Curriculum Vita

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Educational Background

Nov 2011	Doctor of Philosophy. University of Maryland, Baltimore, Program in Neuroscience Thesis Title: *Cognition Following Prenatal Stress and/or Disruption of Neuregulin-1Gene; Implications for Schizophrenia*
2004	Bachelor of Science, Arizona State University, Psychology (Magna Cum Laude)

Professional Experience

2004-present	Graduate Research Assistant, Program in Neuroscience, University of Maryland, Baltimore. Design and run behavioral and molecular experiments. Analyze and interpret results. Write, publish and present findings.
2006	Internship, Maryland Science Center, Baltimore MD. Guide visitors through series of designed experiments, explain and interpret exhibits, design and implement new exhibit.
2002-2004	Research Assistant, Department of Psychology, Arizona State University. Perform experiments. Analyze and interpret data. Assist with writing and presentation of findings.

Honors and Scholarships

Biological Research Experience for Undergraduates (BREU), Arizona State University

Technical Skills

Behavioral Neuroscience:	Morris Water Maze, Prepulse inhibition, Acoustic startle test, Conditioned place preference, Novel object recognition, Social interaction, Open field, Spatial/Visual Object Discrimination test, Self-administration, Acute and chronic stress paradigms, Prenatal stress

Basic Neuroscience:	Cryosectioning, PCR, Western Blotting
Animal Care and Use:	Animal handling techniques to minimize stress response, Indwelling catheter surgery, Intraperitoneal injections, Rat brain extraction
Management:	Assistance with establishment and maintenance of a breeding colony of transgenic rats, Training of graduate and undergraduate students, Management of laboratory supplies; Mountaineering expedition management
Data Analysis and Graphing:	SPSS version 13.0, Graphpad Prism, Photoshop 6.0, Microsoft Office

Teaching Experience

Fall 2010:	Assistant mentor, UMBC student
Guest Lecturer:	Graphpad Prism and Photoshop basic techniques for manuscripts; Markham Laboratory, MPRC, University of Maryland, Baltimore.
Guest Lecturer:	Cognitive Neuroscience (Graduate Level) Drug addiction class; Arizona State University
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Experimental Stations Instructor:	Maryland Science Center Internship, Baltimore MD

Professional Association

Society for Neuroscience
American Neuroendocrine Society

Publications

AR Taylor, SB Taylor, JI Koenig (2011) Disruption of Neuregulin-1 Gene in Rats Causes Specific Cognitive Deficits. In preparation.

SB Taylor, JA Markham, **AR Taylor**, BZ Kanaskie, JI Koenig (2011) Sex-specific Neuroendocrine and Behavioral Phenotypes in Hypomorphic Type II Neuregulin 1 Rats. Behav. Brain Res. Nov 25; 4:173.

AR Taylor, JA Markham, SB Taylor, DB Bell and JI Koenig (2010) Characterization of the Cognitive Impairments Induced by Prenatal Exposure to Stress in the Rat. Front. Behav. Neurosci. 104(2); 205-14.

SB Taylor, **AR Taylor**, JA Markham, AM Geurts, BZ Kanaskie and JI Koenig (2010) Disruption of the Neuregulin 1 Gene in the Rat Alters HPA Axis Activity and Behavioral Responses to Stimuli. Physiol. Behav.

YK Takahashi, MR Roesch, TA Stalnaker, RZ Haney, DJ Calu, **AR Taylor**, KA Burke, G Schoenbaum (2009) The Orbitofrontal Cortex and Ventral Tegmental Area are Necessary for Learning From Unexpected Outcomes. Neuron. 62(2):269-80.

MR Roesch, **AR Taylor**, G Schoenbaum (2006) Encoding of Time-Discounted Rewards in Orbitofronal Cortex is Independent of Value Representation. Neuron. 51(4):509-20.

AT Alleweireldt, RJ Hobbs, **AT Taylor**, JL Neisewander (2006) Effects of SCH-23390 Infused into the Amygdala or Adjacent Cortex and Basal Ganglia on Cocaine Seeking and Self-Administration in Rats. Neuropsychopharm. 31(2): 363-374.

Abstracts

AR Taylor, SB Taylor and JI Koenig. Disruption of Neuregulin-1 Gene Affects Working Memory Ability in Adult Rats. Presentation No. 471.19 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2010. Presenter.

SB Taylor**,** JA Markham, BZ Kanaskie, **AR Taylor**, JI Koenig. Disruption of Neuregulin-1in the Female Rat may alter Hypothalamic-Pituitary-Adrenal Axis Function and Responses to Environmental Stimuli. Presentation No. 697.24 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2010.

SB Taylor, BZ Kanaskie, JA Markham, AM Geurts, **AR Taylor**, JI Koenig. Neuregulin-1May be a Novel Regulator of Behavioral and Neuroendocrine Stress Reactivity. Neurobiology of Stress Workshop, 2010.

AR Taylor, SB Taylor, JA Markham, JI Koenig. Animal Models of Schizophrenia. Schizophrenia Research Forum. Database. Online. (http://www.schizophreniaforum.org/res/models/default.asp), April 2009.

JA Markham, **AR Taylor**, SB Shelton, D Brady-Bell, JI Koenig. (2008) Recent Findings from the Repeated Variable Prenatal Stress Model of Schizophrenia. Abstract for Poster Presentation, American Neuroendocrine Society, 2008.

AR Taylor and JI Koenig. Effects of Prenatal Stress and Time of Day on Learning and Memory. Abstract for Poster Presentation, Society for Neuroscience 2007.

MR Roesch, **AR Taylor**, G Schoenbaum. Neuronal Activity in Orbitofrontal Cortex Covaries with Delay and Reward Magnitude. Abstract for Poster Presentation, Society for Neuroscience, 2005.

AT Alleweireldt, RJ Hobbs, **AT Taylor**, JA Johnson, JL Neisewander. Infusions of the D1 Receptor Antagonist, SCH-23390, Into Caudal Subregions of Amygdala and Striatum Modulate Cocaine Self-Administration and Cocaine-Seeking Behavior in Rats. Abstract for Poster Presentation, Society for Neuroscience, 2004. Co-presenter.

AT Alleweireldt, RJ Hobbs, **AT Taylor**, EM Lungren, JL Neisewander. Blockade of Dopamine D1 Receptors in the Amygdala Attenuates Cue and Cocaine Reinstatement of Extinguished Cocaine-Seeking Behavior in Rats. Abstract for Poster Presentation, Society for Neuroscience, 2003. Co-presenter.

Abstract

Title of Dissertation: Cognition Following Prenatal Stress and/or Disruption of Neuregulin-1 Gene; Implications for Schizophrenia

Adam Taylor, Doctor of Philosophy, 2011

Dissertation Directed by: James I. Koenig, Ph.D., Department of Psychiatry

Schizophrenia is a severe, chronic and disablilitating disease, affecting nearly one percent of the population. Symptoms consist of positive (hallucination, delusions), negative (anhedonia, social withdrawal) and cognitive (memory impairments, attentional deficits). Cognitive symptoms are considered to be a core feature, and contribute greatly to loss of function. Environmental insults, such as prenatal stress, have been shown to increase risk for schizophrenia. In addition, genetic studies have shown that certain genes, such as Neuregulin-1 (*NRG1*), are consistently associated with schizophrenia. Interactions between genetic vulnerabilities and environmental factors are also considered to be important in the development of schizophrenia. Here, using an animal model of repeated variable prenatal stress (RVPS), we have shown that animals exposed to RVPS have deficits in spatial learning and memory in the Morris water maze, deficits in working memory in a spatial/visual object discrimination task, and long-term memory deficits. Utilizing a rat preparation with disruption of the neuregulin-1 gene, which leads to decreased NRG1 mRNA and protein in the brain, we have found no deficit in the spatial learning version of the Morris water maze, but we do see learning impairments in a discrimination task with both visual and spatial components. Finally, we were not able to show a substantial effect of a gene X environment interaction between RVPS and *Nrg1* disruption. Our

results indicate that both prenatal stress and disruption of the neuregulin-1 gene, each risk factors for schizophrenia, result in specific deficits in learning and memory. These deficits are also similar to some cognitive impairments in schizophrenia, indicating our manipulations may be a factor in those symptoms of the disease.

Cognition Following Prenatal Stress and/or Disruption of Neuregulin-1 Gene;

Implications for Schizophrenia

By

Adam Taylor

Dissertation submitted to the faculty of the Graduate School of the

University of Maryland, Baltimore in partial fulfillment

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Doctor of Philosophy

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List of Abbreviations

5HT-1A	Serotonin-1A receptor
BDR	Blind Drunk point mutant mouse
COMT	Catechol-O-methyl-transferase
DISC1	Disrupted in schizophrenia-1
DMXB-A	3-[(2,4-dimethoxy)benzylidelene]anabaseine
DN-DISC1	Dominant negative disrupted in schizophrenia-1
DTNBP1	Dystrobrevin-binding protein-1
EGF	Epidermal Growth Factor
HPA	Hypothalamic-pituitary-adrenal
GABA	Gamma-butyricamino acid
GAP-43	Hippocampal growth associated protein-43
Ig-NRG1	Immunoglobulin domain containing NRG1
LTP	Long-term potentiation
MATRICS	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MhDISC1	Mutated human DISC1
NIMH	National Institute of Mental Health
NMDA	N-methyl-d-aspartic acid
NR	NMDA receptor
NRG1	Neuregulin-1 protein
NRG1/Nrg1	Human/rodent neuregulin-1 gene
$Nrg1^{Tn}$	Hypomorphic Type II NRG1 Rats

PACAP	Pituitary adenylate cyclase-activating polypeptide
PCP	Phencyclidine
PET	Positron emission tomography
PGAP-43	Phosphorylated hippocampal growth associated protein-43
RVPS	Repeated variable prenatal stress
SNAP-25	Synaptosomal-associated protein of 25 kDa
SNP	Single nucleotide polymorphism
THC	Tetrahydrocannibinol
WT	Wild-Type

Chapter 1: Introduction

Schizophrenia Background

Schizophrenia has been known and studied since it was first identified by Kraepelin in 1896 (Sharma and Antonova, 2003) though it is still understood poorly. The prevalence of schizophrenia is generally around one percent across populations. In addition, schizophrenia is also one of the most expensive diseases, combining both medical expenses and loss of productivity due to functional impairment (Knapp, 1997). The disease is both chronic and debilitating and is characterized by positive symptoms (hallucinations, delusions), negative symptoms (anhedonia, social withdrawal) and cognitive symptoms. Illness onset is generally in early adulthood and is usually identified by the appearance of psychotic symptoms, though negative symptoms are usually identifiable as well (Carpenter and Buchanan, 1994). Historically, the positive and negative symptoms have been considered the key facets of the illness. Recently however, it has become clear that the cognitive deficits suffered by schizophrenia patients (such as difficulty with working memory, attention and problem solving) are a core feature of the disease and warrant much further study (Keefe and Fenton, 2007; Bora et al., 2010). While it is certainly not known what causes schizophrenia, it is known that both environmental and genetic factors play a role (The European Network of Schizophrenia Networks for the Study of Gene-Environment Interactions (EU-GEI), 2008).

The underlying pathology of schizophrenia is not fully understood, but much has been learned in the past several decades. The groundbreaking work of Arvid Carlsson in the 1950's, in which the neurotransmitter dopamine was discovered, led to many breakthroughs in both neurobiology and the ability to understand neuropsychological diseases (Iversen and Iversen, 2007). Chlorpromazine and reserpine were, in the 1950's, the first drugs employed as antipsychotics (Kapur and Mamo, 2003). An original study, examining the effects of antipsychotic drugs in animal models, discovered that the antipsychotics effectively acted by blocking dopamine receptors in the brain (van Rossum, 1966). The hypothesis that dopamine was playing a role in the pathophysiology of schizophrenia arose from this and continued to gain traction over the following decades, with dopamine D2 receptors eventually being shown by positron emission tomography (PET) to be increased in the caudate nucleus of schizophrenic patients (Wong et al., 1986). Multiple follow up studies actually did not find an overall increase in D2 receptors in schizophrenia, (Farde and von Bahr, 1990; Pilowsky et al., 1994) though some alterations to the dopamine system were found, including elevated dopamine release following amphetamine treatment (Laruelle et al., 1996; Breier et al., 1997) and Laruelle (Laruelle, 1998), by pooling data from multiple analyses, has shown that schizophrenic patients do indeed show dysregulation in the dopaminergic system, including slightly increased D2 receptor density. However, the antipsychotic drugs did little to help the negative symptoms and as research continued, and the understanding of antipsychotic drugs increased, it became clear that these drugs, such as clozapine, did not only act on dopamine receptors but instead exerted influence on many systems in the brain (Moncrieff, 2009). Recently, new research has continued to espouse dopamine as having a role in schizophrenia, though other systems are also implicated (Winterer, 2006).

One such other system thought to be important in the pathology of schizophrenia is the glutamatergic system. This hypothesis was borne out of the finding that phencyclidine (PCP), an n-methyl-d-aspartatic acid (NMDA) antagonist, can cause a non-schizophrenic person to exhibit each of the symptom clusters, positive, negative and cognitive (Tsai and Coyle, 2002). Other NMDA antagonists, such as ketamine, are also able to increase psychotic symptoms in patients (Lahti et al., 1995) as well as inducing a psychotic state similar to schizophrenia in healthy volunteers (Adler et al., 1999), which was alleviated with the antipsychotic drug clozapine (Malhotra et al., 1997). Furthermore, it has been shown that schizophrenic patients have decreased glutamate concentrations in both the prefrontal cortex and the hippocampus (Tsai et al., 1995). NMDA receptor subunits (NR) have also been shown to be altered in schizophrenia. NR1 was seen to be decreased in the hippocampus of schizophrenic patients (Gao et al., 2000) while NR2D was found to be increased in the prefrontal cortex (Akbarian et al., 1996). Clinical studies have also shown some beneficial effects of agents that modulate the glycine binding site on the NMDA receptor, acting as agonists (Goff et al., 1995; van Berckel et al., 1996; Goff et al., 1999). Thus it seems clear that the NMDA receptors are involved in schizophrenia. However, it is also becoming clear that dopamine and NMDA are certainly not the only players in the pathogenesis of schizophrenia. For instance, galantamine, a modulator of nicotinic receptors, has been shown to have beneficial effects on processing speed and verbal memory (Buchanan et al., 2008). 3-[(2,4-dimethoxy)benzyliddene]anabaseine (DMXB-A), a partial alpha7 nicotinic agonist, has also been shown improve neurocognition in schizophrenic patients (Olincy et al., 2006).

Cognition in Schizophrenia

As mentioned above, the cognitive deficits in schizophrenia are becoming known as an absolute core feature of the disease, as, if not more devastating than either the positive or negative symptom clusters (Keefe and Fenton, 2007). Indeed, as the cognitive symptoms are present early in the disease and are resistant to current treatments they can be a life-long burden for patients (Simpson et al., 2010). Verbal memory, and to a lesser extent, attentional processes, are impaired to an almost identical degree in first-episode unmedicated patients and medication free previous patients compared to healthy controls, indicating a stable deficit throughout (Saykin et al., 1994). Cognitive deficits in schizophrenia are certainly not isolated to verbal memory and learning though. Attention, higher order executive functions such as working memory, processing speed, cognitive flexibility and response inhibition have all been shown to be impaired in schizophrenia (Pietrzak et al., 2009). Meta-analyses have shown that schizophrenia patients have profound deficits in working memory (Lee and Park, 2005; Forbes et al., 2009) and long-term memory (Aleman et al., 1999). A comprehensive meta-analysis, which looked at 204 studies examining spatial ability, executive function, attention, memory and other variables found an overall, broad cognitive impairment in all 22 variables that were tested (Heinrichs and Zakzanis, 1998). The seriousness of cognitive deficits in schizophrenia is such that the National Institute of Mental Health (NIMH) established the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS). This was established with the intention of identifying the subgroups of cognitive phenotypes in which schizophrenia patients have deficits in order to help evaluate and produce more efficacious medications for cognitive symptoms. The seven domains identified by MATRICS were working memory, attention/vigilance, verbal learning and memory, visual learning and memory, reasoning and problem solving, speed of processing and social cognition (Green et al., 2004). Thus it is clear that the cognitive deficits suffered in schizophrenia are of the utmost concern and more understanding of the causes and underlying pathology controlling these deficits are crucial pieces of knowledge.

Animal Models of Schizophrenia

In order to gain a better understanding of the environmental risk factors for schizophrenia and the phenotypes resulting from experiencing those risk factors, animal models where rodents are exposed to the environmental insult associated with schizophrenia have been extensively examined. However, there are limitations to studying a complex illness such as schizophrenia in animal models. Schizophrenia, with its positive symptoms of hallucinations and delusions, is a uniquely human disease. However, the use of animal models lies in their abilities to recreate certain aspects of the disease, rather than attempting to make a truly schizophrenic animal. Matters of the validity of animal models are also important. In order do be acceptable animal models must take into account face, construct and predictive validity as important criteria.

The MATRICS study has identified multiple subsets of cognitive dysfunction in schizophrenia that can be examined in animal models to gain a better understanding of the neurobiological underpinnings of the cognitive deficits. As mentioned above, NMDA receptor antagonists are able to produce a state similar to schizophrenia and have been used in animals to model symptoms of schizophrenia. The 5 choice serial reaction time test is designed to analyze many aspects of cognition, including attention, cognitive flexibility and processing speed. Performance in the 5 choice serial reaction time test is impaired by administration of NMDA antagonists in ways that are similar to cognitive deficits seen in schizophrenia (Amitai and Markou, 2010). Amphetamine sensitization, i.e. repeated exposure to amphetamine, leads to cognitive deficits in attention and flexibility (Featherstone et al., 2007). Neonatal ventral hippocampal lesions lead to deficits in working memory in adult rats that are the same as those seen in adult rats with medial prefrontal cortex lesions (Lipska et al., 2002).

Animal models where either a system known to be involved in schizophrenia is altered or a known risk gene for schizophrenia is mutated are also helpful in determining more clearly the role of these specific systems and genes in specific behaviors. For example, mice lacking either the dopamine D1 or D2 receptor show deficits in spatial working memory (Glickstein et al., 2002) while only the mice lacking D2 showed a deficit in the initial compound discrimination in the attentional set-shifting test (Glickstein et al., 2005). Using inducible and reversible NMDA NR1 receptor knockout mice, it has been shown that NR1, when knocked out during learning or the post-learning period, but not during recall, disrupts long-term (one-month) memory retention (Cui et al., 2005). Specific ablation of the NR2B subunit of the NMDA receptor in the forebrain resulted in spatial and non-spatial memory deficit while ablation in the hippocampus resulted in short-term working memory loss (von Engelhardt et al., 2008). Overexpression of catechol-O-methyltransferase (COMT) resulted in set-shifting deficits as well as recognition and working memory impairments (Papaleo et al., 2008). Mice with a deletion in the dystrobrevin-binding protein 1 (DTNBP1) gene, which leads to a loss of dysbindin, show deficits in long-term memory and working memory (Takao et al., 2008).

Prenatal Stress

How schizophrenia arises, typically it manifests for the first time in late adolescence or early adulthood, is also a question which receives a great deal of examination. One avenue of study has focused on environmental factors that can contribute to the risk of developing schizophrenia and other neuropsychiatric diseases. For instance, it is known that exposures such as infection and nutritional deficits can lead to psychiatric diseases (Brown, 2010). In addition, there have been many environmental risk factors for psychosis that occur during the prenatal period, including prenatal exposure to influenza (Mednick et al., 1988; Kendell and Kemp, 1989; Brown et al., 2004), rubella (Brown et al., 2000; Brown et al., 2001) and toxoplasma gondii (Brown et al., 2005; Mortensen et al., 2007). Prenatal stress, in the form of extreme events such as war, famine or natural disasters, as well as psychological stressors such as bereavement or unwanted pregnancy, has been epidemiologically observed to increase the risk for schizophrenia (Huttunen and Niskanen, 1978; Susser and Lin, 1992; Hoek et al., 1996; Susser et al., 1996; Hoek et al., 1998; van Os and Selten, 1998; St Clair et al., 2005).

Prenatal stress not only increases the risk for schizophrenia, but also has many deleterious outcomes associated with exposure. Infants who had been exposed to prenatal stress have been shown to have poorer cognitive outcomes than those not stressed, as indexed by the Bayley Mental Development Index (Buitelaar et al., 2003; Laplante et al., 2004; Bergman et al., 2007). One recent prospective study found that prenatal cortisol exposure, indexed by amniotic fluid levels, negatively predicts infants' cognitive ability, independent of other prenatal, obstetric, and socioeconomic factors; interestingly, the effect interacts with postnatal environment, as it is only present among children who do not experience a secure infant-mother attachment (Bergman et al., 2010). Another found that the impact of cortisol exposure on cognitive development depends on the timing of prenatal exposure, with elevated levels early in gestation being associated with a slower rate of development over the first year of postnatal life (Davis and Sandman, 2010). Examination of the impact of prenatal stress on cognitive outcomes extending beyond childhood has been very limited, although one study has reported a link between maternal exposure to psychosocial stress during gestation and impaired working memory performance following cortisol treatment in young adult women (Entringer et al., 2009).

Studying the long-term neural and behavioral outcomes following prenatal stress in humans is complicated by numerous other environmental factors which lead to high amounts of individual variation. Animal subjects, however, can be used to establish a cleaner association between prenatal factors and postnatal outcomes, in part because environmental factors can be tightly controlled in the laboratory. Hippocampal Growth Associated Protein-43 (GAP-43) and phosphorylated GAP-43 (pGAP-43), which are involved in neurodevelopmental organization, are increased in prenatally stressed rat pups but not in older rats (Afadlal et al., 2010). Serotonin 1-A (5HT-1A) receptors are reduced in the hippocampus of 1-month old prenatally stressed rats (Van den Hove et al., 2006). Dopamine receptors are increased in frontal cortex and hippocampus while glutamate NMDA receptors are decreased in adult animals that experienced prenatal restraint stress (Berger et al., 2002). Dopamine turnover has been shown to be decreased following prenatal stress (Alonso et al., 1994). Both adolescent and adult rats show increased basal and amphetamine stimulated dopamine output in the nucleus accumbens following prenatal restraint stress (Silvagni et al., 2008). Gene expression levels of NMDA receptor subunits are also altered in young adulthood by prenatal stress (Kinnunen et al., 2003). Dendritic spine density, length and complexity have been shown to be decreased in adolescence following prenatal stress (Murmu et al., 2006) though another study saw no decrease in spine density but rather alterations in ratio of spine type in adult rats (Michelsen et al., 2007).

Much research has also gone into studying the behavioral effects of prenatal stress and many schizophrenia-related phenotypes have been observed in prenatally stressed offspring. Prenatal stress has been shown to induce deficits in spatial learning in adult mice (Son et al., 2006), juvenile rats (Wu et al., 2007), adult rats (Lemaire et al., 2000; Zagron and Weinstock, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Yaka et al., 2007) and guinea pigs stressed at prenatal day 50 but not 60 (Kapoor et al., 2008). In a virtual Morris water maze experiment schizophrenic patients were also shown to have a deficit in this type of spatial learning (Hanlon et al., 2006). A caveat to much research that has been done in the field of prenatal stress is that there are many differing ways of administering the stressors, ranging from single exposures to predators to restraint stress throughout the gestational period. While results are often similar between prenatal stress protocols, it is not uncommon to have differing results across studies. Additionally, stressors in animals are not necessarily comparable to stressors that humans undergo in daily life. A final pitfall to the most oft used prenatal stress paradigm, repeated restraint stress, is that the animals are able to habituate to the stressor, leading to a blunted hypothalamic-pituitary-adrenal (HPA) axis response in the later days of the stress procedure. In an attempt to alleviate this concern our laboratory utilizes a repeated variable prenatal stress (RVPS) paradigm in which pregnant rat dams are subjected to a randomized series of stressors during the final week of gestation, which precludes the possibility of habituation to a single stressor. We have previously shown that male rats exposed to RVPS exhibit phenotypes including hypersensitivity to amphetamine, blunted sensory gating, disrupted social behavior, impaired stress axis regulation, and aberrant prefrontal expression of genes involved in synaptic plasticity (Kinnunen et al., 2003; Koenig et al., 2005; Lee et al., 2007). These RVPS induced alterations have correlates to schizophrenia as well. Dopamine release in the striatum is increased following amphetamine injection in schizophrenic patients and is associated with positive symptoms (Laruelle et al., 1996). Sensory motor gating is decreased in schizophrenia (Braff et al., 2006) and social cognition is one of the subtypes of cognitive deficits in schizophrenia defined by MATRICS (Green et al., 2004).

Specific Aim 1

The first specific aim will involve using control and RVPS animals to test the hypothesis that RVPS results in cognitive deficits similar to certain deficits observed in schizophrenia. We predict that we will see increased latency to find a hidden escape platform in the Morris Water Maze. Further, we predict that RVPS rats will show deficits in both reference and working memory, as well as long-term memory during various iterations of the Can Test, a non-stressful learning test.

Neuregulin-1

The genetics of schizophrenia are proving to be extremely complex, as genome-wide association studies have shown (Duan et al., 2010). However, certain genes have been repeatedly associated with schizophrenia, including the neuregulin-1 gene (*NRG1)* (Stefansson et al., 2002; Stefansson et al., 2003; Liu et al., 2005; Walss-Bass et al., 2006; Alaerts et al., 2009; Nicodemus et al., 2009). *NRG1* codes for a family of growth factors (NRG1 types I - VI) which are involved in several facets of neurodevelopment, many of which are proposed to be involved in the pathogenesis of schizophrenia (Mei and Xiong, 2008).

NRG1 acts on ErbB receptors, which are a family of single transmembrane tyrosine kinases. The receptor ErbB4 is NRG-1 specific and interacts with as well as is activated by NRG1, while ErbB2, in order to be active heterodimerizes with other ErbBs (Tzahar and Yarden, 1998). ErbB3 is able to bind NRG1 but is inactive (Guy et al., 1994). ErbB4, but not ErbB2 or ErbB3 mutations in mice, produce symptoms similar to those seen in schizophrenia (Gerlai et al., 2000). Thus ErbB4 appears to be the most critical NRG1 receptor (Mei and Xiong, 2008). NRG1-ErbB signaling is critically involved in neuronal migration and neuron-glia interactions in the developing cerebellum (Rio et al., 1997). In the cerebral cortex, NRG1 type II promotes cortical neuron migration along radial glial fibers, as well as promoting maintenance and elongation of said glial fibers (Anton et al., 1997). Loss of ErbB4 leads to altered gamma-aminobutyric acid (GABA) interneuron populations in the cortex, by upsetting migration of the interneurons (Flames et al., 2004). NRG1 also has a role in axonal guidance and outgrowth. Gerecke et al., (Gerecke et al., 2004) have shown that NRG1beta stimulates extension of hippocampal axons via signaling through ErbB receptors. In addition, it has been shown that NRG1 is involved in the guidance of the developing thalamocortical axons (Lopez-Bendito et al., 2006).

The Ig-NRG1 isoform, which contains an immunoglobulin domain, induces expression of the beta2 subunit of GABAa receptors, which in turn leads to an increase in functional GABA receptors in cultured granule cells (Rieff et al., 1999). However, it has also been shown that in hippocampal slices, NRG1 actually leads to decreases in GABAa alpha subunits as well as reducing the amplitude of miniature inhibitory postsynaptic currents in CA1 pyramidal neurons (Okada and Corfas, 2004). In cultured cerebellar slices, NRG1beta increases NMDA receptor subunit NR2c mRNA dramatically (Ozaki et al., 1997).

NRG1 is also involved in synaptic maturation and plasticity. Li et al. (Li et al., 2007) have shown that synaptic activity activates the NRG1-ErbB4 signaling pathway which leads to stabilization of the AMPA receptor in the synapse. It is quite interesting that in this study synaptic activity was required for NRG1-ErbB4 signaling while the NRG1-ErbB4 signaling in turn enhanced synaptic function as seen by increased excitatory postsynaptic currents in neurons overexpressing ErbB4 and decreased in neurons underexpressing ErbB4 (Li et al., 2007). ErbB4 is located in the postsynaptic density and interacts with PSD-95 (Huang et al., 2000). NRG1 stimulates phosphorylation of the NMDA receptor subunit NR2b (Bjarnadottir et al., 2007).

Mice with a heterozygous knockout for the transmembrane domain of *Nrg1* (NRG1 TM) have hypophosphorylation of NR2b, as well as altered short term synaptic plasticity and long-term potentiation (LTP), both of which are reversible by incubation with NRG1 (Bjarnadottir et al., 2007). NRG1 also has effects on the GABA system. ErbB4 is located in prefrontal cortical GABAergic neuron terminals and NRG1treatment increased evoked GABA release without affecting basal GABA release, an effect that was not seen in mice with mutated *ErbB4* (Woo et al., 2007). With schizophrenia being seen as a disorder stemming from alterations in normal neuronal development, and the clear role for NRG1 in said development, it has been hypothesized that the underlying pathology of schizophrenia and psychosis could result from aberrant NRG1 signaling (Mei and Xiong, 2008)

Mouse models of decreased NRG1 function have yielded further evidence that NRG1 is involved in various aspects of cognition, which may also be implicated in schizophrenia. Stefansson et al (Stefansson et al., 2002) showed that NRG1 TM mice or *ErbB4* hypomorphic mice were hyperactive and had alterations in prepulse inhibition compared to wildtype (WT) mice, though both of these effects were more robust in the NRG1 TM mice than the ErbB4 mice. In addition, the NRG1 TM mice had fewer functional NMDA receptors than WT mice. The hyperactive phenotype has been confirmed (Karl et al., 2007) and expanded to show that response to social novelty is impaired (O'Tuathaigh et al., 2007), social interactions are altered via increased aggressive following behaviors (O'Tuathaigh et al., 2008), and some subtle cognitive alterations have been observed (Duffy et al., 2010). In addition, NRG1 TM mice have increased levels of serotonin-2A (5HT-2A) receptors (Dean et al., 2008).

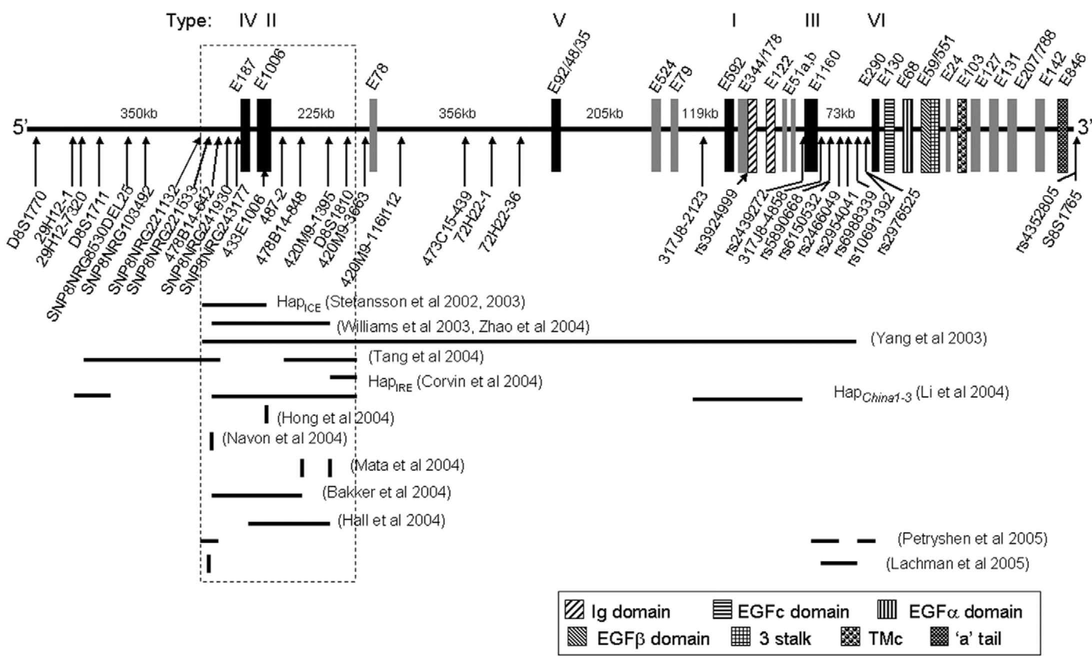
The above studies provide some confirmation that NRG1 may be involved in schizophrenia related behaviors. However, the regions of the gene deleted, i.e. the transmembrane domain, the epidermal growth factor (EGF) domain and the immunoglobulin domain, are in the 3' region of the gene, while the majority of studies have found associations between schizophrenia and the 5' region of *NRG1*, which codes for type II and IV NRG1. An examination of the literature on *NRG1* associations with schizophrenia has shown that 9 studies have found their association residing in the more 5' region of the gene, though one study found an association with virtually the entire gene and 3 studies found their association with regions that were more 3' (Harrison and Law, 2006) (Figure 1.1). We are utilizing a line of rats with disrupted *Nrg1* ($Nrg1^{Tn}$) which were generated with the Sleeping Beauty transposon (Lu et al., 2007). The transposon inserted into the region of the *Nrg1* gene which lies downstream of the coding sequence for Type II NRG1 protein isoforms. Using this unique preparation we have found that the rats do indeed show decreases in both Type II *NRG1* mRNA and protein as well as alterations in stress behavior and physiology (Taylor et al., 2010b).

Specific Aim 2

Utilizing these animals we will test the hypothesis of the second specific aim, that the disruption of *Nrg1*, which leads to a decrease of type II NRG1 protein, results in cognitive deficits similar to certain deficits observed in schizophrenia. Specifically, we predict that *Nrg1*^{Tn} animals will show deficits in spatial learning, as well as deficits in reference and working memory as well as alterations in NMDA NR1.

Figure 1.1: Human Neuregulin-1 Gene Structure

Exons are represented as vertical bars, numbered as per Steinthorsdottir et al (2004). The 5’ exons, which define the “types” of NRG1, are in black, with the corresponding Roman numeral above. Exons giving rise to key functional domains are patterned, per the box; other exons are in grey. Below the gene are the polymorphisms that define the 5’ and 3’ limits of markers used in each schizophrenia study and that define the 5’ and 3’ ends of the various risk haplotypes thus identified. Horizontal lines denote the extent of each risk haplotype. The dashed rectangle shows the region wherein most positive associations have been found. The figure is constructed largely from information in Steinthorsdottir et al (2004) and from Falls (2003b) and Petryshen et al (2005). Reprinted from Biological Psychiatry, Vol. 60, Issue 2, Paul Harrison and Amanda Law, Neuregulin 1 and Schizophrenia: Genetics, Gene Expression and Neurobiology, 132-140, 2006, with permission from Elsevier.



Gene x Environment Interaction

It is generally accepted that schizophrenia arises from an interaction between genetic and environmental risk factors (Tsuang et al., 2004) and much of the heritability may be due to these interactions (van Os et al., 2008). Though many susceptibility genes have been discovered, none actually seem to cause specific functions to cease, but more likely affect pathways leading to enhanced likelihood of developing the disease. In other words, when it comes to psychiatric diseases like schizophrenia, identified genes do not cause the disease, but are involved indirectly (Rutter et al., 2006).

While some estimates place the genetic liability for schizophrenia at around 80 percent, monozygotic twins have only a 50 percent concordance rate, clear evidence that environmental factors must play a critical role (Mittal et al., 2008). A person who is at genetic risk for developing the disorder can be triggered to actually become ill by environmental events. For example, Tienari et al. (Tienari et al., 2004) have shown that adoptees at high genetic risk for schizophrenia are more likely to develop the disorder if the adoptive family is more ‘dysfunctional’. Neither the genetic risk nor the home environment alone predicted schizophrenia, indicating an interaction between the genetic risk and the environmental context. In a Finnish sample, it was observed that offspring of mothers with schizophrenia were more likely to show signs of thought disorder (a common symptom subset in schizophrenia) if they were raised by an adoptive mother who also showed higher levels of 'communication deviance' (Wahlberg et al., 1997). In monozygotic twins, who share 100 percent of their genes, higher levels of obstetric complications have been seen in the twin affected with schizophrenia (McNeil et al., 1994). Clarke et al. (Clarke et al., 2009) showed that prenatal exposure to infection increased the risk for developing schizophrenia only with a family history of the disease. Genetic liability to non-affective psychosis and being raised in a low socioeconomic adoptive household both individually increased the risk for developing psychosis. Having both a genetic liability and being raised in the low socioeconomic household increased the risk more than the combined sum of both risk factors (Wicks et al., 2010).

There have also been experiments conducted in laboratory animals which extend the findings in humans. Animal studies have the advantage over epidemiological studies in humans that conditions in the laboratory can be tightly controlled to study very specific environmental occurrences with very specific genetic mutations. In mice that are lacking pituitary adenylate cyclase-activating polypeptide (PACAP-/-) isolation rearing for two weeks, starting at four weeks old, led to hyperlocomotion and increased aggressive behaviors which were not seen in control mice subjected to the same conditions (Ishihama et al., 2010). Mice with mutated human disrupted in schizophrenia-1 (mhDISC-1) who experienced prenatal immune activation showed behavioral abnormalities, including social behavior alterations and depressive-like behaviors which were not seen in either untreated mhDISC-1 mice or control animals who experienced prenatal immune activation (Abazyan et al., 2010). In addition, mice expressing a dominant-negative DISC1 (DN-DISC1) that experienced prenatal immune activation showed deficits in short term and object recognition memory compared to either WT animals that had prenatal immune activation or DN-DISC1 mice that did not (Ibi et al., 2010). Oliver and Davies

(Oliver and Davies, 2009) showed that the *blind-drunk* point mutant (*Bdr),* mice with a mutation to synaptosomal-associated protein of 25 kDa (Snap-25), have a deficit in prepulse inhibition which is greatly increased if they also experienced variable prenatal stress, and reversed with antipsychotic drug treatment. The prenatally stressed *Bdr* mice also showed alterations in social behaviors which were not seen in either WT mice that had undergone prenatal stress or in control *Bdr* mice. Taken together, the studies conducted in humans and animals clearly point to a role for gene x environment interactions both leading to increases in risk for schizophrenia and psychosis as well as producing phenotypes that may be relevant to symptoms seen in schizophrenia.

NRG1 is a well accepted risk gene for schizophrenia, though there is not yet evidence that it actually causes schizophrenia per se. We have shown that $Nrg1^{Tn}$ rats have altered stress responsively compared to WT rats (Taylor et al., 2010b). In addition, several studies have shown *Nrg1* genotype to interact with environmental factors to produce unique phenotypes (Boucher et al., 2007b; Hintsanen et al., 2007; Karl et al., 2007). *Nrg1* transmembrane domain mutant mice have been shown to be more sensitive to the behavioral effects of tetrahydrocannabinol (THC) than wild type mice (Boucher et al., 2007a). These mice also showed an increase in THC induced c-fos expression in the paraventricular nucleus of the hypothalamus. Mice which had not been treated with THC showed an increase in levels of c-fos in the nucleus accumbens shell and lateral septum. Interestingly, these c-fos level alterations were only observed in mice that had been subjected to behavioral testing (Boucher et al., 2007b). The authors concluded that the stress of testing interacted with the genetic manipulation to produce this effect. This may indicate that an interaction between altered NRG1 signaling and stress is necessary to produce these behavioral and physiological changes. Therefore, *Nrg1* mutant mice show some traits similar to those seen in schizophrenia and in some cases require an environmental interaction for effects to be observed. Additionally, in humans, a particular single nucleotide polymorphism (SNP) for *NRG1* (SPN8NRG221533; T to C) was found to mediate the effect of job strain on atherosclerosis. This indicates that *NRG1* genotype may determine susceptibility for stress induced development of atherosclerosis (Hintsanen et al., 2007).

Specific Aim 3

In order to examine the interactions between an environmental risk factor, prenatal stress, and a genetic risk factor, disrupted *Nrg1*, we have exposed pregnant female $Nrg1^{Tn}$ rats to our RVPS procedure. Using the offspring of RVPS control and $Nrg1^{Tn}$ rats we have examined in the third specific aim the hypothesis that the combined presence of a disruption in *Nrg1* and RVPS results in an additive cognitive deficit than either alone. Specifically we predicted that the rats which are both $Nrg1^{Tn}$ and RVPS would show greater deficits in spatial memory and in reference and working memory.

Significance and Pitfalls

The significance of this work is multi-faceted. As schizophrenia is a devastating, often crippling psychiatric disease, any knowledge gained into the causes of the disease or possible causes of specific symptoms is invaluable information. In this thesis we have studied three different factors that are believed to be involved in either increasing risk for schizophrenia (prenatal stress), a genetic component involved in schizophrenia risk (*NRG1*) and how such risk factors may interact when experienced in tandem.

First, by studying the behavioral outcomes that rats present in adulthood following a prenatal stress regimen that more closely resemble the types of stress experienced by humans, may lead to a more complete understanding of what symptoms of schizophrenia are affected by that risk factor. In addition, it has been shown multiple times in humans that prenatal stress can affect cognitive development. With a more complete understanding of what cognitive systems are affected by prenatal stress it will become more likely to be able to anticipate problems in at risk individuals and attempt preventative treatments to alleviate the development of cognitive deficits.

Secondly, we have examined for the first time a rat with a genetic disruption to *Nrg1*. While mice with mutations to *Nrg1* have been examined in some detail in their neurochemistry and some behavioral tasks, the region of the gene typically knocked out is not generally the same region that associates with schizophrenia in humans. In our preparation the region of the gene that the transposon inserted is in the region that corresponds to most association studies in schizophrenia (Harrison and Law, 2006). In addition, cognitive studies in mice have not yet examined higher order cognitive abilities. In these studies we have examined the change in learning and memory in several challenging behavioral tasks in rats with a mutation to a known schizophrenia risk gene in the region that associates with schizophrenia. The information gained in these studies will not only continue to validate *NRG1* in schizophrenia related behaviors but will also help to understand the more general role of *NRG1* in learning and memory.

Finally, the current direction in studying psychiatric diseases is the idea that these diseases are not 100 percent determined by genetics or environmental occurrences, but instead are a result of a complex interplay of these factors. In order to show how two specific risk factors for schizophrenia might interact we have examined cognitive abilities in animals with disrupted *Nrg1* who have also been subjected to RVPS. Very few studies have yet examined in much detail how genetic risk factors and environmental risk factors may act synergistically in the laboratory, as they must do in typical human life, especially on cognitive endpoints. Therefore this work will be one of a very limited group of experiments to begin to tackle the question of behavioral outcomes following these interactions.

We have utilized behavioral experiments designed to test multiple aspects of learning and memory, which have corollaries to human learning and memory and to deficits in cognition seen in schizophrenia. The Morris water maze is a spatial learning test that is dependant on the hippocampus (Morris et al., 1982) and schizophrenic patients show deficits on this task (Hanlon et al., 2006). The Can Test (Popovic et al., 2001) is a discrimination learning paradigm designed to test reference and working memory and can also be utilized to study long-term memory. We have also adapted the task to be used to test discrimination learning to a specific criteria in multiple ways. Patients with schizophrenia have deficits in working memory (Lee and Park, 2005; Forbes et al., 2009) and are also deficient in long-term memory (Aleman et al., 1999). A two-choice discrimination task, which is similar to the Can

Test, has shown that the dorsal striatum is critical to the learning of the discriminations (Broadbent et al., 2007) and the prefrontal cortex is also implicated in working memory in schizophrenia (Goldman-Rakic, 1995; Ragland et al., 2007; Van Snellenberg, 2009). Thus our experiments will determine if specific risk factors for schizophrenia lead to certain cognitive deficits that are seen in patients with the disease.

Unfortunately, we did experience multiple difficulties during this dissertation, which will be described in more detail in the appropriate sections. Briefly, our first specific aim, namely that RVPS would result in cognitive deficits was performed in Sprague Dawley rats, while the *Nrg1*^{Tn} rats were on a Fischer-344 background. We discovered that while Sprague Dawley rats are capable of intricate behavioral experiments, this was not true of the Fischer rat strain in our hands. Therefore, though many of the same experiments are done in each aim, they had to be tailored for the specific strain that was in each aim. In addition, we had a limited supply of *Nrg1*^{Tn} rats with which to work. This led to the full array of experiments we wanted to perform being curtailed slightly, as well as lowering the number of animals that were available for each experiment, particularly in the gene x environment interaction studies.

Taken in total this thesis puts forth data supporting the hypothesis that RVPS results in cognitive deficits which are similar to certain deficits observed in schizophrenia; that disruption of *Nrg1* results in cognitive deficits similar to certain deficits observed in schizophrenia; and finally, contrary to our hypothesis we present preliminary evidence that the combined presence of a disruption in *Nrg1* and RVPS results in an additive cognitive deficit than either alone.

Hypothesis and Aims

The research in this thesis has been designed to test the hypotheses that RVPS results in cognitive deficits similar to certain deficits observed in schizophrenia.; that disruption of *Nrg1* leading to decreased type II NRG1 results in cognitive deficits similar to certain deficits observed in schizophrenia.; the combined presence of a disruption in *Nrg1* and RVPS results in an additive cognitive deficit than either alone.

Specific aim 1

To determine the learning and memory abilities in adult male rats that have experienced RVPS.

Experiments

(1). To determine the effect of RVPS on hippocampal-dependent spatial memory using the Morris water maze.

(2). To determine the effect of RVPS on reference memory, working memory and long-term memory using multiple versions of the Can Test.

(3). To determine the effect of RVPS on the ability to master a discrimination and switch from different sets of rules using a version of the Can Test which tests days to reaching a specific criteria.

Specific aim 2

To determine the effect of disrupted *Nrg1* leading to decreased type II NRG1 in higher cognitive abilities in adult male rats.

Experiments

(1). To determine the effect of disruption of *Nrg1* on spatial learning ability in adult male rats, using the hippocampal-dependent Morris water maze.

(2). To determine the effect of disruption of *Nrg1* on reference memory and working memory abilities using the spatial/visual object discrimination version of the Can Test.

(3). To determine whether disruption of *Nrg1* alters NMDA receptor subunit NR1 protein expression in prefrontal cortex, dorsal striatum and hippocampus.

Specific aim 3

To examine the gene x environment interaction effect of combined RVPS and disrupted *Nrg1* leading to decreased type II NRG1 in higher cognitive abilities in adult male rats.

Experiments

(1). To determine the interactive effect of RVPS and disrupted *Nrg1* on spatial learning ability using the hippocampal dependent Morris water maze.

(2). To determine the effect of RVPS and disrupted *Nrg1* on reference memory and working memory abilities using the spatial/visual object discrimination version of the Can Test.

Chapter 2: Cognitive Deficits Induced by Prenatal Stress

Introduction

The cause of schizophrenia is unknown, however there is strong evidence that both genetic factors and environmental factors contribute to the development of the disease. Research into the environmental risk factors that contribute to schizophrenia risk has been ongoing for several decades. Many environmental insults have been shown to contribute to risk for schizophrenia, both prenatal and throughout life. It has been shown, through retrospective epidemiological studies, that prenatal exposure to influenza (Mednick et al., 1988; Kendell and Kemp, 1989; Brown et al., 2004), rubella (Brown et al., 2000; Brown et al., 2001) and toxoplasma gondii (Brown et al., 2005; Mortensen et al., 2007) increase risk for schizophrenia. In addition, many other stressful events that occur during pregnancy have also been shown to increase risk for schizophrenia. These stressors include death of the father before birth (Huttunen and Niskanen, 1978) or a close relative during the first trimester of pregnancy (Khashan et al., 2008); if the pregnancy is unwanted (Myhrman et al., 1996); the German invasion and defeat of the Netherlands in 1940 (van Os and Selten, 1998); and exposure to the Chinese famine of 1959-1961 (Xu et al., 2009). However, other studies have failed to find a link between either a deadly flood in the Netherlands in 1953 (Selten et al., 1999) or multiple wars in Israel (Selten et al., 2003) and increased schizophrenia risk in the offspring. In order to be clear, however, it must be noted that prenatal stress is not being considered a cause of schizophrenia; it is more simply an insult during development which has been shown to increase the risk for developing the disease later in life. In addition, prenatal stress may also act as a trigger for the disease in a genetically susceptible individual.

Prenatal stress not only increases risk for developing schizophrenia, but has also been shown to alter many behaviors and physiological indices in both humans and laboratory animals. In humans prenatal stress has been shown to lead to infants having worse cognitive outcomes than seen in infants that were not exposed to prenatal stress, as indexed by the Bayley Mental Development Index (Buitelaar et al., 2003; Laplante et al., 2004; Bergman et al., 2007). Exposure to cortisol during gestation, as measured by amniotic fluid levels, and independent of other prenatal, obstetric and socioeconomic factors, negatively predicts infants cognitive abilities (Bergman et al., 2010). This effect also seems to interact with the postnatal environment as a secure mother-infant attachment ameliorates this effect (Bergman et al., 2010). The timing of exposure to cortisol during gestation is also an important factor as it has been shown that the rate of development over the first year of postnatal life is slower for those individuals who experienced high levels of cortisol early in gestation (Davis and Sandman, 2010). However, another study has shown that children at the ages of either 47 or 81 months of age (approximately 4 or 7 years, respectively) have higher rates of behavioral and emotional problems if their mother experienced high anxiety in the later stages of pregnancy (O'Connor et al., 2002; O'Connor et al., 2003). Prenatal exposure to a severe natural disaster has also been associated with decreased cognition in 5 ½ year old children (Laplante et al., 2008). We have found only one study in humans that has examined the effect of prenatal stress on cognitive abilities outside of childhood. Entringer et al., (Entringer et al., 2009) have shown that healthy young women, mean age 25 years, whose mothers experienced high levels of psychosocial stress, defined as suffering a “major negative life event" during pregnancy, have longer reaction times in an item-recognition task, an indicator of working memory performance, when being tested after a 10mg dose of cortisol.

While the above is clear evidence that prenatal stress has a myriad of unwelcome outcomes throughout life, it is virtually impossible to design experiments in the human population that can answer specific questions about what effects occur from prenatal stress. For instance, the above mentioned studies are retrospectively looking at an entire population that either underwent a stressful situation together and then looked for an increase in schizophrenia in that population or used levels of stress hormones or questionnaires about stress experienced during pregnancy to determine the prenatally stressed group. While these measures are perfectly valid techniques, they are not able to separate out variables such as individual differences to stress vulnerability or that retrospective questionnaires about stress levels during pregnancy can be tainted by incorrect memories about the experience. Therefore studies using animals in the laboratory are very important in untangling the exact outcomes following strictly controlled stress paradigms. However, there are also caveats to be considered using animal models of prenatal stress. First and foremost, laboratory animals are not humans and will necessarily have different reactions to prenatal stress. This is an inescapable factor in animal research that must be accepted. However, the animals are very similar to humans and indeed do generally show similar reactions as seen in humans. Secondly, throughout the literature there are multiple different protocols for administering prenatal stress which can result in different outcomes between laboratories. In addition, the most common form of prenatal stress, namely repeated restraint stress during the final week of gestation, allows for the animals to habituate to the stressor and likely not show as strong of a change in glucocorticoid secretion toward the end of pregnancy. Finally, similar to the first point, stressors used in animal models are not the same as stressors that humans experience in their daily lives. The stress paradigm we use is unpredictable and includes stressors such as restraint, social crowding and swim which may have an emotional aspect to them. Animal studies of prenatal stress also have the advantage of being tightly controlled with specific hypotheses leading to a solid understanding of both physiological outcomes following prenatal stress and behavioral outcomes throughout the lifespan of the prenatal stress exposed animals.

Great amounts of knowledge into the molecular and physiological outcomes following prenatal stress have already been elucidated. For example, two proteins that are involved in neurodevelopmental organization, GAP-43 and pGAP-43, have been shown to be increased in rat pups, but not in older rats that were prenatally stressed (Afadlal et al., 2010). Expression levels of NMDA receptor subunit genes as well as proteins associated with glutamatergic synapses have also been shown to be altered by prenatal stress in young adult rats (Kinnunen et al., 2003). Complexity, length and density of dendritic spines are decreased in prenatally stressed adolescent rats (Murmu et al., 2006). However, Michelsen et al (Michelsen et al., 2007) saw alterations in spine type ratio but no decrease in spine density in adult rats. One-month old prenatally stressed rats also have reduced 5HT-1A receptors in the hippocampus than non-stressed rats (Van den Hove et al., 2006). Prenatal stress decreases dopamine turnover (Alonso et al., 1994) and frontal cortical and hippocampal dopamine receptors are increased while NMDA receptors are decreased (Berger et al., 2002). Basal and amphetamine stimulated dopamine output in the nucleus accumbens is increased in adolescent and adult prenatally stressed rats (Silvagni et al., 2008).

Much research has also gone into studying the behavioral effects of prenatal stress. Prenatal stress has been shown to induce deficits in spatial learning in adult mice (Son et al., 2006), juvenile rats (Wu et al., 2007), adult rats (Lemaire et al., 2000; Zagron and Weinstock, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Yaka et al., 2007) and guinea pigs stressed at prenatal day 50 but not 60 (Kapoor et al., 2008). Anxiety behaviors were found to be increased in rats following prenatal restraint stress, though this study did not find differences in spatial memory (Vallee et al., 1997). A decrease in social interaction has also been seen in adolescent rats following a mild chronic variable prenatal stress paradigm (Cabrera et al., 1999). A pain free, psychological stressor in rats has even been shown to alter anxiety related behaviors in the open field test and immobility in the forced swim test (Abe et al., 2007).

Clearly, prenatal stress has been shown to lead to many behavioral and physiological consequences which can be related to those seen in schizophrenia. However, as mentioned above, the prenatal stress paradigms are varied and may allow for habituation to the stressor if it is a repeated stress. Therefore, in our laboratory we use a random variable prenatal stress paradigm (RVPS) designed to eliminate the possibility of habituation to the stressors. Our paradigm consists of randomizing the presentation of various stressors during the last week of gestation.

Our stressors include 1hr of restraint stress, 15 minutes of forced swim, 6 hours of exposure to a cold room environment, overnight social crowding, 24 hour fasting and 1.5 hours of exposure to an inch and a half of water in an otherwise empty cage (see table 2.1). We have previously shown that male rats exposed to RVPS exhibit phenotypes resembling certain aspects of the schizophrenia symptoms domains including hypersensitivity to amphetamine, blunted sensory gating, disrupted social behavior, impaired stress axis regulation, and aberrant prefrontal expression of genes involved in synaptic plasticity (Kinnunen et al., 2003; Koenig et al., 2005; Lee et al., 2007).

Table 2.1: Repeated Variable Prenatal Stress Protocol

		Time of Day		
Gestation Day		AM	Mid-day	PM
14		Restraint – 60 min	Swim – 15 min	Restraint – 60 min
15		Swim – 15 min	Restraint – 60 min	Swim – 15 min
16		Cold Exposure – 6 hr (0900 hr-1500 hr)		Fast – 24 hr
17		Inch of water in bottom of cage—90min (in at 8:30)	Swim –15 min	Restraint – 60 min
18		Social Stress – 12 hr		
19		Restraint – 60 min	Swim – 15 min	Restraint – 60 min
20		Swim – 15 min	Restraint – 60 min	Swim –15 min, fast overnight
21		Cold Exposure – 6 hr (9 AM-3PM)		
22		Restraint	Swim at noon (unless birthing began)	

Based on the available literature and previous work from our laboratory the first specific aim of this dissertation is based on the hypothesis that RVPS will affect the higher cognitive abilities of male rats in adulthood. We will test this hypothesis by having adult male rats perform the spatial, hippocampal dependent version of the

Morris water maze. We also had adult male rats perform many variations of a visual and/or spatial discrimination learning task known as the Can Test. This test is able to parse several aspects of the learning and memory process, including reference and working memory, the amount of time it takes the animals to effectively 'master' these discriminations and also is very effective at testing the long-term memory retention of the learned discriminations. We predicted that RVPS rats would have decreased latencies in the Morris water maze. Additionally, we predicted that RVPS rats ability to learn discriminations in multiple versions of the Can Test would be decreased via increased errors, as well as their long-term memory being impaired.

Methods

Prenatal Stress

For all experiments, timed pregnant Sprague-Dawley female rats were purchased from Charles River Laboratories and arrived at our animal facility on day two of gestation. All pregnant animals were housed individually in a light-controlled (lights on 0700 hr to 1900 hr) and temperature-controlled facility, and all dams had free access to rat chow (Harlan Teklad) and water. Half of the dams were exposed to prenatal stress according to the procedure described below, while the other (control) dams were exposed to normal animal husbandry procedures. All procedures described herein conform to guidelines for animal research established by the National Institutes of Health, and were approved by the University of Maryland Baltimore School of Medicine Institutional Animal Care and Use Committee.

Beginning on day 14 and continuing through day 21 of gestation, pregnant dams were exposed to a repeated variable stress paradigm (Taylor et al., 2010a). The stressors used in this paradigm were: (1) restraint in a well-ventilated cylindrical plexiglas restrainer (Harvard Bioscience, Boston, MA) for one hour, (2) exposure to a cold environment (4^{o}C) for six hours, (3) overnight food deprivation, (4) prevention of sleep during the light (inactive) portion of the cycle for one hour, via 1 ½ inches of room temperature water in the bottom of an otherwise empty cage, (5) 15 minutes of swim stress, and (6) social stress induced by overcrowded housing conditions during the dark (active) phase of the cycle. Two to three stressors were administered per day in a randomized order (Taylor et al., 2010a). All dams delivered their pups vaginally. Following delivery, the dam and her pups were left undisturbed in their cages until weaning on postnatal day 24-25. At weaning, male and female offspring were housed in same-sex, same-treatment groups of two to three with free access to rat chow and water. The pups were exposed to normal animal husbandry procedures from that point forward until experimental use.

Spatial memory

Animals

Adult male Sprague-Dawley rats of both RVPS and control conditions (age 70 days) were included in this experiment. At the conclusion of testing, Grubbs' test for outliers identified one control animal as an outlier and this animal was excluded from further analyses. The final sample size was 15 animals (n=7 control, n=8 prenatal stress).

Morris water maze

Water Maze training was based on our previously published protocol (Taylor et al., 2010a). Behavioral testing was conducted between 0900 and 1500 each day. Prior to training, animals were habituated to the testing apparatus, which consisted of a circular tank, 1.8 meters in diameter with a depth of 0.9 meters (water depth 0.6 meters). The tank was located in a room with spatial cues external to the maze, including colored objects and posters hanging from the ceiling or taped to the walls. Animals were habituated to the testing apparatus over two days. On the first day, each animal was placed in the tank and allowed to explore it by swimming for two minutes, after which time it was towel dried and returned to its home cage. The following day the rats were exposed for two minutes to the testing apparatus which now contained an escape platform (4 exposures). For habituation to the escape platform and all training trials during the three days of the experiment, the tank water was made opaque by adding non-toxic white tempura paint, and a stable Plexiglas escape platform (13.5cm x 13.5cm) was submerged 2 cm below the water surface in the northwest (NW) quadrant of the maze. Training consisted of four trials per day with a 15 minute inter-trial interval, and continued for three consecutive days. For each trial, the animal was placed in the maze for two minutes, or until the escape platform was located. If after two minutes the animal had not located the escape platform, it was guided to it by the experimenter. Animals remained on the platform for 15 seconds, after which time they were towel dried and returned to their home cage. There were three start locations (NE, SW, SE) and start location was randomized across groups/trials. Following four consecutive days on which no handling or behavioral training occurred, animals were returned to the maze for two "retention" trials (conducted in the same way as the training trials). For both training and retention trials, latency to reach the platform was recorded in seconds, and an average latency per day was determined for each animal.

Discrimination memory

Animals

Adult male Sprague-Dawley rats (60-90 days of age at the beginning of behavioral testing) of both RVPS and control conditions were included in this experiment. In order to determine if being tested during their active phase and inactive phase affected the overall results, animals underwent behavioral testing during either the light/inactive phase (experiments performed between 09.00 and 15.00) or dark/active phase (experiments performed between approximately 17.00

and 03.00; testing room was illuminated with a red light). This resulted in the following groups: control (n=5 inactive, n=7 active) and RVPS (n=7 inactive, n=7 active).

Can test, Version 1

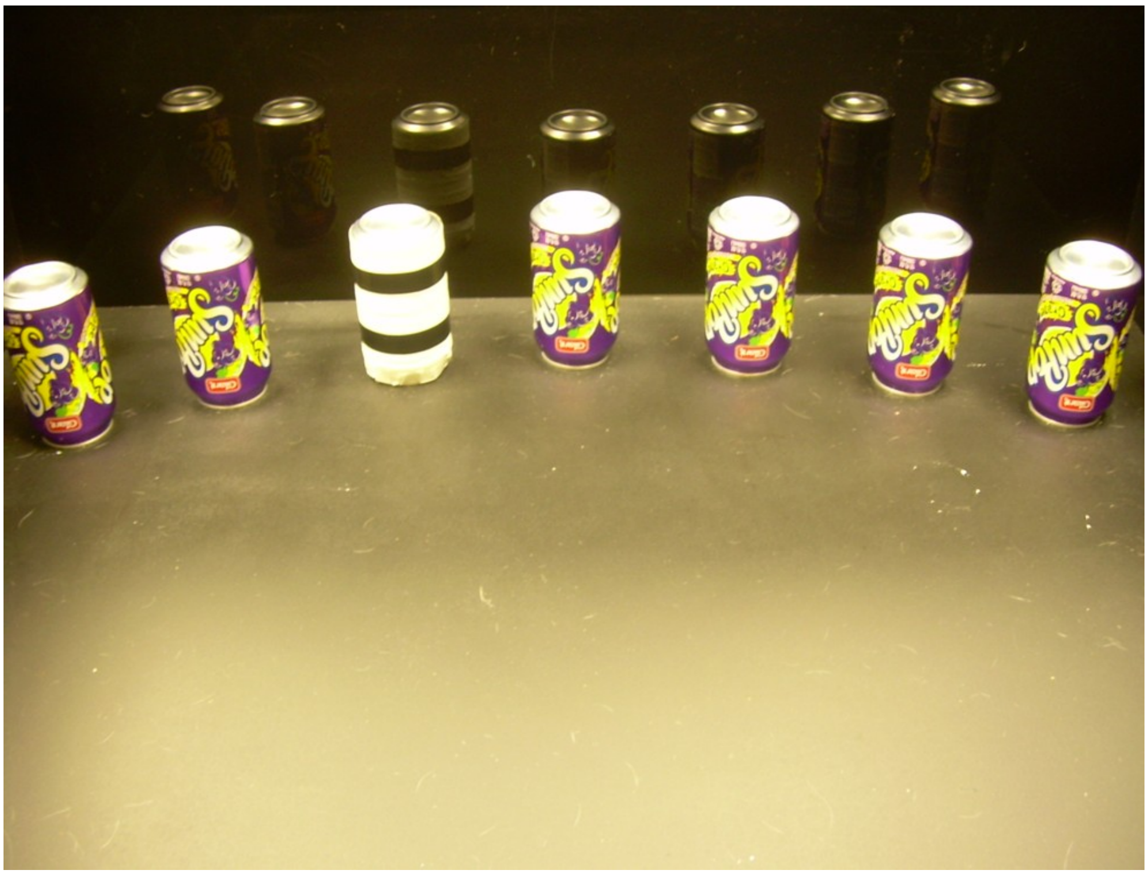
Habituation

The Can Test was first described by Popovic and colleagues (Popovic et al., 2001) and behavioral testing procedures described here were based on that paradigm, and modified by our laboratory (Taylor et al., 2010a). For five consecutive days, animals were handled for one to two minutes each; on the third day, animals were moved from the colony room to the behavioral testing room. Three consecutive days of habituation to the testing apparatus followed the five days of handling. On the first day of habituation each animal was allowed 20 minutes exploration of the behavioral testing apparatus, which consisted of a 100 x 100 x 46 cm box with black plexiglas walls. Animals were returned to the home cage without access to a water bottle, in order to facilitate motivation for finding the water reward during subsequent testing. On the second habituation day, seven identically patterned aluminum cans (filled with cement for weight, 13cm in height) were placed in the apparatus in a fan arrangement so as to be equidistant from the starting point. The cans were placed upside-down, in order to provide a smooth dish to contain a small bolus of water (0.3mL). Water was placed in each of the cans and each animal was allowed to explore the apparatus (with water-containing cans) for 20 minutes. On the third habituation day, the apparatus was set up as above, except with only 3 water-baited cans, and the animal was allowed to explore the apparatus until it found all three water rewards or 10 minutes had elapsed. Following consumption of the final reward, the animal was removed to a holding area while three new cans were baited. The animal was then reintroduced to the apparatus and again allowed to explore until finding the three rewards or ten minutes had elapsed. Following the 3 days of habituation, animals were left undisturbed for 2 days. Water was again removed from the cages the day before testing began (see below). After habituation and testing, rats were allowed access to water for a 45 minute period.

Spatial/visual discrimination test

Seven cans were arranged in the apparatus with the exception that one can was covered in white tape with a design of two black horizontal lines showing (Figure 2.1). Animals were allowed 10 trials per day over a 5 day period to learn that the water reward was always to be found in the unique can, the location of which remained stationary. Thus it was possible for animals to use either spatial or visual cues (or a combination) to learn the location of the water reward. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial, which commenced 15 – 30 seconds after the previous trial ended. A visit to a can was recorded when an animal stood on hind paws and looked for the water reward in the dish created by the can. A correct trial was recorded when the animal visited the rewarded can first. A reference memory error was scored the first time the animal visited a non-rewarded can prior to visiting the rewarded one. A working memory error was scored each time an animal revisited the same non-rewarded can during a given trial. Average correct trials, reference memory errors, and working memory errors were calculated on a per animal basis for each day of training. Following 5 days of training, animals were returned to the colony room for 2 weeks with ad libitum food and water, after which time they were again moved to the behavioral testing room and water-restricted. Twenty-four hours later, retention memory was tested over ten trials (two-week retention test).

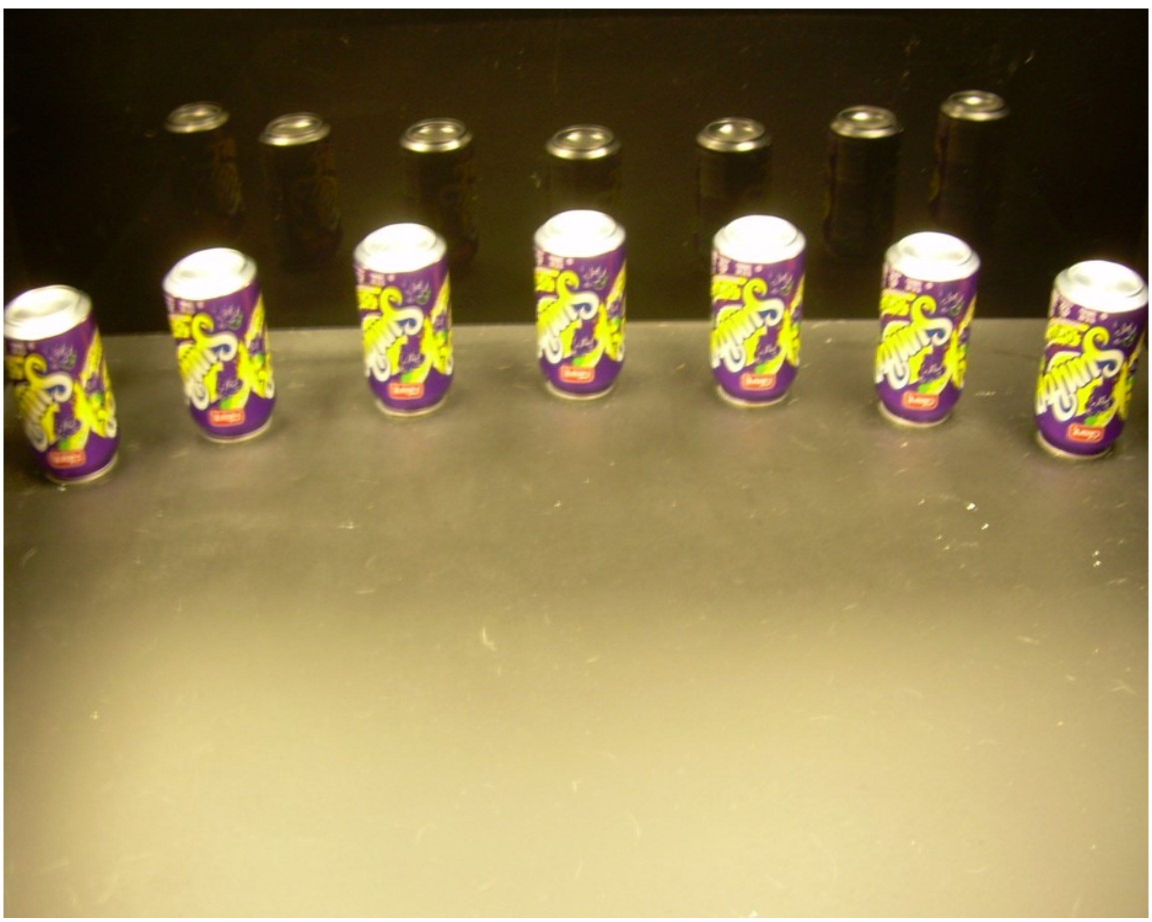
Figure 2.1: Spatial/Visual Discrimination Arena



Spatial discrimination test

Spatial discrimination testing began two days following the retention memory test for spatial/object discrimination. For this test, the testing apparatus was arranged as above, but this time with seven identical cans, thus eliminating the visual pattern cues (Figure 2.2). Animals were allowed 10 trials per day over a five day period to learn that the water reward was always in the same spatial location, a location which was different from the Spatial/Visual discrimination (for Spatial/Visual discrimination the rewarded can was in location 3, for Spatial Discrimination the rewarded can was in location 6). Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial. Correct trials, reference and working memory errors were scored as above. Following five days of training, animals were returned to the colony room for two weeks with ad libitum food and water, after which time they were again moved to the behavioral testing room and water-restricted. Twenty-four hours later, retention memory was tested over 10 trials (two-week retention test).

Figure 2.2: Spatial Discrimination Arena

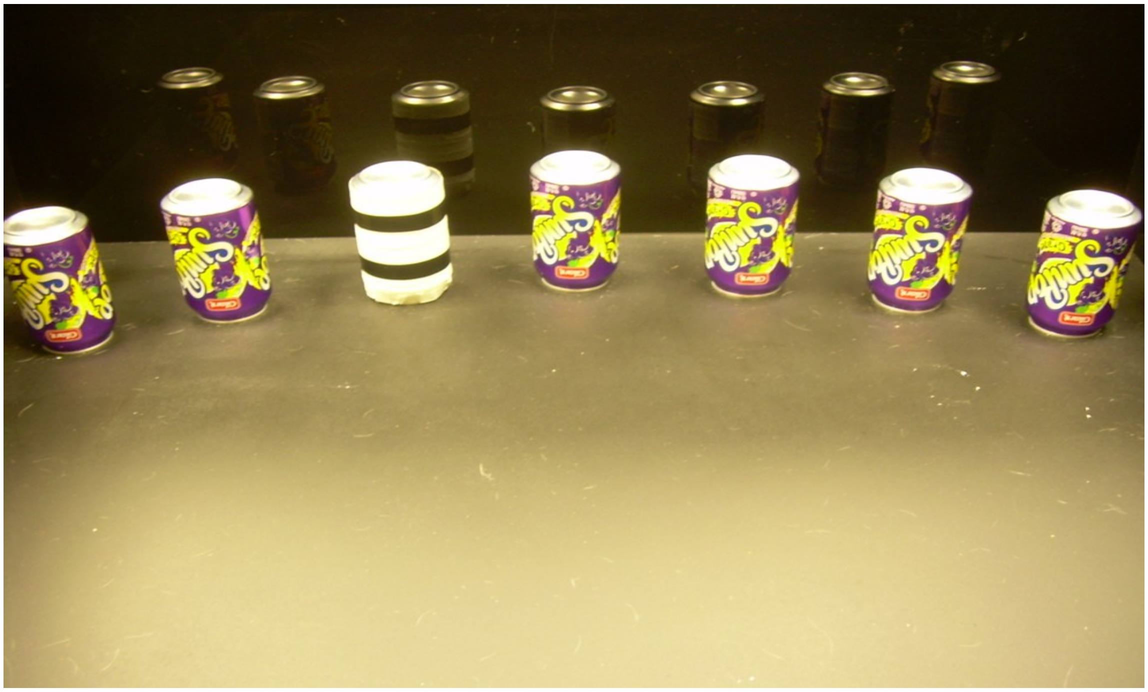


Visual discrimination test

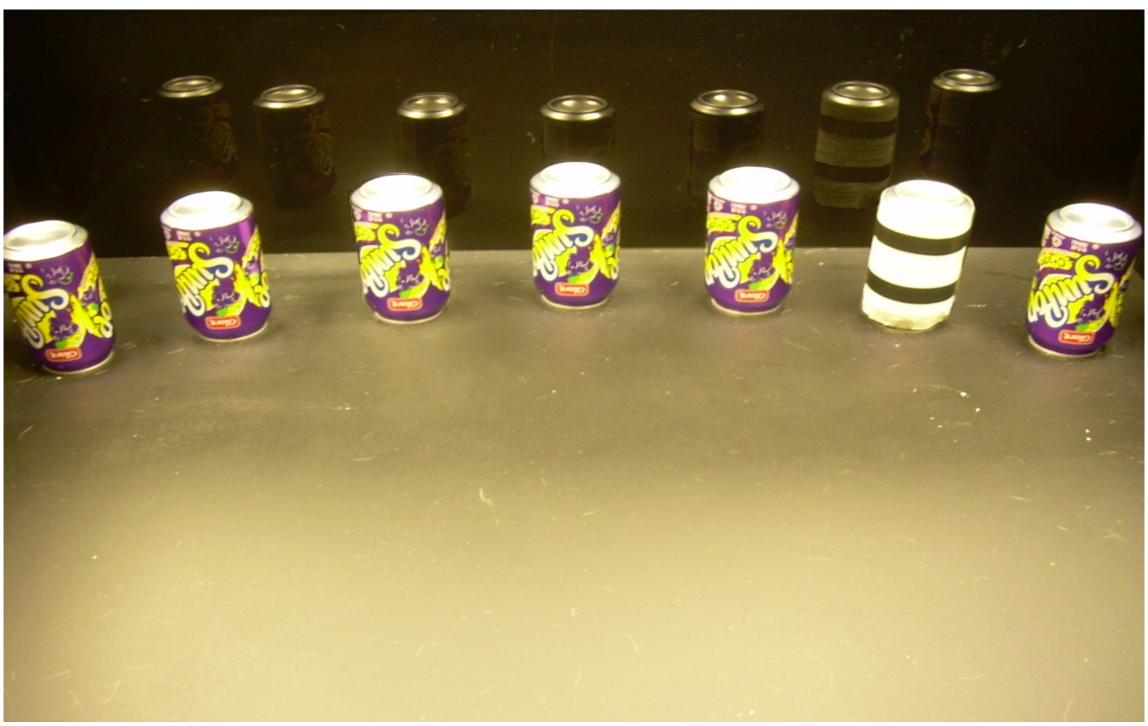
Visual discrimination testing began two days following the retention memory test for spatial discrimination. For this test, the cans were arranged similar to the spatial/visual discrimination test, with one can again covered in white tape with a design of two black horizontal lines showing. This can was again consistently rewarded; however, from trial to trial its location varied (Figure 2.3a and b). Animals were allowed 10 trials per day over a five day period to learn that the water reward was always to be found in the visually unique can, regardless of its spatial location. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited and moved for the next trial. Correct trials, reference and working memory errors were scored as above. Following 5 days of training, animals were returned to the colony room for 2 weeks, after which time they were again moved to the behavioral testing room and water-restricted. Twenty-four hours later, retention memory was tested over 10 trials (two-week retention test). Following the 2-week post-training retention test, animals were returned to the colony room and left undisturbed for a 2-month period, after which time they were again moved to the behavioral testing room for a final retention test of 10 trials (2-month retention test).

Figure 2.3: Visual Discrimination Arena

A



B

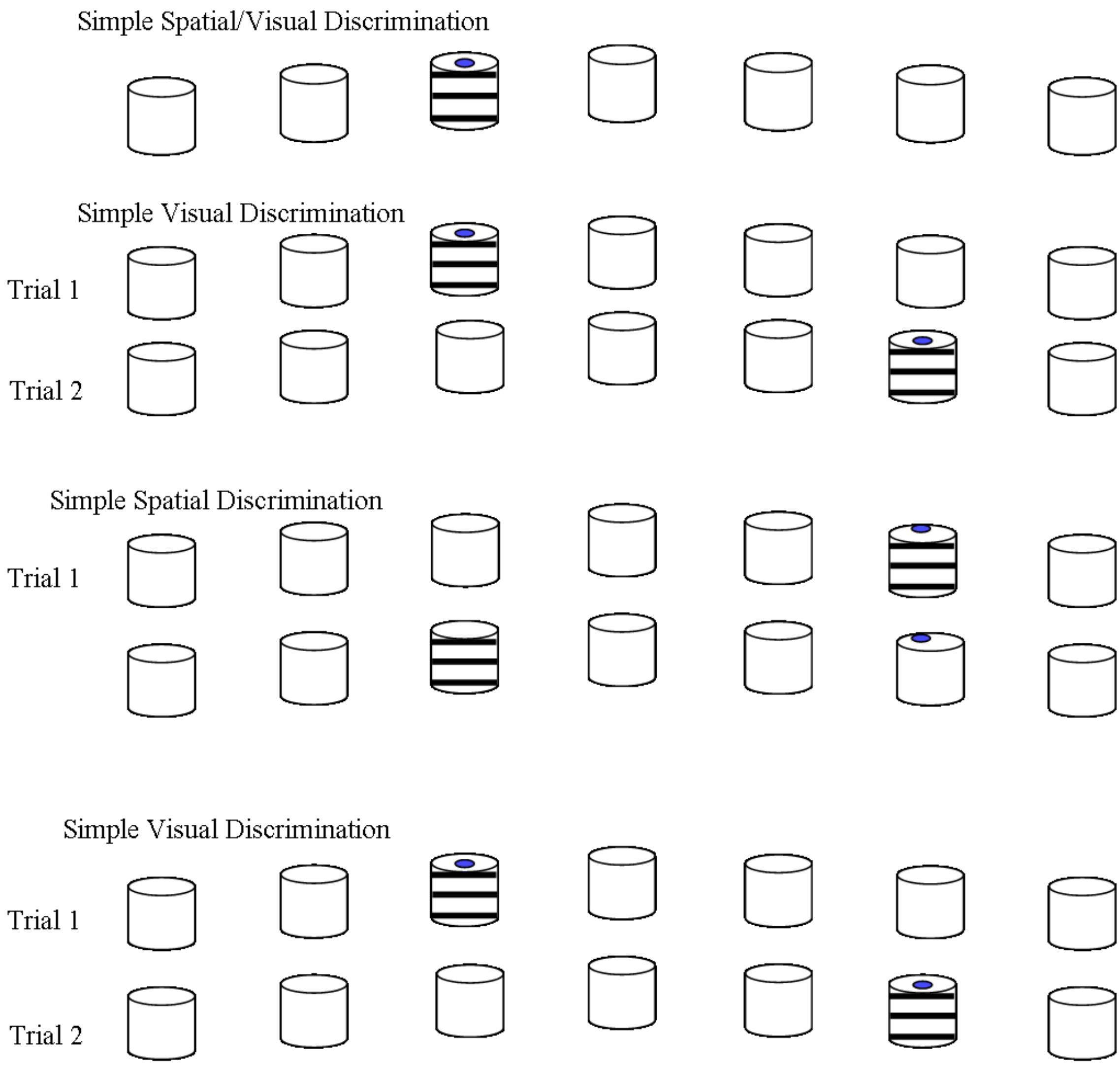


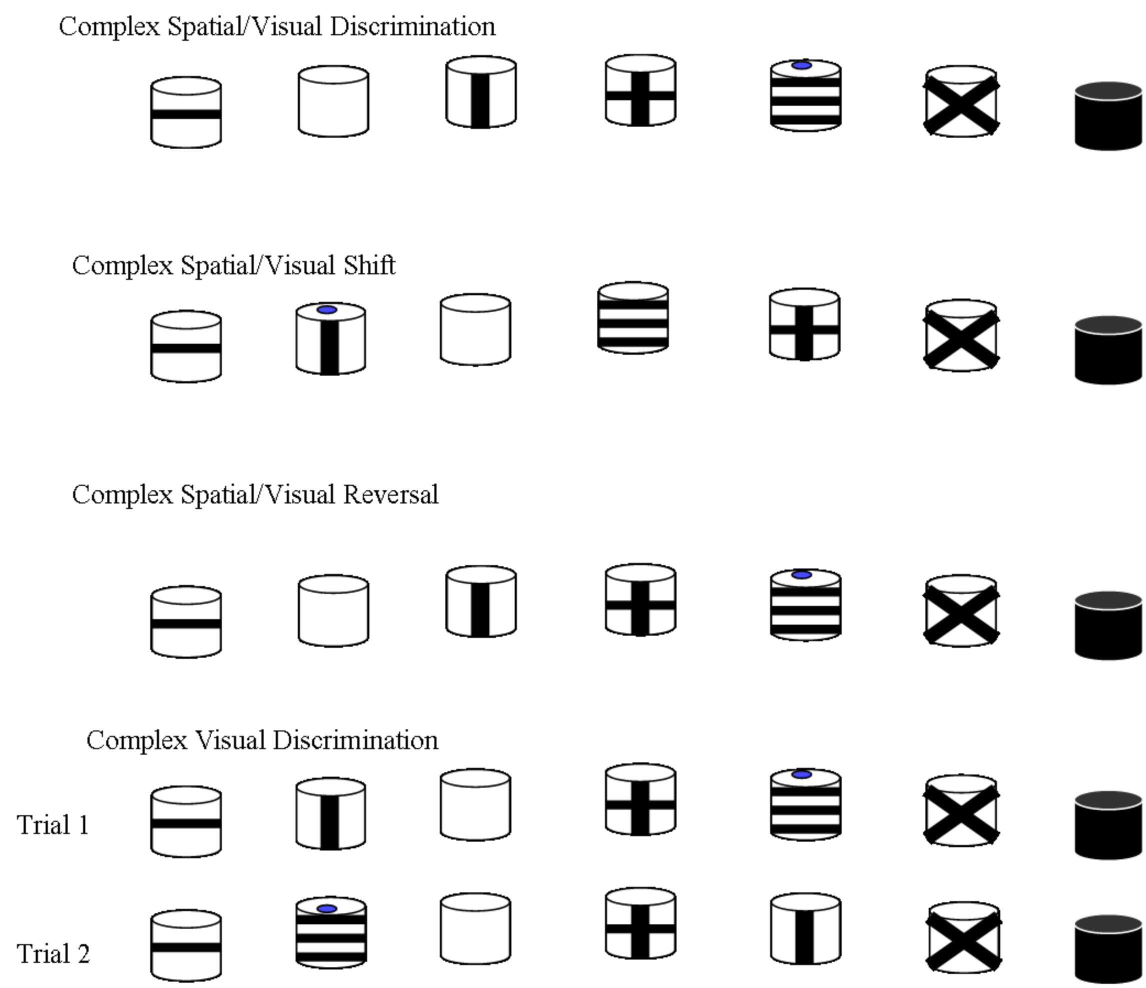
Can Test, Version 2

As a follow up to the above Can Test, we conducted another experiment where the animals were no longer only allowed five days per type of discrimination, but were required to reach a criteria of 8 of 10 trials correct for 2 days in a row to move on to the next discrimination. Correct trials, reference and working memory errors were scored as described above. In addition, multiple different discriminations were added, for a total of eight discriminations, including simple spatial/visual; simple visual; simple spatial shift; simple visual shift; complex spatial/visual; complex spatial/visual shift; complex spatial/visual reversal and complex visual discriminations (Figure 2.4 and see below for detailed descriptions).

Figure 2.4: Can Test Version Two Discrimination Examples

Representations of each discrimination for the second version of the can test. Discriminations where the rewarded can changes location between trials are depicted showing an example of the first 2 trials to show the movement.





Animals

Adult male Sprague-Dawley rats (60-90 days at the beginning of behavioral testing) of both RVPS and control conditions were included in this experiment. All animals underwent behavioral testing during light/inactive phase (experiments performed between 09.00 and 15.00). This resulted in the following groups: control, n = 8 and PNS, n = 6.

Habituation

Habituation was performed following the same protocol as the previous version of the Can Test. For 5 consecutive days, animals were handled for 1 to 2 minutes each; on the third day, animals were moved from the colony room to the behavioral testing room. Three consecutive days of habituation to the testing apparatus followed the five days of handling. On the first day of habituation each animal was allowed 20 minutes exploration of the behavioral testing apparatus, which consisted of a 100 x 100 x 46 cm box with black plexiglas walls. Animals were returned to their home cage without access to a water bottle, in order to facilitate motivation for finding the water reward during subsequent testing. On the second habituation day, seven identically patterned aluminum cans (filled with cement for weight, 13cm in height) were placed in the apparatus in a fan arrangement so as to be equidistant from the starting point. The cans were placed upside-down, in order to provide a smooth dish to contain a small bolus of water (0.3mL). Water was placed in each of the cans and each animal was allowed to explore the apparatus (with water-containing cans) for twenty minutes. On the third habituation day, the apparatus was set up as above, except with only 3 water-baited cans, and the animal was allowed to explore the apparatus until it found all 3 water rewards, or 10 minutes had elapsed. Following consumption of the final reward, the animal was removed to a holding area while three new cans were baited. The animal was then reintroduced to the apparatus and again allowed to explore until finding the three rewards or 10 minutes had elapsed. Following the 3 days of habituation, animals were left undisturbed for 2 days. Water was again removed from the cages the day before testing began (see below). After habituation and testing, rats were allowed access to water for a forty five minute period.

Simple Spatial/visual discrimination

Seven cans wrapped in white tape were arranged in the apparatus with the exception that one can was covered in white tape with a design of three black horizontal lines showing. Animals were allowed 10 trials per day to learn that the can with 3 stripes was the rewarded can (Figure 2.4). Animals continued this discrimination until they performed 8/10 correct trials for 2 consecutive days. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial. The number of trials in which an animal only visited the rewarded can (visited the rewarded can first) was recorded as a correct trial. A reference memory error was scored the first time the animal visited a non-rewarded can prior to visiting the rewarded one. A working memory error was scored each time an animal revisited the same non-rewarded can during a given trial. Average correct trials, reference memory errors, and working memory errors were calculated on a per animal basis for each day of training.

Simple Visual Discrimination

Visual discrimination testing began the day following successfully reaching the criteria in the previous discrimination (i.e., when each rat reaches criteria, it moves on, so some rats started different discriminations on different days). For this test, the cans were arranged similar to the spatial/visual discrimination test, with each can again covered in white tape and one with a design of three black horizontal lines showing. This can was again consistently rewarded; however, from trial to trial its location varied (Figure 2.4). Animals were allowed 10 trials per day and continued this discrimination until they performed 8/10 correct trials for 2 consecutive days. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited and moved for the next trial. Correct trials, reference and working memory errors were scored as above.

Simple Spatial Discrimination

Spatial discrimination testing began the day following successfully reaching the criteria in the previous discrimination. For this test, the testing apparatus was arranged as above, with 7 cans covered in white tape, and 1 with black bar. However, while the can marked with the bars continued to change locations each trial, the water reward was now consistently located in the can in position 6, regardless of if it were pure white or with the striped pattern (Figure 2.4). This forces the animals to adapt their behaviors to use spatial cues only as opposed to visual cues. Animals were allowed 10 trials per day and continued this discrimination until they performed 8/10 correct trials for 2 consecutive days. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial. Correct trials, reference and working memory errors were scored as above.

Simple Visual Discrimination Reversal

Upon successfully reaching the criteria in the previous discrimination rats were reintroduced to the Simple Visual Discrimination and were required to again reach the criteria of 8/10 correct trials for 2 consecutive days. All other features were identical to the original Simple Visual Discrimination Test.

Complex Spatial/Visual Discrimination

Complex Spatial/Visual Discrimination testing began the day following successfully reaching criteria in the previous discrimination. The complex discriminations (this and the following three) retain the same general form as the simple discriminations, with the exception that the non-rewarded cans are no longer simple white but each have a pattern. The cans are still wrapped in white tape but now the black bars take the form of 1 vertical bar; 4 horizontal bars; 3 horizontal bars; a plus (+) shape; pure black; and pure white. For this discrimination the 3 horizontal bars can is the rewarded can and remains stationary in the 5th position (Figure 2.4). For all other details, this discrimination is ran the same as the Simple Spatial/Visual Discrimination.

Complex Spatial/Visual Shift

Complex Spatial/Visual Shift testing began the day following successfully reaching criteria in the previous discrimination. This discrimination consists of the rewarded can no longer being the 3 horizontal bars can in position 5, but is now shifted to the single horizontal bar can in position 2 (Figure 2.4). For all other details, this discrimination is ran the same as the Simple Spatial/Visual Discrimination.

Complex Spatial/Visual Reversal

Complex Spatial/Visual Reversal testing began the day following successfully reaching criteria in the previous discrimination. This discrimination is identical to the first Complex Spatial/Visual Discrimination Test. The rewarded can is again the 3 horizontal bars can in position 5.

Complex Visual Discrimination Test

Complex Visual testing began the day following successfully reaching criteria in the previous discrimination. This discrimination uses the same setup as the previous complex discrimination with the exception that the rewarded can (3 horizontal bars) is no longer stationary in position 5 but now changes locations each trial (Figure 2.4).

Statistical Methods

All statistical analyses were conducted using SPSS statistical package. For behavioral tests analyses of variance (ANOVA) were conducted in which day of training was included as a repeated measures factor. Post hoc tests, conducted using l-matrix contrast statements in SPSS, were used to follow up any significant main effects or interactions. In order to correlate the number of working memory errors committed during training and number of correct trials on the subsequent two-week retention test of the spatial/visual discrimination version of the Can Test, a Pearson product-moment correlation was calculated. For all statistical analyses, $p<.05$ was considered significant.

Results

Morris water maze

The Morris water maze examines spatial memory by hiding a platform in opaque water which the rats must learn the location of through spatial cues. A 2 x 3 repeated measures ANOVA was conducted to determine the influence of treatment (control, RVPS) and training day on latency to reach the escape platform during the three days of training. As expected, latency to find the platform was reduced with increasing days of training ($F_{(2,26)}$= 7.1, p<.01; Figure 2.5A). RVPS impaired performance on this task, as it took RVPS animals significantly longer to find the hidden platform compared to control animals ($F_{(1,13)}$= 5.7, p<.04; Figure 2.5A). For the retention test, planned paired t-tests were used to compare within-group performance between the final day of training and the retention test day, to evaluate whether there was any degradation of memory during the intervening time. Whereas control animals maintained the same high level of performance between the final day of training and the retention test day, RVPS animals took longer to find the platform on the retention test day than they had on the final day of training (t= 2.4, p=.05; Figure 2.5B).

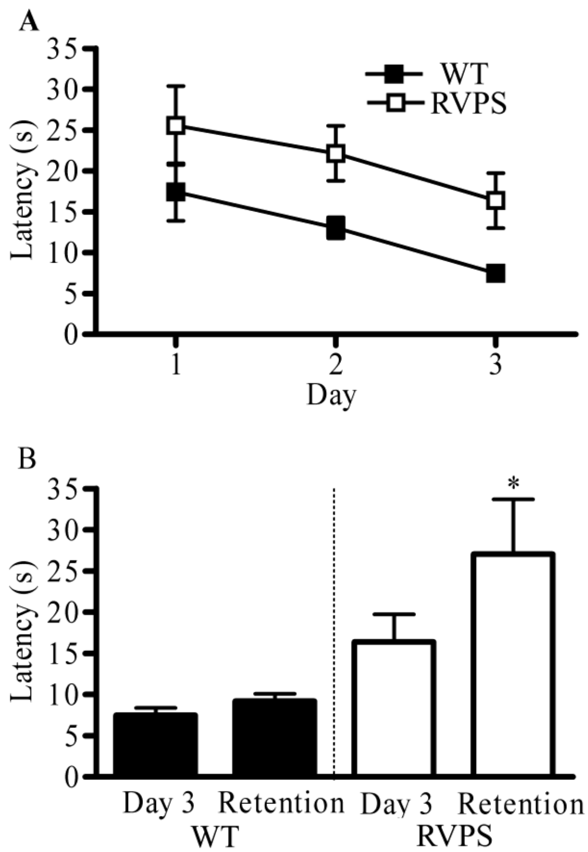
Discrimination memory

Can Test, Version 1

The Can Test interrogates forms of visual and spatial learning and memory. Control Sprague Dawley rats are able to learn the location of the water reward rapidly. Because prenatal treatment and time of training were not found to interact for any measure, and the focus of this chapter is prenatal stress, data are shown

Figure 2.5: Morris Water Maze Results

RVPS male rats took significantly longer to locate a hidden platform over three days of training (*p<.04; A). On the four-day memory retention test RVPS rats had significantly increased latency compared to their final training day (retention latency > day 3 training latency, *p=.05; B) while control rats maintained equivalent performance.



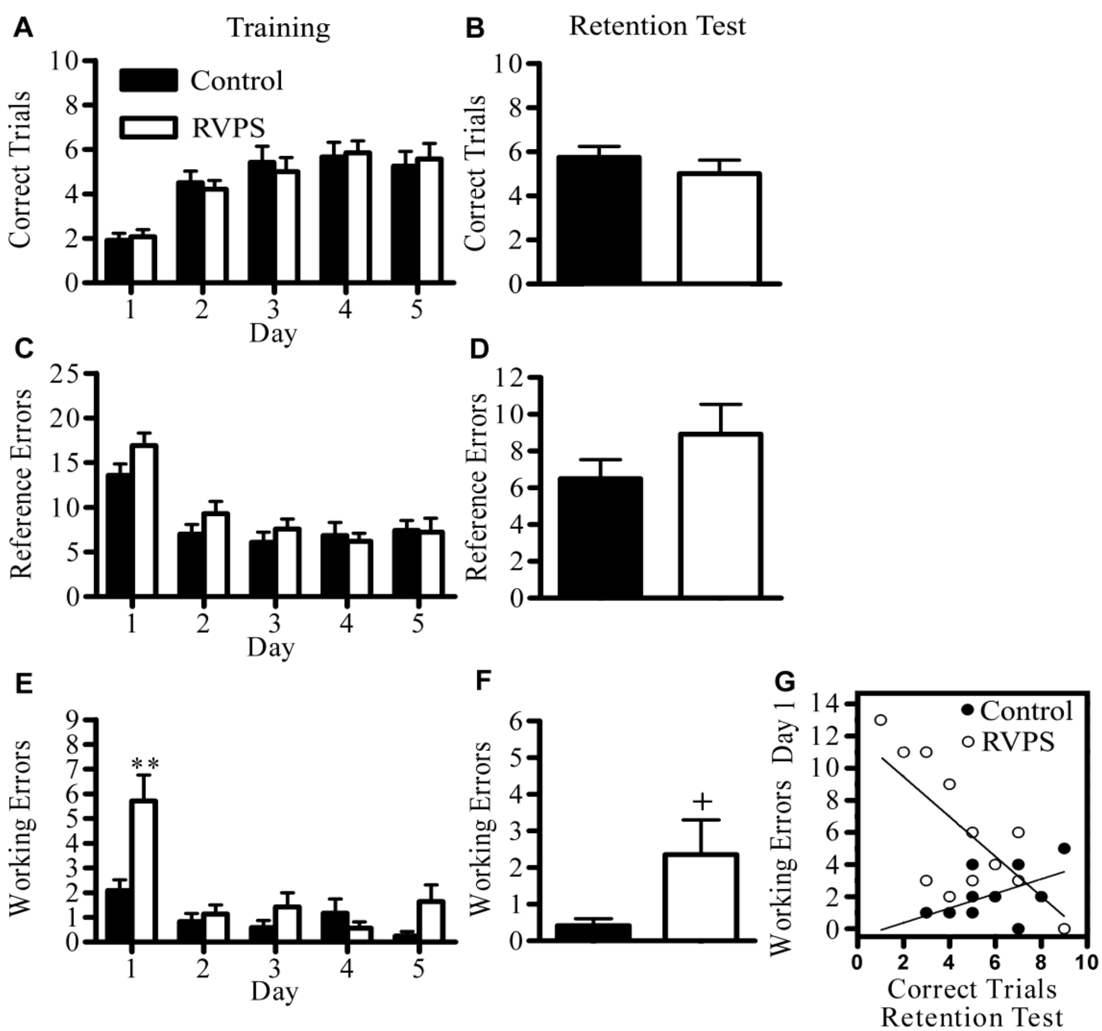
collapsed over time of training to best illustrate the effects of RVPS. Separate 2x2x5 repeated measures ANOVAs (treatment (RVPS vs. control), time of training (active vs. inactive) and training day (days 1-5)) were conducted for number of correct trials, reference memory errors, and working memory errors during training on each version of the test. For the retention tests, separate 2x2 ANOVAs (treatment, time of training) were conducted for the same three performance measures.

Spatial/visual discrimination test

A small water reward is located in the dish created in the top of an upside down soda can which rats are required to learn the location of through visual and/or spatial cues. Animals' performance improved with training, as evidenced by an increase in the number of correct trials ($F_{(4,88)}$= 22.1, p<.0001; figure 2.6A), and concomitant reductions in the number of both reference ($F_{(4,88)}$= 27.9, p<.0001; Figure 2.6C) and working ($F_{(4,88)}$= 14.7, p<.0001; Figure 2.6E) memory errors across training days. RVPS did not impact correct trials (Figure 2.6A, B) or reference memory errors (Figure 2.6C, D) during training or the two-week retention test. Animals exposed to RVPS committed significantly more working memory errors on this test ($F_{(1,22)}$= 5.5, p<.03; Figure 2.6E). The treatment effect interacted with day of training ($F_{(4,88)}$ = 5.3, p<.001) such that the group difference was greatest on the first day of training. There was also a trend for RVPS animals to make more working memory errors than control rats on the two week retention test ($F_{(1,22)}$ = 3.8, p<.066; figure 2.6F). Additionally, a significant negative Pearson product-moment correlation was found between working memory errors committed by RVPS animals on the first day of training and the number of correct trials they made on the two-week retention test (r= -.72, p<.01), whereas no such relationship was found among control animals (Figure 2.6G). Animals trained during the active phase of the light cycle (i.e. when the lights were out) had fewer correct trials ($F_{(1,22)}$ 4.7, p<.05; data not shown) and made more reference memory errors ($F_{(1,22)}$= 10.8, p<.01; data not shown) during training on this task compared to those trained during the inactive phase (lights on). Time of training did not impact performance on the two-week memory retention test (data not shown).

Figure 2.6: Spatial/Visual Discrimination Results

RVPS had no effect on correct trials (A, B) or reference memory errors (C, D) during either training or the two-week retention test. RVPS male rats made more working memory errors during training on this task (*p<.03; E), particularly on the first day of training (**p<.01) and trended to make more working memory errors on two-week memory retention test (+p<.066; F), compared to control animals. Among RVPS animals, a significant negative correlation was found between the number of working memory errors made on the first day of training and the number of correct trials during the Two-week memory retention test (*p<.05; G).

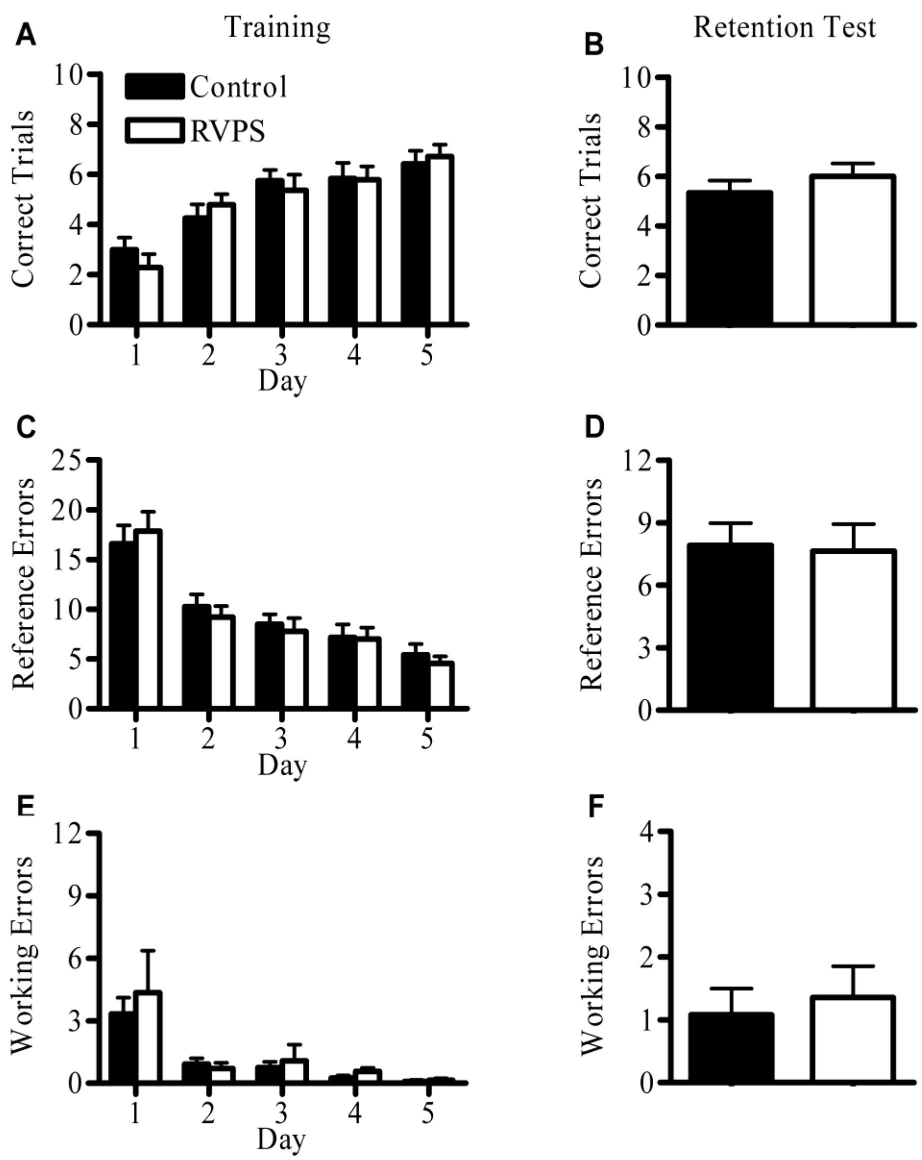


Spatial discrimination test

The number of correct trials increased across training days ($F_{(4,88)}$= 24.7, p<.0001; figure 2.7A), while there was a concomitant reduction in the number of both reference ($F_{(4,88)}$= 39.3, p<.0001; figure 2.7C) and working ($F_{(4,88)}$= 7.9, p<.0001; figure 2.7E) memory errors, indicating that animals' performance on this task improved with training. RVPS did not significantly impact correct trials (Figure 7A, B), reference memory errors (Figure 2.7C, D) or working memory errors (Figure 2.7E, F) during either training or during the two-week retention test. Animals trained during the active phase of the light cycle showed a trend towards committing more reference memory errors than animals trained during the inactive phase ($F_{(1,22)}$= 3.8, p<.065; data not shown); this effect interacted with day of training ($F_{(4,88)}$= 3.0, p<.03) such that the difference between active versus inactive phase trained animals diminished over training (p<.02 on first day of training; comparisons on other days not significant, data not shown). In the two week retention test, animals trained during the active phase made fewer working memory errors than those trained during the inactive phase ($F_{(1,22)}$= 8.4, p<.01; data not shown).

Figure 2.7: Spatial Discrimination Results

RVPS did not have an effect on correct trials (A, B), reference memory errors (C, D) or working memory errors (E, F) during either training or the two-week retention test.

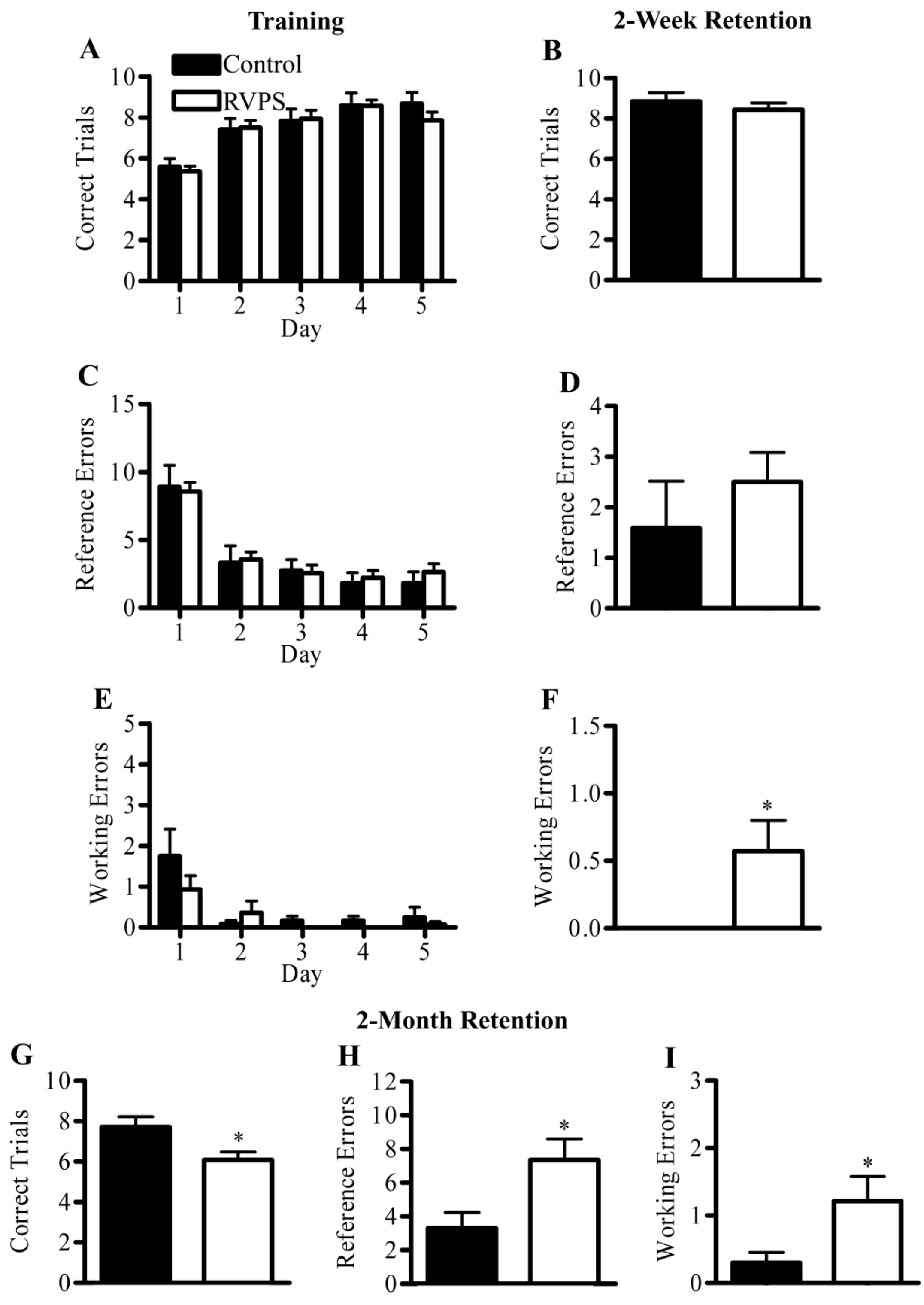


Visual discrimination test

The number of correct trials increased across training days ($F_{(4,88)}$= 18.9, p<.0001; figure 2.8A), while there was a concomitant reduction in the number of both reference ($F_{(4,88)}$= 24.5, p<.0001; figure 2.8C) and working ($F_{(4,88)}$= 11.4, p<.0001; figure 2.8E) memory errors, indicating that animals' performance on this task improved with training. RVPS did not significantly impact correct trials (Figure 2.8A), reference memory errors (Figure 2.8C) or working memory errors (Figure 2.8E) during training. However, RVPS rats made more working memory errors than controls on the two week retention test ($F_{(1,22)}$= 5.0, p<.04; Figure 2.8F) (no control animals made a single working memory error during this test) whereas RVPS did not affect correct trials (Figure 2.8B) or reference memory errors (Figure 2.8D). On the two month (final) retention test, RVPS rats had fewer correct trials ($F_{(1,20)}$= 7.4, p<.02; Figure 2.8G) and made more reference memory ($F_{(1,20)}$ = 6.6, p<.02; Figure 2.8H) and working memory errors ($F_{(1,20)}$= 4.3, p=.05; Figure 2.8I) than control animals. (We were unable to include two animals in this analysis: one control/active period rat died prior to testing, and one control/inactive period rat remained immobile during more than half of the trials) Time of training did not impact performance during training or on the 2-week retention test; however, on the 2-month retention test animals trained during the inactive phase of the light cycle made fewer correct trials ($F_{(1,20)}$= 13.5, p<.01; data not shown), more reference memory errors ($F_{(1,20)}$= 7.6, p<.02; data not shown) and trended toward making more working memory errors ($F_{(1,20)}$ = 3.7, p<.071; data not shown)

Figure 2.8: Visual Discrimination Results

RVPS had no effect on correct trials, reference memory or working memory errors during training (A, C, E). On the two-week retention test RVPS had no effect on correct trials or reference memory errors (B, D) but RVPS rats did make significantly more working memory errors than control rats (F). On the two-month retention test RVPS rats made fewer correct trials (G), more reference (H) and working memory errors (I).



Training
2-Week Retention
A
Control
RVPS
Correct Trials
Day
B
C
Reference Errors
D
E
Working Errors
F
2-Month Retention
G
H
I

Can Test, Version 2

A variation of the Can Test wherein the main endpoint was days to reach a criteria of 8 correct trials for 2 days in a row was ran to determine if overall learning ability was altered in RVPS rats. Because the main focus of this version of the Can Test was to determine days to reaching the criteria of 8 correct trials out of 10 for 2 days, rats reached the criteria in different amounts of days and therefore repeated measures ANOVA for correct trials, reference and working memory errors were only able to be performed for the days that all animals were still running each of the discriminations. As such, the days shown in the analyses for correct trials, reference and working memory are based on when the first animal reached criteria. In other words, if the first rat to reach criteria did so in 5 days and the remaining rats took between 6 and 15 days, analyses for correct trials, reference and working memory are confined to the first 5 days of the discrimination. Amount of days until the first rat reached criteria ranged from 2 days in the Simple Visual and Complex Spatial/Visual Reversal to 12 days for the Simple Spatial Discrimination. Days to criteria were analyzed for each discrimination using students t-test. All days to criteria are shown together in figure 2.9 and all results are summarized in Table 2.2.

Simple Spatial/Visual Discrimination

The number of correct trials increased across training days ($F_{(4,48)}$= 25.755, $p<.001$), while there was a concomitant reduction in the number of both reference ($F_{(4,48)}$= 37.654, $p<.001$) and working ($F_{(4,48)}$= 10.496, $p<.001$) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials ($p = .40$), reference memory errors ($p =$

.49), or working memory errors (p = .80). However, we did detect a day x stress treatment interaction on working memory errors ($F_{(4,48)}$=3.152, p<.03; Figure 2.10A-C). In addition, post hoc tests showed a non-significant increase in working memory errors on the first day for prenatally stressed rats compared to controls (p=.14). Days to criteria did not differ between controls (9.1 ± 1.4) and RVPS rats (7.8 ± 0.70; p=.47; Figure 2.9).

Figure 2.9: Days to criteria

1 = simple spatial/visual; 2 = simple visual, 3 = simple spatial; 4 = simple visual reversal; 5 = complex spatial/visual; 6 = complex spatial/visual switch; 7 = complex spatial/visual reversal, 8 = complex visual.

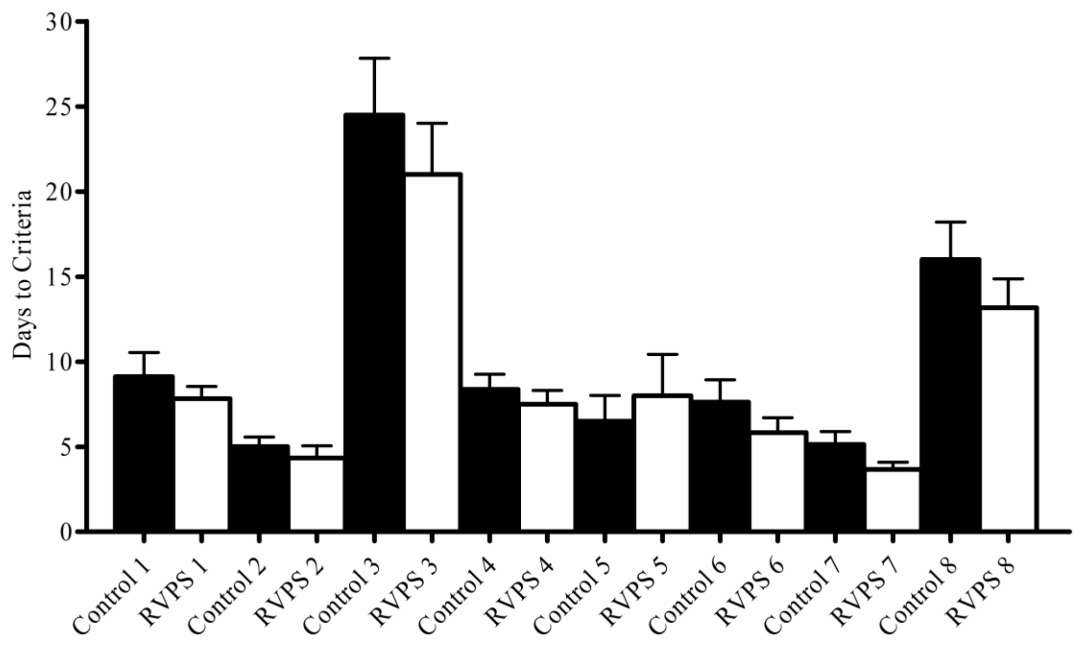
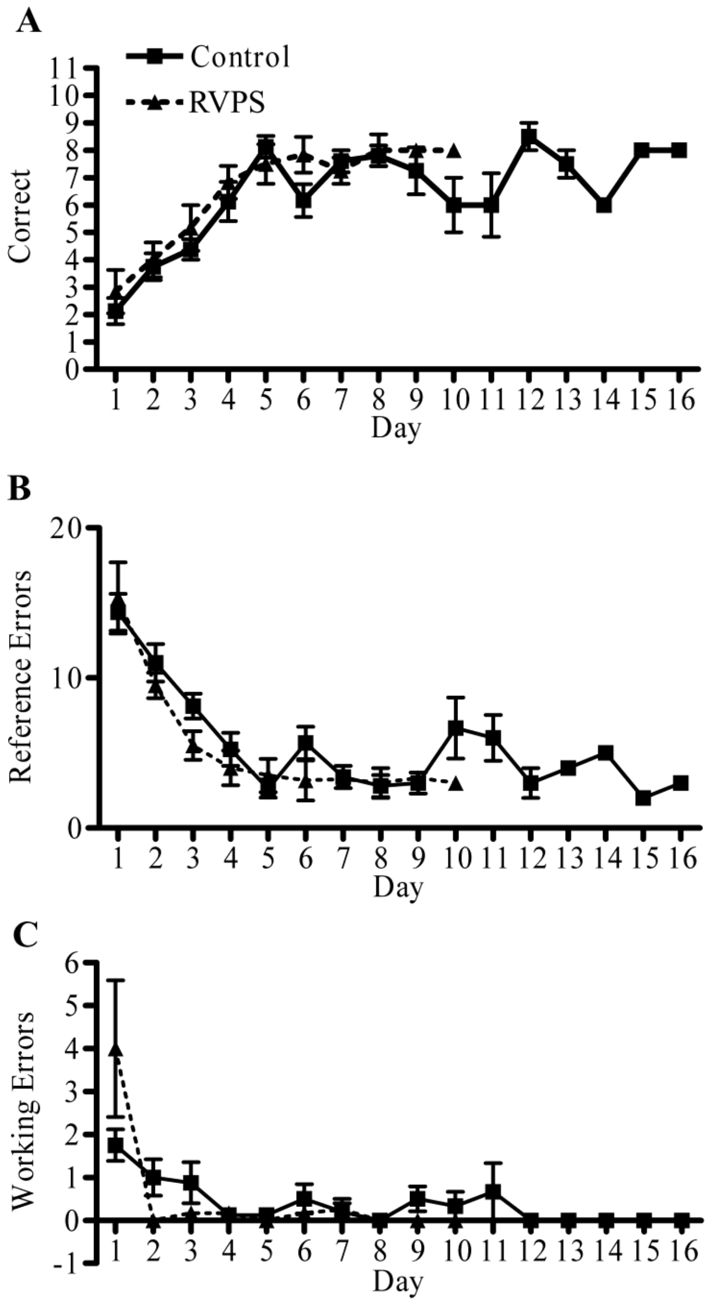


Figure 2.10: Simple Spatial/Visual Discrimination Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).



Simple Visual Discrimination

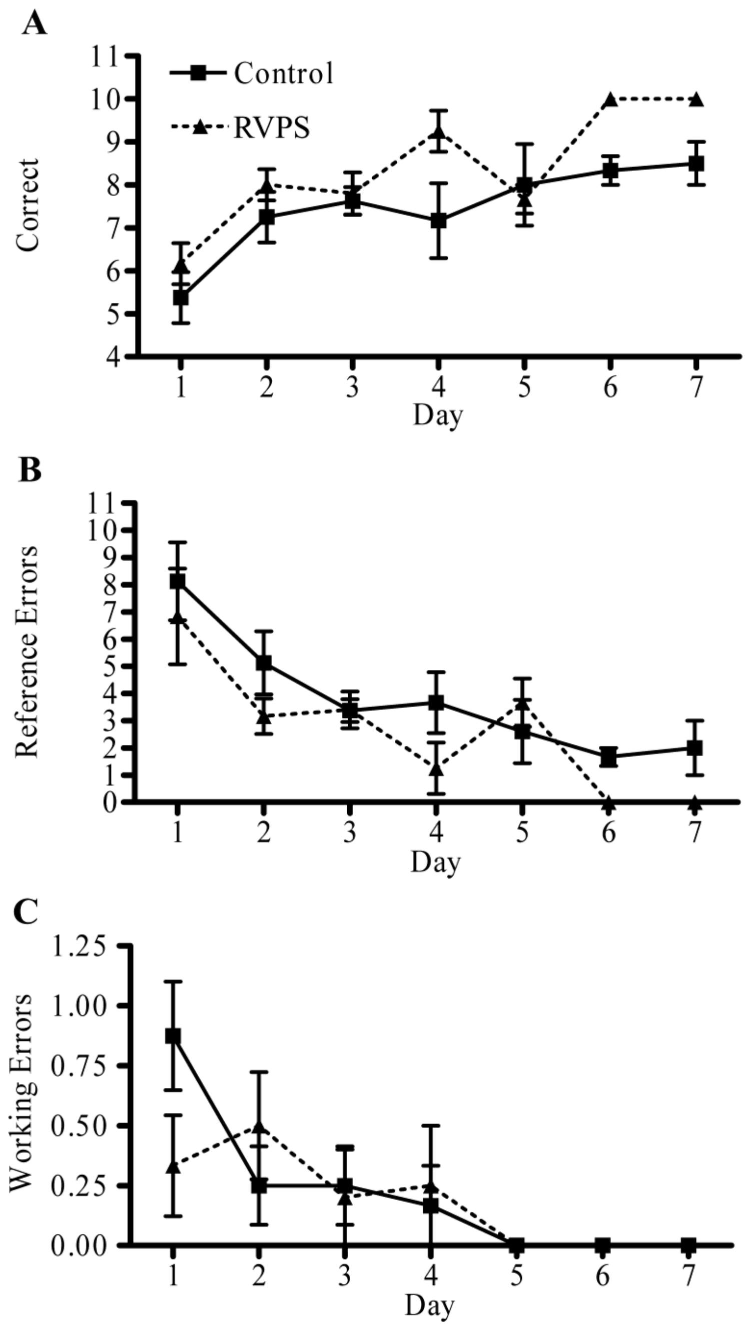
The number of correct trials increased across training days ($F_{(1,12)}$=14.249, p<.004), while there was a concomitant reduction in the number of reference memory errors ($F_{(1,12)}$=6.997, p<.03) indicating that animals' performance on this task improved with training. However, there was not an effect of day on working memory errors (p=.20), which is reasonable as there were only two days before the first rat reached criteria and working memory errors are generally low (means less than 1 each day) for this discrimination. Again, though, we did see an interaction between day and stress treatment ($F_{(1,12)}$=5.477, p<.04). There was no effect of RVPS on number of correct trials (p=.23), reference (p=.27) or working memory errors (p=.56; Figure 2.11A-C). Days to criteria did not differ between controls (5.000 ± 0.5669) and RVPS rats (4.333 ± 0.7149; p=.47; Figure 2.9).

Simple Spatial Discrimination

The number of correct trials increased across training days ($F_{(11,132)}$=14.204, p<.001), while there was a concomitant reduction in the number of both reference ($F_{(11,132)}$=23.435, p<.001) and working ($F_{(11,132)}$=20.621, p<.001) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.93), reference memory errors (p=.41), or working memory errors (p=.11). There was however, a significant interaction between day of testing and stress treatment for reference memory errors ($F_{(11,132)}$=1.932, p<.05; Figure 2.12A-C). Days to criteria did not differ between controls (24.50 ± 3.333) and RVPS rats (21.00 ± 3.011; p=.47; Figure 2.9).

Figure 2.11: Simple Visual Discrimination Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).



Simple Visual Discrimination Reversal

The number of correct trials increased across training days ($F_{(4,48)}$=9.815, p<.001), while there was a concomitant reduction in the number of both reference ($F_{(4,48)}$=18.599, p<.001) and working ($F_{(4,48)}$=.23.006, p<.001) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.37), reference memory errors (p=.36), or working memory errors (p=.43; Figure 2.13A-C). Days to criteria did not differ between controls (8.375 ± 0.8851) and RVPS rats (7.500 ± 0.8062; p=.49; Figure 2.9).

Figure 2.12: Simple Spatial Discrimination Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).

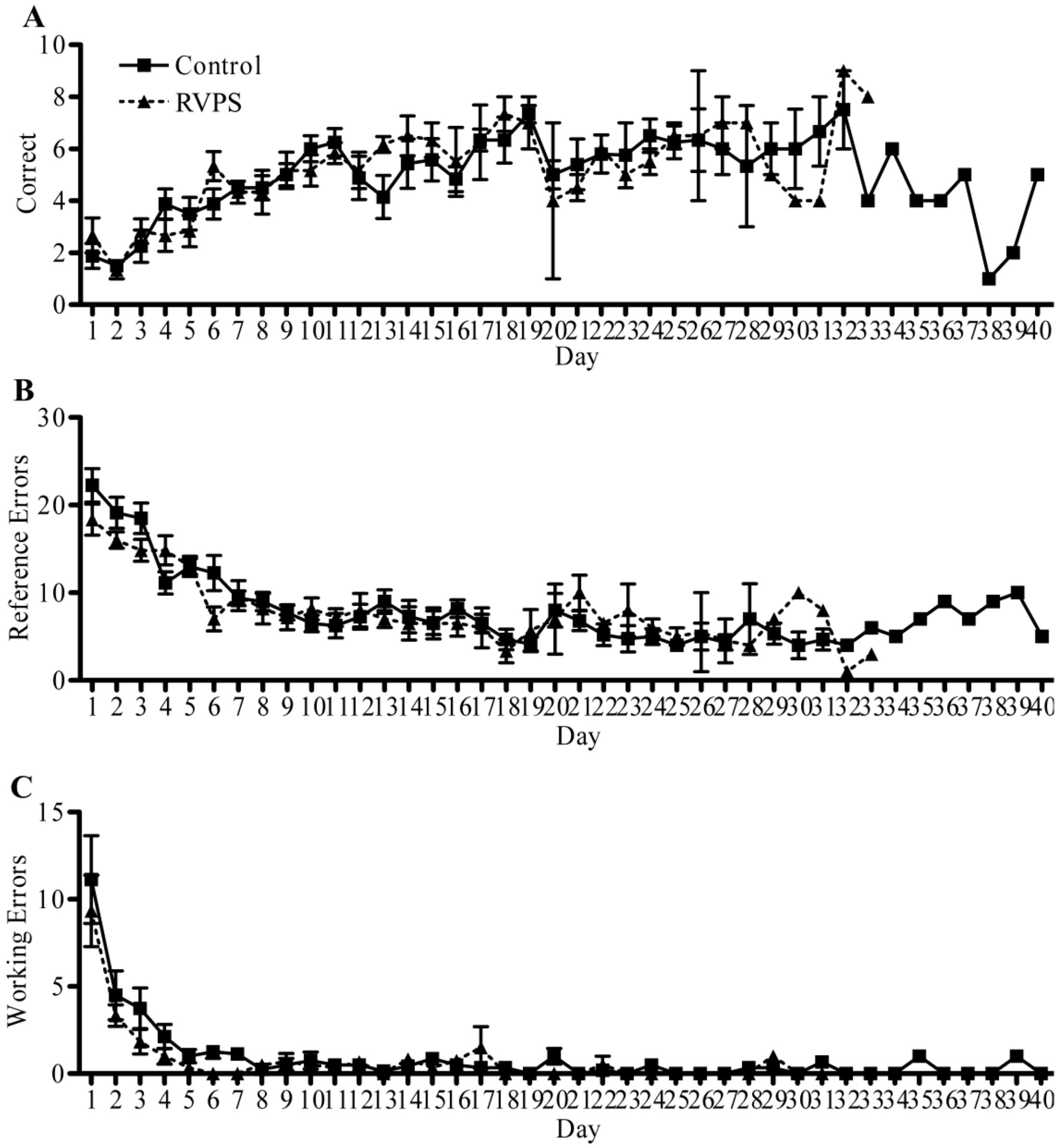
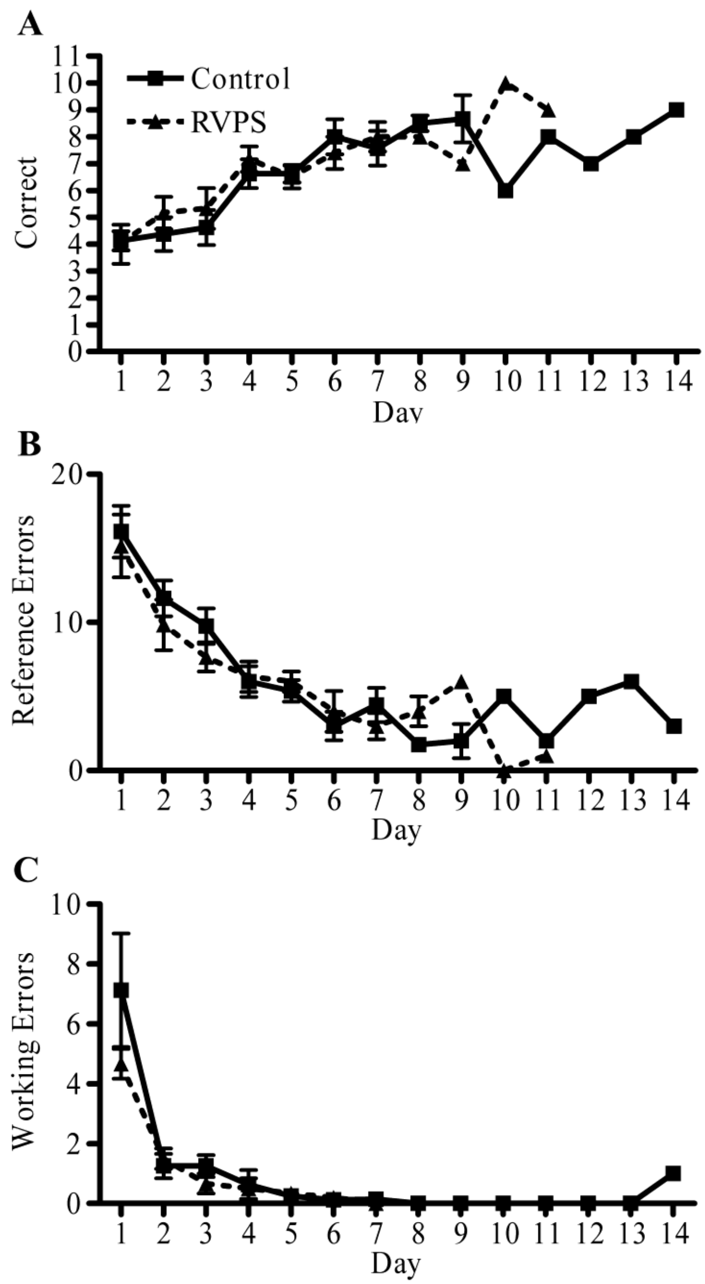


Figure 2.13: Simple Visual Discrimination Reversal Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).



Complex Spatial/Visual Discrimination

The number of correct trials increased across training days ($F_{(3,36)}$=22.561, p<.001), while there was a concomitant reduction in the number of both reference ($F_{(3,36)}$=20.025, p<.001) and working ($F_{(3,36)}$=4.513, p<.01) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.56), reference memory errors (p=.41), or working memory errors (p=.81; Figure 2.14A-C). Days to criteria did not differ between controls (6.500 ± 1.512) and RVPS rats (8.000 ± 2.436; p=.59; Figure 2.9).

Complex Spatial/Visual Discrimination Shift

The number of correct trials increased across training days ($F_{(2,24)}$=20.187, p<.001), while there was a concomitant reduction in the number of both reference ($F_{(2,24)}$=38.44, p<.001) and working ($F_{(2,24)}$=6.950, p<.005) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.97), reference memory errors (p=.42), or working memory errors (p=.51; Figure 2.15A-C). Days to criteria did not differ between controls (7.625 ± 1.308) and RVPS rats (5.833 ± 0.8724; p=.31; Figure 2.9).

Figure 2.14: Complex Spatial/Visual Discrimination Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).

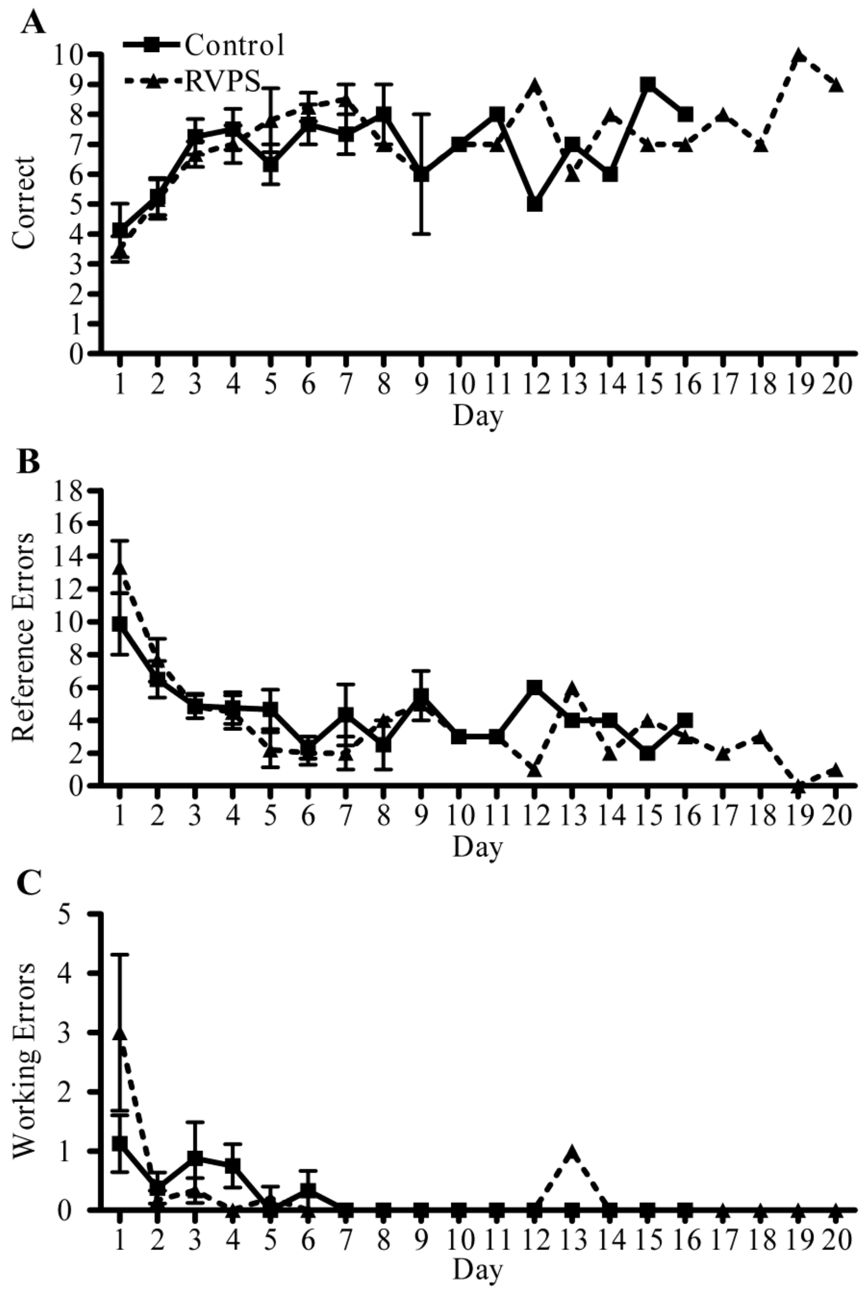
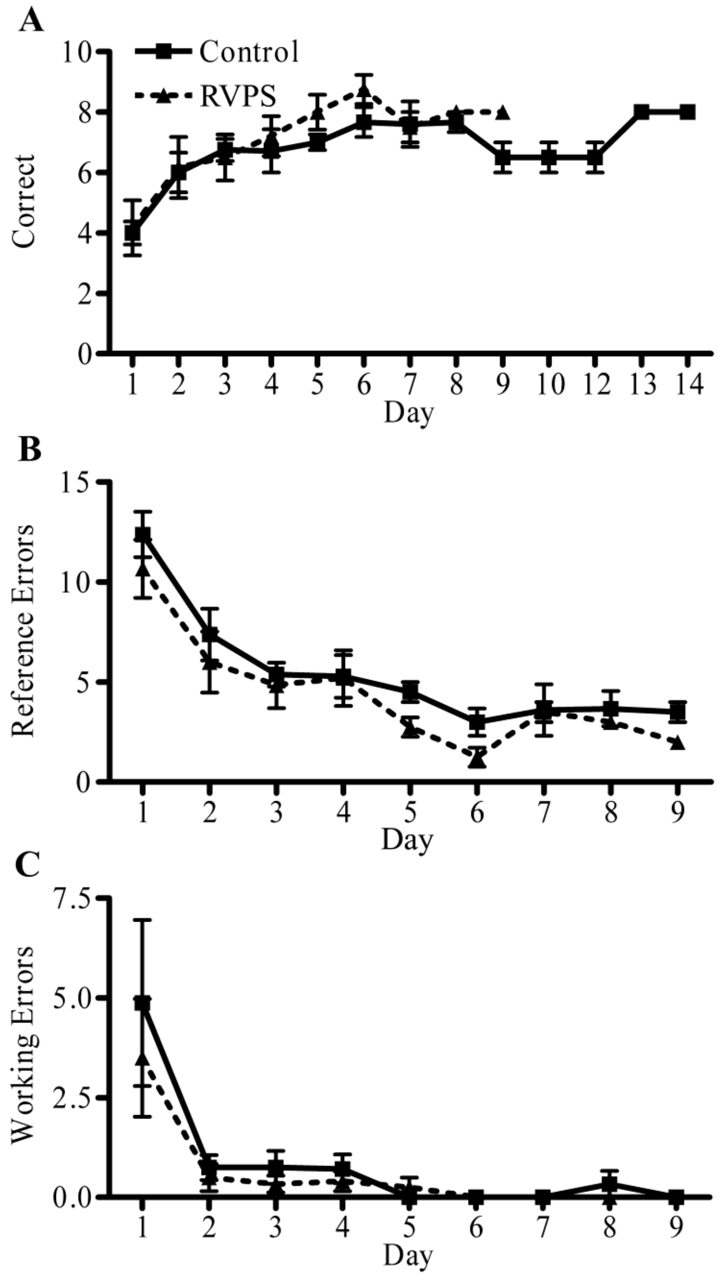


Figure 2.15: Complex Spatial/Visual Switch Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).



Complex Spatial/Visual Discrimination Reversal

The number of correct trials increased across training days ($F_{(1,12)}$=6.071, p<.04), while there was a concomitant reduction in the number of both reference ($F_{(1,12)}$=8.056, p<.02) and working ($F_{(1,12)}$=4.271, p=.06) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.79), reference memory errors (p=.84), or working memory errors (p=.80). We did detect a significant interaction between day of training and stress treatment for both correct trials ($F_{1,12)}$=7.244, p<.03) and reference memory errors ($F_{(1,12)}$=5.768, p <.04; Figure 2.16A-C). Post hoc tests indicate that prenatally stressed rats trended toward making fewer correct trials on the first day ($F_{(1,12)}$=4.550, p=.054). Days to criteria did not differ between controls (5.125 ± 0.7662) and RVPS rats (3.667 ± 0.4216; p=.16; Figure 2.9).

Complex Visual Discrimination

The number of correct trials increased across training days ($F_{(5,60)}$=3.836, p<.005), while there was a concomitant reduction in the number of both reference ($F_{(5,60)}$=4.924, p<.002) and working ($F_{(5,60)}$=3.898, p<.005) memory errors, indicating that animals' performance on this task improved with training. There was no effect of RVPS on number of correct trials (p=.15), reference memory errors (p=.40), or working memory errors (p=.89; Figure 2.17A-C). Days to criteria did not differ between controls (16.00 ± 2.196) and RVPS rats (13.17 ± 1.701; p=.36; Figure 2.9).

Figure 2.16: Complex Spatial/Visual Reversal Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).

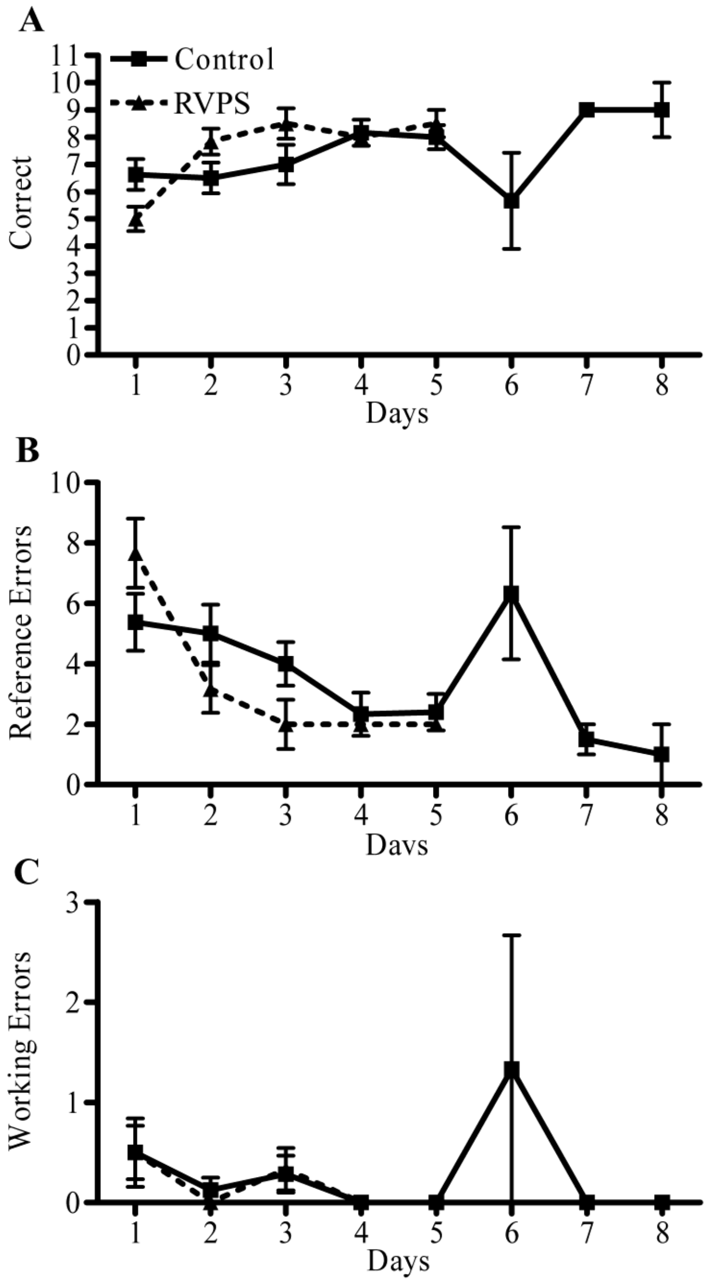


Figure 2.17: Complex Visual Discrimination Results

RVPS had no effect on correct trials (A), reference (B) or working memory errors (C).

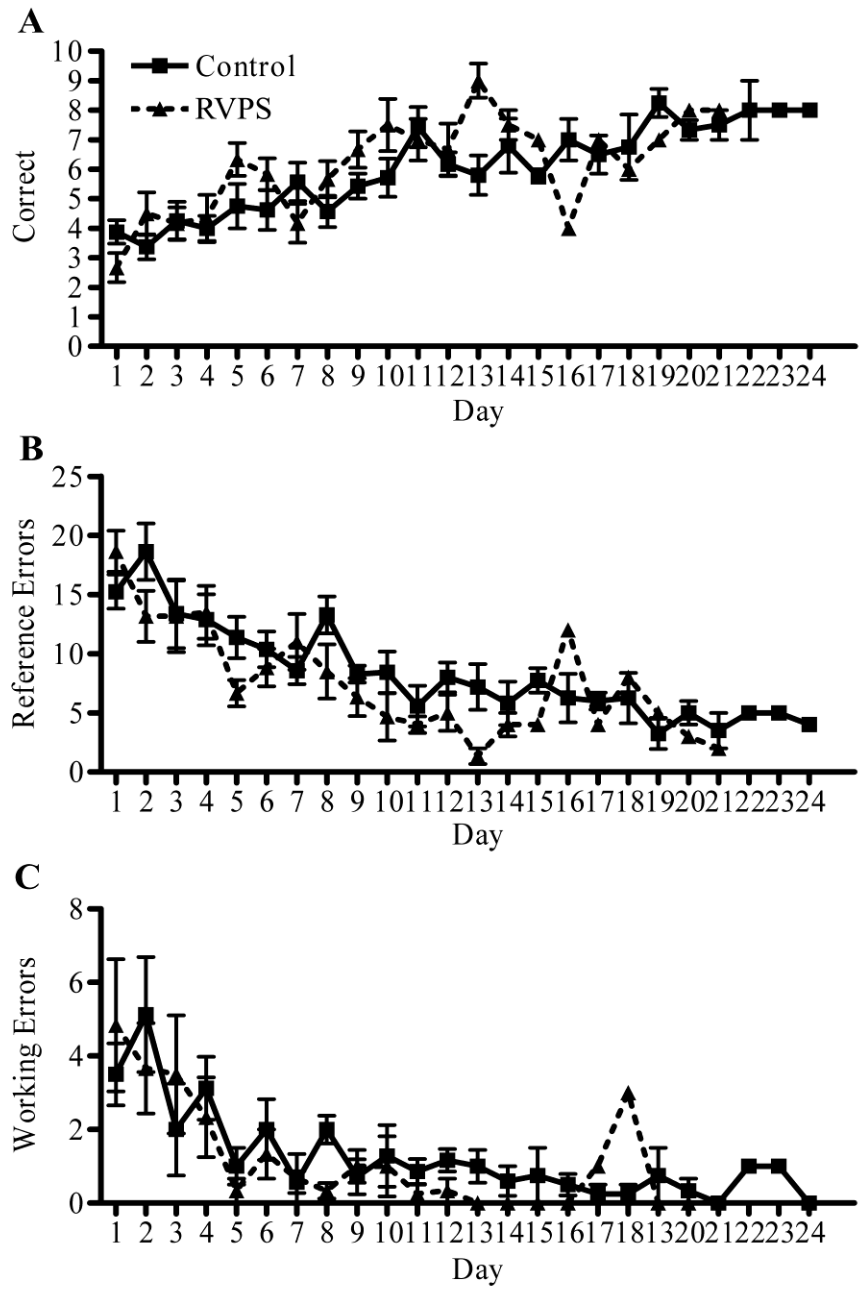


Table 2.2: Summary of Can Test Data

N/E = No Effect; N/A = Not Applicable

Discrimination	Correct Trials	Reference Errors	Working Errors	Interaction
Version 1				
Spatial/Visual	N/E	N/E	**Increase in RVPS ($p<.03$)**	**Day x Stress for Working Errors ($p<.001$); Day 1 increase in RVPS ($p<.01$)**
2-Week Retention	N/E	N/E	**Increase in RVPS ($p=.066$)**	N/A
Spatial	N/E	N/E	N/E	N/E
2-Week Retention	N/E	N/E	N/E	N/A
Visual	N/E	N/E	N/E	N/E
2-Week Retention	N/E	N/E	**Increase in RVPS ($p<.04$)**	N/A
2-Month Retention	**Decrease in RVPS ($p<.02$)**	**Increase in RVPS ($p<.02$)**	**Increase in RVPS ($p=.05$)**	N/A
Version 2				
Simple Spatial/Visual	N/E	N/E	N/E	N/E
Simple Visual	N/E	N/E	N/E	N/E
Simple Spatial	N/E	N/E	N/E	**Day x Stress for Reference Errors**
Simple Visual Reversal	N/E	N/E	N/E	N/E
Complex Spatial/Visual	N/E	N/E	N/E	N/E
Complex Spatial/Visual Shift	N/E	N/E	N/E	N/E
Complex Spatial/Visual Reversal	N/E	N/E	N/E	**Day x Stress for Correct and Reference Errors**
Complex Visual	N/E	N/E	N/E	N/E

Discussion

Prenatal stress is known to be associated with an increased risk for developing schizophrenia as well as with many alterations in cognitive functioning in infants, children and young adults. In addition, prenatal stress has also been shown to alter cognitive behaviors in laboratory animals ranging from young to older animals (Lemaire et al., 2000; Son et al., 2006; Zagron and Weinstock, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Wu et al., 2007; Yaka et al., 2007). Physiological alterations have also been observed in animals that were subjected to prenatal stress. Studies examining higher order cognitive abilities in prenatally stressed rats have not been adequately studied yet though.

The first specific aim of this dissertation was devoted to testing the hypothesis that RVPS results in cognitive deficits similar to certain deficits observed in schizophrenia. We examined this hypothesis by testing control and RVPS rats in a series of relatively simple learning tests (Morris water maze) to increasingly difficult discrimination learning tests (various versions of the Can Test). We predicted that animals that had undergone RVPS would show increased latencies to find the escape platform in the Morris water maze; that animals that had undergone RVPS would show a learning deficit in the Can Test and commit more errors that control rats; and that animals that had undergone RVPS would show a deficit in long-term memory.

Our results indicate that RVPS animals do indeed have a deficit in spatial learning as they have significantly increased latencies to find the platform over the three days of training in the Morris water maze. In addition, we found that RVPS animals, four days after completion of training in the water maze, show increased latencies to find the platform in a memory retention test compared to their final day of training, while control animals performance in the retention test was identical to that of the last day of training. This may indicate that the RVPS animals were not able to remember the location of the platform after four days away from the maze while control animals recall was still intact. Thus we have seen a deficit in spatial learning as well as in long-term spatial memory retention.

In addition, using a comprehensive set of discrimination tests, ranging from a simple visual/spatial discrimination to a complex visual discrimination, collectively know as the Can Test, we have shown that RVPS rats behave generally similar to control rats but with deficits in specific cognitive endpoints. First, RVPS animals, in the initial visual/spatial discrimination test, showed a selective deficit in working memory. The RVPS animals did not have any fewer correct trials nor did they make more reference memory errors than control animals. However they did make significantly more working memory errors than control animals during training. This deficit interacted with day of training such that the RVPS animals made more working memory errors on the first day of training, compared to control animals. In addition, during a two-week memory retention test, the RVPS animals again made more working memory errors than the control animals without showing a deficit in either correct trials or reference memory errors, indicating that the primary deficit is indeed in working memory in this task. Upon completion of the visual/spatial discrimination tests rats were moved to the purely spatial version of the test. In this version of the test RVPS rats did not significantly differ from the control animals in any of the variables tested, namely correct trials, reference and working memory errors. There were also no differences seen in the two-week retention test for this version of the test. Finally, following the two week retention test for the spatial discrimination, animals were moved to the last discrimination test, the purely visual discrimination. During the five days of training the RVPS animals again did not differ from the control animals in any of the variables tested. However, we did again see an effect of RVPS in the two week memory retention test. The RVPS rats made more working memory errors than the control animals. It should be noted that this effect was also affected by the finding that in this test not a single control animal made a single working memory error. Therefore, it seems that after many weeks of training in different versions of the Can Test control rats had learned to simply not make that type of error. RVPS animals on the other hand, while they did not make many, were still prone to commit working memory errors after so many weeks. Upon completion of the two week retention test animals were returned to the colony room for two months before returning to the testing room for a very long-term memory retention test. RVPS rats showed deficits in each of the variables tested. RVPS rats made fewer correct trials, more reference memory errors and more working memory errors than control rats.

In a set of follow up experiments designed to determine if the working memory deficit seen in RVPS rats was not somehow dependent on an overall learning disability, we studied days to reach a criteria of 2 days in a row with at least 8 correct trials out of 10. In this fashion, we were able to still record correct trials and reference and working memory errors but also to see how the animals managed to master specific types of discriminations as well as how they would be able to change their behavior when the rules of the discriminations changed. The animals were ran through eight different discriminations over a period of several months. We found a non-significant increase in working memory errors in RVPS rats on the first day of the first discrimination, again the simple visual/spatial discrimination, which was consistent with our first experiment. Excluding the first day working memory errors, we in fact found no differences between RVPS and control animals. The same amount of correct trials, reference memory errors and working memory errors were recorded for each group of animals in each of the eight different discriminations. In addition, days to reach the set criteria did not differ between groups in any of the eight discriminations. This indicates that RVPS does not alter the animals ability to either learn how to discriminate between the objects or spatial cues. There are several reasons that may be why we did not see the same significant increasein working memory errors in this version of the test. First off, our n was smaller in this set of experiments than in the initial discrimination, as rats were only ran during the light period of the day. Second, the parameters of the experiments were also different. In the first version, the non-rewarded cans were left in their original designs, i.e. with multiple colors and patterns, as opposed to the second version where the non-rewarded cans were covered in white tape, while each time the rewarded can was covered in white tape with horizontal black bars differentiating it from the non-rewarded cans. This difference may have made the second version of the discrimination slightly simpler than the first version, resulting in the difference in errors being more slight the second time. Taken as a whole, these results provide clear evidence that RVPS does lead to certain cognitive deficits which are similar to some deficits in schizophrenia, while other aspects of cognition have been shown to be unaltered by RVPS. Specifically, we have shown that spatial learning, working memory and overall long-term memory are disrupted by RVPS. In schizophrenia, spatial learning is deficient (Hanlon et al., 2006; Girard et al., 2010) working memory is profoundly disrupted (Lee and Park, 2005; Forbes et al., 2009) and long-term memory is also affected (Aleman et al., 1999). It should be noted that while we did see a deficit in spatial learning in the Morris water maze, we did not see a deficit in our spatial version of the Can Test. The most likely explanation of this discrepancy is that in both versions of the Can Test, the spatial discrimination tasks followed either 1 or 2 different spatial/visual discriminations. Therefore, the results of being trained in the former discriminations likely lead to a training effect, as we saw that animals performed better in the spatial and then the visual discrimination in the first version of the test, indicating that familiarity with the task led to enhanced performance.

Our Morris water maze results are in line with multiple studies, which followed various protocols of prenatal stress, tested at different ages and used different strains of animals; adult mice/chronic immobilization stress (Son et al., 2006), juvenile rats/restraint stress (Wu et al., 2007), adult rats/restraint stress (Lemaire et al., 2000; Zagron and Weinstock, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Yaka et al., 2007) and guinea pigs stressed at prenatal day 50 but not 60 (Kapoor et al., 2008); though Alexsandrov (Aleksandrov et al., 2001) found no difference in latency but an over-perseverative strategy following a prenatal social stress protocol. In addition, it should be noted that, to our knowledge, this is the first time a RVPS paradigm consisting of a variety of six stressors over the last week of gestation has been used to study higher executive functioning and long-term memory in rats.

The mechanisms underlying the deficits we have presented here are not known. However, previous work leads to many possibilities of the underlying mechanisms. Most of the research done to date has focused on the hippocampus, as hippocampal dependant learning tasks are often seen to be performed poorly by prenatally stressed animals. Prenatal restraint stress, from prenatal day 15-19, has been shown to reduce hippocampal weight (Szuran et al., 1994) and neurogenesis (Mandyam et al., 2008). Granule cell numbers are decreased in the dentate gyrus of prenatally stressed rats at postnatal day 28 as well as at 22 months of age (Lemaire et al., 2000). It has been shown that approximately 85% of granule cells in rodents are formed after birth (Bayer, 1980), which indicates that the effects of prenatal stress likely are not restricted to fetal development but in fact extend well beyond birth (Charil et al., 2010). In addition, synaptic density has been reported to be decreased in the hippocampus of 35 day old rats that had experienced mild prenatal stressors (Hayashi et al., 1998) as well as synaptic loss and decreased dendritic arborization at 90 days old, following prenatal restraint stress (Barros et al., 2006). These results clearly point to a hippocampal alteration being involved in our rats' deficit in spatial learning in the Morris water maze as well as the long-term memory deficit seen in the Can Test. Unfortunately, to our knowledge no studies have yet examined the hippocampal structure in a prenatally stressed human population, though Laplante and colleagues are beginning to embark upon structural studies in children born during the devastating 1998 ice storm in Quebec and surrounding regions (Charil et al., 2010). It is expected that hippocampal alterations will be observable via MRI in those affected children.

The brain mechanisms underlying the Can Test have not been as well elucidated as those for the water maze. However, it is likely that the prefrontal cortex is involved in the learning of this test and the resultant deficit in working memory following RVPS. Reduction in dendritic arborization and synapses has been observed in the frontal cortex of prenatally stressed rats (Barros et al., 2006). Pyramidal neuron spine densities are also decreased in both the orbitofrontal cortex and anterior cingulate cortex following prenatal stress as well as apical dendritic length being decreased (Murmu et al., 2006). Following a different prenatal stress protocol, i.e. repeated stress as opposed to variable stress, spine type ratio was found to be altered without a change in spine density in the medial prefrontal cortex (Michelsen et al., 2007). The effects of prenatal stress in the above studies on the prefrontal cortex clearly point to a possible neurological deficit which could contribute to the cognitive deficits observed in our study.

Prefrontal cortical disturbances and deficits in working memory are also prominent features of schizophrenia (Castner et al., 2004). The groundbreaking work of Goldman-Rakic and colleagues into the underlying mechanisms of working memory led to the hypothesis that disruption of PFC circuitry and/or connectivity could be involved in the cognitive deficits observed in schizophrenia (Goldman-Rakic and Selemon, 1997). Similar to prenatal stress, neonatal ventral hippocampal lesions result in adult animals with a deficit in working memory (Lipska et al., 2002) which is dependent on the integrity of the medial prefrontal cortex (Aultman and

Moghaddam, 2001). NMDA receptors are also important in working memory. Working memory is disrupted in rats following treatment with the competitive NMDA antagonist D-2-amino-5-phosphonopentanoic acid (AP-5) (Tonkiss and Rawlins, 1991). NMDA receptor antagonism in humans via ketamine is also able to create a specific impairment in manipulation of information in working memory (Honey et al., 2003). It is likely that the deficit observed in our studies stems from a similar mechanism, as we have also shown that RVPS alters prefrontal levels of genes involved in synaptic plasticity and the glutamatergic system (Kinnunen et al., 2003).

In summary, this specific aim was devoted to the hypothesis that repeated variable prenatal stress would result in cognitive deficits. Our hypothesis was confirmed by the findings that RVPS rats did indeed require longer latencies to find the hidden platform in the Morris water maze. The RVPS rats also committed more working memory errors in the spatial/visual discrimination of the Can Test, as well as showing a deficit in long-term memory retention. These deficits were quite specific, as RVPS rats did not show a deficit in reference memory errors, learning discriminations to a criteria level or in their ability to alter their rule set to adapt to alternating discriminations. These findings also further validate the RVPS paradigm as a model of cognitive deficits in schizophrenia. Prenatal stress is a risk factor for schizophrenia insofar as cohorts exposed to prenatal stress have increased rates of schizophrenia (Huttunen and Niskanen, 1978; Susser and Lin, 1992; Hoek et al., 1996; Susser et al., 1996; Hoek et al., 1998; van Os and Selten, 1998; St Clair et al., 2005). Schizophrenic patients have a deficit in a virtual Morris water maze (Hanlon et al., 2006) and meta-analyses have shown consistent impairments in working memory (Lee and Park, 2005) and long-term memory (Aleman et al., 1999). These studies and our results indicate that prenatal stress induces cognitive deficits that are similar to certain deficits observed in schizophrenia.

Chapter 3: Cognition Following Disruption of Neuregulin-1 Gene

Introduction

The search for a gene that is the cause of schizophrenia has been going on for essentially as long as schizophrenia genetics have been studied. Recent studies are conclusively showing that there is no single gene responsible for schizophrenia, but that many genes are involved in the disease. However, though many genes are involved there are some that tend to associate with schizophrenia more often than not and with more regularity than other genes. One such gene, is the neuregulin-1 gene (*NRG1*), which has been shown many times, by multiple groups in multiple populations to associate with schizophrenia (Stefansson et al., 2002; Stefansson et al., 2003; Liu et al., 2005; Walss-Bass et al., 2006; Alaerts et al., 2009; Nicodemus et al., 2009).

Neuregulin-1 is involved in many aspects of schizophrenia, beyond linkage analyses associating the gene with the disease. NRG-1 induced cell migration is significantly decreased in B lymphoblasts taken from schizophrenia patients compared to healthy controls (Sei et al., 2007). The alpha isoform of NRG1 is decreased in prefrontal white matter of schizophrenic patients and neurons expressing NRG1 are decreased in the gray matter of the prefrontal cortex (Bertram et al., 2007). A particular SNP within *NRG1* is associated with increased lateral ventricular volume in schizophrenic patients (Mata et al., 2009). Serum levels of the beta isoform of NRG1, which is more potent and more abundant in the brain than alpha (Harrison and Law, 2006), are decreased in patients with schizophrenia (Shibuya et al., 2010).

NRG1 codes for a family of growth factors (NRG1 types I - VI), which are involved in both developmental processes and adult brain regulation, which may have implications in schizophrenia (Mei and Xiong, 2008). NRG1 I, II, IV and V have a immunoglobulin domain and all have an EGF sequence which in most isoforms is followed by a transmembrane domain (Corfas et al., 2004; Harrison and Law, 2006). NRG1 acts on a family of single transmembrane tyrosine kinase receptors known as ErbB receptors. ErbB4 is the most well characterized ErbB receptor and is the only one known to associate with schizophrenia (Lu et al., 2010). Signaling between NRG1 and ErbB receptors is involved in neuronal migration and neuron-glial interactions in the developing cerebellum (Rio et al., 1997). NRG1 type II promotes migration of cortical neurons along radial growth fibers and promotes maintenance and elongation of the growth fibers (Anton et al., 1997). Alterations to GABAergic interneuron populations have been observed in a preparation lacking ErbB4 receptors, a result stemming from altered interneuron migration (Flames et al., 2004). The NRG1beta isoform, via ErbB signaling, stimulates growth of hippocampal axons (Gerecke et al., 2004). Lopez-Bendito et al., (Lopez-Bendito et al., 2006) have shown that NRG1 is also involved in the guidance of the developing thalamocortical tract neurons. NRG1 induces expression of the GABAa beta2 receptor subunit, leading to increased functional GABA receptors in cultured granule cells (Rieff et al., 1999). In hippocampal slices however, it has been shown that NRG1 decreases GABAa alpha receptor subunits and miniature inhibitory postsynaptic currents amplitude is decreased in CA1 pyramidal cells (Okada and Corfas, 2004). NRG1beta increases NMDA receptor subunit NR2C mRNA in cultured cerebellar slices (Ozaki et al., 1997). Neuronal developmental processes, the glutamate system including NMDA receptors and the GABA system have all been linked to schizophrenia. Mei and

Xiong (Mei and Xiong, 2008) have hypothesized that alterations in NRG1 signaling could underlie the pathology of schizophrenia, as schizophrenia is believed to stem from alterations during development and NRG1 is clearly involved in many aspects of normal development.

The functions of NRG1 are not limited to neuronal and receptor development. NRG1 is also involved in synaptic maturation and plasticity. Long-term potentiation in vitro is suppressed by NRG1 treatment in the hippocampus while basal neurotransmission is not affected (Huang et al., 2000). NMDA receptor subunit NR2B phosphorylation is stimulated by NRG1. In addition, mice with a heterozygous knockout for *Nrg1* transmembrane domain (NRG1 TM) have hypophosphorylation of NR2B and decreased theta burst induced LTP (Bjarnadottir et al., 2007). In addition to being located in the postsynaptic density (Huang et al., 2000), ErbB4 is also located in GABAergic neuron terminals in the prefrontal cortex (Woo et al., 2007). Without affecting basal GABA release, NRG1 treatment was able to increase evoked GABA release, but only in mice with intact ErbB4 receptors (Woo et al., 2007).

Research using various mutant mouse models of NRG1 signaling has examined the behavioral phenotypes that accompany the above mentioned electro-physiological studies. The original mutant mouse model was used in Stefansson et al., (Stefansson et al., 2002) study to complement the finding of association between *NRG1* and schizophrenia. They found that NRG1 TM mice or mice with a mutation to its receptor ErbB4 had impaired prepulse inhibition and a hyperactive phenotype compared to WT mice. These alterations were found in both lines of mice but were more robust in the NRG1 TM mice than the ErbB4 mice. The NRG1 TM mice also had fewer functional NMDA receptors than WT mice. Karl et al. (Karl et al., 2007) confirmed the hyperactive phenotype. In addition, NRG1TM mice have been observed to have altered responses to social novelty (O'Tuathaigh et al., 2007), altered social interactions (O'Tuathaigh et al., 2008), and impaired object recognition and contextual, but not cued, fear conditioning, but not impaired spatial or working memory (Duffy et al., 2010), as well as increased levels of serotonin-2a receptors (Dean et al., 2008). Again, these findings are relatable to what is seen in schizophrenia though a limitation to genetic animal models is that in schizophrenia the genes are not functionally knocked out.

While the previous research, from association studies in humans, to functional studies of NRG1 to examination of mutant mouse lines certainly implicates NRG1 in many aspects of both cognition and brain development and pathology, there is one caveat to consider. In association studies, the region of the *NRG1* gene that is most generally associated with schizophrenia is in the 5' region of the gene, the region that codes for NRG1 types II and IV (Harrison and Law, 2006) (Figure 1.1). However, the majority of transgenic mouse studies have mutated the transmembrane domain, which is located in the 3' region of the gene. While these studies are undoubtedly important and are yielding fascinating information, in order to more closely examine the most oft associated with schizophrenia region of the *NRG1* gene, requires an animal in which the corresponding region is indeed disrupted. In the studies presented herein, we are utilizing such an animal. Our line of transposon-induced disruption of *Nrg1* Fischer rats were generated using a Sleeping Beauty transposon

(Lu et al., 2007) which inserted into the region of the *Nrg1* gene which lies downstream of the coding sequence for Type II NRG1 protein isoforms, the region corresponding to which is associated with schizophrenia. Using this unique preparation we have shown that type II NRG1 mRNA and protein are decreased and that there are alterations in HPA axis secretions and behavioral responses based on environmental stimuli (Taylor et al., 2010b).

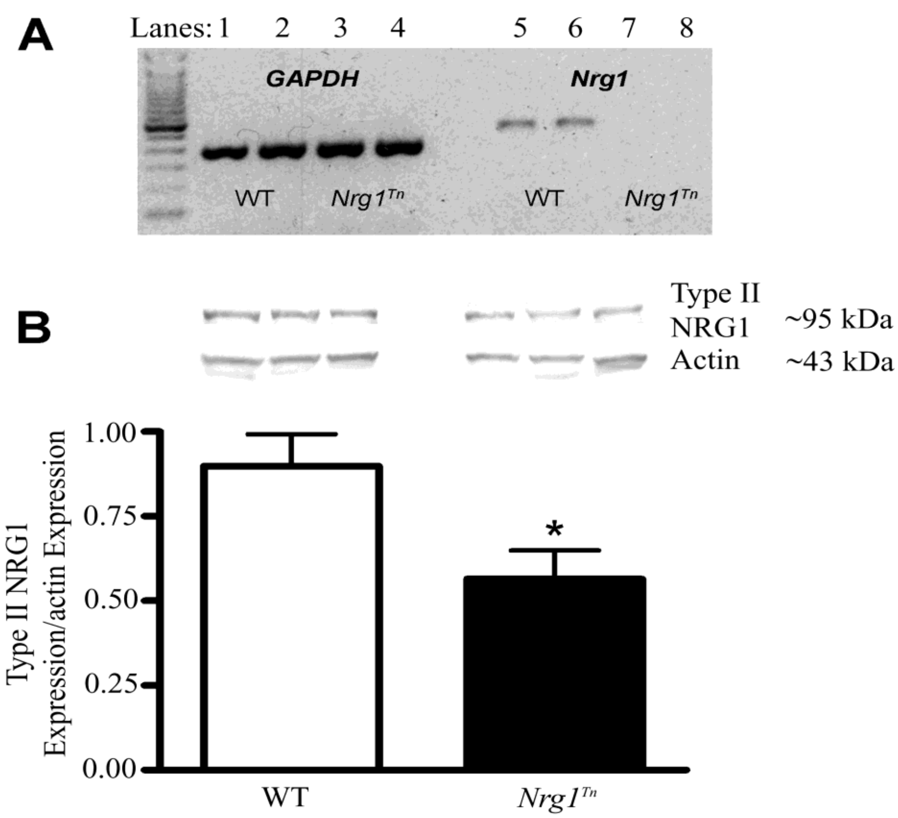
The second specific aim of this dissertation is devoted to testing the hypothesis that disruption of *Nrg1*, leading to decreased NRG1, results in cognitive deficits similar to certain deficits observed in schizophrenia. We will test this hypothesis by using the spatial version of the Morris water maze as well as running rats in the spatial/visual discrimination in the Can Test. Our predictions are that the $Nrg1^{Tn}$ rats will show increased latencies to find the platform in the water maze, as well as disrupted long-term memory in a retention test. We also predict poorer performance in the Can Test, reflected by fewer correct trials and more reference and working memory errors. Finally, we predict that diminished NMDA receptor subunit NR1 levels in prefrontal cortex, dorsal striatum and hippocampus will accompany the behavioral alterations.

Methods

Wildtype and homozygous Fischer 344 $Nrg1^{Tn}$ rats utilized by our laboratory were developed and obtained from the PhysGen Program in Genomic Applications (http://pga.mcw.edu/) at the Medical College of Wisconsin. *Nrg1* disruption was created by using the Sleeping Beauty transposon system (Lu et al., 2007) on a Fischer 344 inbred strain background (F344-$\mathrm{Nrg1}^{\mathrm{Tn(sb\text{-}T2/Bart3)2.183Mcwi}}$) which we have previously shown to result in rats with decreased type II Nrg1 mRNA and protein (Taylor et al., 2010b) (Figure 3.1). The transposon inserted randomly into the first intron, which lies downstream of the coding sequence for Type II NRG1 protein isoforms (see http://www.knockoutrat.com/ and http://rgd.mcw.edu/tools/strains/strains_view.cgi?id=2290103). Animals used in these experiments were housed at the Maryland Psychiatric Research Center in light (lights on at 06:00 hr and off at 20:00 hr) and temperature-controlled facility. With the exception of experimental water manipulations (see below, Can Test) animals were given free access to rat chow (Harlan Teklad, Frederick, MD) and water. All procedures conform to guidelines for animal research established by the National Institutes of Health, and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Figure 3.1: Expression of *Nrg1* mRNA and Type II NRG1 Protein

(A) Representative gel containing RT-PCR products from WT and homozygous *Nrg1*^{Tn} rats. Lanes 1-2: WT GAPDH, lanes 3-4: *Nrg1*^{Tn} GAPDH, lanes 5-6: WT exons 1-2 *Nrg1* mRNA, and lanes 7-8: *Nrg1*^{Tn} exons 1-2 *Nrg1* mRNA. Expression of the first two exons of *Nrg1* in the *Nrg1*^{Tn} rats was greatly reduced (the original gel showed faint bands that could not be captured in an image), while expression of GAPDH mRNA was unaffected by the insertion of the transposon. (B) Upper panel shows a representative Western blot of Type II NRG1 and the re-blot for actin in WT and *Nrg1*^{Tn} rats. Image analysis revealed a significant reduction in Type II NRG1 protein in *Nrg1*^{Tn} rats (*p<0.03). Bars represent mean ± SEM. Reprinted from Physiology and Behavior, Vol. 104, Issue 2, Taylor et al., Disruption of neuregulin 1 gene in the rat alters HPA axis activity and behavioral responses to environmental stimuli, 205-14, 2011, with permission from Elsevier.



Can Test

Habituation

The Can Test is based on our previously published protocol (Taylor et al., 2010a). Behavioral testing procedures described here were based off that paradigm. For five days prior to habituation, adult male rats were handled for 1–2 min each (WT n = 12; *Nrg1*^{Tn} n = 18); on the third day, animals were moved from the colony room to the behavioral testing room. Three consecutive days of habituation to the testing apparatus followed the handling exposures. On the first day of habituation each animal was allowed 20 min to explore the behavioral testing apparatus: a 100 cm × 100 cm × 46 cm box with black plexiglas walls. Animals were returned to their home cage without access to water, in order to facilitate motivation for finding the water reward during subsequent testing. On habituation day two, 7 identically patterned aluminum cans which were filled with cement for weight and stood 13 cm high, were placed in the apparatus in a fan arrangement so as to be equidistant from a starting point. The cans were placed upside-down, in order to provide a dish which could contain a bolus of water (0.3 ml). Water was placed in each of the cans and each animal was allowed to explore the apparatus (with water-containing cans) for 20 min. On the third habituation day, the apparatus was set up as above, except only 3 of the 7 cans were baited with the 0.3 ml of water, and the animal was allowed to explore the apparatus until it found all 3 water rewards, or until 10 min had elapsed. After finding the third reward, the animal was removed to a holding area while water was placed in three different cans. The animal was then reintroduced to the apparatus and allowed to explore until finding the 3 rewards, or until 10 min had elapsed.

Following the 3 days of habituation, animals were left undisturbed for 2 days. Water was again removed from the cages the day before testing began (see below). After habituation and testing, rats were allowed access to water for a 45-min period.

Spatial/visual discrimination test

Seven cans were arranged in the apparatus as above with the exception that 1 can was covered in white tape with a design of 3 black horizontal lines showing. Animals were allowed 10 trials per day over a 5-day period to learn that the water reward was always to be found in the unique can, the location of which remained stationary. Thus it was possible for animals to use either spatial or visual cues (or a combination) to learn the location of the water reward. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial, which commenced 15 – 30 seconds after the previous trial ended. A visit to a can was recorded when an animal stood on hind paws and looked for the water reward in the dish created by the can. A correct trial was recorded when the animal visited the rewarded can first. A reference memory error was scored the first time the animal visited a non-rewarded can prior to visiting the rewarded one during a given trial. A working memory error was scored each time an animal revisited the same non-rewarded can during a given trial. Average correct trials, reference memory errors, and working memory errors were calculated on a per animal basis for each day of training.

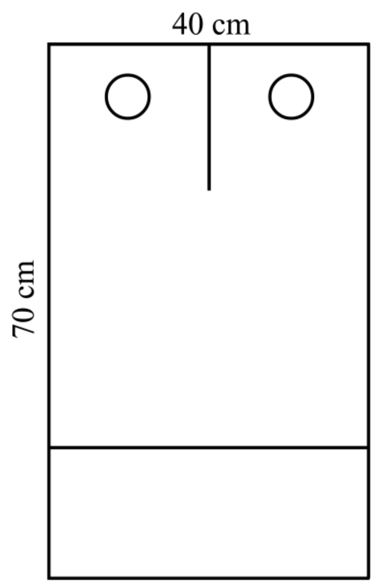
Morris Water Maze

Water Maze training was based on our previously published protocol (Taylor et al., 2010a). Adult male Fischer wild-type (n = 10) and $Nrg1^{Tn}$ (n = 10) were included in this experiment. Water Maze training was performed between 08:00 and 17:00 each day. Before the actual training began, animals were habituated to the environment, consisting of a circular tank 1.8 m in diameter and with a depth of .9 m (.6 m water depth), for 2 days. The tank was positioned in a room with multiple external cues, including colored items and posters either attached to the walls or hanging from the ceiling. On the first day of habituation, each animal was placed in the pool for two minutes, in order to familiarize it with the act of swimming and for exploration of the environment. On the second day, rats were again exposed to the pool, but the water was now made opaque by the addition of non-toxic white tempura paint. In addition, a stable Plexiglas escape platform (13.5 cm X 13.5 cm) was submerged 2 cm below the surface of the water in the northwestern quadrant of the pool. Rats were given 4 trials of up to 2 minutes to learn how to utilize the platform for escape from the water. If rats did not find the platform within two minutes they were gently guided to it by an experimenter. Training began the next day and continued for a total of three days. Training days consisted of 4 trials per day, with a 15 minute inter-trial interval. Rats were allowed up to two minutes to find the platform. If they did not find the platform they were gently guided to it by an experimenter. After fifteen seconds upon the platform rats were removed, toweled off and returned to their home cage. Starting locations were either the NE, SW, or SE quadrant of the tank and were randomized across groups/trials. After the final day of training, rats were returned to the colony room and not handled for six consecutive days. On the seventh day, rats were returned to the water maze for a “retention” test consisting of two trials conducted in the same manner as training. During both training and retention testing, latency to the platform was recorded and an average latency was calculated for each rat.

Attentional Set-Shifting

Adult male Fischer wild-type (n = 2) and *Nrg1*^{Tn} (n = 2) were included in this experiment and were handled for 2 minutes for 3 days before any training began. Rats were maintained at 85% body weight to maintain motivation for food reward, by feeding approximately 9g of rat chow per day. Set-shifting consists of rats learning to find ¼ pieces of cheerios buried in various media in 4cm tall terra cotta pots. The pots are also scented with a couple drops of scented essential oils (see table 2). The experiment involves the rats learning that either the scent on the pot or the media covering the Cheerio bit signal that the reward is located in that pot and they have to learn to a criteria of six correct choices (i.e. not digging in the non-rewarded pot) before moving to the following discriminations (described in more detail below). Because our experience with Fisher rats had shown us that they have difficulty with intricate behavioral experiments, there was necessarily an overabundance of habituation and pretraining that preceded the actual experimental day; this will be discussed briefly. Animals were first introduced to cheerios in their home cage to eliminate neophobia. For several days following this they were trained first to find cheerio bits in terra cotta pots in a large home cage, then to learning gradually to dig through bedding in the pots to find the cheerio bits. When they were successfully digging for cheerios in the large home cage they were moved to an arena designed as in figure 19, with dimensions of 70cm x 40cm x 30cm. The arena consisted of a holding area with a door that could slide up to allow access to the testing chamber where the two pots sat, divided by a middle barrier (Figure 3.2). Several days of habituation to the new arena and continued digging through bedding followed until the rat was consistently digging for the cheerio bits.

Figure 3.2: Setup of the Arena for Attentional Set-shifting



When they reached this stage they were introduced to the various scents on the pot rims and the various media used to bury the cheerio bits in the pots. The following day the rats were exposed to practice digging trials in unscented pots filled with bedding then were introduced to the discrimination learning portion of the test. The rats were required to learn a simple media and odor discrimination. The discriminations were either unscented pots containing either shredded Kleenex or shredded paper, with the shredded paper rewarded and Kleenex not. The odor discrimination was a clove scented pot filled with bedding which was rewarded and a nutmeg scented pot also filled with bedding that was not rewarded. The following day was the actual set-shifting test day. Testing consisted of (see Table 2) first a simple odor discrimination (sd) (e.g. rosewood scent signals reward, lemon does not, and each pot is filled with bedding), then once the rat reaches the criteria of six correct trials in a row, changes to a complex odor discrimination (cd). Each pot will now have four possible combinations for the rats to experience (e.g. rosewood scent filled with either shredded metal basket filler or raffia, and lemon scent filled with either shredded metal basket filler or raffia), and they must learn to ignore the media and continue to dig in only the pots scented with lemon. Upon reaching the criteria of six correct trials in a row in the scented pot the discrimination switches to the reversal discrimination (rev). This is where the four combinations are the same as above, but the rat must now switch his attention to the rosewood scented pot, which now contains the cheerio bit. Upon reaching criteria in this discrimination, rats are moved to the intradimensional shift (id). Here the scents and digging media are all changed (e.g. rosemary scent filled with either shredded felt or shredded fabric and cinnamon scent filled with either shredded felt or shredded fabric) but the same rules still apply, so that the rats must learn that the odor is important, and not the media, and rosemary is the reward. Upon reaching the criteria, animals are moved to the second reversal (rev2) where now the scents and media are the same but cinnamon is now rewarded. Upon reaching the criteria animals are moved to the extradimensional shift (ed). For this discrimination the scents and media are again changed (e.g. citronella scent and either shredded crepe paper or shredded velvet and thyme scent with either shredded crepe paper or shredded velvet). Now the rats must change their behavior and learn that the digging media is associated with the reward. The pots with the shredded crepe paper are now rewarded and the scent is irrelevant. Finally, upon reaching criteria the rats moved to the third reversal (rev3). Here the scents and media are the same except the pots with shredded velvet are now rewarded. Animals can also be ran by using the digging media as the reward symbolizer in the first 5 discriminations and the scent being used to symbolize reward in the extradimensional shift and the third reversal.

Table 3.1: Attentional Set Shifting Schedule

Discrimination		Relevant Stimulus	Irrelevant Stimulus	Rewarded Stimulus	Non-rewarded Stimulus
Simple		Odor	Digging Media	Rosewood	Lemon
Complex		Odor	Digging Media	Rosewood	Lemon
Reversal 1		Odor	Digging Media	Lemon	Rosewod
Interdimensional Shift		Odor	Digging Media	Rosemary	Cinnamon
Reversal 2		Odor	Digging Media	Cinnamon	Rosemary
Extradimensional Shift		Digging Media	Odor	Crepe	Velvet
Reversal 3		Digging Media	Odor	Velvet	Crepe

Western Blotting

Prefrontal cortex, dorsal striatum and dorsal hippocampus samples from animals used in the Morris water maze, (WT n = 10) and (*Nrg1*^{Tn} n = 10), were used to compare expression levels of NMDA receptor subunit NR1. Brain regions were identified using the Paxinos and Watson brain atlas (Gu et al., 2005). Brain tissue samples from the prefrontal cortex, hippocampus and dorsal striatum were isolated from 1mm frozen coronal sections using a 1mm rat brain punch (Stoelting, Wood Dale, IL). Bilateral punches were used for each area with a total of 4 punches for the prefrontal cortex, 6 punches for dorsal hippocampus, and 6 punches for the dorsal striatum. Tissue samples were homogenized via sonication in lysis buffer (50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCL and 1% SDS) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Brain tissue extracts were diluted in Laemmli sample buffer (Bio-Rad, Hercules, CA; 950 μl Laemmli buffer and 50 μl βME / 2-Mercaptoethanol) to a concentration of 20μg of protein per lane and resolved by electrophoresis (Room temperature, 140V for 47 min) on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were electrotransferred (4°C , 30 V for 21h) from the gel onto PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 1X Tris-buffered saline with 0.5% Tween-20 (TBS-T) and 3 % bovine serum albumin (BSA) for 1h and then incubated with the appropriate primary antibody for 24h at 4°C: NR1 (1:1000; sc-1467, Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBS-T and 3% BSA, membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1h at room temperature: rabbit anti-goat (1:2500; AP106P, Millipore, Temecula, CA). Membranes were then washed with TBS-T and the protein detected using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA). Blots were developed by exposure to chemiluminescent film (Amersham, GE Healthcare, Buckinghamshire, UK). Densitometric measurements of the protein bands were made using AIS 6.0 software (Imaging Research Inc., St. Catharines, ON, Canada). Following image collection, membranes were stripped using Restore plus Western blot stripping buffer (Thermo Fisher Scientific, Waltham, MA) and reprobed for actin

(1:10,000; MAB1501, Millipore, Temecula, CA). NR1 expression levels were normalized to actin expression.

Statistical Methods

All statistical analyses were conducted using SPSS statistical package. For behavioral tests analyses of variance (ANOVA) were conducted in which day of training was included as a repeated measures factor. Post hoc tests, conducted using l-matrix contrast statements in SPSS, were used to follow up any significant main effects or interactions. For western blots, t-tests were performed to compare groups. Attentional set-shifting was analyzed using one way ANOVAs. For all statistical analyses, $p<.05$ was considered significant.

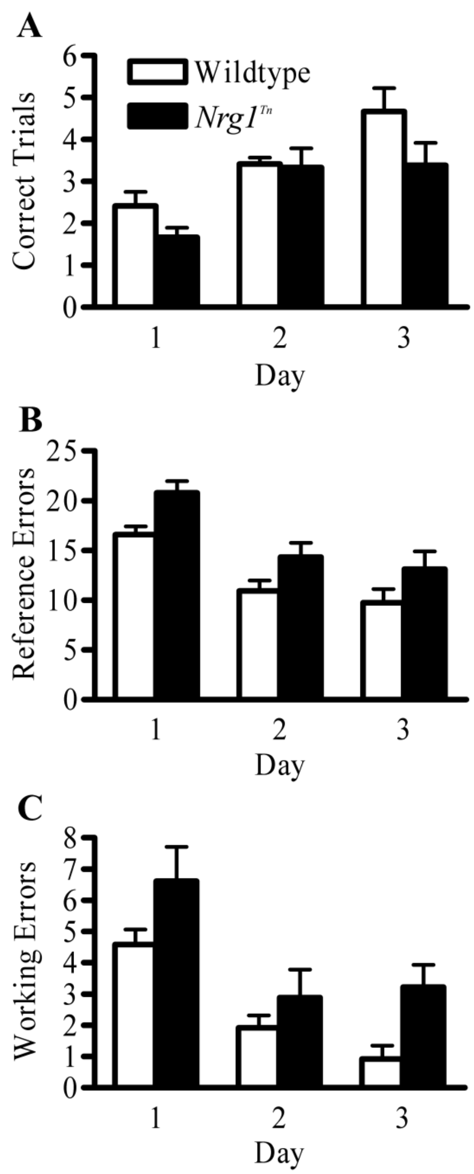
Results

Can Test

Fischer 344 rats were used in this experiment and have been shown to perform more poorly than Sprague Dawley rats in other tasks (Rodriguez et al., 2008). The Can Test was originally designed to be performed over five days of testing in the simple spatial/visual discrimination followed by other more complex discriminations. However, while most rats performed days 1 – 3 of the discrimination, performance continued to decline during the final two days. Therefore days 1 – 3 were analyzed and are presented here. Rats that were inactive more than 5 trials per day were eliminated from behavioral testing and analysis. Rats performance in the spatial/visual discrimination improved over the 3 days of training as shown by a 2 x 3 repeated measures ANOVA, demonstrated by increased correct trials ($F_{2,56} = 12.18$; $p < .001$) and decreased reference errors ($F_{2,56} = 22.31$; $p < .001$) and working errors ($F_{2,56} = 16.08$; $p < .001$). *Nrg1*^{Tn} rats trended toward making fewer correct trials ($F_{1,28} = 2.26$; $p = .077$; Figure 3.3A) than WT animals. *Nrg1*^{Tn} animals also made significantly more reference memory errors ($F_{1,28} = 5.79$; $p < .03$; Figure 3.3B) and made more working memory errors ($F_{1,28} = 4.10$; $p = .05$; Figure 3.3C) than WT rats.

Figure 3.3: Spatial/Visual Discrimination Results

Nrg1[Tn] rats trended toward making fewer correct trials ($p = .077$; A), and made more reference ($p < .03$; B) and working memory errors ($p = .05$; C) than WT rats.

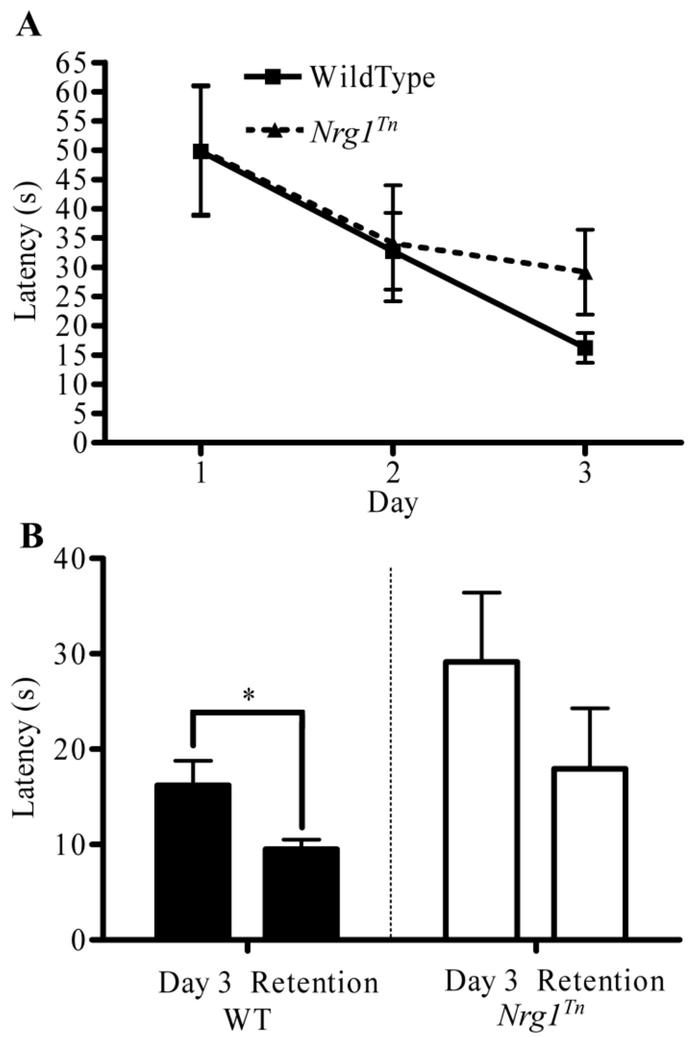


Morris Water Maze

A 2 x 3 repeated measures ANOVA was utilized to determine the effect of both genotype and day of training on the latency to find the platform. All animals decreased latency to find the platform over the 3 days of training ($F_{2,32} = 11.33$; $p < .001$) indicating that they were learning the location of the platform. No effect of genotype on performance during the 3 days of training ($F_{1,16} = .23$; $p = .64$; figure 3.4A) was found. However, planned comparisons between the latency on the final day of training and the latency on the one-week retention test, which were expected to reveal if there was any evidence of a lack of memory for the test, showed that WT rats significantly improved their latency ($t = 2.52$, $p < .05$; Figure 3.4B) while *Nrg1*^{Tn} rats did not have a significant difference in their latency ($t = 1.51$, $p = .17$; figure 3.4B).

Figure 3.4: Morris Water Maze Results

There was no effect of genotype on latency to find the escape platform during the three days of training ($p = .64$; A). Wild-type rats showed shorter latencies on a 7-day retention test ($p < .05$; B) while *Nrg1*^{Tn} rats did not ($p = .17$; B)



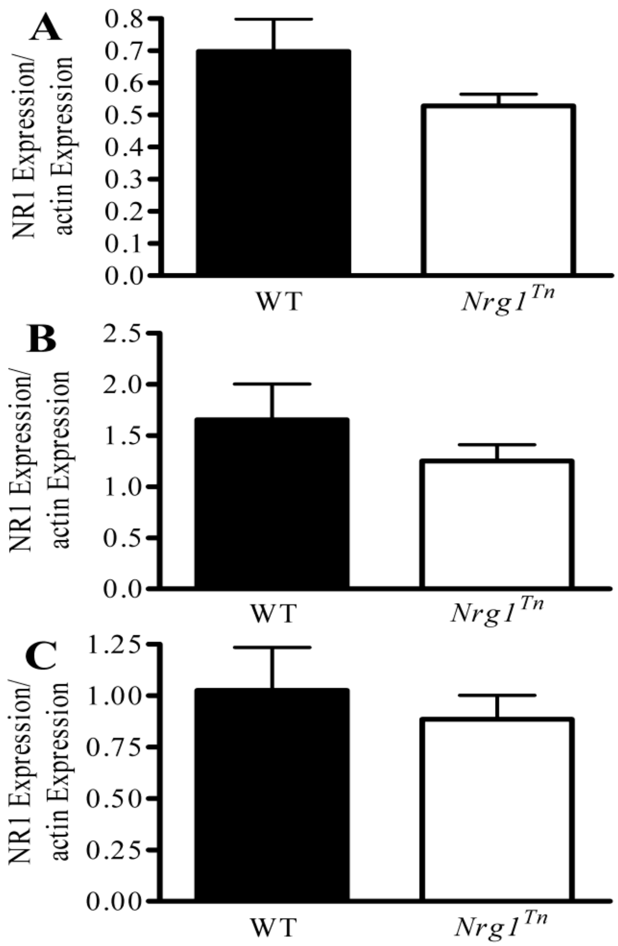
Western Blotting

NMDA receptors are important components of learning and memory and knocking out NMDA receptor subunit NR1 results in memory deficits. T-test comparisons revealed no difference in NR1 levels between WT ($n = 10$) and *Nrg1*^{Tn}

(n = 10) which had been used in the Morris water maze in prefrontal cortex (figure 3.5A; p = .14), dorsal striatum (figure 3.5; p = .31) or hippocampus (figure 3.5C; p = .57).

Figure 3.5: NR1 Protein Levels

NR1 levels do not differ between WT and *Nrg1*^{Tn} rats in either prefrontal cortex (A), dorsal striatum (B) or hippocampus (C).

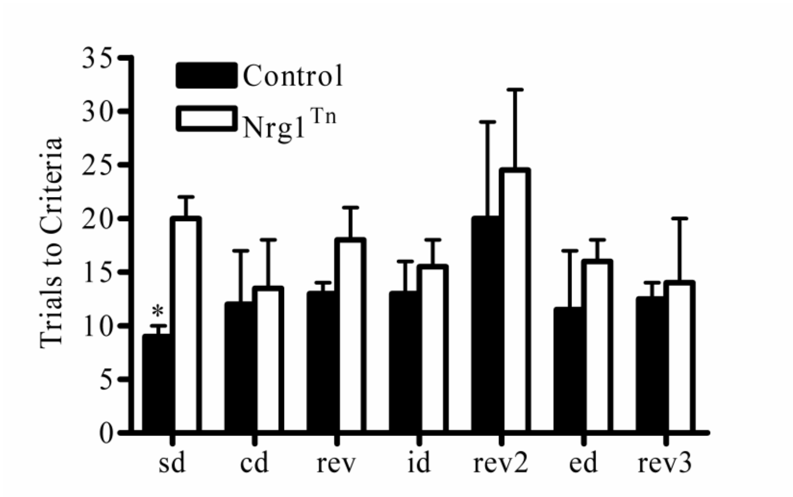


Attentional Set Shifting

Univariate ANOVA were utilized to determine if there was an effect of genotype of trials to criteria for each discrimination. *Nrg1*^{Tn} rats took more trials to reach the criteria of 6 correct trials in a row in the simple discrimination ($t = 4.92$; $p < .04$; Figure 3.6) than WT rats. There was no difference in trials to criteria in any other discriminations.

Figure 3.6: Attentional Set-Shifting Results

Nrg1^{Tn} rats had increased trials to criteria in the simple discrimination ($p < .04$) while there was not an effect of genotype on any other discrimination.



Discussion

In order to test the hypothesis of our second specific aim, that genetically disrupted *Nrg1* would result in cognitive deficits, we tested rats in multiple experiments designed to test their cognitive abilities. The Morris water maze was used to test the prediction that $Nrg1^{Tn}$ rats would show impaired spatial learning compared to WT rats. Contrary to our prediction, we found that $Nrg1^{Tn}$ rats performed identically to WT rats during the three days of training. Following training, rats were given a seven day memory retention test. Because of time constraints, this memory retention test was 7 days as opposed to 4 days in specific aim 2. Again we found that $Nrg1^{Tn}$ rats and WT rats did not have significantly different latencies to find the escape platform. However, we did note that WT rats average latency on the seven day retention test was actually significantly faster when compared to their own average latency on the final day of training. This may indicate that during the seven days when the animals were only in their home cages and were not exposed to the water maze, a further consolidation of memory occurred, an effect we did not see in Sprague Dawley rats. The $Nrg1^{Tn}$ rats latencies did not significantly differ between the final day of training and the seven day retention test, though their latencies were non-significantly quicker.

In order to test our second prediction, that $Nrg1^{Tn}$ rats would have deficits in reference and working memory we ran the rats in the spatial/visual discrimination version of the Can Test. In our experience we have found that Fischer rats are not always particularly good at performing behavioral cognitive tests. The Can Test was originally designed to be 5 days long, but because of attrition, of both WT and

Nrg1^{Tn}, during the experiment we have presented here only the first 3 days of training. *Nrg1*^{Tn} rats made more reference and working memory errors and trended toward making fewer correct trials.

Mice transgenic for the transmembrane domain of *Nrg1* have fewer functional NMDA receptors in the prefrontal cortex (Stefansson et al., 2002) and NRG1 is known to decrease synaptic currents in prefrontal pyramidal neurons (Gu et al., 2005). Western blot analyses were performed in three brain regions of WT and *Nrg1*^{Tn} rats for the obligatory NR1 subunit of the NMDA receptor. This was examined for the known role of the NMDA receptor in learning and memory. NRG1 is involved in NMDA receptor signaling (Li et al., 2007) and NRG1 treatment suppresses long-term potentiation in the hippocampus (Huang et al., 2000). Following from the behavioral results, i.e. no deficit in a hippocampal dependant spatial learning test and a deficit in a presumably more prefrontal and/or striatal task, we anticipated no difference in NR1 levels in the hippocampus but did anticipate a decrease in protein level in the prefrontal cortex and/or striatum. We did not find a decrease in the hippocampus, prefrontal cortex or striatum. It should be noted however, that due to experimental difficulties that will be described in more detail below, the brains that were processed for the western blot analyses were from the animals that performed the water maze and not those that performed the Can Test. Seeing as how there was a non-significant trend toward a decrease in NR1 in the PFC of these rats, who performed a task where there were no differences in learning between the *Nrg1*^{Tn} rats and WT rats, it is conceivable that if the brains from the rats that ran the Can Test, where there were significant differences in learning and memory, were processed we would see a significant difference. In the end however, we do not see any significant differences and therefore we cannot conlclude that altered NMDA receptor signaling mechanisms were responsible for the behavioral differences we did see, though it is also possible that the behavioral training the rats underwent changed the receptor subunit density thereby masking any differences that may have existed as multiple learning tasks have been shown to increase NR1 levels (Cammarota et al., 2000; Zhang et al., 2006). Due to a shortage of *Nrg1*^{Tn} rats, we were unfortunately not able at this time to examine receptor levels in experimentally naïve animals, but future studies will address this question. It may also be the case that NMDA receptor subunit NR2B is involved in the deficit seen here as *Nrg1* mutant mice show hypophosphorylation of NR2B (Bjarnadottir et al., 2007).

We also wanted to test the *Nrg1*^{Tn} rats in attentional set shifting, in order to determine if they had a deficit in cognitive flexibility. Attentional set shifting is a test in which animals are required to form a set of rules, i.e. that a certain scent indicates a food reward, and that other stimuli are irrelevant, and once the set has been sufficiently learned, they are then required to switch to a new set of rules on the fly, to determine if they form attentional sets and if they can shift those sets as required (Birrell and Brown, 2000). However, we discovered that the strain of rat that had the disrupted *Nrg1*, Fischer rats, are virtually incapable of performing a task as cognitively taxing as attentional set shifting. We did manage to have 2 rats from each group, WT and *Nrg1*^{Tn}, complete the entire set shifting experiment and we saw that, with this limited number of animals, *Nrg1*^{Tn} rats took more trials to complete the original simple discrimination than WT rats, and no difference in any of the other discriminations. However, the pattern of the data indicates that the rats are not actually forming sets in the discriminations, because if they were, the extradimensional shift would require many more trials than these rats took, indicating that sets are not being formed. While these results must be considered quite preliminary, it is of note that the first simple discrimination is very similar to the discrimination that the animals learned in the Can Test. In the simple discrimination the rats are learning that the reward is located in a receptacle that is unique in either digging media or scent, while in the Spatial/Visual Discrimination the rats are learning that the reward is in the visually and spatially distinct receptacle. While the actual modalities of stimuli used to discriminate are different, the underlying task is similar. Therefore it is easy to conclude that the deficit does indeed lie in the learning of a simple discrimination as opposed to a deficit in spatial learning. The cognitive deficits we have seen here, i.e. deficit in the Can Test and simple discrimination but not in the water maze are similar to what has been seen in previous work in mutant mice, though results have been conflicting. Mice with a mutation to the EGF domain of *Nrg1* have a deficit in contextual fear conditioning but not in novel object recognition (Ehrlichman et al., 2009). Using the Barnes maze, O'Tuathaigh et al. (O'Tuathaigh et al., 2007) showed that, as in our water maze experiment, mice with mutations to the transmembrane domain of *Nrg1* did not have a spatial learning deficit. However, they also found no evidence of working memory impairment in spontaneous alternation. Another study in transmembrane domain mutant mice also found no deficit in reference or working memory measures in spatial learning in either the radial arm maze or the Y-maze, but did find impairments in contextual fear conditioning and novel object recognition (Duffy et al., 2010). The discrepancies between results can likely be attributed to the fact that different regions of the gene are mutated between studies and different tasks have been employed (with the exception of novel object recognition being impaired in one study and not another). It is clear though, that *Nrg1* mutation, regardless of location does not affect spatial learning, as multiple studies, including ours in rats, have found no evidence of altered spatial learning. Therefore, *Nrg1* is involved in aspects of cognition, but the effects appear to be quite task specific and may be dependent on the region of the gene that is mutated.

Schizophrenic patients show many varieties of cognitive deficits. By studying the behavioral alterations in an animal model of schizophrenia we can gain information into what the pathological roles of those genes are. However, the effects that are seen in animal models are likely more robust than what is seen in human populations as human genetic variants are more subtle variations than outright mutations used in animal models (Banerjee et al., 2010). In addition, in animal research into the roles of *Nrg1*, multiple regions of the gene have been mutated, with often different results stemming from which region of the gene carries the mutation. Mice with mutations to the transmembrain domain of *Nrg1* have altered social interactions (O'Tuathaigh et al., 2007), do not have spatial deficits but do have impairment in novel object recognition (Duffy et al., 2010). Mice with the mutation in the EGF-like domain show a deficit in prepulse inhibition following pharmacological treatment, are hyperlocomotive and have increased habituation than WT mice (Duffy et al., 2008) though another study found that they were not hyperlocomotive nor did they show a PPI deficit, but they did have alterations to social interactions and contextual fear conditioning (Ehrlichman et al., 2009). Mice with mutations to the immunoglobulin domain do not show hyperlocomotion (Rimer et al., 2005). This points to the possibility that *Nrg1* has many functions and that various parts of the gene produce different phenotypic outpoints. Indeed, in humans, multiple regions of the gene and individual SNPs are associated with schizophrenia.

Neuregulin-1 plays many roles in both neural development and ongoing processes in adult animals. In an attempt to discover a mechanism underlying the behavioral deficit seen in the Can Test that the $Nrg1^{Tn}$ animals showed, we examined the NMDA receptor NR1 in prefrontal cortex, dorsal striatum and hippocampus. We chose to examine this receptor for multiple reasons. First, the NMDA receptor complex is well known to be involved in cognitive processes such as learning and memory (Robbins and Murphy, 2006). NRG1-ErbB4 signaling occurs in glutamatergic synapses and NRG1 is involved in receptor expression (Ozaki et al., 1997). A loss of NRG1-ErbB4 signaling in the hippocampus leads to impaired glutamatergic function and development of synapses (Li et al., 2007). While we did not see a change in NR1 levels in any of the regions we examined, there are many other possible explanations for the behavioral deficit we saw. The NR2B subunit may be involved as transmembrane domain mutant mice have been shown to have hypophosphorylation of that subunit (Bjarnadottir et al., 2007). The NR2C subunit might also be involved as NRG1 has been shown to increase that receptor (Okada and Corfas, 2004) and the decrease of NRG1 in our animals may have affected that receptor subunit as well. NRG1 has been found in GABA terminals in the prefrontal cortex and regulates neurotransmission via presynaptic ErbB4 receptors (Woo et al., 2007). NRG1 also seems to play a role in synaptogenesis in GABAergic interneurons (Ting et al., 2011). Therefore it is also possible that our deficits result from an alteration to the GABAergic system.

As mentioned above, behavioral training in Fischer rats is quite challenging. For the water maze, this was not an issue as the rats are virtually guaranteed to do this test, as not performing would result in sinking. However, in appetitive learning tasks, such as the Can Test and attentional set shifting, this is not the case. We were only able to analyze data from three days of training as opposed to the traditional five as we found high levels of attrition as the task continued. The brains of these animals were not processed as the second running of the experiment, which was planned, was the first time we saw mass amounts of attrition, so at the time it was considered an abnormal group of animals and the data was not to be examined. Upon running the experiment multiple more times we discovered this behavioral pattern was not an anomaly.

In summary, the second specific aim of this dissertation was devoted to examining the hypothesis that disrupted *Nrg1* leading to decreased NRG1 Type II results in cognitive deficits similar to certain deficits observed in schizophrenia. Our results both supported and refuted this hypothesis, as our prediction that the rats would perform more poorly in the Can Test than control rats was confirmed, our prediction that the rats would have a deficit in hippocampal dependent learning in the water maze was not confirmed. We also saw, with too few animals for solid conclusions, an increase in trials to criteria in the simple discrimination of attentional set-shifting, which is very similar to the discrimination used in the Can Test. We did not see any alterations in NMDA receptor subunit NR1 in any of the brain regions we examined, indicating that our deficit was not a result of altered NRG1 signaling leading to disruptions of that receptor. However, it is possible that with more animals we would have seen a decrease. This research provides further evidence that NRG1 signaling is involved in specific cognitive behaviors. In addition, in rats with disrupted *Nrg1* leading to decreased NRG1 Type II we found a deficit in each of the cognitive endpoints examined in the Can Test, one of which is working memory, an observed deficit in schizophrenia (Lee and Park, 2005; Forbes et al., 2009).

Chapter 4: Prenatal Stress and Neuregulin-1: Gene x Environment Interaction

Introduction

In the previous chapters we have examined the cognitive phenotypes of animals exposed to an environmental risk factor for schizophrenia as well as animals with a disruption to a gene that has been repeatedly associated with the disease, in order to better understand their role in schizophrenia related behaviors. However, while studying factors such as these in isolation is useful in determining what specific roles they play, in humans genetic and environmental factors necessarily interact during the lifespan of the individual. It is also generally accepted that the interaction of environmental and genetic risk factors for schizophrenia is crucial in the development of the disease (Tsuang et al., 2004; van Os et al., 2008). There have been many studies to identify susceptibility genes for schizophrenia but there does not appear to be cessation of specific functions. It is more likely that genes act to subtly alter pathways that are involved in the development of the disease. Identified genes do not cause the disease, but instead seem to act indirectly (Rutter et al., 2006).

Genetic liability for schizophrenia has been estimated at around 80 percent but monozygotic twins have a 50 percent concordance rate, clear evidence that environmental factors also play a critical role (Mittal et al., 2008). Heritability for schizophrenia has been estimated at around 80% but most of that may be due to gene x environment interactions (van Os et al., 2008). High genetic risk adoptees are more likely to develop schizophrenia if they are raised in a dysfunctional environment (Tienari et al., 2004). An interaction effect is demonstrated by neither genetic risk nor environmental context alone predicting schizophrenia outcome. Thought disorder symptoms are more common in offspring of mothers with schizophrenia if they are adopted away into a family with an adoptive mother who also shows signs of ‘communication deviance’ (Wahlberg et al., 1997). Higher levels of obstetric complications are seen in monozygotic twins that are affected with schizophrenia than those who do not develop the disease (McNeil et al., 1994). In one study, a family history of schizophrenia was necessary to see an increased risk from prenatal exposure to infection (Clarke et al., 2009). Both being adopted and raised in a low socioeconomic environment and genetic liability to non-affective psychosis increased risk while experiencing both in tandem increased risk more than either factor in isolation (Wicks et al., 2010).

Laboratory studies, where conditions can be tightly controlled, have shed more information on how genes and environments can interact to produce unique phenotypes. The limited studies that have been done have been able to examine very specifically how a certain environmental factor and a specific genetic alteration can produce novel outcomes. Isolation rearing for 2 weeks at 4 weeks old in mice lacking pituitary adenylate cyclase-activating polypeptide produced aggressive behaviors and hyperlocomotion not seen in isolation reared control mice (Ishihama et al., 2010). Disrupted in schizophrenia (DISC1), a known risk gene, when mutated in mice, produces depressive behaviors when experienced in tandem with prenatal immune activation. These behaviors are not seen in control mice that undergo prenatal immune activation or in mice with the mutated human DISC1 that do not undergo prenatal immune activation (Abazyan et al., 2010). Another study examined mice that expressed a dominant negative form of DISC1 (DN-DISC1) and were exposed to prenatal immune activation. This study found that DN-DISC1 mice exposed to the immune activation had deficits in short term and object recognition memory. These deficits were not seen in control animals exposed to prenatal immune activation nor in animals with DN-DISC1 that were not exposed (Ibi et al., 2010). Mice with mutated synaptosomal-associated protein of 25kDa that are also prenatally stressed have greater deficits in prepulse inhibition than what is seen with non-prenatally stressed mice carrying the mutation (Oliver and Davies, 2009). This deficit is reversed by treatment with antipsychotic medications. Additionally, social behavior alterations are seen in prenatally stressed mice carrying the mutation that are not seen in animals which are only prenatally stressed or only carry the mutation. The data from both the human studies and experiments in laboratory animals clearly show how important gene x environment interactions are in producing both schizophrenia and related behaviors in humans and in producing complex phenotypes in animals that are not seen, or seen to a greater degree, in animals receiving either only a genetic manipulation or only an environmental manipulation.

As described in the previous chapter, *NRG1* is a well accepted risk gene for schizophrenia, and is involved in many aspects of development and in cognitive behaviors. However, as has also been mentioned, mutations of *NRG1* do not cause schizophrenia, but are associated with increased risk. We have previously shown that *Nrg1*^{tn} rats have altered stress responsively compared to WT rats (Taylor et al., 2010b). Mice with mutations to *Nrg1* have also been shown to be more sensitive to the behavioral effects of tetrahydrocannibinol (THC) than WT mice (Boucher et al., 2007a). C-fos expression differences are seen only in mutant mice subjected to both prior testing and THC, which led the authors to conclude that an interaction between the stress of behavioral testing and the genetic manipulation elicited this effect (Boucher et al., 2007b). Another gene x environment effect was seen in humans, where a particular single nucleotide polymorphism in *NRG1* (SPN8NRG221533; T to C), which is located in the 3' region of the gene (Stefansson et al., 2002), similar to where our transposon inserted, was observed to mediate the effect of job strain on atherosclerosis. Thus, *NRG1* genotype appears to be involved in determining stress induced development of atherosclerosis (Hintsanen et al., 2007).

The third and final aim of this dissertation is devoted to examining the hypothesis that the combined presence of a disruption in *Nrg1* and RVPS results in an additive cognitive deficit than either alone. To test this hypothesis we will run animals in the spatial version of the Morris water maze. In addition animals will be tested in the spatial/visual discrimination version of the Can Test. We predict that animals that have disrupted *Nrg1* and also experience RVPS will have longer latencies to find the platform than RVPS rats, $Nrg1^{Tn}$ rats and control rats. In addition we predict that the RVPS $Nrg1^{Tn}$ rats will perform more poorly on the Can Test than any of the other groups, as indexed by fewer correct trials and more reference and working memory errors.

Methods

Animals

Wildtype and homozygous *Nrg1*^{Tn} rats utilized by our laboratory were developed and obtained from the PhysGen Program in Genomic Applications (http://pga.mcw.edu/) at the Medical College of Wisconsin. *Nrg1* disruption was created by using the Sleeping Beauty transposon system on a Fischer 344 inbred strain background (F344-Nrg1$^{Tn(sb-T2/Bart3)2.183Mcwi}$) (Lu et al., 2007; Taylor et al., 2010b). The transposon inserted randomly into the first intron, which lies downstream of the coding sequence for Type II NRG1 protein isoforms (see http://www.knockoutrat.com/ and http://rgd.mcw.edu/tools/strains/strains_view.cgi?id=2290103).

Prenatal Stress

Pregnant WT and *Nrg1*^{Tn} animals were housed individually in a light-controlled (lights on 0600 hr to 2000 hr) and temperature-controlled facility, and all dams had free access to rat chow (Harlan Teklad) and water. Half of the dams were exposed to prenatal stress according to the procedure described below, while the other (control) dams were not. All procedures described herein conform to guidelines for animal research established by the National Institutes of Health, and were approved by the University of Maryland – Baltimore School of Medicine Institutional Animal Care and Use Committee.

Beginning on day 14 and continuing through day 21 of gestation, pregnant dams were exposed to a RVPS paradigm (Kinnunen et al., 2003; Koenig et al., 2005; Lee et al., 2007) (Table 2.1). Briefly, the stressors used in this paradigm were: (1)

restraint in a well-ventilated cylindrical plexiglas restrainer (Harvard Bioscience, Boston, MA) for one hour, (2) exposure to a cold environment (4^oC) for six hours, (3) overnight food deprivation, (4) prevention of sleep during the light (inactive) portion of the cycle for 90 minutes, via 1 ½ inch of water in the bottom of an otherwise empty home cage, (5) 15 minutes of swim stress, and (6) social stress induced by overcrowded housing conditions during the dark (active) phase of the cycle. Two to three stressors were administered per day in a randomized order (Kinnunen et al., 2003; Koenig et al., 2005; Lee et al., 2007). All dams delivered their pups vaginally. Following delivery, the dam and her pups were left undisturbed in their cages until weaning on postnatal day 24-25. At weaning, male and female offspring were housed in same-sex, like-treated groups of two to three with free access to rat chow and water. The pups were exposed to normal animal husbandry procedures from that point forward until experimental use.

Morris Water Maze

Water Maze training was based on our previously published protocol (Taylor et al., 2010a). Adult male Fischer wild-type (n = 4, from 2 litters), wild-type RVPS (n = 5, from 3 litters), *Nrg1*^{Tn} (n = 4, from 3 litters) and *Nrg1*^{Tn} RVPS (n = 3, from 2 litters) were included in this experiment. Water Maze training was performed between 08:00 and 17:00 each day. Before the actual training began, animals were habituated to the environment, consisting of a circular tank 1.8 m in diameter and with a depth of .9 m (.6 m water depth), for two days. The tank was positioned in a room with multiple external cues, including colored items and posters either attached to the walls or hanging from the ceiling. On the first day of habituation, each animal was placed in the pool for 2 minutes, in order to familiarize it with the act of swimming and for exploration of the environment. On the second day, rats were again exposed to the pool, but the water was now made opaque by the addition of non-toxic white tempura paint. In addition, a stable Plexiglas escape platform (13.5 cm X 13.5 cm) was submerged 2 cm below the surface of the water in the northwestern quadrant of the pool. Rats were given 4 trials of up to two minutes to learn how to utilize the platform for escape from the water. Training began the next day and continued for a total of 3 days. Training days consisted of 4 trials per day, with a 15 minute inter-trial interval. Rats were allowed up to two minutes to find the platform. If they did not find the platform they were gently guided to it by an experimenter. After 15 seconds upon the platform rats were removed, toweled off and returned to their home cage. Starting locations were either the NE, SW, or SE quadrant of the tank and were randomized across groups/trials. During training, latency to the platform was recorded and an average latency was calculated for each rat. After the final day of training, rats were moved to the colony room where they remained undisturbed for the following six days. On the seventh day rats were reintroduced to the water maze and given two retention trials conducted in the same fashion as the training trials.

Can Test

Habituation

The Can Test is based on our previously published protocol (Taylor et al., 2010a). Behavioral testing procedures described here were based off that paradigm. For five days prior to habituation, animals were handled for 1–2 min each; on the third day, animals were moved from the colony room to the behavioral testing room. Three consecutive days of habituation to the testing apparatus followed the handling exposures. On the first day of habituation each animal was allowed 20 min to explore the behavioral testing apparatus: a 100 cm × 100 cm × 46 cm box with black plexiglas walls. Animals were returned to their home cage without access to water, in order to facilitate motivation for finding the water reward during subsequent testing. On habituation day 2, seven identically patterned aluminum cans which were filled with cement for weight and stood 13 cm high, were placed in the apparatus in a fan arrangement so as to be equidistant from a starting point. The cans were placed upside-down, in order to provide a dish which could contain a bolus of water (0.3 ml). Water was placed in each of the cans and each animal was allowed to explore the apparatus (with water-containing cans) for 20 min. On the third habituation day, the apparatus was set up as above, except only 3 of the 7 cans were baited with the .3 ml of water, and the animal was allowed to explore the apparatus until it found all three water rewards, or until 10 min had elapsed. After finding the third reward, the animal was removed to a holding area while water was placed in 3 different cans. The animal was then reintroduced to the apparatus and allowed to explore until finding the three rewards, or until 10 min had elapsed. Following the 3 days of habituation, animals were left undisturbed for 2 days. Water was again removed from the cages the day before testing began (see below). After habituation and testing, rats were allowed access to water for a 45-min period.

Spatial/visual discrimination test

Seven cans were arranged in the apparatus as above with the exception that one can was covered in white tape with a design of three black horizontal lines showing. Animals were allowed ten trials per day over a 3-day period to learn that the water reward was always to be found in the unique can, the location of which remained stationary. Thus it was possible for animals to use either spatial or visual cues (or a combination) to learn the location of the water reward. Following consumption of the reward, the animal was removed to a holding area while the can was re-baited for the next trial. A visit to a can was recorded when an animal stood on hind paws and looked for the water reward in the dish created by the can. A correct trial was recorded when the animal visited the rewarded can first. A reference memory error was scored the first time the animal visited a non-rewarded can prior to visiting the rewarded one during a given trial. A working memory error was scored each time an animal revisited the same non-rewarded can during a given trial. Average correct trials, reference memory errors, and working memory errors were calculated on a per animal basis for each day of training.

Statistical Methods

All statistical analyses were conducted using SPSS statistical package. For behavioral tests analyses of variance (ANOVA) were conducted in which day of training was included as a repeated measures factor. Post hoc tests, conducted using l-matrix contrast statements in SPSS, were used to follow up any significant main effects or interactions. For all statistical analyses, $p<.05$ was considered significant.

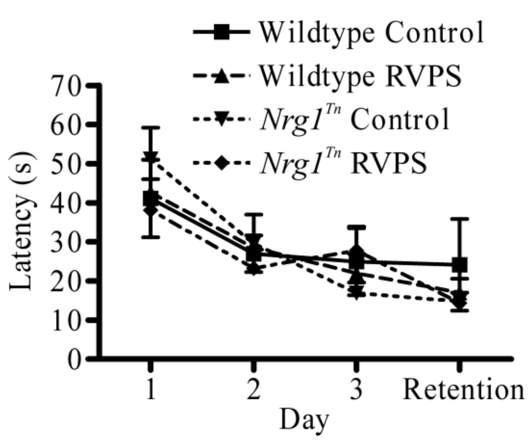
Results

Morris Water Maze

A 2 x 3 repeated measures ANOVA was utilized to determine the effect of both genotype and day of training on the latency to find the platform. All animals decreased latency to find the platform over the 3 days of training ($F_{2,24} = 12.46$; $p < .001$) indicating that they were learning the location of the platform. However we found no effect of genotype ($p = .984$) or stress treatment ($p = .774$) on performance during the 3 days of training, nor an interaction between the two (Figure 4.1). Planned paired comparisons between the final day of training and the seven day retention test revealed that all groups of animals performed identically on the retention test as they did on the final day of training (Figure 4.1; WT $p = .96$; WT RVPS $p = .44$; *Nrg1*^{Tn} $p = .49$; *Nrg1*^{Tn} RVPS $p = .12$)

Figure 4.1: Morris Water Maze Results

There was no effect of genotype or stress treatment and no evidence for a gene x environment interaction

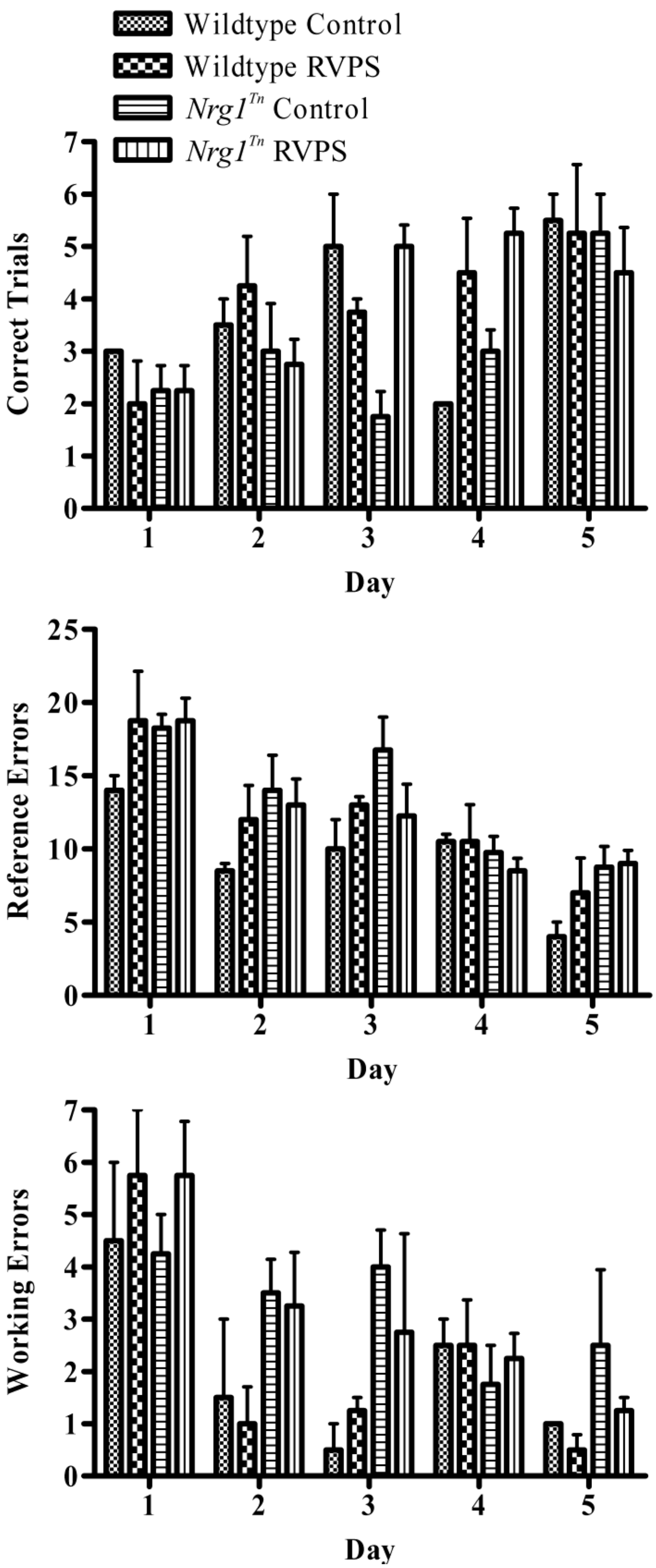


Can Test

All animals performance improved across the five days of training, as evidenced by a 2 x 2 x 5 (genotype x prenatal treatment x day) repeated measures ANOVA indicating increased correct trials ($F_{4,40} = 7.33$; $p < .001$) and decreased reference ($F_{4,40} = 16.86$; p <. .001) and working memory errors ($F_{4,40} = 8.60$; $p < .001$). There was no effect of genotype ($p = .42$) or RVPS ($p = .26$) for correct trials, nor was there evidence for a genotype x RVPS interaction ($p = .42$). There was no effect of genotype ($p = .12$) or RVPS ($p = .52$) for reference memory errors, nor was there evidence for a genotype x RVPS interaction ($p = .13$). There was a trend toward an effect of genotype ($F_{1,10} = 3.71$; $p = .083$) on working memory errors and there was no effect of RVPS on working memory errors ($p = .96$), nor was their evidence for a gene x RVPS interaction (Figure 4.2). However, differing from the previous aim, animals in this experiment finished all five days of training. For a more appropriate comparison between the previous aim and here, analysis was also performed for the first three days of training. There was a trend toward an effect of genotype on correct trials ($F_{1,10} = 5.40$; $p = .079$) as well as a trend toward an interaction between stress and genotype ($F_{1,10} = 5.40$; $p = .079$) as well as an interaction between day of training, RVPS and genotype ($F_{2,20} = 4.02$; $p < .04$). Reference and working memory errors statistics do not appreciably change between examining 3 days or 5 days of testing.

Figure 4.2: Spatial/Visual Discrimination Results

There was no effect of genotype or RVPS, nor evidence for a genotype x stress interaction for correct trials, reference or working memory errors, although there was a trend toward *Nrg1*^{Tn} making more reference memory errors than controls.


Wildtype Control
Wildtype RVPS
$Nrg1^{Tn}$ Control
$Nrg1^{Tn}$ RVPS
Correct Trials
7
6
5
4
3
2
1
0
1
2
3
4
5
Day
Reference Errors
25
20
15
10
5
0
1
2
3
4
5
Day
Working Errors
7
6
5
4
3
2
1
0
1
2
3
4
5
Day

Discussion

The final specific aim of this dissertation was examining the hypothesis that RVPS and a genetic disruption of *Nrg1* in rats would produce an adult rat with a phenotype of cognitive deficits that was more severe than that seen in either manipulation in isolation. To this end we examined rats in the Morris water maze and in a spatial/visual discrimination version of the Can Test. Contrary to our hypothesis we did not find a gene x environment interaction between RVPS and the *Nrg1* disruption. However, because of a shortage of our transgenic animals, our group sizes are small and our results must be taken as preliminary at best.

The Morris water maze that was ran in this aim utilized n's of 4 WT rats from 2 litters; 5 WT RVPS rats from 3 litters; 4 $Nrg1^{Tn}$ rats from 3 litters; and 3 $Nrg1^{Tn}$ RVPS rats from 2 litters. With this limited amount of rats we found no evidence that either RVPS or *Nrg1* genotype had any effect on spatial learning in the water maze or long-term memory in a seven day retention test. In addition we found no evidence for an interaction effect between RVPS and *Nrg1* genotype.

Our second experiment was examining learning ability in the spatial/visual version of the Can Test. For this test we ended with 2 WT control rats, 4 WT RVPS rats, 4 $Nrg1^{Tn}$ control rats and 4 $Nrg1^{Tn}$ RVPS rats. We found that neither RVPS nor *Nrg1* genotype affected performance in this task over five days as represented by correct trials, reference and working memory errors. There was a trend toward $Nrg1^{Tn}$ animals making more reference memory and working memory errors however. In addition, when looking at only the first 3 days of testing, as was the case in chapter 3, correct trials also showed a trend toward being significantly different between genotypes. We also saw a trend for an interaction between genotype and RVPS treatment and a significant interaction between day of testing, genotype and RVPS treatment for correct trials. While these results are not entirely the same as the results from the prior chapter, it should be noted that there is the trend toward more errors and, though not quite a true statistical trend, the p values are .12 and .08 for reference and working memory errors, possibly indicating the same effect as seen previously, as well as correct trials trending toward significance for the first three days of training. In addition, the small amount of animals available make finding statistically significant findings difficult. Finally, in general, the few *Nrg1*^{Tn} that were utilized in this experiment do show a similar trend in error making compared to controls that was seen in the previous experiment and would most likely become significant again if more animals were available. In addition, these results are not the same as our previous RVPS work but the animals in chapter two are Sprague Dawley rats and are Fischer rats in this aim.

Contrary to our hypothesis, we did not find that animals that had both disrupted *Nrg1* and were RVPS performed more poorly than animals with only the gene disruption or that were only RVPS, or control animals, in either behavioral task. This indicates that RVPS and *Nrg1* genotype in these animals do not interact significantly to induce a cognitive deficit. Thus, according to these preliminary results, it seems that having decreased levels of type II NRG1 does not increase vulnerability to RVPS, and nor does RVPS exacerbate any cognitive deficits that arise from the disruption of *Nrg1*. However, we looked at the first three days of the training, as we did in chapter 3, in order to make the comparisons between aims more appropriate, and found slightly different results. When looking at only the first three days we found that *Nrg1*^{Tn} rats trended toward making fewer correct trials and the trends for reference and working memory errors stayed at essentially the same p values. In addition, we saw slight, but exciting, evidence that a gene x environment interaction may have occurred. There was a trend toward a genotype x RVPS interaction for correct trials during the first three days of training, as well as an interaction between day of training, genotype and RVPS. However, due to our limited amount of animals available for experimentation, it is difficult to make any hard and fast conclusions concerning our hypothesis. Our results here must be considered quite preliminary and will need to be reexamined when possible. It will be important in moving forward that we are able to produce animals in numbers that are conducive to more appropriate testing procedures. Future studies can examine further behavioral characterizations as well as looking for physiological alterations. In addition, future experiments should focus on the initial phases of learning as our data indicates that during this period there may be a gene x environment interaction between *Nrg1* genotype and RVPS.

Chapter 5: Overall Discussion

Schizophrenia is a tragic, devastating disease for which there is no cure, for which medication does not effectively treat all symptoms and for which there is no known cause and no true certainty of what the underlying pathology is. Previous research has lead to the general consensus that schizophrenia is a developmental disorder that stems from a complex interplay between genetic and environmental risk factors (Tsuang et al., 2004). The symptomology of schizophrenia is also extremely complex. The three symptom domains are positive symptoms (hallucinations and delusions), negative symptoms (anhedonia, social withdrawal) and cognitive symptoms (working memory, attention, problem solving) (Carpenter and Buchanan, 1994; Keefe and Fenton, 2007; Bora et al., 2010). The cognitive symptoms of schizophrenia are extremely hindering to productivity and can be a life-long burden, as current treatments are not able to properly alleviate them (Simpson et al., 2010). A more thorough understanding of the cause and development of specific aspects of the cognitive deficits will greatly enhance the ability to design and implement progressive treatments. The Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) has defined seven domains of cognition that are altered in schizophrenia: working memory, attention, verbal learning and memory, visual learning and memory, reasoning and problem solving, speed of processing and social cognition (Green et al., 2004). Therefore we have examined the effects on cognition in adult life following prenatal stress, a known environmental risk factor for developing schizophrenia; a genetic manipulation of *Nrg1*, a gene known to be associated with schizophrenia; and we have examined the cognitive outcome in a gene x environment interaction study, where rats with disruption to

Nrg1 were exposed to RVPS. Our experimental paradigms are able to test for rodent homologues to several of the domains specified by MATRICS including working memory, attention and visual learning, as well as spatial learning and long-term memory, which are also altered in schizophrenia (Aleman et al., 1999; Hanlon et al., 2006; Piskulic et al., 2007; Van Snellenberg, 2009; Girard et al., 2010). Other animal models of schizophrenia, such as neonatal ventral hippocampal lesions, amphetamine sensitization and administration of NMDA antagonists have also shown deficits in many of the MATRICS identified domains of cognition (Lipska et al., 2002; Featherstone et al., 2007; Amitai and Markou, 2010). Genetic models of schizophrenia have also been employed. In these models neural systems and specific genes known to be involved in schizophrenia are altered or knocked out.

We have shown that animals exposed to RVPS show specific cognitive deficits. Spatial learning, as assessed by the Morris water maze, was decreased in the RVPS rats, which is consistent with other laboratories results in a variety of species and ages (Son et al., 2006; Zagron and Weinstock, 2006; Hosseini-Sharifabad and Hadinedoushan, 2007; Wu et al., 2007; Yaka et al., 2007; Kapoor et al., 2008) and consistent with deficits in spatial memory seen in schizophrenic patients (Hanlon et al., 2006; Girard et al., 2010). This version of the task is known to be dependent on the hippocampus, which is also known to be abnormal in schizophrenia (Ragland et al., 2007). While we did not perform any analyses on the brain tissue of the RVPS rats, it is well documented in the literature that prenatal stress causes alterations in the hippocampus (Szuran et al., 1994; Hayashi et al., 1998; Lemaire et al., 2000; Barros et al., 2006). RVPS has also been shown to decrease BDNF in the hippocampus in

Sprague Dawley but not Fischer rats (Neeley et al., 2011). Therefore it is very likely that our prenatal manipulation resulted in alterations to the hippocampus that manifest behaviorally as a decreased latency to find the hidden platform. The involvement of the hippocampus in memory (Squire, 1992) and our results from the long term memory retention test in the Can Test also support a hippocampal alteration in the RVPS rats.

We also found that RVPS rats have a working memory deficit in the simple visual/spatial discrimination in the Can Test, particularly on the very first day of training. This deficit was quite specific, as we did not see any differences in correct trials or reference memory errors. In addition, a comprehensive set of discrimination tasks, designed to assess both general learning ability and ability to shift rules were ran. We did not find any differences between RVPS animals and controls on any discriminations days to criteria, indicating learning ability is intact. Working memory is an important part of a cognitive repertoire and is a deficit commonly seen in schizophrenia (Van Snellenberg, 2009). Indeed, it has been argued that working memory deficits may be the underlying cause of many of the cognitive deficits in schizophrenia (Ragland et al., 2007). The prefrontal cortex is important in working memory (Goldman-Rakic, 1995) and previous research has also shown that prenatal stress changes the prefrontal cortex (Barros et al., 2006; Murmu et al., 2006; Michelsen et al., 2007). Therefore, it is likely that our RVPS rats have alterations in their prefrontal cortex as well as the hippocampus.

Schizophrenia genetics are very complex but certain genes, such as neuregulin-1, have been repeatedly shown to be associated with the disease

(Stefansson et al., 2002; Stefansson et al., 2003; Liu et al., 2005; Walss-Bass et al., 2006; Alaerts et al., 2009; Nicodemus et al., 2009). Using a novel rat model in which the Sleeping Beauty transposon (Lu et al., 2007; Taylor et al., 2010b) inserted into the coding region for type II and IV NRG1, the region generally associated with schizophrenia (Harrison and Law, 2006), we have found a variety of cognitive alterations. *Nrg1*^{Tn} animals show a general learning deficit in the spatial/visual discrimination version of the Can Test. This deficit was seen as the *Nrg1*^{Tn} have fewer correct trials and make more reference and working memory errors throughout the three days of training. In the Morris water maze, we found that *Nrg1*^{Tn} rats performed identically over the three days of training compared to WT animals. WT animals, on a memory test seven days after the final day of training, actually showed improvement compared to their performance on the final day of training, while *Nrg1*^{Tn} rats did not significantly improve their performance. Having seen this and knowing that our animals have decreased type II NRG1, and that NRG1 is involved in NMDA receptor signaling, we sought to determine if the disruption to *Nrg1* altered NMDA receptor subunit expression levels. As the water maze is hippocampal dependent and the mechanisms underlying the Can Test discrimination are likely utilizing prefrontal cortex, with the dorsal striatum also being a possible factor, as NR1 deletion in the striatum abolishes LTP (Dang et al., 2006) and inactivation of dorsomedial striatum impairs behavioral flexibility (Ragozzino et al., 2002), we examined the NR1 receptor subunit of NMDA in those regions. Interestingly, we did not find any differences in subunit levels in any region, though levels were non-significantly lower in prefrontal cortex of *Nrg1*^{Tn} rats than in controls and may be significant with more animals added. We had predicted, because we saw no behavioral differences in the hippocampal dependant task, that NR1 levels would not be different in the hippocampus, but would be altered in prefrontal cortex or dorsal striatum as these areas have been implicated in cognitive tests similar to ours and dorsal striatum lesions disrupt discrimination learning to a greater degree than hippocampal lesions (Broadbent et al., 2007; Bissonette et al., 2008). While there was not a significant difference, it is possible that the rats that were trained in the Can Test, i.e. the rats that had behavioral differences in a task that likely involves those regions, would show a decrease in NR1 in PFC, as there was a slight non-significant difference. Additionally, it is possible that the behavioral training itself altered the levels of NR1 in these regions and therefore masked any affect that might have been seen in naïve animals as Morris water maze training has been shown to increase NR1 immunoreactivity (Zhang et al., 2006) and one-trial inhibitory avoidance also selectively increases NR1 expression in the hippocampus (Cammarota et al., 2000). Due to our limited number of transgenic animals it was not feasible during this project to examine the tissue of experimental naïve animals, though future studies will attempt to answer this question.

Genetic factors and environmental factors that put a person at increased risk for developing schizophrenia do not act in isolation in the world at large, while most studies in the laboratory examine the effects of only a genetic manipulation or an environmental insult. The effects of gene x environment interactions in the development of schizophrenia are vast and are likely a major contributor of disease development and symptom presentation. In addition to studies evaluating humans and schizophrenia, laboratory research in animals has also shown that the effects of genetic manipulations and environmental insults can produce phenotypes different from those observed with the genetic manipulation or environmental insult in isolation . Therefore we undertook to examine the effects of our two schizophrenia related manipulations, RVPS and disruption of *Nrg1*, experienced in tandem, thus possibly modeling more realistically the experiences in humans. We did not find a gene x environment interaction in either the Morris water maze or in the spatial/visual version of the Can Test. $Nrg1^{Tn}$ rats that had experienced RVPS performed identically to WT rats, WT RVPS rats and non-stressed $Nrg1^{Tn}$ rats. We did however, find several statistically non-significant trends towards differences in the Can Test, especially when looking over the first three days only instead of the full five days. In this fashion we found trends towards $Nrg1^{Tn}$ rats making fewer correct trials and more reference and working memory errors, in line with the results from chapter three. There was also, when looking only at the first three days, a trend toward an interaction between genotype and prenatal stress treatment. However, due to technical difficulties, our power to uncover a true interaction was severely limited, thus we could not actually fully test this interaction. The trend effect for a gene x environment interaction is exciting though, and future experiments, possibly examining the initial phases of learning, should continue to uncover the true nature of this exciting preliminary result. Our research, due to the extensive nature of our behavioral experiments, has also been exclusively performed using male rats. Experiments examining females that have undergone our environmental and genetic manipulations are also crucial and will need to be addressed by future experiments.

While we have shown that RVPS can lead to cognitive deficits in working memory and long-term memory, as well as our previous research showing social interaction alterations, PPI deficits and recognition memory deficits, we have not ascertained the neurobiological underpinnings of these behavioral alterations. Research in other laboratories has shown alterations to the hippocampus and the PFC, but as our protocol, RVPS, has not been completely examined for its neurobiological outcomes, it is important for future research from our laboratory to focus on the underlying pathology supporting these behavioral changes.

In addition, our research into the roles of *Nrg1* in cognition is still in its infancy. Our *Nrg1*^{Tn} rat line is unique in that it results in a decrease in type II NRG1 by transposon insertion into the gene in the same region as is typically associated with schizophrenia in humans. The majority of previous animal models of decreased *Nrg1* function have focused the mutations in differing regions of the gene, and have been performed entirely in mice, so the research presented here is essentially completely novel. That being said, our results do resemble those seen in previous models, which have shown some subtle cognitive alterations. Future research from our laboratory will continue to examine the behavioral and physiological phenotype induced by decreased NRG1 type II. Research is also currently ongoing studying the interactions between *Nrg1* genotype and the hypothalamic-pituitary-adrenal axis.

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