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DISC1 knockout rats reveal sexually dimorphic patterns of impairment across development

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CONTENTS

Abstract

Schizophrenia is a chronic disorder characterized by three symptom categories: positive (hallucinations, delusions), negative (anhedonia, anxiety), and cognitive (sensory processing and memory deficits). We worked with a genetic model using a biallelic deletion of the Disrupted-in-schizophrenia-1 (DISC1) gene in Sprague-Dawley rats to facilitate our understanding of the biological bases of schizophrenia. Mutations of DISC1 are associated with a higher prevalence of mental illness, especially schizophrenia. Adult DISC1 knockout, compared to wildtype, rats consistently display features of schizophrenia-like outcomes in rodent models: hyperactivity, anxiety, and impaired memory. The present study investigated the progression of the cognitive symptom deficits in prepulse inhibition (PPI) using a longitudinal design, assessing animals at postnatal days 17 (pre-weaning), 26 (post-weaning), 39 (adolescent), and 67 (adult). Cohorts of untested rats were sacrificed at each time to investigate the loss of GABAergic, parvalbumin-containing interneurons which occurs in schizophrenia. Also under examination was the extent to which the emergence and severity of these impairments were different between males and females; schizophrenia onset and symptoms can be sexually dimorphic.The findings were that males DISC1 knockout rats displayed enhanced PPI at the pre-weaning timepoint and impaired at the adult timepoint. Females exhibited little evidence of PPI deficits. PPI is sensitive to females' hormonal cycles and this may have contributed to our findings, thus we also performed a two-hit model with adult females finding that an acute dose of amphetamine could induce deficits in knockout, but not wildtype rats. Additionally, in a cohort of adult animals, male DISC1 knockouts revealed deficits in dendritic complexity while females did not. These findings highlight the importance of including females in the study of schizophrenia.

Introduction

Schizophrenia is a chronic brain disorder that affects about 1% of the world's population cross-culturally, regardless of geographical location (NIMH, n.d.). The universality of the disease makes it difficult to study genetically or environmentally, as there is much variation in causality and symptoms throughout populations, and even between men and women. The symptoms of schizophrenia fit into three categories: positive, negative, and cognitive (American Psychological Association, 2013). Patients may display any combination of these symptoms, and at differing severities. Positive symptoms, such as hallucinations, delusions, or disorganized thought, occur as an addition of behaviors. Negative symptoms, or absences of behavior, include social withdrawal, emotional flattening, and anhedonia. The third category of cognitive symptoms is less studied and poorly understood, and includes deficits in memory, attention, and decision making. This study focused on cognitive symptoms of schizophrenia, and, using a genetic rat model, examined the developmental trajectory of the disorder as a function of biological sex.

The etiology of schizophrenia is just as varied as its symptoms, and the cause, or causes, remain elusive. However, there is evidence of several early life factors that may contribute to the development of schizophrenia. One factor is immunological, where illness during pregnancy may increase risk for development of the disorder. The reason for this is not fully understood, but some hypotheses suggest that viral infections or symptoms such as fever may directly alter the fetus' developing brain, elevated cytokine levels may mediate changes in brain development, or medications taken to treat illness may alter development (Sham et al., 1992; Boska, 2008). Another factor is Vitamin D deficiency during pregnancy, which may lead to an increased risk of schizophrenia in the offspring, though the mechanism for this has not been fully explored

(McGrath, 1999; McGrath et al., 2003). A third major factor is genetics, as research has also identified and studied several genes that place people at risk for developing schizophrenia. For example, mutation in the C4 gene may cause over-pruning of synapses during development, which later contributes to pathology and altered levels of neurotransmission (Sekar et al., 2016). Other examples of candidate risk genes for schizophrenia include COMT, DTNBP1, NRG1, and DISC1, which is the gene used in the this study's model of schizophrenia (Farrell et al., 2015). However, these risk genes are not guarantees that schizophrenia will develop. Early life impacts or predispositions, such as those which occur during pregnancy or are inherited, can act as an initial "hit" or predisposition, which, depending on severity, may or may not cause symptoms. The two-hit hypothesis of schizophrenia posits that this initial hit may create a vulnerability which requires a second, later life hit to cause pathology (Maynard et al., 2001). This second hit could be anything from a traumatic event to drug use. The two-hit hypothesis provides many targets for investigation in models of schizophrenia, as the interactions between risk factors can become considerably varied and complex.

These risk factors may cause the occurrence of schizophrenia in any number of combinations, thus resulting in a wide variety of biological symptoms. Neurochemically, schizophrenia involves elevated levels of dopamine in the ventral tegmental area, through the mesocortical pathway (Laruelle et al., 1999). However, more recent research suggests a more complicated story, as abnormalities seen in the dopaminergic system may be a result of increases in prefrontal cortex glutamate levels, which in turn decrease GABA transmission, ultimately increasing dopamine activity in the ventral tegmental area (Marsman et al., 2014; Volk et al., 2010; Coyle et al., 2004). Another biological hallmark of schizophrenia is decreased parvalbumin in GABAergic interneurons, which are important for neural inhibition, both in the

hippocampus and throughout the cortex (Nakazawa et al., 2012). Experimental parvalbumin deficiencies in mice caused deficits in prepulse inhibition behavior, similar to those seen in schizophrenia (Popelář et al., 2013). Other hypotheses also incorporate serotonin, and many combinations of all the suspect neurotransmitters (Silver et al., 2009). Another biological symptom is structural; deficits in neural proliferation and alterations of dendritic morphology during development also occur in schizophrenia (Feron et al., 1999; Arnold, 1999; Reif et al., 2006). These deficits are likely involved in cognitive symptoms, as neurogenesis and the hippocampus are highly linked to learning and memory (Gonçalves, Scahfer, & Gage, 2016). In sum, the symptoms of schizophrenia are an excellent example of the interconnectedness of neural networks, and how problems in one area can have widespread effects. This biological complexity can create challenges in the study of schizophrenia, as it is difficult to fully understand how small changes cause such broad impact.

In addition to the many questions that remain about the causes and biology of schizophrenia, there is also mounting evidence that these are importantly impacted by biological sex. Research on schizophrenia often overlooks sex as a biological factor, and frequently studies are conducted solely in males. Schizophrenia is more common in males, as well as having more severe and different kinds of symptoms in general (McGrath, 2006). Positive symptoms tend to be most severe in females, while cognitive symptoms tend to be most prevalent in males, although the validity of these distinctions is far from well-established (Ring et al., 1991; Leung & Chue, 2000). Even the progression of the disorder is sexually dimorphic, with male age of onset slightly earlier in adolescence (Leung & Chue, 2000). Females with schizophrenia may also have a second onset of symptoms at menopause, suggesting a modulatory role for estradiol (Holder & Wayhs, 2014; Leung & Chue, 2000). Estradiol may be neuroprotective, changing the

incidence, severity, and kinds of symptoms which females experience, even across the menstrual cycle (Riecher-Rossler et al., 1994). Thus, though both males and females may have the same genetic risk factors, they may exhibit different presentations of symptoms, which was a key focus of the present study.

The symptom of schizophrenia we chose to focus on for this study was deficits in prepulse inhibition (PPI). PPI is a sensorimotor gating phenomenon, by which temporarily related stimuli are associated to modify subsequent behavior. Impairment in prepulse inhibition is a common cognitive symptom, as deficits in ordering and associating information occur frequently in schizophrenia (Koch, 1998; Swerdlow, Hartman & Auerbach, 1997). However, despite its prevalence, PPI impairment is relatively understudied and not a commonly known symptom. There is some indication that PPI may be related to other symptoms in different categories, including auditory hallucinations, though this currently remains speculative (Smith et al., 2013; Thoma et al., 2017). Studies do not often consider sex when studying PPI in schizophrenia; however, there is some evidence in humans that females with schizophrenia do not always display this symptom. One study found that males show deficits in PPI but females do not in a small population of people with schizophrenia, potentially due to a variation of PPI across the menstrual cycle (Kumari, Aasen, & Sharma, 2004). Chemically, PPI behavior relies on the dopaminergic system, which is abnormal in schizophrenia. Some studies demonstrate that artificially modelling schizophrenia through excess dopamine causes PPI deficits (Auclair et al., 2006). These deficits can also be counteracted by both typical and atypical antipsychotic treatment, suggesting that deficits in PPI may be a result of the core causes of schizophrenia (Auclair et al., 2006; Hoffman & Donovan, 1994). The specific brain areas associated with both dopamine and PPI include the nucleus accumbens and the medial prefrontal cortex (mPFC)

(Swerdlow, Geyer, & Braff, 2001). The hippocampus, commonly known for its involvement in the cognitive functions of learning and memory is also heavily involved in PPI (Freedman et al., 1996). Additionally, mutations in the alpha-7 nicotinic acetylcholine receptor, which are prevalent in the hippocampus, are linked to a higher risk of developing schizophrenia (Xu et al., 2001), while treatment with nicotine can relieve PPI deficits (Hong et al., 2008). Some studies associate normalized PPI in patients with schizophrenia who smoke, subsequently inferring that patients self-medicated cognitive symptoms with nicotine as an acetylcholine agonist (Rabin, Sacco, & George et al., 2009; Song et al. 2014). Therefore, PPI as a cognitive symptom of schizophrenia can be tied directly to the pathology in several brain areas.

Animal models of schizophrenia are an excellent way of learning about causality and the various symptoms trajectories, such as PPI. Current rodent models in particular are extremely varied, and include genetic, behavioral, and drug manipulations. Dopamine agonists, such as amphetamine, can artificially raise dopamine levels to replicate the biological state of a brain with schizophrenia and thus symptoms (Ham et al., 2017). The NMDA receptor antagonist MK-801 is also used in animal models, artificially creating lowered glutamatergic activity which leads to downstream dopamine release (Young, Zhou, & Geyer, 2010). Knockout models, by contrast, allow for genetic replication of specific human conditions, and can be completed for any number of schizophrenia risk genes, such as DISC1. Genetic manipulations can also alter specific proteins related to schizophrenia pathology, such as alpha-7 nicotinic acetylcholine receptors (Gass et al., 2016). Researchers examine these models, looking for symptoms which match to the human experience of schizophrenia, such as memory and learning deficits, hyperactivity, anxiety, altered social behavior, and deficits in prepulse inhibition (Kilts, 2001; Crawley & Paylor, 1997). PPI is uniquely versatile, as it can be studied and analyzed almost

identically in humans and rodents, with very similar biological underpinnings (Swerdlow et al., 1994).

This study utilizes one key candidate risk gene for its model, DISC1, or Disrupted-in-schizophrenia-1, which is a plasticity gene whose mutation has been associated with schizophrenia and bipolar disorder. The gene's importance was originally discovered in a family in Scotland with a high incidence of schizophrenia, where pathology was associated with a balanced translocation between chromosomes 1 and 11 (t(1;11)(q42;q14)) (Blackwood et al., 2001; Johnstone et al., 2011). Not all of DISC1's impacts on brain function are fully understood, however, we do know that it encodes a multifunctional protein that influences neuronal development and adult brain function, including neurite architecture, neuronal migration, intracellular transport, and synaptic transmission (Johnstone et al., 2011; Wu et al., 2013). On the cellular level, the DISC1 protein is involved in many processes, including neurite outgrowth, mitochondrial development, and cytoskeletal operation (Miyoshi et al. 2003; Millar et al. 2005). There is some evidence that the N-terminus head of the protein is important for the mitochondria, while the C-terminal domain has other varied functions (James et al., 2004; Millar et al. 2005).

Mutations in DISC1 may contribute to the pathology of schizophrenia by disturbing normal neural development and proliferation. DISC1 is most highly expressed in the hippocampus of the adult brain, with specific localization to the dentate gyrus (Lee et al., 2015; Austin et al., 2004; Meyer & Morris, 2008). The hippocampus is highly plastic, and the dentate gyrus is one of the few brain areas that gives rise to new neurons, so it is likely that DISC1 plays a role in plasticity and its associated behavioral correlates, such as learning and memory. Therefore, DISC1 likely plays an important role in biological processes that support healthy cognitive function during development. Greenhill et al. (2016) found that a DISC1 mutant mouse

model displayed a lack of long-term potentiation, which is important for adult plasticity and cognition. The authors posit that the retained presence of long-term depression until the beginning of adulthood as a form of plasticity accounts for the delayed symptoms in schizophrenia. These effects could not be reversed through a reintroduction of the protein, indicating that DISC1 is involved in irreversible developmental changes. Additionally, DISC1 mutant mouse models have displayed a decrease in parvalbumin neurons in both the mPFC and the hippocampus, suggesting a role for DISC1 in GABAergic development, and mediating inhibitory transmission (Hikida et al., 2007; Wei et al., 2015). Thus, DISC1 fits squarely into the known biology of schizophrenia, regarding alterations in proliferation and dendritic complexity connecting to DISC1's cytoskeletal role, and changes in GABAergic development ultimately leading to the hallmark excess dopamine.

The present study examines the sex differences in cognitive and biological pathology in a DISC1 knockout (DISC1 KO) model in Sprague Dawley rats. The specific knockout is a result of a CRISPR-Cas9 20 base deletion in the DISC1 gene on chromosome 19, causing an early stop codon which causes termination at exon 6 out of 14. Rats were studied longitudinally for PPI to determine cognitive trajectory over development. Tracking symptoms repeatedly over time is particularly important for gathering information about age of onset, the consistency of symptoms, and the severity of symptoms, while considering sex differences in each of these factors. Additional untested groups of rats were sacrificed at each timepoint to examine parvalbumin positive GABAergic interneurons as a function of the DISC1 knockout independent of behavioral testing effects. To further explore the nuances of this model, additional experiments were conducted in separate groups of adult animals to examine dendritic morphology and the two-hit hypothesis in females.

1. Experiment 1: Developmental Trajectory of PPI and Parvalbumin

This experiment was used to track PPI across development in DISC1 KO and wildtype (WT), male and female rats. Rats were assessed at four timepoints: PD 17 (pre-weaning), PD 26 (post-weaning), PD 39 (adolescence), and PD 67 (adulthood) (Figure 1). Brain pathology was also examined by sacrificing age matched rats at each of the four original timepoints to examine GABAergic interneurons through parvalbumin.

1.1 Experiment 1: Methods

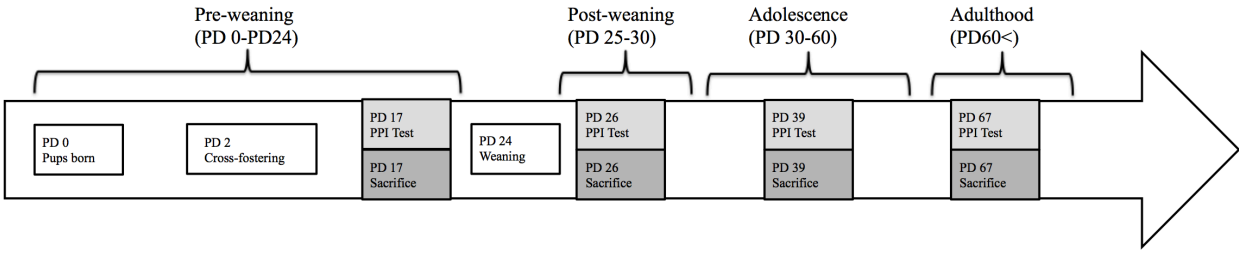

Fig. 1. A timeline of Experiment 1 events. Light gray indicates PPI testing in the same group of animals at pre-weaning, post-weaning, adolescence, and adulthood. Darker gray indicates sacrifice of separate groups of rats at each of the four timepoints for immunohistochemistry analysis.

Colony Conditions

All rats were housed in individually ventilated, clear polycarbonate cages (30.5 x 30.5 x 18.5 cm, Thoren Caging Systems, Inc., Hazleton, PA) lined with corncob bedding. During pregnancy, dams were housed individually. Except for dams, all rats were housed in same-sex cage pairs. The colony room was maintained at 20-24°C with 15-45% humidity and a 12-hour light/12-hour dark cycle with lights on at 8:00 am daily. All rats had ad libitum access to food

(commercialized rat chow, Harlan Teklad, Madison, WI) and water. Body weights were taken once a week. All testing procedures were approved by the Colby College Institutional Animal Care and Use Committee and performed in accordance with federal standards. All testing was conducted with the principal investigator blind to genotype conditions.

Subjects

Sprague Dawley rats were bred and born on site. These rats were the seventh generation of offspring from original breeding pairs obtained from SAGE® Labs (TGRA6370, Boyertown, PA). After all dams gave birth, on PD 2, pups were sexed, toe-clipped to denote their genetic background, and cross-fostered among all dams so that each litter contained a mix of female and male wildtype and knockout rats. On PD 24, all rat pups were weaned into same-sex cage pairs; 44 males and 44 females were used in this experiment.

Behavioral Testing: Prepulse Inhibition Assessment

Prepulse inhibition procedures were performed with 12 WT males, 12 DISC1 KO males, 13 WT females, and 11 DISC1 KO females. Each rat was tested at four timepoints, PD 17, PD 26, PD 39, and PD 67. Procedures were performed using an automated SRLab startle system (San Diego Instruments, San Diego, CA, USA). We used two sound-attenuated acoustic startle chambers, with dimensions of 28 x 28 x 28 cm. Each chamber contained a Plexiglass tube (10cm diameter x 20 cm length) that was closed on both ends. The cylinder sat on top of an accelerometer that measured vertical displacement. Acoustic stimuli and white noise were generated by the SRLab software running on an adjacent PC. Data were recorded on the adjacent computer using the Acoustic Startle software (Startle, SDI).

Habituation. Rats were placed in the cylinder in the chamber with the lights and fan on but without any acoustic stimuli for 15 minutes.

Assessment. The day following habituation, rats underwent assessment of their response to acoustic stimuli. To do this, ten acoustic startle trials at 120 dB for 40 ms each and 3 trials of each prepulse, including 75 dB, 80 dB, and 85 dB, for 20 ms each, were randomly presented, with the entire session lasting 13 minutes. No stimuli were presented as prepulses.

Testing. The testing session occurred the day following assessment. To examine PPI in the rats, there were 10 recordings without an acoustic stimulus for 40 ms each, 10 acoustic startle trials at 120 dB for 40 ms each, and 10 trials of each prepulse, including 75 dB, 80 dB, and 85 dB, lasting 20 ms, presented 100 ms before the acoustic startle at 120 dB, lasting 40 ms. Trials were randomized with an inter-trial interval of 10-30 seconds, with the entire procedure lasting 21 minutes. Percent PPI was calculated for each animal using the following equation:

%PPI= (Acoustic Startle Response-PPI) /Acoustic Startle Response x 100.

Preparation of Tissue for Immunohistochemistry

At each timepoint (PD 17, PD 26, PD 39, and PD 67) 10 female and 10 male rats (n=5 for WT and DISC1 KO), were sacrificed. Rats were deeply anesthetized with isoflurane in 1.5% O_2 and decapitated. The brains were extracted and hemisected: one hemisphere was frozen by holding the tissue in methylpentanol over dry ice for 10 seconds and then stored at -70^{o} C and the other was post-fixed in 4% paraformaldehyde in 1M phosphate buffer (PB) at 4°C and then cryoprotected in 30% sucrose in paraformaldehyde at 4°C and left for 2 days before cryostat sectioning. During sectioning, the cryostat was kept at -21°C and the stage was kept at -17°C.

Series of consecutive 40 μm coronal sections were taken from the prefrontal cortex through the hippocampus and stored in 0.1% sodium azide at 4°C until staining was performed.

Parvalbumin Immunohistochemistry

Parvalbumin immunohistochemistry was used to identify GABAergic interneurons in the prefrontal cortex and hippocampus. Ten sections of the prefrontal cortex and ten hippocampal sections were selected at random for staining. The free-floating sections were rinsed in phosphate-buffered saline (PBS), incubated in a 10% normal horse serum (NHS; Vector Laboratories) and 0.2% triton-X-100 in PBS solution (PBST) for 1 hour at room temperature, and then incubated with the primary parvalbumin antibody in rabbit (1:2,500; Thermo Fisher Scientific), 2% NHS (Vector Laboratories), and 0.2% PBST. The sections remained in this primary antibody solution overnight on a shaker kept at 4°C. The following day, the tissue was rinsed with PBS and then incubated with the secondary anti-rabbit made in horse (1:200; Vector Laboratories), 3% NHS, and 0.2% PBST for one hour at room temperature. After the incubation, the sections were rinsed in PBS and transferred to an avidin-biotin complex (ABC; Vector Laboratories) for one hour at room temperature. The tissue was rinsed in PBS and Vector ImmPact SG solution (Vector Laboratories) was used to visualize the stained neurons. Tissue was then rinsed sequentially in PBS, dH2O, and finally stored in PBS at 4^{o}C until mounting. Tissue was then mounted on 1% gelatin-coated slides, cleared in xylenes, and coverslipped.

Quantification of Parvalbumin Positive Neurons

For each brain, five coronal sections of the prefrontal cortex (Bregma 3.2-2.7 mm) were selected for analysis. Five sections of the hippocampus (Bregma -3.30- -4.20 mm) were also

selected. Images were taken at 40X magnification and analyzed using the ImageJ computer program to quantify the number of punctate, parvalbumin-stained cells (see Additional Figures for representative images). A 500 pixel square box was created around the infralimbic region of the prefrontal cortex. A freehand line was drawn around the dentate gyrus in the dorsal hippocampus, as this area is most associated with learning, memory, and other cognitive processes (Kheirbek & Hen, 2011). Image threshold was adjusted to maximize cells labeled while minimizing background and a particle analysis was conducted within the enclosed shape. After analyzing the labeled particles, the number of points (cells) was recorded for each.

Statistical Analyses

Means and standard errors were calculated and displayed in figures for all behavioral and histological results including percent PPI and number of parvalbumin positive cells. 2x4 mixed ANOVAs (one each for males and females) were conducted to analyze percent PPI, with the between subjects factor of Genotype (WT, DISC1 KO) and the within subjects factor of Postnatal Day (PD 17, PD 26, PD 39, PD 67). 2x4 between subjects ANOVAs (one each for male and female prefrontal cortex and hippocampus) were conducted to analyze parvalbumin data, with the between subjects factors of Genotype and Postnatal Day. For all analyses, planned one-tailed t-tests were conducted separately for males and females at different timepoints, under the hypothesis that the genetic manipulation would reduce percent PPI and number of parvalbumin positive neurons. No comparisons were made directly between male and female animals, as fundamental sex differences were not the focus of the current study. The significance level was 0.05 for all tests.

1.2 Experiment 1: Results

Prepulse inhibition at 80dB

A 2x4 mixed ANOVA on percent PPI for males revealed a significant main effect of Postnatal Day (F [3,95] = 45.43, $p < 0.0001$) and interaction effect of Postnatal Day and Genotype (F [3,95] = 2.81, $p = 0.046$). There was no main effect of genotype ($p > 0.05$). Planned comparisons revealed significantly greater inhibition at PD 17 ($p = 0.036$) and lower inhibition at PD 67 ($p = 0.031$) in DISC1 KO males in comparison to WT rats (see Figure 2a). For females, the analysis revealed a significant main effect of Postnatal Day (F [3,95] = 43.19, $p < 0.0001$), but no main effect of Genotype and no interaction effect of Postnatal Day and Genotype, and no significant planned comparisons (all p's > 0.05; see Figure 2b).

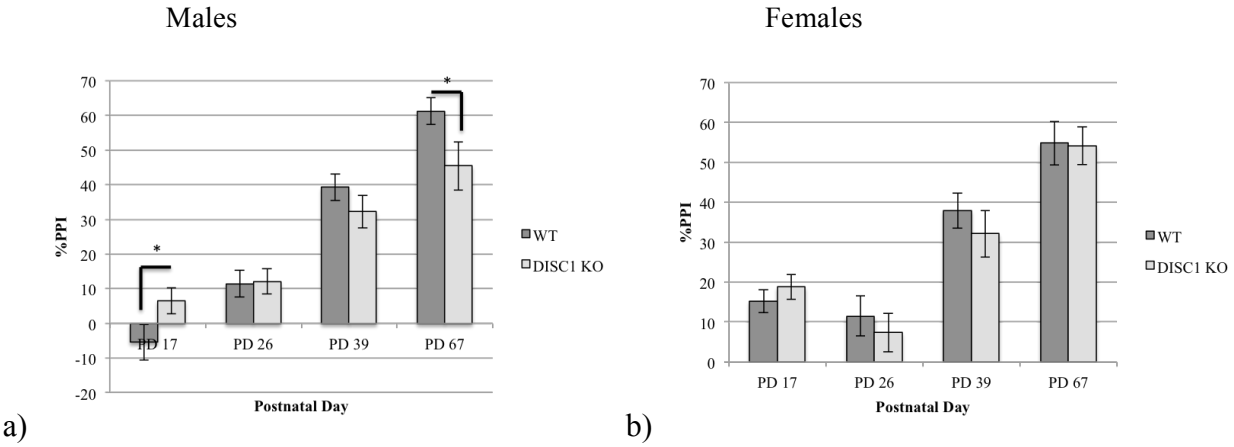

Fig. 2. Mean percent prepulse inhibition at 80 dB over 10 trials for a) male and b) female wildtype and DISC1 knockout rats over development. * p<0.05. Error bars signify ± SEM.

Parvalbumin positive neurons in the prefrontal cortex and the hippocampus

A 2x4 between factors ANOVA in males revealed significant main effects of Postnatal Day on number of parvalbumin positive cells in the infralimbic prefrontal cortex (F [3,39] = 20.03, $p < 0.0001$). This same result was found in the dentate gyrus of the hippocampus (F [3,39]

= 3.13, $p = 0.039$). There was no main effect of Genotype or interaction effect of Postnatal Day and Genotype (all p's > 0.05). Planned comparisons revealed significantly less parvalbumin positive neurons at PD 17 in both brain areas (prefrontal cortex $p = 0.013$; hippocampus $p = 0.047$) in DISC1 KO males in comparison to WT rats (see Figure 3a and c). For females, the analyses revealed significant main effect of Postnatal Day in the prefrontal cortex only (F [3,39] = 26.01, $p < 0.0001$; hippocampus $p > 0.05$). There was no main effect of Genotype or interaction effect of Postnatal Day and Genotype, and no significant planned comparisons (all p's > 0.05; see Figure 3b and d).

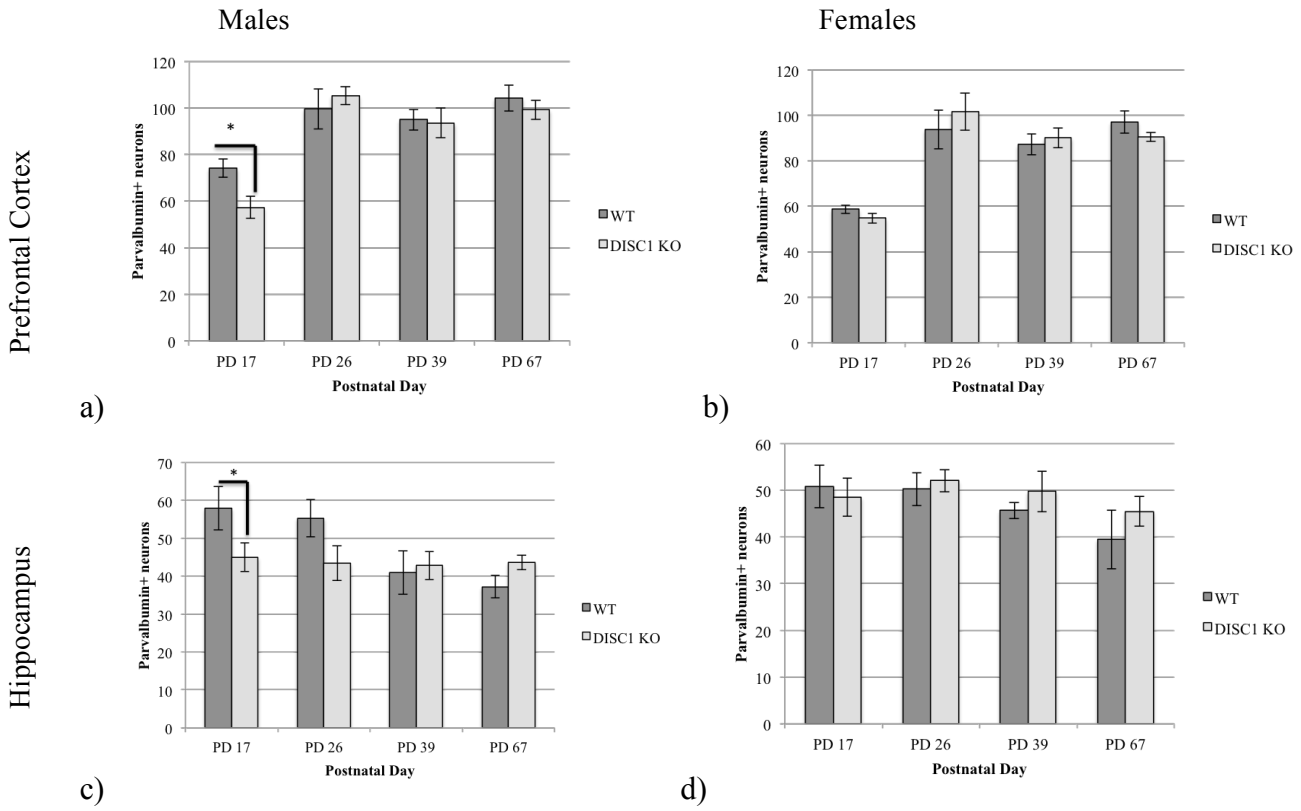

Fig. 3. Mean number of parvalbumin positive neurons for a) male and b) female prefrontal cortex and c) male and d) female hippocampus, comparing wildtype and DISC1 knockout rats over development. * p<0.05. Error bars signify ± SEM.

1.3 Experiment 1: Discussion

The first experiment in this study examined prepulse inhibition as a cognitive symptom and parvalbumin positive GABAergic interneurons as a biological symptom of schizophrenia over development. Based on prior research in humans with schizophrenia, we expected to see deficits in prepulse inhibition as well as a reduction of parvalbumin cells in the prefrontal cortex and hippocampus (Beasley et al., 2002; Koch, 1998; Swerdlow, Hartman, & Auerbach, 1997; Beasley & Reynolds, 1997). These symptoms have been replicated in mouse models also utilizing the DISC1 gene, but have only been considered in adulthood. Specifically, Clapcote et al.'s (2007) DISC1 missense mouse model demonstrated deficits in PPI while Umeda et al.'s (2016) DISC1 KO mouse model demonstrated decreases in parvalbumin-positive cells. We expected these cognitive and biological symptoms to occur at least in adulthood, however, this study was one of the first to examine a model of schizophrenia across development, as well as in both sexes. Generally, the trends in percent PPI in Experiment 1 increased across development in both males and females, which is consistent with findings in the literature regarding sensorimotor gating (Moran et al., 2016; Parisi & Ison, 1979; 1981). Our results regarding genotype, however, were sexually dimorphic, with only males DISC1 KO showing any symptoms. Even within the male group, symptoms in both PPI and parvalbumin were not consistent throughout development.

In this experiment, PPI abnormalities occurred only in male DISC1 KO, and not female DISC1 KO rats. This finding supports general knowledge about schizophrenia's higher prevalence and more severe symptoms in the male population, suggesting that females may be somewhat less susceptible to certain symptoms. In this case, regarding prepulse inhibition, the extent to which females display deficits is under debate, which is further confounded by variation in PPI across the menstrual cycle, where PPI is reduced in the luteal stage in

comparison to the follicular stage (Plappart, Rodenbucher & Pilz, 2005; Swerdlow et al., 1997). Some studies in humans demonstrate that females with schizophrenia show PPI deficits (Kumari, Aasen, & Sharma, 2004) while other do not (Braff et al. 2005). Our model of schizophrenia works with the DISC1 KO as a risk factor, which may not be enough to induce deficits in the female animals. Human studies are most often conducted in populations of people diagnosed with schizophrenia without regard for varied etiologies or at risk populations. With regards to modelling PPI deficits in females at risk for schizophrenia, this experiment would benefit from work with the two-hit hypothesis, which was conducted in Experiment 3.

With the male rats of this experiment, we observed an interesting pattern of PPI across development. At pre-weaning (PD 17), DISC1 KO rats actually demonstrated an increase in PPI, which disappeared during the next two timepoints, and became a deficit during adulthood (PD 67). The adulthood deficit is consistent with PPI deficits seen in humans with schizophrenia and in other animal models, however the initial increase is a unique finding in animal research. One study conducted in a human population found that at risk subjects (at a slightly older comparative age to our finding) who later developed psychosis initially displayed enhancements in PPI (Cadenhead, 2011). This unexpected directionality may be due to an early compensatory measure in the brain which fails to last into adulthood, accounting for the lack of symptoms of schizophrenia in early life. The absence at the two other timepoints may be due to a success in these initial compensatory measures, which do not last through the brain changes which occur during adolescence. In adolescence, synaptic pruning, or the removal of excess neuron processes, occurs as part of the maturation of executive function and normal brain development (Selemon, 2013; Semple et al., 2013). This process is thought to occur too much in schizophrenia, causing damage to the communication between brain systems (Keshavan, Anderson, & Pettigrew, 1994).

The present study may not capture this exact period, so future experiments should expand the adolescent timepoint to more closely examine the PPI trajectory. The initial increase in PPI at PD 17 could be an indication of early brain circuitry changes, and could point toward a window of opportunity for early intervention with treatment.

Similar to PPI, this experiment demonstrated parvalbumin neuron decreases only in DISC1 KO males, again with a different pattern across development. This indicates that females may be resistant to even the biological brain changes which occur with schizophrenia risk factors. In an electrophysiological study of tissue from mice carrying mutant forms of DISC1, Holley et al. (2013) found that male mice were more impaired by the mutation than females, demonstrating a sex specific decrease in cortical GABAergic neurotransmission. In our study, the male DISC1 KO rats demonstrated decreases in parvalbumin positive GABAergic interneurons in both the hippocampus and the prefrontal cortex only at pre-weaning (PD 17). These early decreases may induce structural and neurochemical changes which contribute to later pathology, despite the reemergence of normal GABAergic activity. This finding has not been supported in animal model or studies of human brain tissue, both of which find decreased parvalbumin in adulthood (Volk & Lewis, 2014). It is important to note that although our last timepoint is in the adulthood of the rat, it is an earlier time in comparison to when human samples would be taken (following natural death). There may be a reemergence of the decrease in inhibitory control as cognitive function normally declines in aging, which this study may have missed due to early adulthood being the last timepoint.

In males, Experiment 1 demonstrated abnormalities in both PPI and parvalbumin at the earliest, pre-weaning, timepoint. Although previous experiments have shown that disruption of GABAergic signalling causes decreases in PPI, these models only focus on adult animals (Brown

et al., 2015; Popelář et al., 2013). This study showed deficits in PPI in adulthood with normal numbers of parvalbumin cells, suggesting that the two may not be immediately related, but rather parvalbumin may play more of a developmental role which contributes to later pathology.

2. Experiment 2: Analysis of Dendritic Morphology

This experiment's goal was to investigate the effects of the DISC1 knockout on dendritic morphology in female and male rats using the Golgi-Cox staining procedure. DISC1 is a plasticity gene associated with cytoskeletal elements, and therefore likely alters dendritic morphology and complexity. These changes could contribute to the cognitive effects seen in Experiment 1.

2.1 Experiment 2: Methods

Colony Conditions

Colony conditions were the same as in Experiment 1.

Subjects

In this experiment, 8 adult male and 8 female adult rats were used for Golgi-Cox procedures (n=4 for WT and DISC1 KO, PD 268). These rats were born and bred on site as the fourth generation of offspring from original breeding pairs obtained from SAGE® Labs (TGRA6370, Boyertown, PA). After all dams gave birth, on PD 2, pups were sexed, toe-clipped to denote their genetic background, and cross-fostered among all dams so that each litter contained a mix of female and male wildtype and knockout rats. On PD 24, all rat pups were weaned into same-sex cage pairs.

Golgi-Cox Staining

Animals were given an overdose of sodium pentobarbital by intraperitoneal injection (65 mg/kg) then transcardially perfused with 200 mL of 0.9% saline followed by 200 mL of 4% paraformaldehyde in saline. The brains were extracted and immediately placed in Golgi-Cox solution at room temperature in the dark, for 2 days. Golgi-Cox solution consisted of 5 volume parts each 5% potassium dichromate and 5% mercuric chloride, 4 volume parts 5% potassium chromate, and 10 volume parts deionized water, kept at room temperature in the dark for 5 days to allow precipitate to sink. Solution was replenished at this point, and brains were again left in the dark for 12 days. Brains were then transferred to a 30% sucrose solution, and remained in a light-proof container at 4^{o}C for about 4 days, or until tissue had sunk. One hemisphere per brain was sectioned coronally on a vibratome at 200 μm in 6% sucrose solution. Each section was immediately mounted on a double coated 2% gelatin-slide and stored in a damp, light-proof container until the following steps. The tissue was developed in Kodak Fix, cleared in xylenes, and coverslipped.

Morphological Analysis

Tissue was observed at 600X magnification with oil using the Neurolucida computer program (Microbrightfield Inc., Williston, VT). 6 granule cells per brain were randomly selected from the dentate gyrus in the dorsal portion of the hippocampus. Each cell body and process was traced by hand in the program. The resulting trace models were then analyzed in the Neuroexplorer component of Neurolucida for dendritic complexity index which normalizes

across different sizes of neurons, using the equation DCI=∑ (branch tip orders + # branch tips) x (total dendritic length/total number of primary dendrites) (Alexander et al., 2017).

Statistical Analyses

Means and standard errors were calculated for all histological results including dendritic complexity. A 2x2 between factors ANOVA was used to analyze PPI data resulting from Experiment 2, with between subjects factors of Genotype (WT, DISC1 KO) and Sex (male, female). For all analyses, planned one-tailed t-tests were conducted as appropriate, under the hypothesis that the genetic manipulation would reduce dendritic complexity. No comparisons were made directly between male and female animals, as fundamental sex differences were not the focus of the current study. The significance level was 0.05 for all tests unless otherwise stated.

2.2 Experiment 2: Results

Dendritic morphology in the dentate gyrus

A 2x2 between factors ANOVA was conducted on the dendritic complexity of cells in the dentate gyrus, revealing a significant main effect of Genotype (F [1,15] = 23.59, p = 0.0004) and interaction effect of Genotype and Sex (F [1,15] = 5.47, p = 0.038). There was no main effect of Sex ($p > 0.05$). A planned comparison revealed significantly less dendritic complexity in DISC1 KO males in comparison to WT rats (p = 0.0008; see Figure 4a). For females, the comparison revealed no significant difference ($p > 0.05$; see Figure 4b).

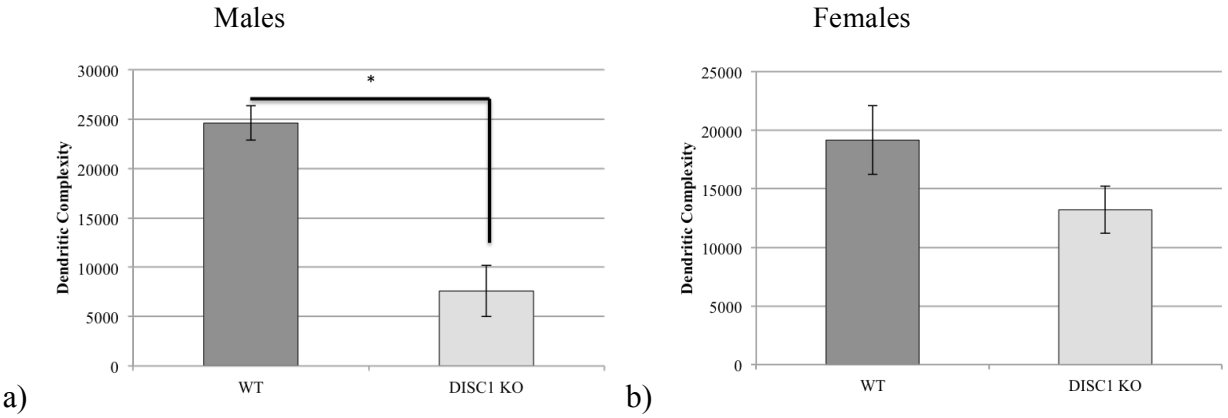

Fig. 4. Mean dendritic complexity of cells in the dentate gyrus for a) male and b) female wildtype and DISC1 knockout rats. * $p<0.05$. Error bars signify ± SEM.

2.3 Experiment 2: Discussion

The second experiment in this study examined dendritic morphology to study DISC1's cytoskeletal involvement in plasticity and dendrite outgrowth. The specific area under investigation was the dentate gyrus, both because DISC1 is expressed there and because it is one of the only highly plastic areas of the brain in adulthood (Austin et al., 2004). Pointing to DISC1's role in this brain area, a DISC1 deletion in mice decreased neurogenesis and caused mispositioning of new cells from the dentate (Lee et al., 2015). As hypothesized, we observed deficits in dendritic complexity in male DISC1 KO rats. However, this result was not observed in females, where there was no difference between DISC1 KO and WT groups. These findings correspond well with the results from Experiment 1, with abnormalities as a result of DISC1 as a risk factor occurring only in males, again pointing toward a need to examine the two-hit hypothesis (Experiment 3).

Evidence from animal modelling indicates that estradiol may have a protective role with regard to dendritic morphology. Ovariectomized female rats experience decreases in neurogenesis, suggesting the importance of ovarian hormones in plasticity (Simonyan &

Chavushyan, 2016; Tuscher et al., 2016). Wang, Zhu, & Xu (2018) found that estradiol treatment in adult female rats promoted dendritic spine growth, which is related to learning, memory, and other cognitive functions which have been implicated in schizophrenia. Therefore, females may again be protected from a biological symptom of schizophrenia, supporting the results from Experiment 1. Future examination of the Golgi-Cox stain in this study could also incorporate other measures of cellular plasticity, such as spine analysis and Scholl analysis. Additionally, this experiment only examines adult rats, which means the rats were beyond the adolescent period of synaptic pruning, which is suspect in schizophrenia (Selemon, 2013; Semple et al., 2013; Keshavan, Anderson, & Pettigrew, 1994). More information regarding the developmental trajectory of dendritic morphology in this model could provide insight into the biological underpinnings of the cognitive symptoms we observed in Experiment 1.

3. Experiment 3: DISC1 KO and Second-Hit Amphetamines as Schizophrenia Risk Factors in Females

This experiment expanded on an aspect of schizophrenia that was unexplored in Experiments 1 and 2: the two-hit hypothesis. Given the negative results in females in Experiments 1 and 2, it is possible that the genetic predisposition alone is not sufficient to produce PPI deficits, and the addition of a second "hit" may be needed. In this model, the first hit is the DISC1 KO, and the second hit is an acute dose of the dopamine agonist amphetamine. The purpose of this experiment was to investigate the extent to which female DISC1 KO rats, compared to female WT rats, exhibit PPI deficits in response to a dopamine agonist given in adulthood.

3.1 Experiment 3: Methods

Colony Conditions

Colony conditions were the same as in Experiment 1 and 2.

Subjects

22 adult wildtype and 29 DISC1 KO female rats (PD 200 at testing) were used for this experiment. These rats were born and bred on site as the sixth generation of offspring from original breeding pairs obtained from SAGE® Labs (TGRA6370, Boyertown, PA). On PD 25, all rat pups were weaned into same-sex cage pairs.

Amphetamine Exposure and PPI Assessment

PPI procedures were the same as used in Experiment 1. 15 minutes prior to their the PPI test session, 11 wildtype and 14 DISC1 KO rats received an intraperitoneal amphetamine injection (1 mg/kg, Sigma Aldrich). The remaining 11 wildtype and 15 DISC1 KO rats received saline injections of equivalent body weight volume.

Statistical Analyses

Means and standard errors were calculated for percent PPI. A 2x2 between factors ANOVA was used to analyze PPI data resulting from Experiment 3, with between subjects factors of Genotype (WT, DISC1) and Drug Treatment (control saline, amphetamine). For all analyses, planned one-tailed t-tests were conducted under the hypothesis that the genetic manipulation and the amphetamine treatment would reduce percent PPI. The significance level was 0.05 for all tests unless otherwise stated.

3.2 Experiment 3: Results

Prepulse inhibition at 80 dB

A 2x2 between factors ANOVA revealed a significant main effect of Drug Treatment (F [1,50] = 13.23, p = 0.0007) on percent PPI. There was no main effect of Genotype or interaction effect of Genotype and Drug Treatment (all p's > 0.05). A planned comparison revealed significantly less inhibition in amphetamine treated DISC1 KO rats in comparison to control DISC1 KO rats (p = 0.001). For wildtype, there was no significant difference between treatment groups (p > 0.05; see Figure 5).

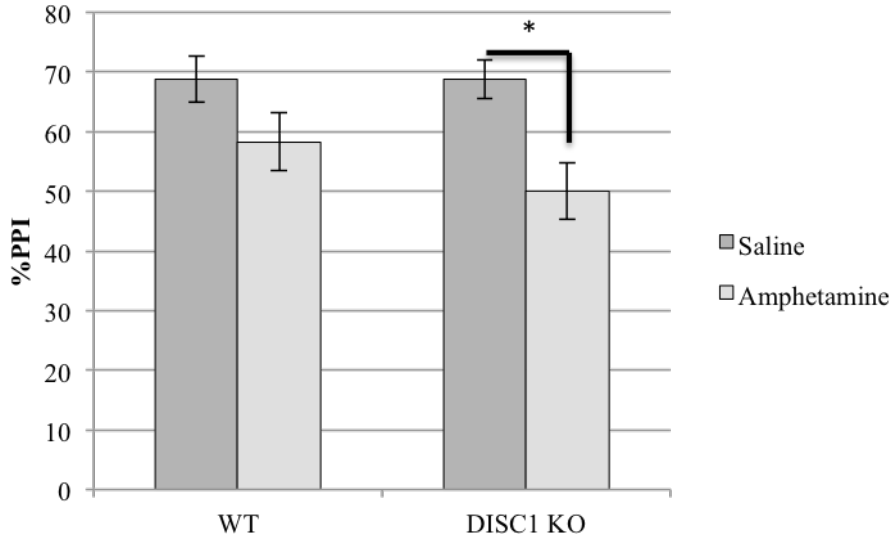

Fig. 5. Mean percent prepulse inhibition at 80 dB over 10 trials for female wildtype and DISC1 knockout rats treated with saline (control) or an acute dose of amphetamine. * p<0.05. Error bars signify ± SEM.

3.3 Experiment 3: Discussion

Based on the results from prior experiments, we expected to see little reduction in PPI when comparing DISC1 KO to WT adult female rats, which is what the results from Experiment 3 demonstrate. This replication helped validate our previous sexually dimorphic PPI results.

Additionally, with this consistency, it is unlikely that our results were influenced by the females' estrous cycle phase, especially since each cage was individually ventilated, preventing the syncing of cycles and randomizing the phases. Experiment 3 most importantly examined the two-hit hypothesis, where the DISC1 KO acted as a risk factor, which must be combined with another hit to produce discernible symptoms. We specifically used the dopamine agonist amphetamine, hypothesizing that at a low, acute dose, we could produce deficits in DISC1 KO but not WT rats (Swerdlow et al., 2003). Our findings support this hypothesis, suggesting that females at risk for schizophrenia may become vulnerable to this cognitive symptom following a second later-life factor.

Other studies have utilized different risk factors to produce two-hit models which focus on PPI as a cognitive symptom of schizophrenia, but were not female specific. Unlike our study, where neither the DISC1 KO nor the amphetamine on its own caused abnormalities, most other models use an initial hit which causes PPI deficits, which are then exacerbated by a second hit. For example, Choy et al. (2009) found that maternal deprivation lowered baseline PPI which was worsened by young-adult stress. Lim et al. (2012) found that neonatal MK-801 combined with isolation rearing produced profound PPI deficits, while isolation on its own only moderately reduced PPI. However this other study was only conducted in male animals, and was therefore limited. The present Experiment 3 uses the DISC1 KO, whose mutation in humans is associated with schizophrenia, and therefore may be a more etiological model than other manipulations. The DISC1 KO seems to be a risk factor rather than an absolute cause of schizophrenia symptoms, requiring a second "hit", consistent with the two-hit hypothesis in humans. The model may not directly compare to humans diagnosed with schizophrenia, but rather an at risk population.

General Discussion

The results from Experiments 1 and 2 in this study indicated that DISC1 KO males, but not females, demonstrated abnormalities in PPI and parvalbumin-positive GABAergic interneurons at different points during development, as well as showing deficits in dendritic morphology in adulthood. Experiment 3 then sought to target this lack of vulnerability in females by combining the DISC1 KO risk factor with acute amphetamine exposure in a two-hit model. This second "hit" was able to induce PPI deficits in the DISC1 KO animals. Overall, this study suggests that with regards to PPI and these specific biological brain changes, male rats are more vulnerable to the DISC1 KO, which is consistent with the prevalence of schizophrenia in humans. However, the longitudinal nature of Experiment 1 limited behavioral observations to what could be repeat tested, i.e. a sensorimotor gating phenomenon such as PPI. Other symptoms would be highly useful for characterizing the developmental sex differences further, but would require separate cohorts for each timepoint. Examples of behavioral tests which could be studied using this model include locomotion/hyperactivity in the open field test for positive symptoms; the elevated plus maze, social interaction test, and sucrose preference test of anhedonia for negative symptoms; and the Morris Water Maze task or radial arm maze for working and spatial memory for other cognitive symptoms. Biologically, brain tissue samples could be used to study actual amounts of parvalbumin in the brain, as well as looking at measures of neurogenesis, dopaminergic activity, and glutamatergic activity.

Additionally, the nature of the sex differences found in this experiment need to be studied. As in Experiment 3, the two-hit model could be very useful to determine what additional "hits" are necessary to cause pathology in the females. These studies could combine the DISC1

KO with other risk factors, like early life stress or maternal separation, chronic drug use, or a chronic mild stress paradigm. Gondal removal and hormone replacement could also provide information about the roles of sex hormones like estradiol in schizophrenia.

In summary, this study points toward validity of the DISC1 KO rat as a risk factor model for schizophrenia, as well as the importance of studying schizophrenia over development in both sexes.

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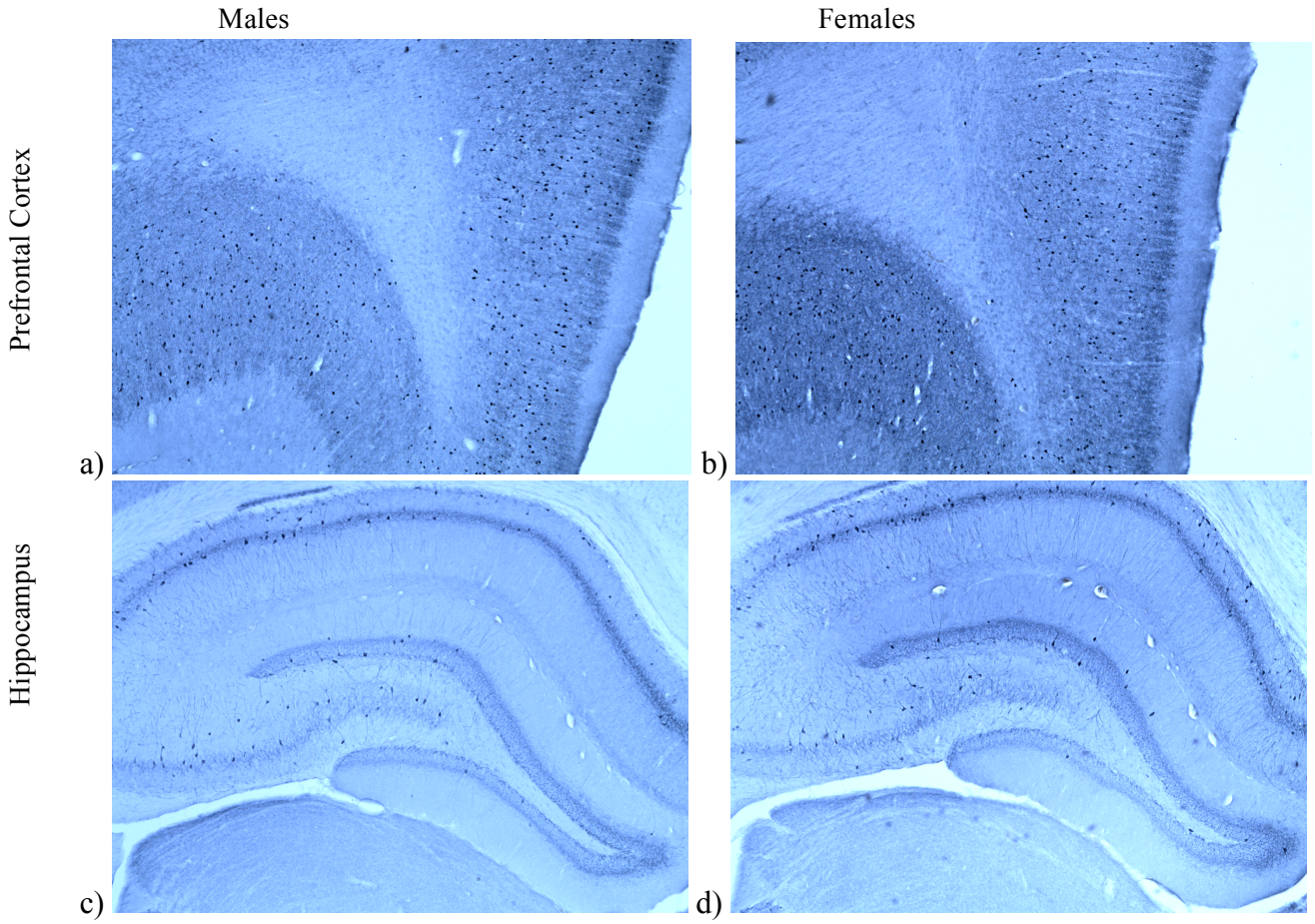
Additional Figures



Representative parvalbumin staining for a) male and b) female prefrontal cortex and c) male and d) female hippocampus, in wildtype adult (PD 67) rats.

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