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Project Title: Exploring Parent-of-Origin Effects on Contextual and Cued Conditioned Threat Learning in Type III Neuregulin 1 Transmembrane Domain Mutant Mice

Category: Animal Sciences

A. Rationale

Schizophrenia is a complex psychiatric disorder caused by both genetic and environmental factors (Sullivan et al., 2003). Genetic disruptions and prenatal complications suggest that schizophrenia is a neurodevelopmental disorder (Murray et al., 1987; Walsh et al., 2008). Interactions between multiple genes and environmental factors alter brain development in ways that increase the risk of developing the disorder (Tsuange, 2000).

Genome wide association studies (GWAS) of populations in Iceland and Scotland identified Neuregulin 1 (Nrg1) as a schizophrenia susceptibility gene (Stefansson et al., 2002). Nrg1 encodes for a family of signaling molecules that play fundamental roles in the development of the nervous system (Harrison et al., 2005). When Nrg1 signaling is disrupted *in vivo*, there are morphological and psychological defects, as evidenced by a variety of mouse models. Mice heterozygous for a Nrg1 nonsense mutation that truncates the protein in the transmembrane domain display a hyperactive phenotype (Karl et al., 2007). Additionally, synapse maintenance is disrupted in mice with a mutation that eliminates expression of Type III Nrg1 (Wolpowitz et al., 2000). Nrg1 mice with partial deletion of epidermal growth factor like domains display decreased sociability and reduced freezing during contextual fear conditioning (Ehrlichman et al., 2007). These distinct phenotypes indicate that a variety of brain functions are affected by mutations within Nrg1. There are a number of phenotypes unique to Type III Nrg1 mutant animals. In Type III Nrg1 mutant mice, axon pathfinding is disrupted, and there are deficits in Schwann cell development (Hancock et al., 2011; Birchmeier et al., 2008). Type III Nrg1 mutant mice also exhibit impaired memory and sensorimotor gating (Chen et al., 2008).

The Type III Nrg1 cell receptor spans the cell membrane in a hairpin-like fashion. There are two transmembrane domains, one which is a cysteine-rich domain and one

which is located at the c-terminus (Fleck at al., 2013). Type III Nrg1 goes through a series of complex proteolytic cleavages (Fleck at al., 2013). After juxtacrine signaling, the intracellular domain (ICD) at the c-terminus is liberated by gamma secretase and enters the nucleus, where it interacts with other proteins to alter gene expression in a process known as back-signaling (Bao, 2003). A valine to leucine hypomorphic mutation, or a mutation causing partial loss of gene function, in the transmembrane domain disrupts cleavage by gamma secretase and is associated with morphological defects in the development of cortical neurons (Chen et al., 2010).

This valine to leucine substitution in the human Nrg1 gene was originally identified in an isolated population in Costa Rica and was shown to be associated with an increased risk of psychosis, a key symptom of schizophrenia (Walss-Bass et al., 2006). Among the symptoms of schizophrenia are delusions, hallucinations, disorganized speech and behavior, and negative emotional response (American Psychiatric Publishing, 2013). The areas of the brain most connected to these behaviors are the hippocampus and the amygdala, the former of which has structural abnormalities in chronic schizophrenics (Velakoulis et al., 2006). Associative, or conditioned threat, learning, analyzes functions that require both the amygdala and hippocampus (Clark et al.,1998; Goosens et al., 2001). The paradigm teaches animals to associate neutral sensory inputs (e.g. a general context or a discrete tone) with an aversive stimulus and monitors their subsequent response to these sensory cues (Curzon et al., 2009). In mice heterozygous for a mutation that eliminates expression of Type III Nrg1, abnormalities in contextual threat conditioning were found to be influenced by both sex (males showed a phenotype, females did not) and the parent-of-origin of the mutation (Shang et al., 2017). The hypothesis that schizophrenia is influenced by parent-of-origin is supported by a study analyzing a locus on chromosome 2p12-q11 that is associated with schizophrenia (Francks et al., 2003) and supporting evidence that the locus contains an imprinted gene, LRRTM1 (Ludwig et al., 2009).

B. Research Questions

1. Is there a link between the valine to leucine mutation in the transmembrane domain and a behavioral phenotype across wild type, heterozygous and mutant mice?

2. Is the phenotype influenced by the parent-of-origin of the mutation?

These questions were asked due to the strong evidence that schizophrenia is influenced by parent-of-origin. Additionally, the study by Shang et al. demonstrated that the parent-of-origin of a different mutation within Neuregulin 1 influenced associative learning.

C.1. Procedures

1. Associative Learning

This study will utilize both contextual and cued conditioned threat learning in order to examine both contextual associative learning and cued associative learning. The conditioned threat learning protocol will be performed for six consecutive days, each test 24 hours after the previous. Throughout the procedure, a white noise generator will be used to eliminate background noise. The behavioral paradigm has 4 distinct phases: handling, training, contextual recall, and cue recall:

1.1 Handling

The first three days of the procedure will consist of handling sessions to reduce extraneous anxiety. On each day, individual animals will be handled (cupped in researcher's hands with minimal or no restraint) for a total of five minutes.

1.2 Training

On the fourth day of the procedure, mice will be trained. Individual mice will be placed in fear conditioning cubicles with silver walls and metal grid floors, both scented with ethanol. The protocol will consist of 120 seconds of free exploration, a 30 second long 80 decibel tone, and a 2 second 0.8 mAmp shock presented via the metal floor. Note that the shock will be presented during the last 2 seconds of the tone. At 240 and 360 seconds into the session, the tone and foot shock pairing will be repeated. After the final pairing, mice will remain in the chamber for an additional 90 seconds.

1.3 Context Recall

Twenty-four hours after the training session, animals will be returned to the same conditioning chamber and their freezing was recorded during a 480 second session. Freezing will be defined as lack of movement aside from breathing. This session will provide a measure of the animals' freezing in the same context in which they will be trained.

1.4 Cue Recall

Forty-eight hours after training, mice will be placed in a cubicle with black and white striped walls and a mesh floor that will be scented with lemongrass citrus. This will alter visual, tactile, and olfactory cues, relative to the training environment. After 120 seconds of free exploration, mice will be subjected to the same tone used during the training session for 30 seconds. As opposed to the training protocol, mice will not be shocked, and the tone will only be administered once. Following the single tone presentation, mice will remain in the chamber for an additional 150 seconds. Freezing will also be recorded during cue recall.

C.2. Risk and Safety

No hazardous chemicals will be used. There are no potential risks. Gloves and a lab coat will be worn when handling the animals.

C.3. Data Analysis

1. Video Analysis

During the training and both recall sessions, mice will be filmed with cameras connected to Freeze Frame 4 trial recorder software. The software analyzes motion with a motion detection algorithm (ActiMectrics Software, 2018). After the videos are recorded, Freeze Frame 4 trial viewer will be used to identify and record the times at which each

mouse froze. The protocols will be broken up into 30 second time bins and analyzed for the percentage of time spent freezing during that time bin.

2. Statistics

The percentage of time spent freezing in 30 second time bins will be exported from Freeze Frame 4 first into a Microsoft Excel workbook and then into an IBM SPSS file. All graphs will be created in Microsoft Excel, and statistical analyses will be performed in IBM SPSS. Differences between groups of wild type, heterozygous (parent-of-origin unknown), and mutant mice during training will be analyzed with a repeated measures (RM) analysis of variance (ANOVA) for each 30 second time bin. The same statistical analyses will be used when comparing heterozygous mice with mutant mothers (MM) and mutant fathers (MF). Differences between groups of male and female mice within genotypic groups during training will be analyzed with RMANOVA. Genotypic effect on percent freezing across the full 480 seconds of context recall will be averaged and then analyzed using a one way ANOVA. The same statistical analyses will be performed when comparing heterozygous mice MM and MF. Differences between male and female mice within genotypic groups during context recall will also be analyzed with a one way ANOVA. The percent freezing data for cue recall will be averaged into three time bins: pre-tone, tone, and tone. Differences between groups of heterozygous (parent-of-origin unknown), mutant, and wild type mice during cue recall will be analyzed with a RMANOVA for each of the three time bins. The same statistical analyses will be performed when comparing heterozygous mice with MM and MF. Differences between groups of male and female mice within genotypic groups during cue recall will be analyzed with RMANOVA as well. Response to context will be isolated by comparing the naïve portion of training (i.e. the first 120 seconds in the novel context), the first two minutes of context recall, and the naïve portion of cue recall (i.e. the first 120 seconds in the novel context). Differences between groups of heterozygous (parent-of-origin unknown), mutant, and wild type mice will be analyzed with a RMANOVA each time bin. The same statistical analysis will be performed when comparing heterozygous mice with MM and MF. Differences between groups of male and female mice within

genotypic groups during training will be analyzed with RMANOVA as well. Significance will be defined as $p < .05$.

1. Human participants research

Non-applicable

2. Vertebrate animal research

a. Flies are an alternative to mice; however, they do not have a Neuregulin 1 gene. They have a similar gene, but it does not function the same. Therefore, in order to do phenotypic analysis, mice must be utilized.

b. This study will contribute to the wealth of investigations into the functions and mechanisms of Nrg1, a schizophrenia susceptibility gene. Additionally, because genome databases do not contain parent-of-origin information, a parent-of-origin effect for Nrg1 would impact any Nrg1 genome wide association study (GWAS).

c. All procedures are detailed in the "Procedures" portion of the research plan. Potential discomfort will be minimized with a short and tightly controlled circuit. Therefore, the mice will not accidentally receive a strong shock. Additionally, the shock will only occur for 2 seconds at 0.8 mAmps.

d. There will be 42 mice. The species is Mus Musculus. The strain is C57 black 6. They will be around 120 days old because that is around the adolescent stage of a mouse. There must be about eight mice in each experimental group so that the data can be significant.

e. The use of animals was approved by the Institutional Animal Care and Use Committee of State University of New York, Stony Brook (Stony Brook, NY, USA). Procedures are in accordance with NIH guidelines. Animals were weaned on postnatal day 21. Animals were maintained in a vivarium at Stony Brook University (Stony Brook, NY, USA). Housing facilities were temperature- and humidity-controlled. Food and water were

available *ad libitum.* Animals were kept on a 12 hour light/dark cycle with the light cycle running from 7AM to 7PM. Cages had a maximum of four animals; no animals were single housed prior to behavioral testing. Animals were tested at 10-14 weeks of age. The experimenter was blind to genotype throughout.

f. Mice will be turned over to other researchers for another investigation.

3. Potentially hazardous biological research

Non-applicable

4. Hazardous chemicals, acitivites & devices

Non-applicable

D. Bibliography

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Addendum

No changes were made.