavior. Finally, we administered (+)-Doxanthrine (DOX), a D1R agonist, to induce hyperactivity in the Behavioral Pattern Monitor (BPM), hypothesizing that; 3) DOX would stimulate activity in GFP- but not D1RshRNA-treated mice.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice ($n = 30$; 4–7 months old, 20–35 g) were supplied by the Jackson Laboratory (Bar Harbor, ME) and housed in facilities that are approved by the American Association for Accreditation of Laboratory Animal Care and meet all federal and state requirements for care and treatment of laboratory animals. Mice were group-housed (3–4 per cage) in a climate-controlled animal colony with a reversed day/night cycle (lights on at 19:00 h, off at 07:00 h) and tested during the dark phase between 08:00 and 17:00 h. Food (Harlan Teklad, Madison, WI) and water were available *ad libitum* except throughout training and testing, when food was restricted to maintain mice at ~85% full body weight. All procedures were approved by the University of California San Diego (UCSD) Animal Care and Use Committee.

2.2. Construction of D1RshRNA

The target sequence for the D1RshRNA was designed by *in silico* selection. Two web-based siRNA-selection programs were used for the designs of D1RshRNA (MIT Whitehead siRNA Selection Web Server: http://jura.wi.mit.edu/bioc/siRNAext/; siDirect 2.0: http://sidirect2.rnai.jp/) [33,34]. The target region was chosen from 1892 to 1914 of Drd1 mRNA, 5′ AAGAGCATATGCCACTTTGTATT 3′ (Fig. 1a). The final processed 19mer sequence had 42% GC with a Gibbs free-energy value (ΔG) of −4.0, which indicates the predominant selection of the antisense strand as the guide strand for the RNA-induced silencing complex (RISC). The *in silico* analysis of potential off-target effects was conducted online, and the chosen D1RshRNA had relatively low potential off-target effects in the mouse genome [33–35]. For example, siDirect 2.0 lists 11 near-matches to the guide strand and 20 near-matches to the passenger strand, none of which have any obvious relevance to the phenotypes observed in this study, and zero exact matches.

The D1RshRNA expression cassette was directly cloned into a pU6-shRNA vector (Talron Biotech). The D1RshRNA expression cassette driven by the U6 promoter was then subcloned into a scAAV-shuttle vector with a GFP marker gene (scAAV5, Fig. 1b). The scAAV5 virus was produced by the UCSD Vector Development Core. The titer of the scAAV5 was about 10^{12}, measured by quantitative PCR.

2.3. shRNA delivery

Mice were matched into two groups based on rate of responding on the fixed-ratio of 1 (FR1) schedule, as well as breakpoint in the PRBP (described below). The matched groups were randomly assigned injection of GFP- or D1RshRNA-carrying scAAV. Two injection sites (bregma +0.86 mm, medial-lateral ±1.65 mm) at both dorsal (−2.45 mm) and (−3.8 mm) ventral striatum were selected to maximize the area of transduction within the striatum. A 1 μL volume of the assigned virus was injected slowly (over 2 min) using stereotaxic delivery at each site. Mice were not trained or tested in the 5-choice apparatus (described below) during the 40 days after scAAV delivery to allow adequate time for recovery and gene expression.

2.4. Immunohistochemical analysis

Forty days after surgery, mice that received either GFP or D1RshRNA virus ($n = 2$ per group) were randomly selected for the analysis of D1R expression in striatum. Brains were fixed with paraformaldehyde (4%, PBS buffered) and embedded in paraffin. Immunohistochemical staining was conducted with rabbit anti-DA D1 polyclonal antibody (Chemicon) as described [36].

2.5. 5-hole apparatus

Sound-insulated 5-hole operant chambers (15; 25 × 25 × 25 cm; Med Associates, Inc., St. Albans, VT) were used. Each chamber had a house light near the ceiling and was ventilated by a fan that provided a low level of background noise. There was an array of 5 square holes (2.5 × 2.5 × 2.5 cm, 2.5 cm above the grid floor)

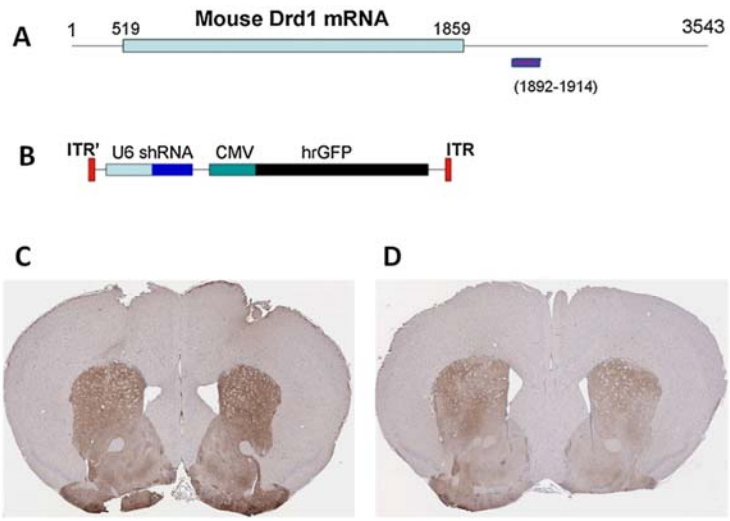

Fig. 1. D1RshRNA expression cassette in scAAV vector and suppression of D1R expression. A. Targeted mouse Drd1 gene. B. D1RshRNA expression cassette driven by a U6 promotor in scAAV5 virus carrying GFP marker gene. ITR: inverted terminal repeat. C, D. Striatal D1R expression in brain slices from GFP- (C) and D1RshRNA-treated (D) mice.

arranged horizontally on a curved wall so that each was equidistant from a food delivery magazine (Lafayette Instruments, Lafayette, IN) on the opposite panel at floor level. Each hole had a light-emitting diode (LED) at the back and infrared beams, mounted vertically 3 mm from the opening of the hole to detect responses. The food delivery magazine contained a well in which liquid reinforcement (strawberry Nesquik® plus non-fat milk, 30 μL) was delivered by a peristaltic pump (Lafayette Instruments, Lafayette, IN). The magazine had an LED at the top. An infrared beam, mounted horizontally 5 mm from the floor and recessed 6 mm into the magazine, was used to detect magazine entries. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates, Inc.) using custom programming.

2.6. Fixed-ratio responsive training

Mice were trained to poke their noses ("hole-poke") into a single recessed lit hole for 5 days/week as described previously [37,38]. Mice were first acclimated to the reinforcer. Days 1–3; milk was dispensed every 15 s into the magazine well while the magazine was lit. Head entry into the magazine extinguished the magazine light until the next reinforcement delivery. Day 4 onward; mice were required to hole-poke the center hole to obtain reinforcement on a fixed ratio of 1 (FR1) schedule, until >70 responses were recorded within 30 min for 2 consecutive days. Upon meeting criterion, mice were trained in this session only 2 days per week to minimize over-training.

2.7. Progressive ratio breakpoint study

Once the mice had reached a stable level of responding on the FR1 schedule, they were challenged with the PRBP [39,40]. On the PRBP schedule, the number of hole-pokes required to receive reinforcement increased by a step that grew by 1 every three trials (1, 2, 4, 7, etc.). The breakpoint was the last ratio completed by the end of the 1-h trial (Fig. 2a).

2.8. Probabilistic learning task

Forty days after surgery, mice were retrained on the FR1 schedule for 12 sessions then trained on a probabilistic learning paradigm, based on previous reports [41,42]. In the probabilistic learning task, holes 2 and 4 were illuminated. A response at the "target" stimulus, hole 2 or 4 counterbalanced across mice, resulted in a reward (one drop of milk) or punishment (4 s timeout) in a 80/20 ratio (Fig. 3a). A response at the "nontarget" stimulus resulted in a reward or punishment in a 20/80 ratio. A response in holes 1, 3, or 5 had no consequence. Each session terminated after 30 min or after 200 rewards were obtained. Mice were trained until target responding was >85% for two consecutive sessions. Hence, the primary outcome measure for probabilistic learning was the number of sessions required to achieve criterion.

2.8.1. Decision-making analyses from probabilistic learning: win-stay and lose-shift ratio analysis

The decision-making of mice following reward or punishment for each trial, t, was also analyzed accordingly:

$$win(t) = \begin{cases} 0 \textit{ for an omitted or punished trail} \\ 1 \textit{ for a rewarded trial} \end{cases}$$

$$loss(t) = \begin{cases} 0 \textit{ for an omitted or rewarded trail} \\ 1 \textit{ for a punished trial} \end{cases}$$

Following a rewarded trial, for which win(t) = 1, a "win-stay" event for trial $t+1$ was counted if a mouse made a response at the same hole (target or nontarget) that was rewarded in trial t.

$$shift(t+1) = \begin{cases} 0 \textit{ for an omission or switching to the side opposite to that rewarded} \\ 1 \textit{ for staying on the side that was rewarded} \end{cases}$$

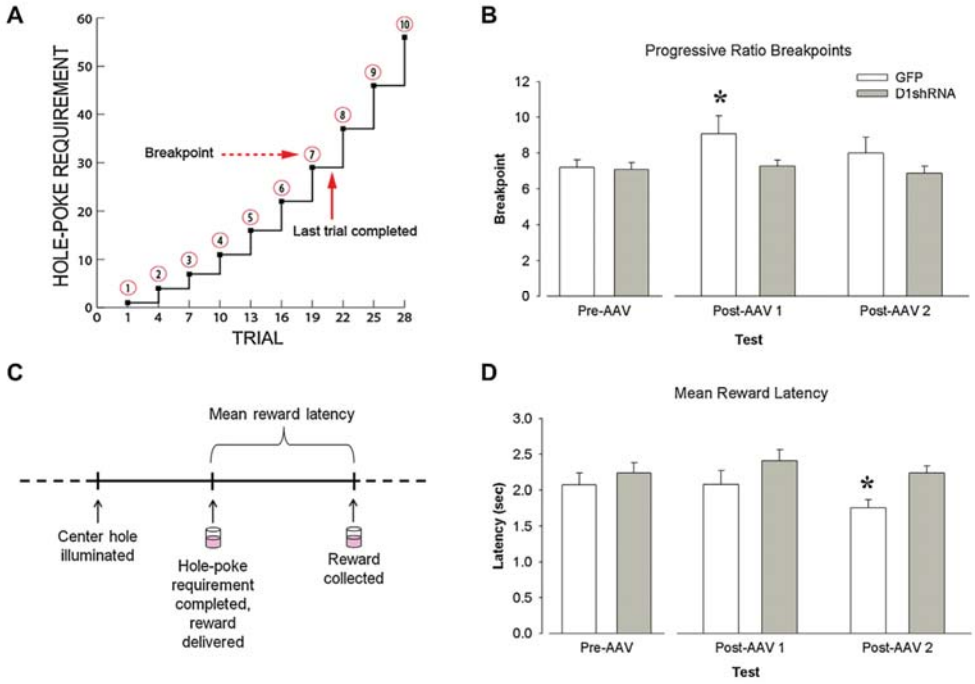

Fig. 2. Progressive ratio breakpoint and mean reward latency before and after AAV transduction. There were no significant differences between groups for either measure during any of the three tests. A. Schematic of PRBP test. The number of hole-pokes required to receive a reward increased every three trials. The breakpoint was the number of the final ratio completed in 1 h. B. During the first PRBP test after AAV treatment ("Post-AAV 1"), the breakpoint for the GFP-treated mice was significantly greater than that before treatment ("Pre-AAV"). C. Schematic of trial timeline. Mean reward latency is the time between reward delivery and collection. D. During the second PRBP test after AAV treatment ("Post-AAV 2"), the mean latency to obtain the reward was significantly shorter for the GFP-treated mice than before treatment. Data are presented as mean ± SEM. * denotes $p < 0.05$ vs. GFP-treated mice Pre-AAV.

Following a punished trial, for which loss(t) = 1, a "lose-shift" event for trial $t+1$ was counted if a mouse made a response at the opposite hole (target or nontarget) that resulted in punishment in trial t.

$$shift\,(t+1) = \begin{cases} 0 \textit{ for an omission or staying on the side that was punished} \\ 1 \textit{ for switching to the side opposite to that punished} \end{cases}$$

The probabilities of win-stay and lose-shift events were calculated as follows:

$$P(stay|win) = \frac{\sum stay}{\sum win}$$

$$P(shift|loss) = \frac{\sum shift}{\sum loss}$$

2.9. Mouse behavior pattern monitor (BPM)

Spontaneous locomotor and exploratory behavior was tested using the mouse BPM [43–50]. There were 8 Plexiglas BPM chambers (30.5 × 61 × 38 cm), each enclosed within a ventilated and sound-attenuated cabinet. A grid of 12 × 24 infrared photobeams 1 cm above the floor was used to detect the mouse's position in an x-y coordinate system every 55 ms. Ten holes (2.5 cm in diameter; 3 in each long wall, 1 in each short wall, and 3 along the center of the floor) contained infrared photobeams to detect investigatory holepokes. An array of 16 infrared photobeams 2.5 cm above the floor was used to detect rearing behavior. As described previously, each cabinet was equipped with a house light that was on for the duration of the testing period.

The testing room was kept dark, under red light, with a white noise generator producing background noise at 65 dB. Mice were brought into the testing room under black cloth and acclimated to the testing room for 60 min. After being weighed, they were placed in their respective testing chambers and allowed to habituate for 30 min. They then received saline (VEH) or (+)-doxanthrine HCl (DOX; see "(+)-Doxanthrine treatment" below) and were placed immediately into the BPM chambers for 30 min. The primary outcome measure was distance traveled.

2.9.1. (+)-Doxanthrine treatment

DOX HCl (1.0 mg/kg, dissolved in saline) was selected based on previous reports that it induces hyperactivity [51]. On day 1 of testing, half of the animals were treated with VEH before testing and the other half were treated with DOX at 5 mL/kg intraperitoneally immediately before testing. One week later, treatments were switched.

2.10. Barnes maze

The BM was built according to previous reports [52]. An opaque Plexiglas disk (75 cm diameter) was used as a platform. Twenty

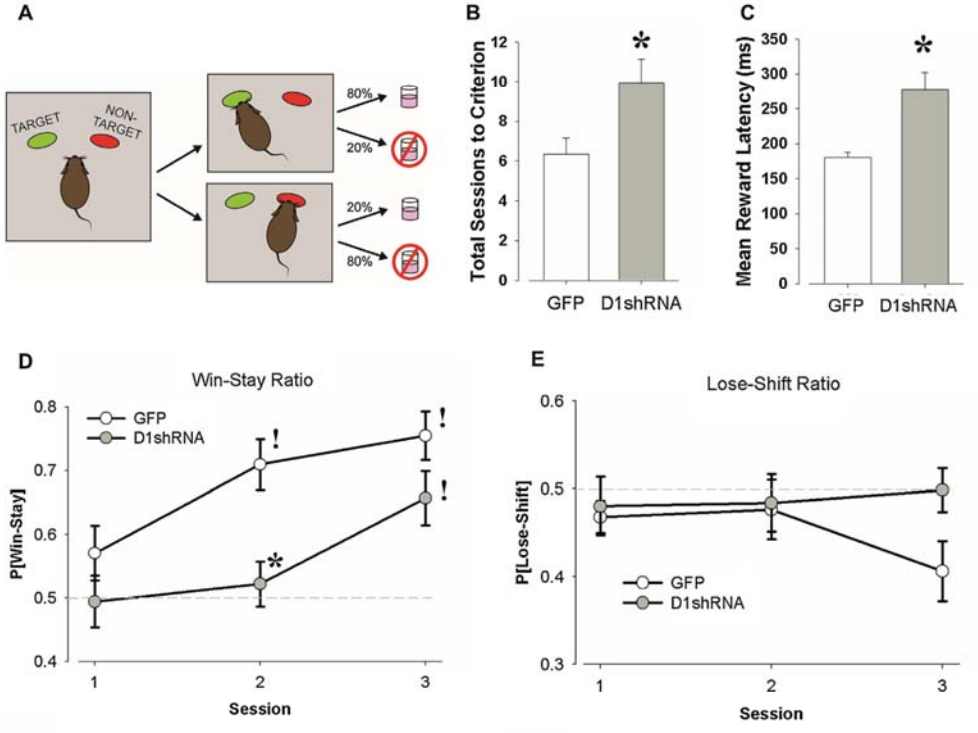

Fig. 3. Probabilistic learning performance after AAV transduction. A. Schematic of the probabilistic learning task. A response at the target stimulus resulted in a reward or punishment in a 80/20 ratio. A response at the nontarget stimulus resulted in a reward or punishment in a 20/80 ratio. Training continued between days until >85% target responses were recorded for 2 consecutive sessions (max. 15 sessions). B. D1RshRNA-treated mice required significantly more sessions to reach criterion than GFP-treated mice. C. D1RshRNA-treated mice had greater latency to retrieve the reward after delivery. D. The win-stay ratio was significantly lower for D1RshRNA-treated mice than for GFP-treated mice. There was a main effect of session and a trend toward a significant session × group interaction. E. There was no main effect of group, no main effect of session, and no session × group interaction on lose-shift ratio. Data are presented as mean ± SEM. * denotes $p < 0.05$, ! denotes $p < 0.05$ when compared to Day 1.

holes (5 cm diameter) were distributed evenly around the perimeter 5 cm from the edge of the disk (Fig. 4a). A black Plexiglas escape box (19 × 8 × 7 cm) was placed under one of the holes. Distinct spatial cues were located around the testing room and were kept constant throughout the study. On days 1–4 of testing, each mouse was placed in the escape box for 1 min prior to the training session. At the beginning of the session, the mouse was placed in the center of the platform in a cylindrical blue start chamber (10 cm high). A fan and a room light were turned on 30 s after the start of the trial, and the start chamber was removed, enabling the mouse to explore the maze freely. The session ended when the mouse entered the escape hole or after 3 min elapsed. When the mouse entered the escape hole, the fan and room light were turned off, and the mouse was allowed to remain in the dark for 1 min. When the mouse did not enter the escape hole by itself after 2 min, it was placed in the escape box for 1 min. The escape box was always located underneath the same randomly determined hole for each mouse. For the training sessions, the number of primary errors (number of holes visited before the mouse visits the escape hole), primary latency (time to reach the escape hole for the first time), and total latency (time taken to escape) were recorded.

For the probe test on day 5, the escape box was removed, and the mouse was allowed to explore the maze freely for 3 min. The time spent in each quadrant was determined and the percentage of time spent in the target quadrant, which originally contained the escape box, was compared with the average time spent in the three remaining quadrants. Number of errors (entries into non-escape holes), latency to locate the escape hole, and latency to enter the escape hole were recorded during training. Number of entries and time spent in each quadrant were recorded during the probe trial.

2.11. Statistics

Data from each experiment were analyzed using analysis of variance (ANOVA) with virus as a between-subjects factor and time (probabilistic learning and BPM studies), DOX treatment (for BPM), and training trial or quadrant (for BM) as within-subjects factors. Tukey post hoc analyses were conducted on all significant main effects and interactions. Sessions to criterion of the FR1 task and PRBP were analyzed using Student's *t*-tests. The alpha level was set to 0.05. All statistics were performed using SPSS (19.0, Chicago, IL, USA).

3. Results

3.1. Suppression of D1R in the striatum

The DA D1R gene is abundantly expressed in mouse striatum and was not affected by the co-expression of GFP protein in cells

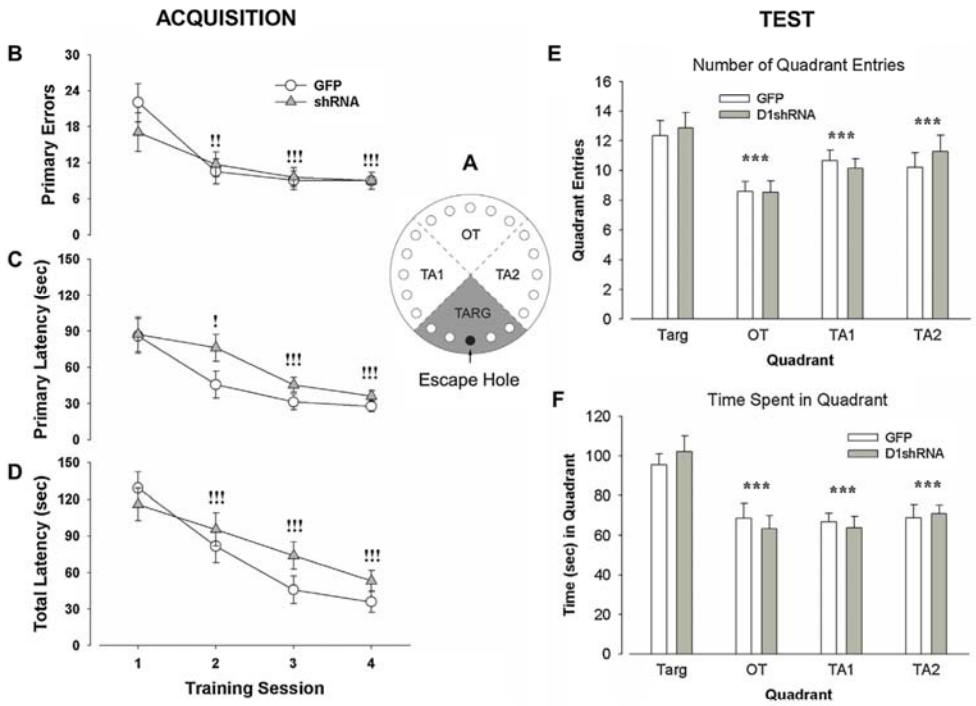

Fig. 4. Frequency of quadrant entries and time spent in each quadrant during probe trial. A. Schematic of Barnes Maze. Targ = target quadrant; OT = quadrant opposite to target; TA1 and TA2 = quadrants adjacent to target. B–D. There was a main effect of session but neither a main effect of group nor on primary errors trial × group interactions (B), primary latency (C), and total latency (D). E, F. There was a main effect of quadrant but neither a main effect of group nor a quadrant × group interaction on number of quadrant entries (E) and time spent in each quadrant (F). Entries into and time spent in the target quadrant were significantly greater than entries into and time spent in the remaining quadrants. The number of entries into the quadrant opposite the target quadrant was significantly lower than time spent in the other quadrants for both groups. Data are presented as mean ± SEM. ** denotes $p < 0.01$, and *** denotes $p < 0.001$ when compared to target quadrant. ! denotes $p < 0.05$, !! denotes $p < 0.01$, and !!! denotes $p < 0.001$when compared to target quadrant.

infected with the scAAV5 vector virus (Fig. 1c). Immunohistochemical analysis revealed however, that D1shRNA-treated mice exhibited reduced D1R expression (Fig. 1d).

3.2. Effect of D1RshRNA suppression of D1Rs on motivation

To assess whether D1R suppression affected motivation, we tested GFP- and D1RshRNA-treated mice using the PRBP. Although there was no significant difference in breakpoint between groups "post-AAV," the GFP-treated group had a significantly higher breakpoint after virus delivery than they did "pre-AAV," whereas the D1RshRNA-treated group did not have a significant difference in breakpoints pre- and post-AAV. Latency to obtain the reward did not differ. It was possible that the elevated breakpoint of the GFP-treated mice may have been due to learning, an effect possibly blocked in D1RshRNA-treated mice. Hence, we retested both groups in the PRBP ("post-AAV2"). The breakpoint of both groups decreased, resulting in no difference between groups, hence learning was unlikely a factor, and not differently affected by group since there was only a main effect of test day on breakpoint ($F_{(2,56)} = 3.6$, $p < 0.05$; Fig. 2a) and a trend-effect on mean latency to obtain the reward ($F_{(2,56)} = 2.5$, $p = 0.09$; Fig. 2b) with no day × group interaction for either measure. Latency to obtain rewards was significantly greater for mice treated with D1shRNA compared with GFP-treated mice on day 3. Although GFP-treated mice obtained rewards significantly faster during the third test when compared to the first test, the D1RshRNA-treated mice showed no increase in speed over time.

3.3. Effect of D1RshRNA suppression of D1Rs on probabilistic learning

In the probabilistic learning task, all of the GFP-treated mice (n = 15) reached criterion by Day 14 of testing, but only 12 of 15 D1RshRNA-treated animals reached criterion by this time. Due to time constraints, testing was concluded after 15 days. For those animals that did not reach criterion (two consecutive days >85% target responding) by Day 15, the number of sessions to criterion was set to 15 for analysis. The D1RshRNA-treated mice required significantly more sessions to reach criterion than the GFP-treated mice ($F_{(1,28)} = 6.4$, $p < 0.05$; Fig. 3b). For all other analyses, only those animals that completed at least one stage were compared.

Because several (7 of 15) GFP-treated mice reached criterion within the first three days of testing, they were moved to the reversal learning stage and hence these data were no longer applicable to the initial acquisition of the task. Hence, complete datasets for all mice in initial task acquisition were only available over the first three days of testing. During this time period, before any mice had reached criterion, the percentage of rewarded trials increased over time ($F_{(2,50)} = 20.9$, $p < 0.001$) but did not differ between groups, although there was a trend toward a session × group interaction

Table 1
Additional measures from the probabilistic learning task. There were no significant differences between groups.

Measure	AAV-GFP (n=15, Mean ± SD)	AAV-D1RshRNA (n = 12, Mean ± SD)
Total rewards	398.33± 190.97	430.00 ± 154.83
Percentage of trials rewarded (%)	63.49 ± 7.00	62.56 ± 2.71
Percentage of premature responses	1.15 ± 1.16	0.52 ± 0.70
Average perseverative responses	8.63 ± 3.98	15.17 ± 20.07
Average time-out perseverative responses	21.47 ± 7.80	20.63 ± 11.46
Average target latency (sec)	4.51 ± 1.93	5.40 ± 1.90
Average non-target latency (sec)	6.28 ± 3.32	7.25 ± 3.54

($F_{(2,50)} = 2.5$, $p = 0.09$). The D1RshRNA-treated mice had longer average latency to retrieve the reward however (Fig. 3c). Additional measures for which there were no significant differences are listed in Table 1.

The win-stay and lose-shift ratios for the first three sessions were analyzed to assess reward- and punishment-associative learning before mice had reached criterion. Although win-stay ratios (Fig. 3d) improved over time ($F_{(2,50)} = 22.1$, $p < 0.001$), there was also a trend toward a session × group interaction ($F_{(2,50)} = 2.5$, $p = 0.09$). This interaction was driven by overall lower win-stay ratios for D1RshRNA- than GFP-treated mice ($F_{(1,25)} = 6.7$, $p < 0.05$). Importantly, on Day 1 the win-stay ratio for both groups did not differ from each other or chance. By Day 2, there was a significant difference between groups ($t_{(25)} = 3.6$, $p < 0.005$), revealing that only the GFP control group had a ratio significantly greater than chance ($t_{(14)} = 5.5$, $p < 0.001$). By Day 3, there was only a trend difference between groups ($t_{(25)} = 1.8$, $p < 0.09$), with both groups having a ratio significantly greater than chance (GFP: $t_{(14)} = 6.9$, $p < 0.001$; D1RshRNA: $t_{(11)} = 3.9$, $p < 0.005$). Essentially, these data reflect that GFP-treated mice learned to stay after wins more often and faster than D1RshRNA-treated mice. When comparing the lose-shift ratios for the first 3 days of testing (Fig. 3e), there was no main effect of group or session (Fs < 1, *ns*), or session × group interaction ($F_{(2,50)} = 2.0$, *ns*), and so post hoc analysis was not conducted.

3.4. D1RshRNA suppression of D1Rs did not affect aversely motivated learning and memory in the Barnes maze

For the four training sessions in the BM, there was a main effect of session on all measures (primary errors: $F_{(3,84)} = 11.0$, $p < 0.001$; primary latency: $F_{(3,84)} = 15.1$, $p < 0.001$; total latency: $F_{(3,84)} = 31.0$, $p < 0.001$; Fig. 4b–d) but neither main effects of group (Fs < 2.5, *ns*) nor trial × group interactions (Fs < 2, *ns*). Hence, acquisition of the task during training was equivalent for the two groups. For the probe trial, the number of entries per quadrant and time spent in each quadrant were analyzed using repeated measures ANOVA across quadrant and group. For both measures, there was a main effect of quadrant (quadrant entries: $F_{(3,84)} = 19.3$, $p < 0.001$; quadrant time: $F_{(3,84)} = 10.8$, $p < 0.001$; Fig. 4e, f) but no main effect of group (Fs < 1.8, ns) or quadrant × group interaction (Fs < 1, *ns*). Both groups entered and spent more time in the target quadrant compared to the other quadrants.

3.5. D1RshRNA suppression of D1Rs decreased sensitivity to doxanthrine-induced hyperactivity

During the 30-min BPM session following DOX treatment, compared to the 30-min prior to treatment, there was a main effect of time bin ($F_{(5,140)} = 25.4$, $p < 0.001$) and a significant drug × bin interaction ($F_{(5,140)} = 4.1$, $p = 0.002$; Fig. 5). There was no main effect of group (F < 1.1, *ns*) and only a trend toward a significant main effect of drug ($F_{(1,28)} = 3.7$, $p = 0.06$). No drug × group, bin × group, or drug × bin × group interactions were observed (Fs < 1, *ns*). *A priori post hoc* repeated measures ANOVAs for each group revealed that only the GFP-treated mice exhibited a drug × bin interaction (GFP: $F_{(5,70)} = 4.8$, $p = 0.001$; D1RshRNA: F < 1, *ns*). DOX increased activity in the first two time bins for the GFP group (0–5 min: $t_{(14)} = 2.6$, $p < 0.05$; 6–10 min: $t_{(14)} = 2.5$, $p < 0.05$), but did not affect activity in any bin in the D1RshRNA-treated group.

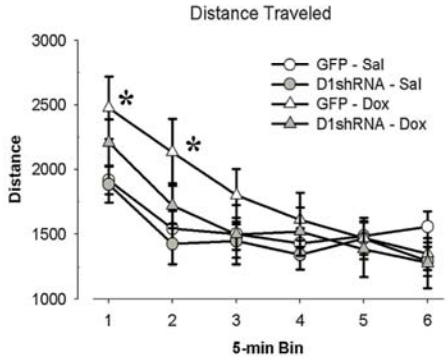

Fig. 5. Distance traveled in the BPM before and after VEH or DOX treatment. There was a main effect of 5-min bin and a significant drug × bin interaction. There was a trend toward a significant main effect of drug but no main effect of group and no drug × group, bin × group, or drug × bin × group interactions. Data are presented as mean ± SEM. * denotes p < 0.05 when compared to GFP-Sal control mice in each time bin.

4. Discussion

With localized suppression of D1R in the striatum, we demonstrated that striatal D1Rs are required for reward- but not punishment-associative learning using a paradigm that is also used to assess learning in neuropsychiatric patients. Further support for the selective deleterious reward-associative effects of D1R suppression came from the more ethologically relevant aversively motivated BM task wherein learning was not affected by D1R suppression. Finally, the lack of D1R agonist-induced hyperactivity in the D1R-suppressed mice – observed in GFP-treated mice – supports the D1R specificity of our D1RshRNA.

DA is vital for learning, as suggested by the DA reward prediction error hypothesis [7,9]. Numerous aspects of learning exist however, and so identifying the mechanisms underlying the specific learning deficits seen in neuropsychiatric patients is important, such as impaired reward-associative learning in the probabilistic task in patients with schizophrenia [53]. A primary site of action of DA is striatal D1Rs. Attempts to understand the function of this receptor using D1R knockout (KO) mice have been difficult however, especially separating effects of learning from the motivational deficits of these mice. Previous studies have found that striatal D1Rs are important for mediating the effect of reward magnitude on learning (i.e., faster learning for a greater reward), as well as willingness to work for a reward [54–56]. Consistent with these findings, D1R KO mice have low rates of responding in the PRBP, which could

be due to either impaired learning or amotivation [40,57]. These mice exhibit deficits in spatial and operant learning [40,58], and striatal-specific D1R KO mice exhibit reduced acquisition of contextual fear conditioning [59]. Because these D1R KO mice never had D1Rs from conception, it is impossible to determine if these deficits are due to the immediate absence of the D1Rs or a compensatory mechanism during development. Importantly, our viral shRNA technique rectified this experimental confound because we trained the mice to respond prior to virus delivery and D1R suppression. Hence, prior to AAV treatment, there were no differences between groups in PRBP or latency to obtain rewards. After AAV-treatment, FR1 performance did not differ between groups when measured as proportional to the rewards earned before surgery. PRBP did differ between groups after AAV-treatment however, as the GFP-treated mice had a higher breakpoint than the D1RshRNA-treated mice, due to increased responding in GFP-treated mice. This increase in breakpoint may have been due to learning from the previous trial in the GFP- but not D1RshRNA-treated mice. Upon retesting however, the breakpoint for both groups were comparable to their initial baseline test, and the difference between groups disappeared. These results suggest that the D1shRNA did not affect motivation as measured by the PRBP.

Probabilistic learning tasks have been used extensively in rodent models and patient populations to investigate the neural substrates associated with reversal learning, perseveration, and reward- versus punishment-associative learning [42,60–64]. Although the effects of genetic and pharmacological manipulation of D1Rs have been tested in simple reinforcement learning tasks [21,27], we wished to examine the contributions of striatal D1Rs to learning in a manner consistent with human testing in response to reward versus punishment. Therefore, the mice were tested in a probabilistic learning task. D1R suppression impaired learning with 20% of D1RshRNA-treated mice unable to achieve criterion. This effect was not attributable to a lack of responses by these mice (they averaged 46 responses over 30 min, comparable to the GFP-treated mice). The D1RshRNA-treated mice that did achieve criterion completed significantly fewer trials per session than the GFP-treated mice, likely as a result of lower baseline levels of responding and/or making more nontarget responses, thus incurring more delays due to punishing time-outs. Hence, D1RshRNA-induced suppression of D1Rs negatively affected learning in the task. Given that learning could occur via reward- or punishment-related feedback, further analyses of the decision-making of these mice were conducted [41,28]. To examine the decision-making processes of the mice, we analyzed their choices (to stay on the same side or switch sides) after rewards or punishments. Thus, the win-stay and lose-shift strategies were analyzed over the first three days of learning (limited to the first three days because some GFP-treated mice reached criterion by day 4). Importantly, the poorer learning of D1RshRNA-treated mice was driven by the slower elevation of their win-stay ratio, which did not differ from chance until day 3. In contrast, the GFP-treated mice learned to stay after being rewarded with a probability greater than chance by day 2. In other words, it took the D1RshRNA-treated mice longer to learn to return to the same stimulus for which they had just been rewarded. Because D1RshRNA treatment did not affect the lose-shift ratios of the mice, these data support the hypothesis that reward-associative learning was deleteriously affected in these mice. Hence, D1R suppression impaired learning via deleteriously affecting reward-associative learning, whereas punishment-associative learning appeared intact. Such findings are not dissimilar to patients with schizophrenia who exhibit impaired learning in response to rewards but normal learning in response to punishment [5]. In contrast however, patients with bipolar disorder mania exhibit impaired learning driven by a hypersensitivity to reward, preferring high-reward options [12]. Similar probabilistic learning outcomes across species indicate that the altered learning of these patients could reflect lower D1R expression in schizophrenia, versus perhaps an over-expression/stimulation in mania patients.

The normal punishment-related learning of D1R suppressed mice in the probabilistic learning task was observed in response to a 4 s time-out with a bright light. An alternative explanation for the normal punishment-related learning of these mice could be that this 'punishment' was not sufficiently salient to drive behavior. Longer time-outs and punishing shocks can be more aversive [65,66]. Alternatively, to examine more ethologically relevant and hence salient aversely motivated learning, we also tested the learning and memory of these mice in the BM. The BM puts mice in a brightly lit elevated platform with only one escape location [67]. In contrast with the operant-based probabilistic learning task, in which behavior is shaped by positive reinforcement, the Barnes maze utilizes negative reinforcement (escape from aversive conditions). The saliency of this aversive environment is supported by the speed of escape learning of these mice and is widely used in the literature to examine such learning [68]. Although patterns of DA release during both positive and negative reinforcement may be similar, these processes have differing underlying mechanisms, differentially involving the direct and indirect pathways, respectively [20,24,69]. Learning from negative reinforcement—like learning from punishment—likely involves D2Rs but not D1Rs [20]. Therefore, we did not expect performance on the Barnes maze to be disrupted by striatal D1RshRNA. Consistent with this hypothesis, the D1RshRNA-treated mice did not differ in learning or memory for the escape location compared with the GFP-treated mice. In addition to supporting the normal punishment-related learning in the D1RshRNA-treated mice, the results of the BM suggest intact hippocampal function, providing further evidence that the deficits in the probabilistic learning task are due to striatal D1Rs and not hippocampal D1Rs.

Initially, immunohistochemical analyses confirmed that the D1RshRNA reduced expression of striatal D1Rs. (+)-DOX is a full D1R agonist, with a 200-fold greater selectivity for D1-like over D2-like receptors, that induces hyperactivity in mice and rats [51,70]. To confirm functional suppression of D1Rs, we demonstrated that DOX induced hyperactivity in GFP-treated by not striatally D1R suppressed mice (Fig. 5). These data support findings of the strong striatal component to stimulant-induced hyperactivity and DOX-induced action on the direct striatal pathway in rats [71]. These data support the immunohistochemical analyses that the striatally administered D1RshRNA suppressed D1R expression. Furthermore, these data support the D1R selectivity of DOX [70] and that DOX-induced hyperactivity is mediated via striatal D1Rs.

Herein, we showed that striatal D1Rs are important for reward-associative learning using tasks with cross-species relevance [72]. These data support the assertion that D1R mediate reward-associative learning, likely via the direct MSN pathway of the basal ganglia [27,73]. Although our virus was delivered throughout the dorsal and ventral striatum, it is likely that D1R suppression in the nucleus accumbens (NAc) was particularly important for mediating the decrease in reward learning, because inputs to NAc from the prelimbic cortex (PL) of the prefrontal cortex and the basolateral amygdala (BLA) are important for driving responses through reward [74,75]. In addition, inactivation of the NAc in rats has been shown to decrease win-stays during a risk-discounting task when rewards are associated with high risk [76]. In particular, there is evidence that D1Rs—but not D2Rs—mediate the interactions between BLA and NAc that are necessary for the formation of reward associations [77]. Interestingly, however, blockade of D1Rs in NAc was shown to increase lose-shift behavior and not win-stays [78], but in the risk-discounting task where "loss" was the absence of high reward and the switch was made to a guar-

anteed (small) reward, so it is difficult to make a comparison to lose-shifts in our probabilistic learning task [79]. In the same study, D2R blockade did not influence preference for reward, which is consistent with our findings. Suppression of D1Rs in the dorsal medial and dorsal lateral striatum (DMS and DLS, respectively) could also affect reward learning in various ways. The DMS is associated with action-outcome learning and suppression could thus impact task acquisition, while the DLS is associated with habit formation and suppression could impact behavior maintenance [22]. In addition to these localized differences in overall DA function, D1Rs specifically have been implicated in reward learning and habit formation [20,21,26]. More localized application of the D1RshRNA and tasks designed specifically to dissociate action-outcome versus habitual behavior impairments will help elucidate the importance of specific DA receptors in each region of the dorsal striatum. Overall, these data provide support for recent positron emission tomography evidence that individual approach and avoidance learning rates in humans are predicted by variability in striatal D1R and D2R binding [80]. Increased regional specificity of D1R suppression, as well as greater information regarding the connectivity of the D1R neurons, will be important for better understanding the system underlying reward-associative learning.

The D1R has been a target for cognition enhancement in schizophrenia, as highlighted by MATRICS [81]. Our studies provide support and guidance as to what aspects of cognition should be targeted in clinical trials. In addition, however, poor reward-associative learning in patients with schizophrenia has been associated with negative symptoms in schizophrenia, specifically amotivation [53]. Thus, these data support the notion that the D1R may be a viable target for treating motivational impairments in schizophrenia patients [82,83], or as a pharmacological augment to cognitive training [6,84].

Confirmation that striatal D1R stimulation can enhance reward-associative learning will first be required. Furthermore, given that poor reward-associative learning of patients with psychiatric illnesses could negatively impact psychotherapeutic interventions, such as cognitive training, pharmacologically augmenting such learning could synergistically enhance the effectiveness of cognitive training [6,84]. The techniques described here will enable direct assessment of the cross-species sensitivity of D1R stimulation on reward-associative learning prior to full clinical trials [72].

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Appendix A, in full, is a reprint of the material as it appears in Higa KK, Young JW, Ji B, Nichols DE, Geyer MA, Zhou X. *Striatal dopamine D1 receptor suppression impairs reward-associative learning.* Behavioural Brain Research. 2017 Apr 14;323:100-110. The dissertation author was the primary investigator and author of this paper.

Appendix B:

Nicotine withdrawal-induced inattention is absent in α7 nAChR knockout mice

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ORIGINAL INVESTIGATION

Nicotine withdrawal-induced inattention is absent in alpha7 nAChR knockout mice

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Abstract
Rationale Smoking is the leading cause of preventable death in the USA, but quit attempts result in withdrawal-induced cognitive dysfunction and predicts relapse. Greater understanding of the neural mechanism(s) underlying these cognitive deficits is required to develop targeted treatments to aid quit attempts.
Objectives We examined nicotine withdrawal-induced inattention in mice lacking the α7 nicotinic acetylcholine receptor (nAChR) using the five-choice continuous performance test (5C-CPT).
Methods Mice were trained in the 5C-CPT prior to osmotic minipump implantation containing saline or nicotine. Experiment 1 used 40 mg kg^{-1} day^{-1} nicotine treatment and tested C57BL/6 mice 4, 28, and 52 h after pump removal. Experiment 2 used 14 and 40 mg kg^{-1} day^{-1} nicotine treatment in α7 nAChR knockout (KO) and wildtype (WT) littermates tested 4 h after pump removal. Subsets of WT mice were killed before and after pump removal to assess changes in receptor expression associated with nicotine administration and withdrawal.
Results Nicotine withdrawal impaired attention in the 5C-CPT, driven by response inhibition and target detection deficits. The overall attentional deficit was absent in α7 nAChR KO mice despite response disinhibition in these mice. Synaptosomal glutamate mGluR5 and dopamine D_4 receptor expression were reduced during chronic nicotine but increased during withdrawal, potentially contributing to cognitive deficits.
Conclusions The α7 nAChR may underlie nicotine withdrawal-induced deficits in target detection but is not required for response disinhibition deficits. Alterations to the glutamatergic and dopaminergic pathways may also contribute to withdrawal-induced attentional deficits, providing novel targets to alleviate the cognitive symptoms of withdrawal during quit attempts.

Keywords Response inhibition · α7 Nicotinic acetylcholine receptor · Five-choice continuous performance task · Attention · mGluR5 · Dopamine D_4 receptor

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Introduction

Tobacco smoking is the leading cause of premature and preventable death, disease, and disability in the USA (United States Department of Health and Human Services 2014). Although the percentage of US adults who smoke cigarettes has declined from 20.9% in 2005, it was still ~16.8%, or ~40 million adults as of 2014 (Jamal et al. 2015). These high rates of tobacco use reflect, to some extent, the difficulty in quitting. Although two thirds of smokers report a desire to quit, half relapse within the first week after attempting to quit and ~90% relapse within a year of quitting (Garvey et al. 1992; Powell et al. 2010; Ashare and Hawk 2012; Jamal et al. 2015).

It is now commonly accepted that the difficulty of smoking cessation is, in part, due to the cognitive deficits associated with nicotine withdrawal (Hall et al. 2015). Patients report difficulty concentrating and confusion with quantified nicotine withdrawal-induced impairments to working memory, attention, response inhibition, reward processing, and reaction

time (Hughes 2007; McClernon et al. 2015; van Enkhuizen and Young 2016). In fact, some of these cognitive impairments, including attention deficits and impulse control, before and during withdrawal can predict relapse (Pomerleau et al. 2003; Dolan et al. 2004; Rukstalis et al. 2005; Krishnan-Sarin and Reynolds 2007; Culhane et al. 2008; Powell et al. 2010). Understanding the mechanism(s) underlying withdrawal-induced cognitive deficits may be key toward developing targeted treatments that will remediate these deficits and aid quit attempts (Hall et al. 2015).

Nicotine is the primary psychoactive agent in tobacco smoke. Nicotine is the prototypical ligand of the nicotinic acetylcholine receptors (nAChRs), the most abundant of which are α4β2 and α7 nAChRs (Gotti et al. 2006). Nicotine is a full agonist at both of these nAChRs, although at a higher affinity for α4β2 nAChRs (Gotti et al. 2009). Importantly, nicotine administration can improve cognitive dysfunction during tobacco withdrawal, as can varenicline, a partial agonist of α4β2 and full agonist of α7 nAChRs (Patterson et al. 2009; Ashare and McKee 2012). The efficacy of nicotine and varenicline in improving cognition implicate both the α4β2 and α7 nAChRs in nicotine withdrawal-induced cognitive deficits. Yildirim et al. (2015) found that ABT-089 (a partial α4β2 agonist), but not ABT-107 (an α7 agonist), alleviated the symptoms of withdrawal-induced deficits in contextual fear conditioning in mice. These results importantly suggest that α4β2 antagonism may *treat* withdrawal-induced cognitive deficits in humans. Unfortunately, these findings are limited to this hippocampal-dependent task. To our knowledge, no studies have examined the *necessity* of either of these receptors in the development of nicotine withdrawal-induced cognitive deficits. Conducting such studies in humans would prove difficult. Determining the necessity of these receptors in the effects of withdrawal can however be readily studied in transgenic mice (e.g., Stoker et al. 2012a), enabling more targeted examination of putative underlying mechanisms.

Many studies exist determining the necessity of nAChRs in cognitive functioning using transgenic mice. For example, null mutation of the α7 nAChR revealed the importance of this nAChR for reward learning and sustained attention, as measured by the five-choice serial reaction-time task (5CSRTT), although these mice exhibited normal motivation and punishment-associated learning compared with their wildtype (WT) littermates (Young et al. 2004, 2007, 2011a; Keller et al. 2005; Hoyle et al. 2006; Levin et al. 2009). Nicotine withdrawal studies in these and β4 nAChR null mice revealed the importance of the α7 and β4 subunits in the somatic signs and anhedonic-like state of nicotine withdrawal (Stoker et al. 2012a). The roles of the α7 and β4 nAChR subunits in withdrawal-induced cognitive dysfunction have yet to be determined. In rat studies, nicotine withdrawal impaired sustained attention (Shoaib and Bizarro 2005; Semenova et al. 2007), an effect that was remediated by varenicline treatment (Jackson et al. 2016). Although measuring sustained attention, the 5CSRTT has no response inhibition component, which is common to human attentional tests (Young et al. 2011b; Lustig et al. 2013) and important during nicotine abstinence (Tsaur et al. 2015). The development of the five-choice continuous performance test (5C-CPT) for mice (Young et al. 2009), rats (Barnes et al. 2012; Hayward et al. 2016), and humans (Young et al. 2013a) has enabled the assessment of sustained attention and response inhibition by adding a non-target stimulus requiring inhibition, consistent with established human tests (Romberg et al. 2013). The use of the 5C-CPT enabled the double dissociation of response inhibition and premature responses (early responding to no stimuli) driven by reduced dopamine D_4 receptor expression and 5-HT_{2C} antagonism, respectively (Young et al. 2011b).

Despite its utility, to date, no studies have used the 5C-CPT to investigate the necessity of specific nAChRs on nicotine withdrawal-induced deficits in attention and response inhibition. Furthermore, few studies have investigated the molecular changes that occur in the brain due to nicotine withdrawal. Given that reduced expression of dopamine D_4 receptors has been associated with response disinhibition (Young et al. 2011b), we assessed their levels in mice during chronic nicotine administration and withdrawal. In addition, group I metabotropic glutamate receptors (mGluR1 and mGluR5) play an important role in maintaining the reinforcing effects of drugs like nicotine and may undergo adaptations during chronic drug exposure, potentially contributing to the withdrawal-induced changes in behavior (Kenny and Markou 2004). For example, reduced function of mGluR5 receptors may contribute to withdrawal-induced anhedonia and somatic symptoms (Liechti and Markou 2007; Stoker et al. 2012b), since chronic nicotine increased mGluR5 expression during treatment, and levels were restored to baseline 1 day after withdrawal (Pistillo et al. 2016). Hence, we also examined whether nicotine withdrawal-induced changes in mGluR1 and mGluR5 expression might be associated with inattention and assessed receptor levels during chronic nicotine treatment and withdrawal.

In this study, we assessed the cognitive deficits associated with nicotine withdrawal in healthy mice and those lacking α7 nAChRs, hypothesizing, based on rat and human studies (Shoaib and Bizarro 2005; Semenova et al. 2007; Harrison et al. 2009; McClernon et al. 2015), that attention would be disrupted during withdrawal. Furthermore, we hypothesized that mice lacking α7 nAChRs would not exhibit nicotine withdrawal-induced deficits in attention. Finally, we hypothesized that nicotine withdrawal would result in decreased expression of dopamine D_4 and glutamate mGluR1 and mGluR5 receptors.

Materials and methods

Mice

Male C57BL/6J mice ($n = 27$) and male α7 nAChR knockout mice (KO, $n = 26$) and their WT littermates ($n = 28$) were bred in-house, the latter generated from heterozygous breeding pairs on a C57B/6J background as previously described (Young et al. 2011a; Stoker et al. 2012a). Training began at 3 months old (baseline weight for all mice, 23–35 g, consistent with previous reports, there was no difference in weights between the WT and KO mice). All mice were group-housed (maximum four per cage) and maintained in a climate-controlled vivarium with a reversed day/night cycle (lights on at 20:00 hours, off at 8:00 hours) and tested during the dark phase between 9:00 and 18:00 hours. Food (Harlan Teklad, Madison, WI) and water were available ad libitum except during training and testing, when food was restricted to maintain mice at ~85% full body weight (20–30 g). All mice were maintained in an animal facility that meets all federal and state requirements for animal care and was approved by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the University of California San Diego (UCSD) Animal Care and Use Committee.

Five-hole apparatus

Sound-insulated five-hole operant chambers (25 × 25 × 25 cm; Med Associates, Inc., St. Albans, VT) were used as described previously (Young et al. 2015). Each chamber had a house light and fan, with an array of five square holes (2.5 × 2.5 × 2.5 cm, 2.5 cm above the grid floor) arranged horizontally on a curved wall opposite the liquid delivery magazine (Lafayette Instruments, Lafayette, IN). Each hole had a light-emitting diode (LED) at the back and infrared beams, mounted vertically 3 mm from the opening to detect responses. The food delivery magazine contained a well for liquid reinforcement (30 μl; strawberry Nesquik® plus non-fat milk), delivered by a peristaltic pump (Lafayette Instruments, Lafayette, IN), with an LED at the top. The magazine also contained an infrared beam 5 mm from the floor, recessed 6 mm to detect head entries. The control of stimuli and recording of responses were managed by a SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates, Inc.) using custom programming.

Mouse 5C-CPT

Mice were trained as previously described. Mice were trained initially to retrieve rewards from the magazine, after which they were trained in a fixed ratio 1 (FR1) schedule. During this FR1 schedule, all five holes opposite the magazine were illuminated and mice were rewarded for nose poking any of the five holes. Sessions lasted 30 min or until 150 trials were completed. FR1 criterion was 70 responses for two consecutive days. Once criterion was reached, mice were only trained 2 days/week while other mice were trained 5 days/week in order to minimize over-training in FR1. After all mice had reached stable performance at criterion, training began on the 5CSRTT, in which only one of the five nose-poke holes was illuminated. Initially, mice had to make the correct choice (hole poking in the illuminated hole) within 10 s (stimulus duration (SD)) or the house light was illuminated for a 4-s time out. The intertrial interval (ITI) was held at a constant 4 s, and sessions lasted for 30 min or until 120 trials were completed. Criterion for this stage of training was 30 correct trials with mean correct response latencies less than half the current SD s for two consecutive sessions. Mice then progressed to 8, 4, and 2 s SD trials when meeting the same criterion. Once at 2 s, a variable ITI (3–7 s) was introduced to limit the use of a temporally mediated strategy (Cope et al. 2016) and increase the attentional load of the task. After reaching criterion, mice progressed to the 5C-CPT, in which target trials were the same as in the 5CSRTT (response required in a single lit hole) but non-target trials were added in which all five holes were illuminated (requiring the inhibition of responding), with the SD consistent between the two trial types. If during the non-target trial the nose poke was inhibited for 2 s, the mouse was rewarded, but a response was punished with a 4-s timeout. Once responding at less than 1.5 s mean correct latency for two consecutive days, mice were moved to a 1.5 SD. Criterion was set at least 30 correct trials, mean overall correct latency less than 1.5 s, and less than 50% false alarm rate for two consecutive sessions.

After stability in performance was achieved, challenge sessions were used. These sessions were identical to training sessions but lasted for 250 trials with 3–7 s variable ITI and 1.5 s SD. Extended session challenges can be used to assess vigilance decrements (Young et al. 2009). Mice continued to be trained in 30 min or 120 trial sessions between challenge sessions.

Primary outcome variables of the 5C-CPT were hit rate (p[HR], the proportion of correct target responses to missed targets) representing target detection, false alarm rate (p[FA], the proportion of inappropriate responses to the non-target stimulus to correct withdrawals to the non-target stimulus) representing response inhibition, and d' (a composite parametric measure for the difference between p[HR] and p[FA]) representing vigilance. Secondary outcome variables include the responsivity index (RI) to represent bias of responding, as well as accuracy (the proportion of correct responses to incorrect responses), latencies to correct target responses (mean correct latency (MCL)), total trials, the percentage of omitted trials, and the %percentageof premature responses representing motoric impulsivity/temporal discrimination (Cope et al. 2016). The calculation for each variable is thoroughly described elsewhere (Cope and Young 2016).

For experiment 1, mice were tested 1 week before pump implantation and counterbalanced based on d', % omissions, p[FA], and MCL for treatment with saline or nicotine (40 mg kg^{-1} day^{-1}). Mice were tested on the same challenge 2 and 27 days after pump implantation, as well as 4, 28, and 52 h after pump removal (Fig. 1) but trained in the standard task every other day. The 4-h time point was chosen based on previous findings of withdrawal-induced anhedonia after 3–6 h of withdrawal from 40 mg kg^{-1} day^{-1} of chronic nicotine (Stoker et al. 2008; Stoker et al. 2012a). Additional assessments were made 24 and 48 h after the initial testing in keeping with the mice's normal training/testing schedule.

For experiment 2, α7 mutant mice were trained in the 5C-CPT and stability established at a 3–7-s variable ITI and 1.5-s SD over 3 days. These data were averaged and used to counterbalance into treatment groups (saline, nicotine at 14 mg kg^{-1} day^{-1}, and nicotine at 40 mg kg^{-1} day^{-1}) based on average d', p[HR], and MCL. These mice were also challenged in the extended 250-trial session 33 days after pump implantation and 4 h after pump removal. To assess receptor expression during nicotine treatment and withdrawal, nine WT mice (n = 3 per treatment group) were removed 33 days after pump implantation (before pump removal) and nine additional WT mice (n = 3 per treatment group) were removed after the final extended 5C-CPT session (during withdrawal). Performance in the 5C-CPT was assessed only in the mice with a full data-set for all 5C-CPT tests, before, during, and after nicotine.

Drug

(−)Nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline solution and pH adjusted to 7 ± 0.5 with sodium hydroxide (Sigma-Aldrich). Nicotine was infused through subcutaneous osmotic minipumps at concentrations of 14 and 40 mg kg^{-1} day^{-1} (Model 2004, ALZET, Palo Alto, CA). Doses were chosen based on previous reports of effects in mice (Stoker et al. 2008; Portugal and Gould 2009; Hall et al. 2015).

Osmotic minipump implantation and removal surgery

The ALZET mini-osmotic pump Model 2004 has a reservoir volume of 200 μl and delivers solutions at a pumping rate of 0.25 μl/h (±0.05 μl/h). Pumps were filled and primed in 0.9% saline solution at room temperature for 40–48 h before insertion. Mice were anesthetized with isoflurane (1–3% in oxygen). Before inserting the minipump, the area around the back of the neck was shaved and sterilized with betadine. An incision was made and a pouch large enough for the pump was blunt dissected into the back using scissors. The pre-filled pump was inserted into the pouch with the flow modulator directed posteriorly, away from the wound. The incision was closed with 9 mm wound clips (MikRon Precision, Inc., Gardena, CA), and baytril (5 mg/kg) and flunixamine (2.5 mg/kg) were injected subcutaneously to minimize chances of infection and alleviate pain, respectively. After 28 or 33 days, the nicotine minipumps were surgically removed under isoflurane anesthesia and the wound was stapled using aseptic surgery techniques with administration of baytril and flunixamine, as described above.

Cotinine assessment

Cotinine, a major metabolite in the urine, is a commonly used marker of nicotine exposure (Haufroid and Lison 1998), given the short half-life of nicotine. Therefore, to confirm the presence of nicotine in these mice at levels comparable with human smokers, on day 26 of experiment 1, urine was collected from mice for assessment of cotinine levels. Samples were shipped to Millenium Health Laboratories (San Diego, CA) for analysis.

Synaptosomal isolation and western blot

Synaptosomal isolation and western blots were performed as previously described (Naviaux et al. 2013, 2015). Briefly, cerebral samples were collected, homogenized, and synaptosomes

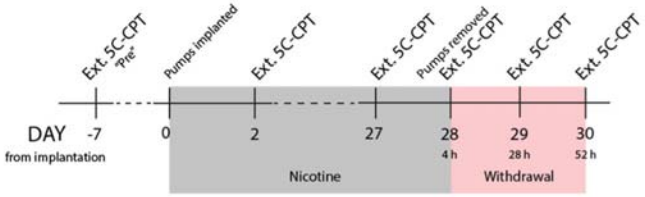

Fig. 1 Timeline of chronic nicotine testing procedure. Mice were split into two matched treatment groups based on an extended 5C-CPT session (Ext. 5C-CPT) 1 week (−7 days, *pre*) before implantation of minipumps (saline or 40 mg kg^{-1} day^{-1} nicotine). Mice were tested again 2 and 27 days after pump implantation. Minipumps were removed 28 days after implantation, and the mice were tested at 4, 28, and 52 h after pump removal

isolated by discontinuous Percoll gradient centrifugation. Twelve micrograms of cerebral synaptosomal protein was loaded. Blots were probed with primary antibodies overnight in a cold room using anti-mGluR1 (no. ab27199 from Abcam, Cambridge, MA), mGluR5 (no. ab76316 from Abcam), and dopamine D_4 receptor (no. ADR-004 from Alomone Labs, Jerusalem, Israel) antibodies. After washing, the membranes were blotted with goat anti-rabbit secondary antibody (no. 31460 from Pierce, Rockford, IL). The proteins of interest were visualized by ECL reagent (no. 32109) or Pierce SuperSignal™ West Femto Maximum Sensitivity Substrate (no. PI-34095), and the immunoblots were exposed to X-Omat Blue films and scanned. The target protein density was normalized by Ponceau S staining and analyzed in GraphPad Prism 6.0 (La Jolla, CA).

Statistics

All primary (d', p[HR], p[FA]) and secondary outcome measures (RI, accuracy, MCL, total trials, percentage of omitted trails, and % of premature responses) in the 5C-CPT from experiment 1 before and during nicotine administration were analyzed using one-way analysis of variance (ANOVA) with treatment group as the between-subject factor. The same measures from experiment 1 after withdrawal were analyzed using ANOVA with treatment group as a between-subject factor and time after withdrawal as a within-subject factor. Since we hypothesized a priori that the effect of withdrawal would diminish over time, we conducted planned ANOVAs to assess differences in the primary outcome measures specifically at the 4-h time point after withdrawal with treatment group as the between-subject factor. Primary and secondary 5C-CPT measures from experiment 2 during and after nicotine administration were analyzed using ANOVA with treatment group and genotype as between-subject factors. We conducted a priori planned ANOVAs on the primary outcome measures to compare the saline-treated mice and 40 mg kg^{-1} day^{-1} nicotine-treated group specifically (consistent with experiment 1) with treatment group and genotype as between-subject factors. Receptor concentrations were normalized by protein, and the concentration of receptor in the saline-treated animals was taken as the 100% reference. Samples were analyzed using two-way ANOVA with treatment group and time point (before or after withdrawal) as between-subject factors. Tukey post hoc analyses were conducted on all significant main effects and interactions. The alpha level was set to 0.05. All statistics were performed using SPSS (19.0, Chicago, IL) or GraphPad Prism 6.0 (La Jolla, CA).

Results

Experiment 1: the effects of nicotine administration and withdrawal on 5C-CPT performance in C57BL/6J mice

We examined the effects of nicotine administration and withdrawal on 5C-CPT performance in mice, with vigilance represented by d' (Fig. 2a). Before nicotine administration, there was no significant difference between groups assigned to receive saline or nicotine. Two days after pump implantation, however, there was a trend toward greater d' for the mice that received nicotine ($F_{(1, 25)} = 3.2$, $p < 0.1$). This trend was gone by 27 days after pump implantation. ANOVA across the three time points following withdrawal (4, 28, and 52 h after pump removal) revealed a trend toward a main effect of nicotine ($F_{(2, 50)} = 3.7$, $p < 0.1$), but no main effect of time nor a nicotine × time interaction on d'. An a priori planned comparison revealed that consistent with measurement of anhedonia-like behavior in mice 4 h after pump removal, those withdrawn from nicotine exhibited poorer d' compared with control mice ($F_{(1, 25)} = 4.2$, $p < 0.05$) with a modest to strong effect size (Cohen's $d = 0.8$).

Further analyses revealed significant effects of nicotine on p[HR] ($F_{(1, 25)} = 4.1$, $p = 0.05$, Fig. 2b) and on p[FA] ($F_{(1, 25)} = 11.5$, $p < 0.003$, Fig. 2c) 2 days after pump implantation —with both p[HR] and p[FA] lower for mice receiving nicotine—but no significant effects of nicotine on either measure before treatment or 27 days after pump implantation. Although reduced p[HR] and elevated p[FA] were observed during withdrawal, these results were not significant. Specific analysis on p[FA] during only the 4-h withdrawal time point did, however, reveal a significant effect of nicotine withdrawal to increase p[FA] ($F_{(1, 25)} = 3.1$, $p < 0.01$) with a modest effect size (Cohen's $d = 0.6$). At this time point, though it was not statistically significant, the reduction in p[HR] also had a modest effect size (Cohen's $d = 0.5$).

Two days after pump implantation, there was a main effect of nicotine on RI ($F_{(1, 25)} = 10.2$, $p < 0.001$, Fig. 2d), whereby the mice receiving nicotine had a significantly more negative RI than mice receiving saline, indicative of a reduced response rate. The effect of nicotine on RI was not present before nicotine administration, 27 days after pump implantation, or during withdrawal. There was no main effect of nicotine on accuracy before or during nicotine administration, but ANOVA revealed a main effect during withdrawal ($F_{(1, 25)} = 7.3$, $p < 0.02$, Fig. 2e), with reduced accuracy in animals that received nicotine treatment. Finally, there was no main effect of nicotine on MCL before, during, or after nicotine administration (Fig. 1f). There were neither main effects of time nor nicotine × time interaction on RI, accuracy, or MCL after withdrawal.

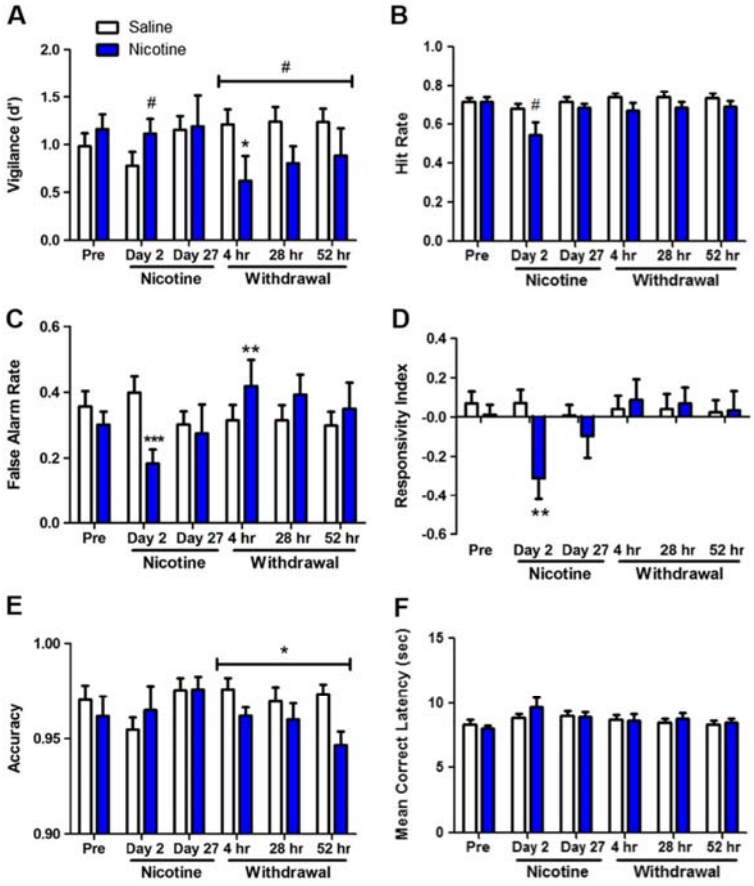

Fig. 2 Effects of chronic nicotine (40 mg kg^{-1} day^{-1} vs. saline) administration and withdrawal on performance of C57BL/6J mice in the 5C-CPT. Before nicotine minipump implantation (*pre*), there were no significant differences between groups on d' (**a**), hit rate (**b**), false alarm rate (**c**), responsivity index (**d**), accuracy (**e**), or mean correct latency (**f**). Two days after pump implantation, there were trends toward greater d' and lower hit rate for mice that received nicotine. Nicotine treatment also resulted in significantly lower false alarm rate and more negative responsivity index. No trends or significant effects of nicotine were observed 27 days after nicotine treatment. After pump removal, there were main effects of withdrawal from nicotine treatment on d' and accuracy, which were both lower for mice that had received nicotine. Data are shown as mean ± SEM, $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{*}p < 0.01$, $^{***}p < 0.001$ when compared with saline

Results and statistics for additional secondary measures can be found in Table 1. There were no significant main effects of nicotine or time on total trials. There was a significant effect of nicotine treatment on % omissions 2 days after pump implantation ($F_{(1, 25)} = 4.2$, $p = 0.05$)—with increased omissions for nicotine-treated mice—but not 27 days after treatment. After pump removal, the nicotine-treated animals exhibited increased % omissions ($F_{(1, 25)} = 3.0$, $p = 0.1$), but this increase was not significant. After pump removal, there was a significant main effect of nicotine withdrawal on premature responses ($F_{(1, 25)} = 9.9$, $p < 0.004$), whereby the nicotine-treated mice had a higher percentage of premature responses. This difference was not observed during nicotine treatment, but before nicotine administration, the nicotine-treated group already exhibited more premature responses than the saline-treated group ($F_{(1, 25)} = 3.2$, $p < 0.1$). Therefore, it is unclear from these data if the effect during the withdrawal period was due to nicotine treatment.

Experiment 2: the effects of chronic nicotine administration and withdrawal on 5C-CPT performance in α7 nAChR KO mice and WT littermates

In this study, the mice were tested in the 5C-CPT before pump implantation and 33 days later. Because the study included the assessment of nicotine withdrawal-induced changes in receptor expression, requiring the loss of a large subset of WT mice, all mice were tested in the 5C-CPT only 4 h after pump removal.

There were no significant differences in rewards collected or days to reach criterion in the stages before 5CSRTT training. When the animals reached asymptotic performance on the

Table 1 Effects of chronic nicotine (40 mg kg^{-1} day^{-1} vs. saline) administration and withdrawal on secondary performance measures for C57BL/6J mice in the 5C-CPT

Measure		Pre-treatment group			Nicotine administration				Nicotine withdrawal									
					Day 2 nicotine			Day 27 nicotine			4 h		28 h		52 h		Nicotine	Time		Nic × Time	
		Mean ± SEM	$F_{(1, 25)}$	p	Mean ± SEM	$F_{(1, 25)}$	p	Mean ± SEM	$F_{(1, 25)}$	p	Mean ± SEM	p	Mean ± SEM	p	Mean ± SEM	p	$F_{(1, 25)}$	p	$F_{(2, 50)}$	p	$F_{(2, 50)}$	p
Total trials	Saline	235.50 ± 4.91	<2	ns	231.00 ± 6.06	<2	ns	238.93 ± 3.34	<1	ns	239.00 ± 3.44	ns	234.00 ± 5.28		237.86 ± 4.84		3.1	0.09	<1	ns	<1	ns
	Nic (40 mg kg^{-1} day^{-1})	243.38 ± 3.77			213.23 ± 14.28			238.32 ± 3.87			223.00 ± 8.70		224.77 ± 5.96		229.15 ± 5.23							
% Omissions	Sal	27.88 ± 2.20	<1	ns	31.02 ± 2.54	4.2	0.05	27.75 ± 2.10	<2	ns	10.83 ± 0.99	ns	10.98 ± 1.23		11.04 ± 1.12		3.0	0.1	<1	ns	<1	ns
	Nic (40 mg kg^{-1} day^{-1})	27.41 ± 2.44			44.85 ± 6.73			30.98 ± 2.05			15.81 ± 3.37		13.82 ± 1.37		13.05 ± 1.23							
% Premature responses	Sal	3.09 ± 0.53	3.2	0.09	2.85 ± 0.85	<1	ns	1.66 ± 0.46	<1	ns	1.81 ± 0.34	ns	2.14 ± 0.52		2.44 ± 0.62		9.9	0.00	2.9	0.06	<2	ns
	Nic (40 mg kg^{-1} day^{-1})	6.42 ± 1.93			3.40 ± 1.64			3.00 ± 1.08			4.99 ± 1.48		4.69 ± 1.30		7.83 ± 1.80							

There were no significant main effects of nicotine treatment or time on total trials. There was a significant effect of nicotine withdrawal 2 days after pump implantation but not 27 days after treatment. After pump removal, there was a trend toward increased omissions for the nicotine-treated animals. Before pump implantation, there was a trend toward increased premature responses for mice assigned to the nicotine treatment group. This trend was not observed during nicotine treatment but was significant after pump removal

5CSRTT with a constant ITI for four consecutive days, KO mice exhibited a lower accuracy than WT mice ($F_{(1, 56)} = 6.3$, $p < 0.02$). There was no effect of genotype on the percentage of omitted trials. These findings are consistent with results from Hoyle et al. (2006) using the same task design in α7 nAChR KO mice. A variable ITI was then introduced to limit the use of a temporally mediated strategy and increase the attentional load of the task. When the mice attained stable performance for 4 consecutive days, the KO mice again exhibited a lower accuracy than WT mice ($F_{(1, 56)} = 6.2$, $p < 0.02$). The KO mice omitted a greater percentage of trials than the WT mice ($F_{(1, 56)} = 4.3$, $p < 0.05$). These results are consistent with the report of Young et al. (2004) on the same task with a variable ITI. As described below, the KO mice continued to exhibit lower accuracy throughout training on the 5C-CPT. No differences to attain criterion in the 5C-CPT stage were observed.

Baseline assessment of performance in mice in the 5C-CPT revealed neither main effects of genotype or nicotine nor assigned nicotine group × genotype interactions on d', p[HR], p[FA], RI, or MCL ($F < 1$, ns). Subsequent analyses of these measures therefore focus on the effects of nicotine 33 days after pump implantation and after 4 h withdrawal.

During chronic nicotine treatment on day 33, there were neither main effect of nicotine, genotype, nor nicotine × genotype interactions on d'. In contrast however, 4 h after pump removal, the nicotine-treated groups exhibited lower d' than the saline-treated group ($F_{(2, 39)} = 2.5$, $p < 0.1$, Fig. 3a). Given our a priori hypotheses from experiment 1, we analyzed the effects of the same dose (40 mg kg^{-1} day^{-1}) on performance in contrast to saline-treated mice. Consistent with experiment 1, a significant main effect of nicotine 4 h after withdrawal was observed ($F_{(1, 26)} = 4.6$, $p < 0.05$), as was a modest nicotine × genotype interaction ($F_{(1, 26)} = 3.6$, $p < 0.1$). Post hoc analyses revealed that there was no significant effect of genotype for mice treated with saline alone. The effect of nicotine withdrawal after the 40-mg kg^{-1} day^{-1} treatment compared with saline-treated mice, however, was significant only in the WT mice ($F_{(1, 10)} = 8.2$, $p < 0.05$) but not the KO mice ($F < 1$, ns). The lack of nicotine effect on the KO mice suggests that nicotine withdrawal-induced impaired attention/vigilance requires the α7 nAChR.

Thirty-three days after pump implantation, there were neither main effect of nicotine nor genotype but there was a significant nicotine × genotype interaction on p[HR] ($F_{(2, 39)} = 4.1$, $p < 0.05$, Fig. 3b). Post hoc analyses revealed that there was a main effect of nicotine on p[HR] in WT mice ($F_{(2, 16)} = 6.1$, $p = 0.01$)—whereby mice receiving the low dose of nicotine exhibited higher p[HR] than mice receiving saline ($F_{(1, 10)} = 4.0$, $p < 0.1$) and the high dose of nicotine ($F_{(1, 12)} = 18.0$, $p < 0.001$)—but that this effect was absent in KO mice ($F < 2$, ns). Furthermore, the genotypic difference between mice was observed whereby low-dose nicotine-treated WT mice exhibited higher p[HR] compared with KO mice

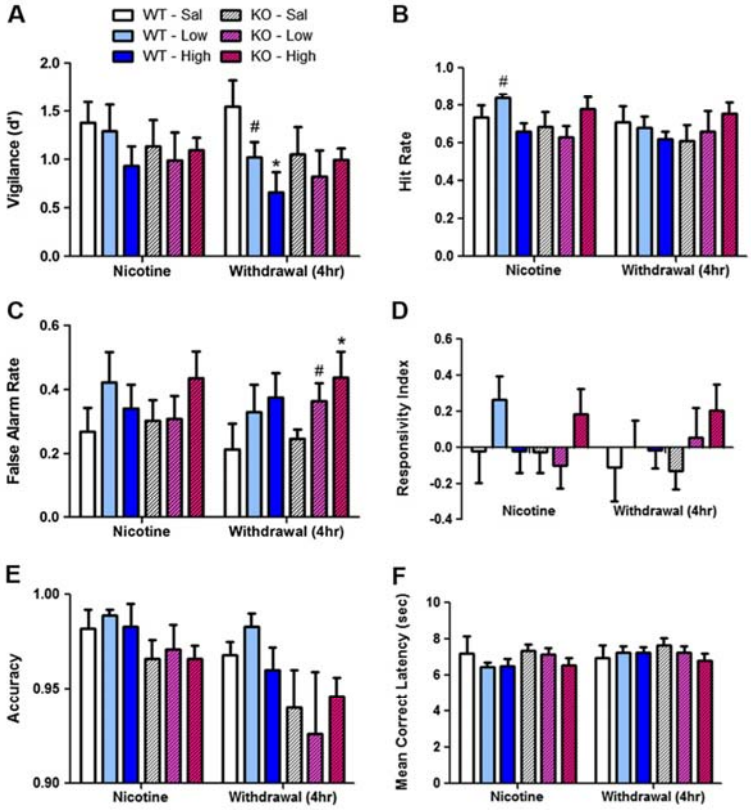

Fig. 3 Effects of chronic nicotine ("low" 14 or "high" 40 mg kg^{-1} day^{-1} vs. saline) administration and withdrawal on performance of WT and α7 nAChR KO mice in the 5C-CPT. Thirty-three days after nicotine minipump implantation (*nicotine*), there were no main effects of nicotine treatment or genotype on d' (**a**), p[HR] (**b**), p[FA] (**c**), RI (**d**), and MCL (**f**). There was a significant nicotine × genotype interaction on p[HR] (**b**), since WT but not KO mice receiving the low dose of nicotine exhibited elevated p[HR]. Four hours after pump removal (*withdrawal*), there were no main effects of nicotine treatment or genotype nor nicotine × genotype interactions on p[HR], RI, or MCL. After pump removal, there was a main effect of withdrawal from nicotine treatment on p[FA] (**c**), and there was a trend toward an effect of nicotine withdrawal on d' (**a**) whereby the effect of nicotine withdrawal to decrease d' was significant for WT but not KO mice. During and after nicotine treatment, there was a main effect of genotype on accuracy (**d**) with KO mice exhibiting lower accuracy than WT littermates. Data are shown as mean ± SEM, $^{\#}p < 0.1$, $^{*}p < 0.05$ when compared with saline

($F_{(1,\ 13)} = 11.0$, $p < 0.01$). This genotype-specific effect suggests that improvement in attention due to chronic nicotine at a low dose relies on α7 nAChRs. Four hours after pump removal, there were neither main effects of nicotine or genotype nor nicotine × genotype interactions on p[HR]. An analysis of the high dose revealed no main effects but a slight modest nicotine × genotype interaction ($F_{(1,\ 26)} = 2.5$, $p < 0.1$) since nicotine withdrawal was associated with a statistically insignificant reduction in p[HR] in WT mice but an increase in p[HR] for KO mice.

No significant effects of nicotine, genotype, or their interactions on p[FA] were observed during nicotine treatment. Four hours after pump removal however, there was a main effect of nicotine ($F_{(1,\ 39)} = 3.6$, $p < 0.05$, Fig. 3c). Post hoc analyses revealed that both the low dose ($F_{(1,\ 25)} = 4.2$, $p = 0.05$) and high dose of nicotine ($F_{(1,\ 26)} = 7.3$, $p < 0.02$) resulted in significant nicotine withdrawal-induced increases in p[FA]. There was no main effect of gene or nicotine × genotype interaction. Hence, irrespective of genotype, withdrawal from chronic nicotine resulted in response disinhibition.

No significant effects of nicotine, genotype, or their interactions on RI were observed during nicotine treatment or after pump removal ($F < 1$, ns, Fig. 3d). On Day 33 after pump implantation, there was potentially a modest nicotine × genotype interaction ($F_{(2,}$

$_{39)} = 2.9$, $p < 0.1$). No post hoc analyses revealed any significant effects however. No significant effects of nicotine, genotype, or their interactions on MCL were observed during nicotine treatment or after pump removal (Fig. 3f).

There was a significant main effect of genotype on accuracy at baseline ($F_{(1, 39)} = 6.6$, $p < 0.02$), during nicotine treatment ($F_{(1, 39)} = 5.0$, $p < 0.05$), and 4 h after nicotine withdrawal ($F_{(1, 39)} = 4.9$, $p < 0.05$) (Fig. 3e). This main effect of genotype was consistent across these three time periods with KO mice exhibiting lower accuracy than their WT littermates throughout testing. There was no main effect of nicotine or nicotine × genotype interaction on accuracy.

There was no main effect of genotype or nicotine on any of the additional secondary outcome measures (total trials, percentage of omissions, and percentage of premature responses), shown in Table 2. There was a significant nicotine × genotype interaction on the percentage of omitted trials 33 days after pump implantation ($F_{(2, 39)} = 4.1$, $p < 0.05$)—whereby the low dose of nicotine resulted in fewer omitted trials compared with saline ($F_{(1, 10)} = 3.3$, $p < 0.1$) and the high nicotine dose ($F_{(1, 12)} = 17.6$. $p = 0.001$) for the WT mice (overall: $F_{(2, 16)} = 6.1$. $p < 0.02$)—but there was no effect of dose on omissions for the KO mice ($F < 2$, ns). This reduction in omitted trials after chronic nicotine administration, like the effect on p[HR], may be reliant on the presence of α7 nAChRs.

Cotinine assessment

Urine cotinine levels were assessed after 26 days of saline or nicotine (40 mg kg^{-1} day^{-1}) treatment in C57BL/6 mice. Urine cotinine was detected (464.9 ± 73.9 ng/ml) for mice treated with 40 mg kg^{-1} day^{-1} nicotine but not for those that received saline (0 ± 37.0 ng/ml).

Biochemical analysis

Synaptosomal western blot analyses revealed a main effect of nicotine on mGluR1 receptors during nicotine administration and 4 h after withdrawal ($F_{(1, 8)} = 9.0$, $p < 0.02$; Table 3). There was no main effect of time point ($F < 2$, ns) or nicotine × time point interaction ($F < 1$, ns). These findings suggest that nicotine treatment alters mGluR1 and does not normalize within the first 4 h of withdrawal. There were no main effects of nicotine ($F < 1$, ns) or time point ($F_{(1, 8)} < 1$, ns) on mGluR5 expression. There was, however, a slight a nicotine × time point interaction ($F_{(1, 8)} = 3.9$, $p < 0.1$; Table 3) whereby nicotine-treated mice had reduced mGluR5 expression during nicotine administration but not during withdrawal. Similarly, there were no main effects of nicotine ($F < 1$, ns) or time point ($F < 2$, ns) on dopamine D_4 receptor expression ($F < 2$, ns), but the nicotine × time point interaction was significant ($F_{(1, 8)} = 7.3$, $p = 0.03$; Table 3), again due to reduced D_4 receptor expression during nicotine administration but not during withdrawal. Post hoc analyses revealed no significant pair-wise differences in expression of mGluR5 and D_4 receptors (split by time point or by treatment group), likely due to low sample size.

Discussion

Withdrawal from nicotine impaired attention/vigilance in mice when measured in a continuous performance test (CPT). Vigilance (d') can be impaired due to increased misses to targets (inattention) and/or increased responding to non-targets (response disinhibition). The nicotine withdrawal-induced deficit was driven by both. Experiment 1 demonstrated that, consistent with findings in humans, these deficits gradually disappear over time, with deficits in nicotine withdrawal mice observed after 4 but not 52 h after withdrawal. WT mice in experiment 2 were similarly affected 4 h after nicotine withdrawal. In contrast, mice lacking α7 nAChRs did not exhibit the same nicotine withdrawal-induced deficits due to modest elevations in both target and non-target responding in these mice. Hence, nicotine withdrawal-induced response disinhibition occurred irrespective of the presence of α7 nAChRs, in contrast with withdrawal-induced inattention. Therefore, different mechanisms may underlie withdrawal-induced cognitive deficits in inattention vs. response disinhibition.

Importantly, nicotine withdrawal exerted consistent effects across both studies. Both a reduced hit rate and elevated false alarm rate were observed and although not necessarily significant alone, when combined (a common practice in human CPT testing), significant deficits in performance were observed (Figs. 2a and 3a). This profile of inattention and response disinhibition is consistent with human studies demonstrating delayed reaction times to target stimuli and increased false alarms in CPTs during nicotine abstinence (Harrison et al. 2009; Powell et al. 2010), as well as impaired response inhibition in a stop-signal task, go/no-go tasks, and the Stroop task (Harrison et al. 2009; Kozink et al. 2010; Ashare and Hawk 2012). Also consistent with human testing is the apparent attenuation of withdrawal-induced performance deficits of mice over time. Figure 1a demonstrates that the greatest deficit during nicotine withdrawal was 4 h after pump removal. Mice undergoing nicotine withdrawal gradually improved in performance over time to the point where there was no difference from their saline-treated controls 52 h later. This gradual improvement was mirrored in the reduction in false alarm rates over time. Hence, we have demonstrated that the use of the CPT in mice during withdrawal produces results that are consistent with human withdrawal/CPT studies and can be useful in the study and development of treatments of withdrawal-induced cognitive deficits.

In addition to nicotine withdrawal effects, experiment 1 demonstrated that on the second day of nicotine treatment d' was greater for mice receiving nicotine compared with those receiving saline. Considering d' alone, the 'better' performance of these

Table 2 Effects of chronic nicotine ("Nic" at 14 or 40 mg kg^{-1} day^{-1} vs. saline, "Sal") administration and withdrawal on secondary performance measures for WT and α7 nAChR KO mice in the 5C-CPT

	Pre-treatment						Nicotine (Day 33)						Withdrawal (4 h)					
	Mean ± SEM	Genotype		Treatment group		Genotype × group		Mean ± SEM	Genotype		Nicotine		Genotype × nicotine		Mean ± SEM	Genotype		Nicotine		Genotype × nicotine	
		$F_{(1, 39)}$	p	$F_{(2, 39)}$	p	$F_{(2, 39)}$	p		$F_{(1, 39)}$	p	$F_{(2, 39)}$	p	$F_{(2, 39)}$	p		$F_{(1, 39)}$	p	$F_{(2, 39)}$	p	$F_{(2, 39)}$	p
Total trials																					
WT-Sal	113.07 ± 3.44	<1	ns	<1	ns	2.2	ns	243.20 ± 2.01	<2	ns	<1	ns	<2	ns	237.80 ± 6.99	<1	ns	<1	ns	<2	ns
WT-Nic (14)	116.76 ± 1.71							245.00 ± 0.00							220.00 ± 17.86						
WT-Nic (40)	116.95 ± 1.69							227.00 ± 12.54							207.29 ± 19.48						
KO-Sal	117.70 ± 1.23							227.56 ± 11.68							219.00 ± 9.29						
KO-Nic (14)	108.50 ± 5.93							224.50 ± 12.64							222.13 ± 21.97						
KO-Nic (40)	113.74 ± 2.43							236.56 ± 4.72							239.00 ± 5.60						
% Omissions																					
WT-Sal	22.03 ± 4.59	<1	ns	<1	ns	<2	ns	26.22 ± 6.67	<1	ns	<1	ns	4.1	0.02	28.06 ± 8.09	<1	ns	<1	ns	<2	ns
WT-Nic (14)	15.25 ± 1.38							15.66 ± 1.73							31.43 ± 6.07						
WT-Nic (40)	20.41 ± 3.11							33.51 ± 4.26							37.03 ± 4.01						
KO-Sal	16.43 ± 1.79							30.50 ± 7.38							37.84 ± 8.53						
KO-Nic (14)	21.04 ± 4.69							36.44 ± 6.31							32.66 ± 10.73						
KO-Nic (40)	18.04 ± 4.18							21.56 ± 6.61							23.39 ± 6.01						
% Premature responses																					
WT-Sal	3.79 ± 0.81	<1	ns	<1	ns	<1	ns	4.13 ± 1.13	<1	ns	<1	ns	<1	ns	3.96 ± 1.71	2.0	ns	<1	ns	<1	ns
WT-Nic (14)	8.18 ± 3.66							3.50 ± 1.01							4.47 ± 1.83						
WT-Nic (40)	5.37 ± 1.92							4.79 ± 2.99							6.03 ± 3.35						
KO-Sal	5.79 ± 2.03							4.73 ± 1.85							8.04 ± 3.19						
KO-Nic (14)	6.18 ± 2.02							4.50 ± 1.98							6.45 ± 2.24						
KO-Nic (40)	6.25 ± 2.94							5.29 ± 1.97							12.31 ± 5.66						

There was no main effect of genotype or nicotine on total trials, % omissions, or % premature responses. There was a significant nicotine × genotype interaction on % omissions 33 days after pump implantation since the low dose of nicotine resulted in fewer omitted trials compared with saline and the high nicotine dose for the WT mice but not KO mice

Table 3 Effect of chronic nicotine (40 mg kg^{-1} day^{-1}) treatment on mGluR1, mGluR5, and dopamine D_4 receptor expression

Receptor	Saline		Nicotine		*N* per group	2-way ANOVA interaction $F_{(1, 8)}$	*p* value
	Pump on % Control ± SEM	Pump off % Control ± SEM	Pump on % Control ± SEM	Pump off % Control ± SEM			
mGluR1	100 ± 3.7	107. ± 2.3	117 ± 6.3	127 ± 9.8	3	0.05	0.82
mGluR5	100 ± 9.8	99 ± 8.0	79 ± 6.2	110 ± 8.5	3	3.9	0.08
D_4R	100 ± 6.2	88.5 ± 12	75 ± 2.0	101 ± 5.1	3	7.3	0.03

After 33 days of chronic nicotine treatment ("pump on") and 4 h after pump removal ("pump off"), a subset of WT mice were removed and levels of synaptosomal mGluR1, mGluR5, and D_4 receptor expression were analyzed. There was a significant effect of nicotine on mGluR1 expression during nicotine administration and during withdrawal. Significant interactions between synaptosomal expression during nicotine (pump on) and withdrawal (pump off) for mGluR5 (trend) and dopamine D_4 receptors were observed, though no significant post hoc differences between levels were observed

nicotine-treated mice could be driven not by improved performance on nicotine but by a poorer response of the saline-treated mice to pump implantation. This difference may be due to residual pain from recent surgery or exposure to isoflurane treatment that can differentially affect gene expression and networks (Lowes et al. 2016). The procognitive (Semenova et al. 2007; Wallace and Bertrand 2013) and antinociceptive (Han et al. 2005; Jackson et al. 2009) effects of nicotine in rodents have been well established and may have ameliorated the reduction in performance observed in the saline-treated animals. As described previously, however, vigilance (d') is a composite measure accounting for responses to targets (p[HR]), as well as inhibition during non-target trials (p[FA]). Although d' was increased, we found that both p[HR] and p[FA] were reduced in mice that received nicotine. Furthermore, RI was decreased and % omissions increased in these mice, indicative of reduced responding to both target and non-target stimuli. These effects may be due to physical rather than cognitive differences between groups. For example, it is possible that the acidity of the nicotine solution may have contributed to post-operative discomfort that may have affected performance. In any case, all differences in performance between saline- and nicotine-treated groups disappeared by day 27. Hence, after long-term exposure, no improvements in cognition were observed, consistent with a lack of improvement in chronic smokers vs. non-smokers. It is possible that repeated testing under the influence of nicotine may have altered the baseline performance of the mice, compromising the observed effects of withdrawal on performance. Our previous studies, however, observed remarkable stability in baseline performance after multiple tests with the administration of various drugs, including nicotine (Young et al. 2013b), hence the current design. Although we cannot definitively rule out the potential for continued influence of nicotine, it remains unlikely that repeated testing during this administration significantly affected these withdrawal results.

Though not significant across or within every time point during withdrawal, p[HR] was consistently lower and p[FA] consistently higher for mice receiving chronic nicotine treatment compared with mice receiving saline. Specifically at the 4-h time point, withdrawal-induced elevation in p[FA] for mice that received nicotine was statistically significant. The subtle effect of withdrawal on both measures may have been masked due to small sample size. Overall, this experiment illustrates that the 5C-CPT—which includes inhibition from responding to non-target trials—can be used to disassociate sustained attention and disinhibition measures in mice, enabling comparisons with similar measures in humans. Indeed, our finding that impaired attention during nicotine withdrawal in mice is consistent with similar findings in human studies (McClernon et al. 2015; van Enkhuizen and Young 2016).

Further relevance to human testing comes from observations that treatment with 40 mg kg^{-1} day^{-1} nicotine resulted in urine cotinine levels (~450 ng/ml) comparable with the lower range of urine cotinine levels for human smokers, where <10 cigarettes/day = 646 ng/ml, while >20 cigarettes/day = 1100 ng/ml (Wall et al. 1988; Haufroid and Lison 1998; Parker et al. 2002). Support for using higher doses of nicotine for 28 days comes from studies using lower chronic nicotine levels (14 days at 6.3 mg kg^{-1} day^{-1}) which produce plasma cotinine levels of 60–180 ng/ml (Portugal et al. 2012), that are low relative to 260–300 ng/ml in human smokers (Benowitz et al. 1983; Shoaib and Stolerman 1999). Hence, the current technique for chronic nicotine treatment with osmotic minipumps resulted in cotinine levels comparable with those seen in human smokers.

We also examined the effects of nicotine withdrawal on 5C-CPT performance in α7 nAChR KO mice and their WT littermates. Consistent with our initial findings, 4 h after pump removal, d' was lower for nicotine-treated mice than saline-treated mice. More specifically, d' was significantly lower for mice that received the high dose of nicotine (40 mg kg^{-1} day^{-1}) compared with mice that received saline providing reproducible evidence for an effect of nicotine withdrawal on cognitive performance. Importantly here however, there was also a modest nicotine treatment × genotype interaction whereby there was a significant effect of nicotine withdrawal on d' in WT mice but not KO mice. The lack of nicotine treatment effect in the KO mice suggests that α7 nAChRs are required for nicotine withdrawal-induced inattention, which may contribute

to the difficulty of quitting smoking in humans. Further analyses revealed that, consistent with experiment 1 and human studies, the probability of false alarms (p[FA]) is significantly affected by nicotine withdrawal—with elevated p[FA] for mice that received nicotine vs. saline. As noted above, d' incorporates both p[FA] and p[HR]. Given that withdrawal affects p[FA] in both WT and KO mice, the difference in d' may stem from differential effects of withdrawal on p[HR] in these genotypes. Indeed, there was a modest nicotine × genotype interaction on p[HR] whereby the WT mice that received the high dose of nicotine exhibited reduced p[HR] 4 h after nicotine withdrawal while the KO mice that received nicotine exhibited a slight increase in p[HR]. Although the effects were not statistically significant, even modest effects on p[HR] in combination with changes in p[FA] can profoundly affect overall attentional performance. Lack of significance may be due to the removal of WT mice to assess potential alterations in receptor expression that result from chronic nicotine and its withdrawal, described below. Thus, both inattention and response disinhibition should be considered for remediation to improve cognition during withdrawal.

Our findings support the hypothesis that an α7 nAChR antagonist during nicotine withdrawal may help alleviate the effects of withdrawal on sustained attention. Certainly, α7 nAChR antagonist treatments have improved attention and cognitive performance (Hahn et al. 2011; Burke et al. 2014). Of course, consistent with previous studies, the KO mice exhibited an overall impairment in attention throughout the experiment, as measured by accuracy, as well as slower training (Young et al. 2004; Keller et al. 2005; Hoyle et al. 2006), perhaps driven by reward-related learning deficits (Young et al. 2011a). Therefore, KO mice may have altered baseline behavior that could confound the interpretation of these data. Furthermore, since the null mutation of α7 nAChRs in these mice is constitutive, whether or not the temporary disruption of α7 nAChRs via conditional KO or pharmacological antagonism would also affect accuracy remains to be tested. Nonetheless, the consistency of withdrawal effects in WT mice in both experiments and comparable effects of withdrawal on p[FA] in both WT and KO mice suggest that the differential effects on p[HR] warrant further investigation.

Previous studies have shown that subchronic nicotine treatment improves attention by increasing target responding (Young et al. 2013b). Consistent with these findings, we observed an improvement in p[HR] and reduced omissions due to chronic low—but not high—dose nicotine administration in WT mice. Importantly, we observed that the nicotine-related improvements were observed in WT mice only and not KO mice. This genotype-specific effect further suggests that the cognition-enhancing properties of low doses of nicotine treatment may rely on α7 nAChRs. Combined, these data highlight the fact that although acute nicotine treatment improves attention by increasing target responding, withdrawal from nicotine impairs performance primarily by increasing response disinhibition and reducing accuracy, as observed in the 5CSRTT. Since nicotine withdrawal-induced disinhibition does not seem to rely on α7 nAChRs, another target must be identified for the remediation of this cognitive deficit.

Synaptosomal western blots revealed that mGluR5 and D_4 receptor levels were decreased in mice that received chronic nicotine treatment compared with those that received saline. Four hours after nicotine pump removal, however, these expression levels were elevated for mice that received nicotine. In contrast, levels of mGluR1 were elevated in mice that received nicotine during administration, as well as during withdrawal. The elevated levels of mGluR5 and D_4 receptor after nicotine withdrawal may be overcompensation during the recovery from lack of nicotine. Of course, these are not the only changes associated with withdrawal, and we do not know if the α7 nAChR KO mice would exhibit the same changes in receptor expression. Hence, it is unclear if either of these changes would be suitable targets to treat withdrawal-induced disinhibition. Given that mice with reduced dopamine D_4 receptor expression exhibit response disinhibition (Young et al. 2011b), however, agonists at this receptor may remediate response disinhibition that occurs during withdrawal. In fact, D_4 receptor agonist treatment improved response inhibition in rats with high p[FA] during 5C-CPT testing (Tomlinson et al. 2015).

Since the success of quitting attempts can be increased by two- or threefold with the aid of counseling and medications, it is imperative to better understand and develop more accessible treatments to aid smoking cessation (Fiore et al. 2008; Polosa and Benowitz 2011; Ashare and Schmidt 2014). Our studies suggest that a combination of therapeutic interventions—to target the α7 nAChR-dependent changes in target detection (p[HR]) and possibly dopamine D_4 receptors in response inhibition (p[FA])—may be necessary to address multiple impairments to attention in people experiencing nicotine withdrawal.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Appendix B, in full, is a reprint of the material as it appears in Higa KK, Grim A, Kamenski ME, van Enkhuizen J, Zhou X, Li K, Naviaux JC, Wang L, Naviaux RK, Geyer MA, Markou A, Young JW. *Nicotine withdrawal-induced inattention is absent in α7 nAChR knockout mice.* Psychopharmacology (Berl). 2017 Feb 28;234:1573–1586; © Springer-Verlag Berlin Heidelberg 2017, with kind permission of Springer. The dissertation author was the primary investigator and author of this paper.