Receptor-Associated Protein (RAP) Models In Vivo Reelin Haploinsufficiency: Implications in Schizophrenia

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Receptor-Associated Protein (RAP) Models In Vivo Reelin Haploinsufficiency: Implications in Schizophrenia

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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ABSTRACT

The “two-hit” schizophrenia hypothesis suggests genetic and environmental abnormalities interrupt early CNS function. This increases vulnerability of a “second hit” and schizophrenia onset. Chronic stress and decreased Reelin signaling are reportedly associated with schizophrenia. Heterozygous Reeler Mice (HRM) show a 50% reduction in Reelin and display major schizophrenia phenotypes. Receptor-Associated Protein (RAP) blocks ligand-association to Reelin receptor Apolipoprotein E receptor 2 (ApoER2). In this study, we sought to replicate major heterozygous reeler mouse (HRM) phenotypes using in vivo RAP studies to establish an experimental in vitro model. Using an in vitro model, we investigated the effects of chronic stress and decreased Reelin signaling on AMPAR subunit expression.

Implantable Alzet osmotic pumps allowed bilateral ventricular 7nM RAP perfusion in 12-14 week-old mice. An assay revealed significant Dab-1 phosphorylation reduction in RAP-perfused animals. These results correspond with learning and memory and associative-fear conditioning abnormalities. Overall activity, sensory perception, and nociception remained unaltered. RAP-perfused mice displayed deficits in pre-pulse inhibition to acoustic startle, and therefore sensory-gating deficits. A significant decrease in Glur1 and Glur2/3 expression was observed in primary hippocampal/cortical neurons following chronic RAP and CORT exposure.

Collectively, our results show postnatal Reelin signaling disruption produces physiological, biochemical, and behavioral phenotypes similar to the HRM model. The exact mechanism of
Reelin-dependent AMPAR insertion remains unclear. Glur1 and Glur2/3 appear to be inserted by differing mechanisms. Glur1 is reported to be inserted with Reelin activation of phosphoinositol-3-kinase (PI3K) signaling. Glur2/3, whose mechanism of insertion is unknown, has not been shown to be inserted via PI3K. Our findings also demonstrate the usefulness of in vitro RAP use, in which apolipoprotein E receptor 2 (ApoER2) expression is predominant compared to other lipoprotein receptors that may be affected with RAP application.
CHAPTER ONE:
INTRODUCTION

Two-Hit Hypothesis and Schizophrenia

Schizophrenia is a widespread, complex, neurodevelopmental disorder that adversely affects its victims as well as their families. Unfortunately, the underlying molecular cause for this disorder remains unclear [1,2]. The development of brain imaging and advanced diagnosis has led to correlative associations between abnormal brain function and schizophrenia. However, identifying the exact causal pathway of this disorder has been hindered by the failure to identify biological predictors that would effectively allow diagnosis prior to the occurrence of symptoms. For example, the dopamine hypothesis links increased mesolimbic dopaminergic activity to episodes of psychosis, which could result in the auditory and visual hallucinations associated with this disorder [3-5]. While this specific model provides a possible mechanism for the development of psychotic episodes, it does not address the mechanisms underlying the plethora of other symptoms associated with the disorder, in particular the cognitive impairment. The cognitive deficits associated with schizophrenia development include abnormalities in learning, working memory and executive memory, which rely primarily on the pre-frontal cortex and the hippocampal formation [6-8]. Thus, neuronal proteins associated with normal cognitive function within these two important CNS regions and also found altered in schizophrenia are likely causative players in schizophrenia etiology and/or cognitive alteration.
It is well established that cognitive disruption and behavioral abnormality can occur from temporally dependent environmental pressures, in particular prenatal insult. Environmental pressures and specific genetic alterations may not obligate for a person to show overt symptoms of neurologic insult; however, this could predispose the CNS to become increasingly sensitive to otherwise innocuous types of insult. This in-turn results in notable shifts of overall brain activity and explicit behavioral manifestations [9]. The ‘two hit’ hypothesis for schizophrenia suggests that genetic or environmental factors disrupt normal central nervous system (CNS) developmental or alters neuronal connectivity. Importantly, this alteration does present in an overt phenotype. However, these initial developmental disruptions create an increased vulnerability to encountering a ‘second hit’, eventually leading to the manifestation of several neurological disorders, including schizophrenia. This ‘second hit’ can be environmental, such as chronic stress or viral infection, or due to increased normal neuronal action, such as hormonal imbalance or necessary synaptic pruning. This ‘two hit’ hypothesis has implicated a number of proteins including a well-studied neuronal protein called Reelin. Abnormalities in Reelin protein correlate strongly with the development of schizophrenia. A 50% reduction in Reelin protein and mRNA have been observed in all post-mortem analyses of schizophrenic brains [10,11]. Reelin plays a vital role in neuronal migration and is essential for normal early brain development [12,13]. Reductions in Reelin during brain development may result in disrupted connectivity due to the mis-migration of certain neurons throughout the cortex and hippocampal formation. This makes Reelin a suitable candidate as a potential ‘first hit’ in schizophrenia development as it may predispose the CNS for a future ‘second hit’. In addition to having a role in early brain development, Reelin also regulates synaptic plasticity and neuronal function in the adult brain [14-16]. Therefore, altered Reelin in the adult brain has the potential
to provide a ‘second hit’ within the brain and contribute to the development of schizophrenia. Recent research into the role of Reelin in the developing and post-natal brain fit nicely into the ‘two hit hypothesis’. In fact, as described in this dissertation, Reelin may play the role of both ‘first’ and ‘second’ hit or be sufficient to cause behavioral aspects of schizophrenia through adult Reelin signaling disruption.

The Reelin signaling pathway has been shown to contribute to the regulation of learning and memory in both the hippocampal- and the pre-frontal cortex-dependent memory tasks [16-18]. Abnormalities in the function of this signaling pathway have been linked to several neurological disorders, including bipolar disorder, autism, and schizophrenia [11,19,20], each of which has a cognitive disruption component. While deficiencies and abnormalities in Reelin signaling have been linked to the development of schizophrenia, the exact mechanism through which this occurs is not completely understood [20-22]. Mice heterozygous for the Reelin gene (Rln) have been shown to exhibit many neurological deficits that are associated with schizophrenia [23,24]. These abnormalities include reductions in dendritic spine density, and pre-pulse inhibition (PPI) [23,25,26]. In addition to abnormalities in Reelin signaling, chronic stress is also been shown to be linked with schizophrenia development and also has the potential to be a ‘second hit’ for schizophrenia development. Interestingly, chronic stress is shown to be linked with some of the same neuropsychiatric disorders as Reelin deficiency such as, bipolar disorder, and depression [27-29].

In the current dissertation I have tested the hypothesis that Reelin signaling disruption will recapitulated the major phenotypes of the Reelin heterozygous mouse (HRM), a loose model of schizophrenia, through the use of *in vivo* studies utilizing receptor-associated protein (RAP). This dissertation also provides experimental evidence of an *in vitro* model that will be useful to
investigate the effects of chronic stress and stress coupled with decreased Reelin signaling on potential biochemical changes in the neuron, including, but not limited to, AMPA receptor subunit expression. Replicating these phenotypes with the use of RAP will further validate the potential role of Reelin in specific behavioral changes in the adult mouse, provide a greater understanding on the contribution of defective Reelin signaling to the development of schizophrenia and provide a potential model without the caveat of developmental alterations contributing to behavioral changes. Furthermore, Reelin signaling is shown to control AMPAR insertion in both iv vitro and in vivo models, which correlates nicely to alterations in AMPA receptor insertion associated with schizophrenia [30,31]. To date, there is no evidence to show how up-stream mechanisms underlying the cognitive deficits associated with schizophrenia, and other cognitive disorders may involve Reelin signaling in the adult brain.

This introduction will provide a primer to the general areas Reelin biology, the down-stream pathways of Reelin signaling and how receptor associated protein (RAP) can be used to modulate this signaling system. The clinical and behavioral manifestations of schizophrenia will be discussed, as well as the abnormalities that occur in the glutamatergic system in schizophrenic patients. The role of animal models to better understand molecular mechanisms and the deficiencies of current available models for schizophrenia will be explored in the context of Reelin signaling and human post mortem biochemical analysis. Finally, I will provide a description of the biological mechanisms underlying stress, and the disruptions that can occur in the hypothalamic adrenal pituitary (HPA) axis when the experience of stress duration is shifted from an acute to a chronic state. I will describe how stress experienced for extended durations can be linked to the development of schizophrenia, in particular the phenomenon of AMPA receptor trafficking, what is unknown about its mechanisms, and its link to Reelin signaling and
PI3K. While there is much known about these specific systems, the current research will attempt to bridge gaps in our understanding of schizophrenia etiology in general and specifically regarding the schizophrenia and its association with Reelin signaling, stress, and AMPA receptor insertion. Chapter 2 will provide scientific data that explores some of the important research questions posed in the first chapter. Chapter 3 will summarize data collected in the area of induced-pluripotent stem cells (iPSCs) created from fibroblasts both heterozygous and homozygous for Reelin. Chapter 4 will provide a summary and of the scientific and the significance of these findings.

**Reelin Signaling Pathway**

Reeler mice have a natural mutation that causes them to be Reelin deficient. These mice have been vital in studying the function of Reelin. An unstable and ataxic gait is characteristic of the homozygous reeler mouse. In this mouse, normal cortical layering of neurons is reversed. Mutations in the disabled-1 (Dab-1) gene and deletions in both the apolipoprotein receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR) produce defects in cortical layering identical to that of the reeler mouse. These mice, which differ genetically but are identical in phenotype, have been used to investigate the signaling pathways that Reelin uses to modulate neuronal function during both CNS development and adulthood.

When fetal brain development occurs, the neocortex is formed from the preplate. The Cajal-Retzius cells are among the cells in this preplate. When brain development occurs normally, Cajal-Retzius cells migrate into the marginal zone, which is the location where these cells produce and secrete Reelin. During development Reelin functions as a molecular guiding cue for ventricular migrating neurons in the neocortex, hippocampus, and cerebellum. Disruption of Reelin function
during embryonic brain development produces defects in the gross morphology of affected brain regions. In these mice, instead of forming a dense granular layer, dentate granule cells form a loose distribution throughout the hilar region. After development, Reelin is secreted by certain GABA (γ-aminobutyric acid) containing interneurons in the hippocampus and cortex following the gradual abatement of the cajal-retzius cells. This change in expression of this is affiliated with a differential function that is not associated with development. In the postnatal brain, Reelin activates several signaling pathways in the CNS that regulate synaptic plasticity and function. A lack in the expression of Reelin or its receptors, ApoER2 and VLDLR, is linked with abnormalities in associative and spatial learning as well as defects in hippocampal long-term potentiation (LTP) and changes in dendritic spine morphology.

The mouse Reelin protein is a 400 kDa glycoprotein consisting of 3461 amino acids and eight internal repeats of amino acids. Each internal repeat has a cysteine-rich epidermal growth factor (EGF)-like motif that is flanked by two subdomains. When Reelin binds to its receptors, this causes a clustering of ApoER2 and VLDLR. Following this clustering, Dab-1 undergoes tyrosine phosphorylation by the Src family of non-receptor protein tyrosine kinases (SFKs), which increases the activation of Dab-1. SFKs have a role in regulating synaptic plasticity. The tyrosine phosphorylation of Dab-1 leads to the phosphorylation of N-methyl-D-aspartate receptor (NMDAR). A following increase of calcium influx induces depolarization of the post-synaptic membrane. This calcium influx also leads to increased α-amino-3-hydroxy-5-methylisoxazolepropionic acid receptor (AMPAR). The influx of calcium and depolarization leads to increased phosphorylation of CREB and synthesis of proteins, which is increased with increased learning, memory and synaptic plasticity. Dab-1 phosphorylation also results in the activation of
phosphatidylinositol-3-kianse (PI3K), protein kinase B (PKB/Akt), and inhibition of glycogen synthase 3 beta (GSK3β).

Both ApoER2 and VLDLR are members of the low density lipoprotein receptor (LDLR) family. LDL receptors are multifaceted cell surface proteins that participate in extracellular protein endocytosis, regulation of synaptic function, and cross-membrane signal transduction. This family of receptors has seven mammalian member, which in addition to ApoER2 and VLDLR, include low-density- lipoprotein receptor (LDLR), multiple epidermal growth factor (EGF) repeat-containing protein-7 (MEGF7), LDL-related protein (LRP), LDL-related protein-1B (LRP1B), and megalin (Qiu). Each of these receptors has a ligand binding domain, an EGF homology domain, a single transmembrane segment, and a minimum of one cytoplasmic NPxY motif that functions as an interaction site for intracellular adaptor proteins. These adaptor proteins give LDL receptors the ability to couple to particular pathways of signal transduction. These signaling pathways use MAP kinases, tyrosine kinases, lipid kinases, and ligand-gated ion channels. Investigation of these receptors in their roles in synaptic plasticity and memory have involved the use of gene knockout studies. Mice lacking VLDLR and ApoER2 exhibit deficiencies in spatial learning, impaired associative fear conditioned learning, and slightly disrupted hippocampal LTP in area CA1.

Defective Reelin signaling is believed to contribute to the development of schizophrenia [9,10,19,32,33]. Several studies have shown a reduction of up to 50% in Reelin mRNA and protein levels in post mortem brain of schizophrenics and individuals with bipolar disorder with psychosis [10,24,34]. Heterozygous Reeler mice, +/-rl, which carry a mutation of one allele for the Reelin gene, have been shown to exhibit neurophysiological and behavioral characteristics of patients with schizophrenia [35]. Heterozygous Reeler mice have been shown to show many
neuroanatomical and cognitive deficits affiliated with schizophrenia, which include alterations in synaptic density and behavioral abnormalities [36,37]. Behavioral deficits include declines in pre-pulse inhibition (PPI) and exploration in the elevated maze, and apathy directly after a 5-week period of social isolation [23,24,38]. Collectively, these reports propose that a deficiency in Reelin has the potential to be critical to the development of schizophrenia, in addition to other neurological disorders [20,33]. However, there is a main caveat that the +/-rln mice have half the available Reelin protein during CNS in utero development. It is impossible using this model to determine how much of the overt phenotype is due to reduced Reelin signaling or connectivity defects due to altered neuronal migration.

**Receptor Associated Protein (RAP)**

Receptor-Associated Protein fused with glutathione S-transferase (GST-RAP) is an artificial fusion protein that has been used as a universal antagonist of ligand binding to the family of LDL receptors [15,16]. Importantly, GST-RAP acts as a non-competitive antagonist of Reelin for the ApoER2 and the VLDL receptors, thereby blocking Reelin from binding to its receptors and activating the downstream effects of the Reelin signaling pathway [12]. The RAP protein is an intracellular ER protein that functions as a molecular chaperone for LDL receptors [17]. When RAP binds to the LDL receptors, it helps these receptors to fold properly [39], and also prevents these receptors from binding prematurely to ligands for the receptors prior to membrane insertion [40]. Extracellular application of RAP results in binding to and blocking receptor signaling from the family of receptors [41]. RAP is shown to effective block all of the actions of Reelin on NMDARs, AMPARs and Dab-1, Src and PI3K signaling [42].
Absence of Reelin, ApoER2 or Dab-1 effectively prevents long-term potentiation (LTP) induction, but the developmental defect caveat exists for all of mouse models with genetic alterations in Reelin, ApoER2 or Dab-1. The exogenous application of RAP can be incredibly useful to examine the contribution of lipoprotein receptors in neuronal function. For example, Guojun Bu and colleagues revealed that high frequency stimulation (hfs)-induced synaptic plasticity in area CA1 can not be induced after the addition of RAP to wild-type hippocampal slices [39]. Taken together, this suggests that RAP acts on the same mechanism as the absence of Reelin signaling components to prevent hfs-induced LTP. One potential mechanism for this action of RAP is the prevention of NMDA receptor modulation, and subsequent decrease in NMDAR conductance of calcium. This is supported by recent work showing that RAP treated neurons resulted in only 19 percent of neurons responding to glutamate compared with the 64 percent of control neurons that were treated with Reelin [42]. GST-RAP is not a physiological extracellular ligand but may act to internalize Reelin receptors as well as blocking Reelin binding [43]. It is shown that internalization of ApoER2 and VLDLR occur via clathrin coated pit initiation [44] and GST-RAP application results in receptor internalization as well [45]. Thus, GST-RAP may work to block ligand binding and remove the surface associated receptor.

**Schizophrenia**

Schizophrenia is a devastating illness that adversely affects 26 million people worldwide and accounts for 1% of the global burden of disease [46,47]. This mental illness is characterized by the onset of one or more episodes of psychosis, which consists of mental distortions in the reality of an individual [48,49]. Schizophrenia is a psychotic disorder that triggers severe disruptions in thought, speech, or behavioral patterns [50,51]. Schizophrenia is associated with...
positive, negative, and cognitive symptoms. Positive symptoms of schizophrenia often consist of characteristics that typically “add” features to an individual’s perception [52]. Examples of positive symptoms include delusions, auditory, and visual hallucinations [53]. Negative symptoms often result from a “lack of” a particular characteristic that would normally be present in an individual from the general population [54]. Examples of negative symptoms include a lack of motivation, apathy, and withdrawal [55]. The cognitive symptoms associated with schizophrenia are often more difficult to detect than both positive and negative symptoms [56]. Cognitive symptoms therefore in some cases often remain unaddressed. Executive functioning (EF), which is also referred as cognitive control, refers to the ability of an individual to engage in and execute goal-oriented behavior [57]. Activities with executive function include: working memory, mental flexibility, and self-control [58,59]. Mental flexibility gives one the ability to plan and multi-task. Self-control gives one the ability to set priorities and avoid impulsive behaviors. In addition to deficits in executive functioning, cognitive deficits of schizophrenia also include impairments in learning and declarative memory.

**Animal models of Schizophrenia**

Schizophrenia is a very complex disorder involving patients that possess incredibly varied symptom combinations, making diagnosis and treatment often difficult. With all complex disorders involving numerous genetic alterations and likely combinations of environmental factors make it exceedingly difficult to model in a lower order mammalian host. Moreover, schizophrenia is marked by psychotic episodes that have no behavioral correlate in a rodent model. Therefore, finding an animal model that closely mirrors the heterogeneities associated with this illness would be a very valuable scientific tool for research. Animal models are useful
in general because they provide an opportunity to follow the progression of a disease over a shorter period of time compared with humans. Animal models also allow the use of physically intrusive techniques to investigate biological pathways linked with disease pathology. Although there are many benefits to using animal models, there are many obstacles to using these models as well. A major hurdle encountered in using animal models for schizophrenia due to its complexity is the inability to replicate certain symptoms that human-specific, including psychotic thoughts and memories. Another problem encountered with animal models is the lack of a gold standard treatment that alleviates all of the symptoms associated with schizophrenia [60]. Therefore, there is not currently a treatment that can be used as a positive control for studies involving schizophrenia. There are approximately 20 animal models that have been developed for schizophrenia [61]. These animal models fall into four primary categories: developmental, drug-induced, lesion, or genetic manipulation [62]. Initially, most of the animal models for schizophrenia were based upon dopamine abnormalities [61]. However, many studies with the progression of time revealed the need to develop animal models with glutamate dysfunction as a basis of the manifestation of this illness [63,64]. Specific examples of commonly used animal models include using methylazoxymethanol (MAM), neonatal hippocampal lesions, isolation rearing from weaning, and chronic phenylcyclidine (PCP) administration [65]. These models have been shown to exhibit both cortical and dopaminergic abnormalities. They have also shown to be linked with deficits in sensorimotor gating from abnormalities in cortical and limbic systems. The positives and negatives of each of the four categories of schizophrenia animal models is beyond the major focus of this dissertation; however, as an example of the utility of one such widely used model is notable and relevant to the current study. Systemic injection of
phenylcyclidine (PCP) is widely used to produce a schizophrenia model and falls under the category of a “drug-induced” model. Application of PCP shows behavioral symptoms in non-schizophrenics that closely resemble schizophrenia [64]. Manifestations that include positive and negative symptoms include paranoia, auditory hallucinations, apathy, and social withdrawal. Symptoms produced that resemble cognitive deficits include impairments in working memory. In research animals, PCP and other antagonists of NMDA receptors such as ketamine have been shown to produce several of the cognitive deficits exhibited in schizophrenia, which include impaired working memory in performance tasks, altered social behavior, hyperactivity, and abnormalities in sensory gating. The major caveat of using this “drug-induced” model is that the effect of PCP may be mimicking symptoms of schizophrenia without tapping into the same molecular mechanisms underlying schizophrenia. In other words, this model may lead researchers to better understand PCP effects on the brain, but unfortunately not the underpinnings of schizophrenia, per se. The use of genetic models have a greater direct relationship with the basis of schizophrenia as these genes have been associated with the disorder. Of course, the major caveat here is the effect of altered expression of genes and when this may be occurring in the human condition versus disruption in knockout animal models where absence of the gene may affect developmental processes. This is certainly the care with the heterozygous Reeler mouse. mouse model for schizophrenia [37]. HRM have been shown to share several behavioral and neuroanatomical similarities with schizophrenic patients [24]. These similarities include decreased spine density in the pre-frontal cortex (PFC), increased neuronal packing density, apathy development after a 5-week social isolation, declines in exploration in the elevated plus maze, and decreases in pre-pulse inhibition and olfactory learning [23,26,36].
The link between schizophrenia development with certain forms of environmental exposure, such as chronic stress, has made it difficult to determine the biological mechanisms affiliated with this disorder, and therefore difficult to develop efficient therapeutics to treat patients [66]. Several scientific theories have been developed to describe the association between abnormal brain function and schizophrenia. However, identifying the exact causal pathway of this disorder has been hindered by the failure to identify biological predictors for the occurrence of symptoms.

**Chronic Stress and Schizophrenia**

Chronic stress is one environmental factor that has been shown to be linked with the development of schizophrenia [67-69]. Chronic stress experienced during pregnancy, childhood and adolescence have been reported to be linked with a higher risk of schizophrenia development, which typically manifests in early adulthood. [32,69]. Several studies have also proposed that abnormal responses to stress activate the manifestation of many other mental illnesses [70]. It has been inferred that individuals who carry genetic risk factors for schizophrenia are more susceptible to the effects of stress [71]. Several studies have shown that a large percentage of individuals who experienced their first episode of psychosis experienced a traumatic event or series of events prior to having the psychotic episode [72]. Also, elevated cortisol levels have been detected in several studies in the post-mortem brains of schizophrenic patients [73,74]. The fundamental constituents of the stress cascade, which are excessive cortisol production, hippocampal damage, and impairment in hippocampal cognition, have all been shown to be abnormal in schizophrenia [75-77]. These studies have demonstrated that diurnal rhythms of cortisol are disrupted in schizophrenia [78]. In these studies a bilateral reduction was found in the hippocampus by 4 percent [79]. It has therefore been postulated that stress,
especially when experienced chronically, is linked with the development of schizophrenia. Currently, no reliable forms of treatment have been formulated to alleviate the cognitive symptoms of schizophrenia that are linked with chronic stress exposure [80,81]. In many instances, positive symptoms are successfully alleviated in schizophrenic patients by dopamine D2 antagonists [5,82,83]. Unfortunately, effective treatments for negative and cognitive symptoms are not as prevalent. This problem is extremely relevant for patients with schizophrenia who suffer from cognitive symptoms.

Learning, spatial memory, episodic memory, and declarative memory are regulated primarily by the hippocampus and have been shown to be adversely affected in schizophrenic patients [85,86]. It has been suggested that reductions in the hippocampal volume of schizophrenic patients occurs through neurotoxicity induced by glucocorticoids from increased stress [76]. These reductions in hippocampal volume are consistent with deficits in declarative memory, and episodic memory [87]. Declarative memory consists of the ability to recall typical facts and experiences [88]. Episodic memory refers to the ability of an individual to recall memorable personal experiences [89]. Learning and memory formation has been shown to be governed in part by the dentate gyrus region of the hippocampus [90]. Spatial learning has been shown to be disrupted by stress [91]. In an experiment with rats that experienced maternal separation, the rats were unable to distinguish between objects that were in the same location and in a different location [92]. The dentate gyrus within the hippocampus receives direct inputs from the entorhinal cortex [93]. The entorhinal cortex sends projections to the CA3 region of the hippocampus, which is considered to be the gateway to the hippocampus [94]. GABAAergic interneurons largely exist in the dentate gyrus [95].
These neurons are also abundant within the hilus and the CA3 region of the hippocampus. It has been suggested that abnormalities with inhibitory neurons in the DG have been linked with abnormalities in pattern separation and with the manifestation of schizophrenia [96]. The executive functions of the brain are primarily regulated and performed by the pre-frontal cortex (PFC) [97]. Lesions to the PFC have been shown to be linked with manifestations of forgetfulness, impulsivity, disorganization and distractibility [58]. Post-mortem studies have shown schizophrenia patients to exhibit a thinner PFC compared with normal individuals [7,97]. In the PFC, excitatory neurons in the PFC consist primarily of neurons [98]. Cortical pyramidal neurons are separated into different subtypes depending upon their target axonal projections, morphology, and physiology [99]. Several studies have shown decreased spine density of pyramidal neurons in layer III of the dorsolateral prefrontal cortex of schizophrenic patients [100]. In addition to glutamatergic pyramidal neurons, the PFC also contains inhibitory GABAergic interneurons that form connections with each other as well as with pyramidal neuronal networks [100]. Cortical interneurons are divided into subcategories based upon their electrophysiological, chemical and morphological properties.

The Glutamate Hypothesis For Schizophrenia and AMPA Receptor Modulation

The glutamate hypothesis for schizophrenia is continuing to evolve as it gains more experimental support. This hypothesis was formulated after schizophrenia-like symptoms were observed in normal individuals after the intake of NMDA receptor antagonists phenylcycloidine (PCP) and ketamine [64]. Glutamate is responsible for activating both NMDA receptors and AMPA receptors [101]. When a cell is at resting membrane potential, glutamate molecules bind to the recognition sites on NMDA receptors and AMPA receptors [48]. When AMPA receptors
are activated, they admit sodium ions into the cell. The entry of these sodium ions results in the depolarization of the cell. This depolarization causing magnesium ions blocking NMDA receptors to be dislodged. When these magnesium ions are dislodged, calcium ions are given the ability to enter the cells through NMDA receptors. This depolarization leads to an insertion of more AMPA receptors into the post-synaptic membrane [41,102].

Abnormal extracellular glutamate levels have been observed in a number of disorders, including schizophrenia, Alzheimer’s disease, obsessive-compulsive disorder, parkinson’s disease [103]. Post-mortem studies of brain observation have revealed a link between the glutamatergic system and schizophrenia. These studies have shown declines in NMDA receptors, AMPA receptors, glutamate and Nacetyl- a-linked acidic dipeptidase (NAALADase), which is the enzyme that cleaves NAAG into NAA [104]. These studies have also shown increased levels of NAAG, in both the pre-frontal cortex and hippocampus [105]. These declines are correlated with decreases in glutamate receptor activity in brain regions that have been linked to the pathology of schizophrenia. LTP, being governed by NMDA receptors, is adversely affected by these abnormalities in glutamate receptor activity [8,106]. This deficit in LTP activity is linked to abnormalities in working memory and other cognitive deficits controlled by affected brain regions [8].

The subunits of AMPA receptors are encoded by four different genes: GLUR1, GLUR2, GLUR3, and GLUR4 [107]. AMPA receptor subunits have multiple isoforms as well due to alternative splicing. GLUR1-GLUR4 AMPA receptor subunits are joined together in different combinations; these different combinations determine how each channel functions. GLUR1-GLUR3 AMPA receptor subunits are generally located in the hippocampus, cerebral cortex, amygdala, and the basal ganglia [108]. The GLUR4 subunit is generally present in lesser
quantities than the three other AMPA receptor subunits, except in the areas of the cerebellum and the reticular thalamic nuclei [109]. In the cerebellum and the reticular thalamic nuclei, these subunits are present in abundant quantities [108]. AMPA receptor subunits are manufactured and assembled in the rough endoplasmic reticulum (ER). After AMPA receptors are assembled, they move across the golgi apparatus and are inserted into the plasma membrane [110]. Abnormalities in AMPA receptor trafficking, which occurs through the processes of AMPA Receptor Insertion and AMPA receptor endocytosis, have been linked to the development of schizophrenia [111]. AMPA receptor trafficking is very critical for proper synaptic function [112]. Therefore, it is necessary to study intracellular pathways linked with aberrant AMPA receptor function to determine the abnormal functionality of these pathways is linked with the development of schizophrenia. On a neuronal level, homeostatic plasticity is a response that uses negative-feedback that is used to make compensations for imbalances in neuronal activity [113]. Homeostatic synaptic plasticity, which is also known as synaptic scaling, is primarily regulated by alterations in the activity of AMPA receptors [114]. When homeostatic regulation occurs, AMPA receptor numbers are either up-regulated at the post-synaptic surface through the process of AMPA trafficking process of receptor insertion when there is a deficit in AMPA receptor activity. However, when there is an over-activity in receptor activity, there will be a down-regulation of AMPA receptors at the post-synaptic surface through the AMPA receptor trafficking process of receptor internalization [115]. The process of synaptic scaling becomes active when there are disturbances in the nervous system triggering imbalances in AMPA receptor activity [101]. For example, the neurological effects of stress can be detrimental to processes such as learning, cognition, and memory. These
neurophysiological effects of chronic stress activate the homeostatic synaptic plasticity negative-feedback responses to compensate for disturbances in neurological activity [112]. While the neurological effects of chronic stress can be detrimental, the neurological effects of acute stress can be quite beneficial to the nervous system [116]. The effects of acute stress have been shown to be adaptive with positive responses such as improvements in cognitive performance and vigilance. Acute stress has been shown to activate NMDA receptors, and by increasing long-term potentiation (LTP) with increased Ca+2 entry, there is an increased insertion of GLUR1 AMPA receptor subunits onto the plasma membrane. An increased insertion of GLUR1 subunits onto the plasma membrane has been shown to occur more quickly than the insertion of GLUR2 subunits [117]. Most AMPA receptors have been shown to possess at least one GLUR2 subunit, and therefore only allow the entry of sodium into the cell to depolarize the membrane potential. When AMPA receptors are formed without any GLUR2 subunits, they allow the entry of calcium in addition to sodium, thereby contributing to LTP enhancement along with NMDA receptors within the brain [101]. AMPA receptors that lack GLUR2 receptors, such as GLUR1 receptors, have been shown to have an increased permeability to Ca+2 than receptors that possess a GLUR2 receptor subunit. Traditional LTP can coincide with LTP induced from acute stress by GLUR2-lacking receptors, namely GLUR1. This increased insertion of GLUR1-containing AMPA receptors induced by acute stress has been shown to be dependent upon protein kinase A (PKA). In neurons from the prefrontal cortex, acute stress and acute corticosterone (CORT) exposure has been shown to cause increases in synaptic transmission by increased NMDA and AMPA receptor activity [112]. When neuronal firing has been under acute suppression, homeostatic plasticity causes the activity of synapses to
be strengthened [31] However, when neuronal firing becomes chronically activated, homeostatic plasticity causes the activity of synapses to be weakened [110].

**Signaling Pathways and Molecules That Regulate AMPA Receptor Trafficking**

Several signaling pathways have been shown to participate in the processes of AMPA receptor trafficking [101]. For example, TNFα and the PI3K-Akt pathway have been linked to an up-regulation in AMPA receptors in the post-synaptic surface by receptor insertion [117]. However, the PICK1 postsynaptic scaffolding protein, is linked with the down-regulation of AMPA receptors in the post-synaptic surface by post-synaptic surface receptor internalization.

**GluR1 Knockout Mice**

A genetic deficit of the GLUR1 subunit has been shown to be linked with severe abnormalities in hippocampal function. Pyramidal neurons in the CA1 region of the hippocampus lacking the GLUR1 subunit exhibited defects in extra synaptic currents from AMPA receptors. These results reveal that the GLUR1 subunit is essential for synaptic scaling. These results also communicate that the GLUR1 subunit is very critical for the maintenance of the preservation of an extra- synaptic pool of AMPA receptors. Behaviorally, mice that like the GluR1 subunit have been shown to show deficits in spatial working memory and spatial learning. Bilateral hippocampal lesions in mice that were deficient of GluR1 displayed defects in spatial learning due to subpar performances in both the elevated plus-maze and Y-maze, which are both dependent on hippocampal function. These mice were also reported to be more aggressive, anxious, and hyperactive than wild-type mice. These mice did not show any deficits in their sensorimotor behavior.
**GluR2 and GluR2/3 Knockout Mice**

Unlike GluR1 knockout mice, GluR2 knockout mice have been shown to have extreme abnormalities in their development and behavior. These abnormalities include a lack of motivation, deficits in learning, and declines in sensory stimulus processing. These mice have been shown to have deficits in their neocortical, cerebellar, and hippocampal functions. Mice lacking GluR2 have been shown to have reduced synaptic currents regulated by AMPA receptors. These results indicate that GluR2 is critical for transmission regulated by AMPA receptors. GluR1, however, has been shown to be more essential for synaptic plasticity that is activity-dependent.

GluR2/3 double knockout mice have been shown to display a similar phenotype to GluR2 knockout mice, in addition to decreased survival and retardation. These double knockout mice did not have any reported abnormalities in LTP and LTD, which shows that GluR1 is capable of establishing LTP and regulating synaptic plasticity in neurons in the CA1 region of the hippocampus. Because of the severe retardation exhibited by these double knockout mice, it has been difficult for researchers to gather learning and memory behavioral data from them.

**Phosphoinositoltidyl-3 Kinase (PI3K), Reelin, and AMPA Receptor Trafficking**

The mechanisms that regulate AMPA receptor transmission, particularly regarding AMPA receptor insertion, remain under debate. Both Reelin and Phosphoinositoltidyl-3 Kinase (PI3K) have been shown to be linked to AMPA receptor insertion. The signaling of PI3K participates in a wide-array of cellular processes, which include cell growth, apoptosis inhibition, and endocytosis [118]. The activation of PI3K in neurons is facilitated by phosphorylated Dab1 with the p85α subunit of PI3K. SFKs have been shown to be activated independently of PI3K,
and SFK function is required for successive PI3K activation. When Reelin binds to the ApoER2 and VLDR receptors, this leads to the phosphorylation of Dab1, PI3K activation, protein kinase B (Akt) phosphorylation, inhibition of GSK-3B, and declines in Tau phosphorylation. A study reported that the bath perfusion of recombinant Reelin onto cultured hippocampal slices was linked with increases in NMDA receptor phosphorylation, increased NMDA receptor whole-cell currents, and therefore increases in LTP. The Reelin treatment of hippocampal slices was shown to be linked with an increase in miniature excitatory post-synaptic currents in AMPA receptors. The treatment of these hippocampal slices with Reelin was also linked with an increase in AMPA receptor expression in the CA1 region of the hippocampus as well as a reduction in NMDAR silent synapses [95]. Hippocampal primary neurons from reeler embryos were reported to have a decrease in GLU1 and NR1 subunits compared with primary neurons prepared from wild-type embryos. Wild-type organotypic hippocampal cultures that were chronically treated with Reelin (over 5 days) showed an increase in AMPA receptor insertion and spine density. Reelin exposure in CA1 pyramidal neurons has been shown to be associated with AMPA receptor insertion through a mechanism dependent upon phosphoinositide-3-kinase (PI3K). PI3K activity was detected using a western blot, in which phosphorylation changes to serine/threonine protein kinase Akt were detected. PI3K and Akt inhibition has been shown to interfere with dendrite branching that is induced by Reelin exposure. Increases in AMPA receptor expression are shown to be inhibited by PI3K inhibitor Wortmannin and LY294002. NMDA receptor activation has been shown to trigger AMPA receptor insertion in the GLU1 subunit, which is dependent upon PI3K, but not GLU2 [119]. PI3- K has the ability to bind to all of the GLUR subunits. [120]. The activation of NMDA receptors causes an increase in Ca+2 entry, and the calcium becomes complexed with
calmodulin. The calcium-calmodulin complex activates PI3K, which is associated with AMPA receptors, and facilitates an increase in AMPA receptor insertion.

**Inhibitors of PI3K**

Wortmannin, which is a fungal metabolite, in addition to LY294002, are both well-known inhibitors of PI3K [121]. Wortmannin binds to PI3K at the p110 subunit in a non-competitive and irreversible manner, which inhibits the enzyme. Wortmannin at a concentration of 100 nM has been shown to inhibit PI3K by up to 95 percent [121]. Wortmannin has been shown to inhibit PKB/Akt phosphorylation as well as increase levels of apoptosis. Wortmannin has been shown to inhibit the cell survival that is promoted by PI3K-PKB/Akt. Wortmannin is a very potent inhibitor of PI3K that has been memory and spatial learning.

**Biological Mechanisms Underlying Stress**

A stressor is defined as an experience or an encounter that threatens the capacity of the individual to adapt [122]. Stress results from physiological or psychosocial stressors that threaten homeostasis [123]. Examples of physiological stressors include physical trauma, dehydration, and starvation [124]. Examples of psychosocial stressors include emotional trauma, unemployment, and loss [125]. Acute stress is often positive, as it increases the strength of adaptation mechanisms [126]. Chronic stress, however, can be negative when it surpasses the ability to cope and can lead to behavioral and physical abnormalities. This harmful type of stress is referred to as distress. Distress, which is believed to be linked to the development of many psychiatric illness, often causes manifestations of confusion, poor concentration, and anxiety [127].
Hypothalamic Pituitary Adrenal (HPA) Axis

The hypothalamic pituitary adrenal (HPA) axis is a system that mediates the response of the body to stress [67,128]. This reaction enables an animal to adapt quickly during acute conditions. However, under chronic stress, there may be abnormalities in this response [67]. When stress is experienced, this triggers the secretion of the corticotropin-releasing hormone (CRH) from the hypothalamus. The presence of the corticotropin-releasing hormone (CRH) leads to the secretion of adrenocorticotropin hormone (ACTH) from the pituitary gland [129]. The secretion of ACTH into the blood activates the release of glucocorticoids, including corticosterone, from the adrenal gland. The feedback of corticosterone onto the hypothalamus and pituitary negatively regulates the secretion of ACTH and CRH, which is referred to as the negative feedback loop [130]. Glucocorticoids cortisol (in humans) and corticosterone (in rodents) are two of the most vital regulators of the HPA axis. If the negative-feedback loop of the HPA-axis goes unchecked, the activation of this system can be detrimental.

Disorders of the HPA Axis

Many schizophrenic patients have been reported to have HPA defects. For example, many of these patients did not achieve suppressed corticosterone levels following dexamethasone administration [32]. Many of these patients were also reported to have increased basal corticosterone levels. Increased levels of corticosteroids are often found in many individuals who have been diagnosed with depression and anxiety[131]. Affective disorders have been shown to trigger hyperactivity of the HPA axis, which is believed to result from defects in the negative feedback of corticosterone.
Hypercortisolaemia (excessive cortisol) has been shown to be linked to depression [101]. Unfortunately, the biological pathways by which corticosterone triggers symptoms of depression are not well understood [132]. Hypocortisolaemia (insufficient cortisol) has been shown to be linked to post-traumatic stress disorder. [69]. In several studies, mice exposed to corticosterone during both gestational and post-natal periods demonstrated brain abnormalities analogous with those observed in schizophrenia [32].

**Glucocorticoid (GR) and Mineralcorticoid (MR) Receptors**

Corticosterone has the potential to produce long-term effects in cognitive and emotional procedures by activating both glucocorticoid (GR) and mineralcorticoid (MR) receptors [133]. Corticosterone (CORT) binds to both mineralcorticoid (MR) and glucocorticoid receptors (GR), which are located in the hippocampus, prefrontal cortex, and amygdala [78]. The glucocorticoid (GR) receptor functions in hippocampal-dependent spatial memory using a genomic mechanism [89]. In addition to hippocampal-dependent spatial memory, GRs are also involved in contextual fear memory, which is regulated by the hippocampus and amygdala. The MR and GR receptors are members of the nuclear receptor superfamily [27]. When these receptors are not bound to ligands, both MRs and GRs are present in the cytoplasm, and joined in a complex with heat shock proteins [67]. Heat shock proteins are molecular chaperones that help to fold the MRs and GRs into their correct confirmations. The binding of CORT causes these receptors to be translocated into the nucleus, where they will bind specific sequences of DNA, which are called hormone responsive elements [69]. This binding causes the transcription or
suppression of specific genes. When CORT binds to the GR receptors, both non-genomic and genomic responses occur [124]. The genomic response is typically slower than the non-genomic response and usually takes place after several hours [134]. The non-genomic response, however, is usually much quicker than the non-genomic response and usually occurs after seconds or minutes.

**Learning and Memory Formation during Stress**

Many animal and human studies have reported that acute stress triggers the formation of powerful and long-term memories [129]. Chronic stress exposure to glucocorticoids has been shown to be linked to cognitive deficiencies and atrophy in the hippocampus [77]. Several studies have shown that repeated stress exposure in male juvenile rats has adverse effects on the development of temporal order recognition memory, which is controlled by the prefrontal cortex [69]. Stress experienced for acute durations of time has been shown to increase associative learning. However, chronic stress has been shown to be linked to malfunctions in working memory and impairments in pre-frontal cortex malfunction.

**Neuronal Structural Remodeling From Stress**

Chronic stress has been shown to induce shrinkage of dendritic spines in the CA3 region and dentate gyrus in the hippocampus as well as spine loss in CA1 neurons [134]. The dentate gyrus also experiences a decrease in cell number following chronic doses of corticosterone and chronic stress [123]. Neuronal damage has been
reported to occur following the chronic exposure of corticosterone, especially in the hippocampus, which has many corticosteroid receptors [91]. Several studies involving repeatedly stressed animals have shown a decrease in both AMPA and NMDA receptor expression and synaptic transmission mediated by these receptors [66]. This decrease in glutamatergic receptor expression and synaptic transmission was facilitated by increased ubiquitin/proteasome facilitated degradation of GluR1 and NR1 subunits [69]. Many studies have shown that proteasome inhibition averted a decrease in AMPA and NMDA receptor expression and recognition memory in repeatedly stressed animals. Continual stress has been shown to adversely affect recognition memory. Chronic stress has also been shown to cause dendritic atrophy in the CA3 region of the hippocampus. Chronic stress has also been shown to suppress neurogenesis in the dentate gyrus, as well as impair long term potentiation (LTP).

There are several downstream mechanisms that are reportedly affected by acute and chronic stress [101]. When acute stress is experienced, there is an increased delivery of NMDA receptors and AMPA receptors to cell surface, which is activated by serum and glucocorticoid inducible kinase (SGK) and Rab4 [69]. Several studies suggest that this decrease in glutamatergic receptors after chronic stress may occur through increased ubiquitin/proteasome degradation of GluR1 and NR1 subunits. Typically, the activation of the HPA axis and/or the sympathetic nervous system results in waking up. In humans and rodents, these hormones are associated with attention and vigilance [71]. Typically, the activation of the HPA axis and/or the sympathetic nervous system results in waking up. In humans and rodents, these hormones are associated with attention and vigilance [71].
Stress and CORT

Corticosterone (CORT) has been shown to adversely affect hippocampal neurogenesis [102]. Chronic CORT exposure has been shown to cause a significant decrease in the cell number and proliferation in hippocampus of the rodent brain [135]. CORT has been shown to decrease the complexity of dendrites in newborn neurons [130]. Several studies have shown that repeated applications of stress adversely affect several hippocampal functions, which include decreased neurogenesis, abnormal LTP, and a lowered dendritic complexity in dentate granule cells and CA3 pyramidal cells [136].

Specific Aims and Goals

The ultimate goal of this dissertation is to discuss our experimental efforts to use an in-vivo model with RAP to replicate major phenotypes of the heterozygous reeler mouse (HRM), which is an established schizophrenia mouse model. I also aim to discuss the results of a RAP in vitro model used to examine how AMPA receptor insertion in the prefrontal cortex and hippocampus is affected by chronic stress simulated by CORT when combined with chronic RAP exposure. Abnormalities in Reelin signaling have been shown to be linked with schizophrenia development. Post-mortem studies have observed a reduction of up to 50% of Reelin mRNA and protein levels in the brains of schizophrenic patients. Heterozygous reeler mice (HRM) exhibit many behavioral and physiological characteristics of patients with schizophrenia, including PPI deficits, decreased elevated plus maze exploration, and apathy following social isolation. The Receptor-Associated Protein (RAP) is a non-competitive antagonist of Reelin for the ApoER2 and the VLDL receptors that functions by blocking Reelin from binding to its receptors and
activating its downstream effects. In this study, we aimed to determine if a reduction in Reelin signaling produces a similar phenotype to the HRM model, which is an established mouse model for schizophrenia. We intend to assess how hippocampal synaptic plasticity, prepulse inhibition, and associative fear conditioned learning and memory are affected in mice undergoing ventricular GST-RAP-perfusion compared with control mice, and how these differences correlate with the HRM phenotype.

The ‘two hit’ hypothesis for schizophrenia states that both genetic and environmental factors interrupt normal developmental central nervous system (CNS) function. This initial disturbance occurring during development results in an increased vulnerability to experiencing a ‘second hit’, which is ultimately linked with the development of schizophrenia. Reelin could be potentially be involved in the ‘first hit’ in schizophrenia development due to its role in early brain development. However, Reelin also participates in signaling in the adult brain, and therefore has the potential to participate as a ‘second hit’ to schizophrenia as well. Chronic stress has also been linked to the development of schizophrenia. In this study, we used an in vitro model, to investigate the effects of chronic stress and decreased Reelin signaling on AMPAR subunit expression.
Figure 1-1. The Reelin Signaling Pathway and AMPA Receptor Insertion. Reelin binds to tapolipoprotein receptor 2 (ApoER2) and very-low density lipoprotein receptor (VLDLR). This binding activates Dab-1 tyrosine phosphorylation. Dab-1 phosphorylation leads to NMDA receptor phosphorylation. NMDA receptor phosphorylation activates an increase of calcium influx. This depolarization activates an increase in AMPA receptor insertion.
Figure 1-2. *The Hypothalamic Pituitary Adrenal (HPA) Axis*. Stress triggers corticotropin-releasing hormone (CRH) release from the hypothalamus. CRH release leads to the secretion of the adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH secretion activates the release of glucocorticoids. Glucocorticoid feedback onto the hypothalamus and pituitary negatively regulates the secretion of ACTH and CRH, which is referred to as the negative feedback loop.
References


CHAPTER TWO:
RECEPTOR-ASSOCIATED PROTEIN PERFUSION ALTERS ASSOCIATIVE LEARNING, SYNAPTIC PLASTICITY, SENSORY MOTOR GATING

Introduction

Schizophrenia is an intricate neurodevelopmental disorder that adversely affects approximately 1% of the global population [142,143]. Identifying the predominant causal pathways for this illness has been hindered by the difficulty of identifying biological predictors for the occurrence of symptoms. The ‘two hit’ hypothesis for schizophrenia development [9] proposes that an initial hit, such as a genetic mutation, chronic stress, or drug abuse, induces the manifestation of neurological abnormalities. These presumed ‘hits’ increase the susceptibility of an impending second hit occurring later in life, thereby increasing the probability of schizophrenia development. Reelin fits nicely as a ‘first hit’ candidate due to its role in early brain development and the identification of reduced Reelin protein and mRNA by approximately 50% in the post mortem schizophrenic brain [9,144]. Reelin signaling is also present in the adult brain, controlling multiple aspects of neuronal function, where Reelin changes may actually represent a potential ‘second hit’. Among several other risk factors for the development of schizophrenia, prenatal stress has been identified as crucial and may be associated with reduced Reelin signaling [145]. In addition, chronic stress is shown to be linked with schizophrenia development and making stress an attractive ‘second hit’ candidate as well [146-149]. It is widely accepted that multiple factors play a role in neuropsychiatric disorder development. The recent identification
of Reelin and stress in epigenetic regulation and prefrontal cortical function make development of better cellular and animal models essential in investigating their molecular roles in specific neuropsychiatric disorders.

Reelin binds to, and signals through, ApoER2 and very-low-density lipoprotein receptor (VLDLR). Reelin signal transduction occurs through interaction of disabled-1 (Dab-1) with the intracellular NPxY motif of ApoER2 and VLDLR, resulting in tyrosine phosphorylation of Dab-1, activation of the SFKs (Src family nonreceptor tyrosine kinases), PI3K, CDK5 and ERK signaling proteins [32,150]. During early CNS development, Reelin is secreted by Cajal-Retzius cells and is responsible for normal neuronal migration and proper structural development of the cortex, hippocampus and cerebellum [150-152]. Following CNS development, the Cajal-Retzius cells expire and Reelin expression in the postnatal CNS is seen via interneurons. Secreted Reelin is widely distributed throughout the CNS and is intimately associated with normal neuronal signal transduction, hippocampal synaptic plasticity and multiple forms of memory formation in the adult brain [153,154]. Reelin regulates synaptic plasticity by modulating NMDA receptor conductance and maturation, and by controlling AMPA receptor insertion into the membrane, reducing silent synapses [17,155-157]. Thus, Reelin signaling in the adult mammalian brain can modulate multiple neuronal processes essential for normal cognitive function and potentially contributes to the schizophrenia phenotype via postnatal Reelin signaling defects.

To model the 50% reduction in Reelin mRNA and protein levels found in the schizophrenic brain, heterozygous Reelin mice (HRM), which carry a mutation of one allele for the Reelin gene, exhibit many neurophysiological and behavioral characteristics of schizophrenic patients [23,43]. These abnormalities include: alterations in synaptic density, reduced pre-pulse inhibition (PPI), decreased elevated plus maze exploration, and apathy following social isolation.
In addition, there is a significant reduction in hippocampal synaptic plasticity induced with high frequency stimulation (hfs) and paired neuronal depolarization [156]. Interestingly, nearly all of the phenotypes of the HRM model can be rescued in vivo with direct exogenous application of Reelin into the ventricles of adult HRM [24]. It is unknown if the ~50% reduction in Reelin expression in the schizophrenic brain occurs during in utero CNS development or sometime after birth. In the current investigation, we sought to determine if reduced Reelin signaling in the adult mammalian CNS resulted in a similar phenotype to the established HRM model.

The intracellular expressed Receptor-Associated Protein (RAP) is an endoplasmic reticulum (ER)-associated protein that binds to the LDLR family of lipoprotein receptors. RAP acts as a molecular chaperone to newly translated lipoprotein receptors, preventing ligand binding from the ER to the golgi. The extracellular application of recombinant RAP is shown to effectively block normal ligand association to the Reelin receptors ApoER2 and VLDLR. Effective knockdown of Reelin signaling with RAP is shown to disrupt hfs-induced long-term potentiation (LTP) in the mouse hippocampus and reduce spatial learning in rats injected with RAP in the lateral entorhinal cortex [150,160]. This data suggests that postnatal Reelin signaling disruption, and not prenatal Reelin-dependent neuronal migration, is involved with development of neuropsychiatric disorders ranging from autism to bipolar disorder and depression [20,161-164].

In addition to using an in vivo model to determine if a decrease in Reelin signaling generated a phenotype resembling the HRM model, an in vitro model was also used in this study. This model could investigate how decreased Reelin signaling, in combination with chronic physiological stress induced by corticosterone (CORT), altered AMPA receptor insertion into the post-synaptic membrane in the cortical and hippocampal regions of the brain. These regions are adversely
affected in the cognitive symptoms of schizophrenia. Stress leads to the activation of the hypothalamic adrenal pituitary (HPA) axis, which activates elevated secretion of glucocorticoids into the plasma [165]. In humans, stress induces an increased secretion of cortisol, and in rodents there is an increased secretion of CORT [142]. Abnormal HPA-axis function has been identified in schizophrenic patients using the dexamethasone suppression test (DST) [63,166]. In these studies, schizophrenic patients exhibited higher cortisol and lower percentages of DST suppression of cortisol in comparison to healthy control individuals [167,168].

**Materials and Methods**

**Hippocampal slice preparation**

All animal experiments were performed according to the standards approved by the Institutional Animal Care Use Committee at the University of South Florida. C57/BL6 mice and ApoER2 deficient mice (Jackson Laboratories, Bar Harbor, ME) were bred for at least 5 generations. Mice were euthanized by decapitation, and brains removed rapidly to ice-cold high-sucrose cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 28 mM NaHCO$_3$, 0.5 mM CaCl$_2$, 5 mM D-glucose, 7 mM MgCl$_2$ and 0.6 mM l-ascorbate). All solutions were saturated with 95% O$_2$ and 5% CO$_2$. Hippocampal slices were made with the use of a vibratome (Leica VT1200) and equilibrated in a 50% cutting saline-50% artificial cerebrospinal fluid (ACSF) solution (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 26mM NaHCO$_3$, 10 mM D-glucose, 2 mM CaCl$_2$, and 1 mM MgCl$_2$) at room temperature for a minimum of 30 minutes.

**Electrophysiology**

The recording chamber was maintained at 30° C with an ACSF laminar flow rate of 1 mL/min. Post excitatory postsynaptic potentials (pEPSPs) were recorded from the area CA1 *stratum*
radiatum with glass micropipettes pulled to a tip with a 1 µm diameter (1-4MΩ) filled with ACSF. Each response was generated by stimulating fibers from the CA3 region. Stimulation was performed using a Teflon-coated bipolar nichrome electrode. The 100-Hz stimulation protocol consisted of two trains of 100 Hz frequency stimulation for 1 second with each train separated by a 20 second interval. Stimulus intensities were adjusted to give population excitatory postsynaptic potentials (pEPSP) with slopes that were ≤ 50% that of the maximum determined from an input-output curve. The calculated 50% maximum stimulus was used for the 100 Hz LTP-inducing protocol. Potentiation was quantified as the normalized increased of mean pEPSP following tetanic stimulation normalized to mean pEPSP for the duration of the baseline recording. Experimental results were obtained from slices that displayed stable baseline synaptic transmission for a minimum of 30 minutes prior to the delivery of hfs. Fc-RAP, Fc, or control medium was diluted in oxygenated ACSF and perfused onto hippocampal slices at 1 mL/min for 20 minutes before hfs and continued for another 20 minutes.

Cell culture and protein production
The Dab-1 phosphorylation assay was performed as previously described [32]. All brains from embryonic day 15 mouse embryos were homogenized in Hank’s balanced salt solution (HBSS), centrifuged (200 x g) for 4 minutes, resuspended in HBSS, centrifuged (200 x g for 4 minutes), resuspended in medium (Dulbecco’s modified Eagle medium-nutrient mixture F-12 [Ham] containing B27 supplement [Gibco BRL], 10 mM glutamine, and antibiotics), and plated onto poly-L-ornithine-coated 6 cm diameter dishes. After 3 days in culture, the cells were washed with HBSS and incubated with different media containing the indicated ligands. After 20 minutes at 37°C, cells were washed again, scraped into 350 µL of radioimmunoprecipitation assay (RIPA) buffer (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 2 mM EDTA, 50 mM...
NaF, 2 mM Na$_2$VO$_4$, 1% B-mercaptoethanol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and complete protease inhibitor cocktail [Roche]), and lysed for 30 minutes on ice. The lysates were centrifuged at 20,000 x g for 30 minutes, and the supernatants were immediately used for immunoprecipitation of Dab-1 with 4 µL of 2720 antiserum. After 2 hours at 4°C, 20 µL of a suspension containing protein A beads (Amersham) was added for 2 hours at 4°C. The beads were washed with RIPA buffer and boiled in reducing Laemmli buffer to SDS-PAGE blotting. The 211 amino acid glutathione transferase (GST) protein was expressed by itself or fused with RAP. The GST protein was used for GST-RAP purification with a GSH column. Protein purity and concentration was verified through Western blot analysis.

**Intraventricular infusion**

Three-month-old male C57/BL6 (WT) mice were implanted with a brain infusion cannula connected with an osmotic mini-pump (Alzet). The pumps were incubated in sterile saline for 48 hours before implantation at 37°C to prime the pumps. Mice were anesthetized with isoflurane and placed in a stereotaxic frame for implantation. Guide cannulas were implanted into both left and right ventricles (anteroposterior, 1.0mm; mediolateral, -0.5 mm; dorsolateral, -2.5mm) and linked to the osmotic pump (Model 1004; Alzet; pumping rate, 0.11 µL/h; total volume, 100 µL) inserted subcutaneously. GST-RAP, GST, or sterile-filtered ACSF was infused through the cannula, which was linked to the mini-pump. The infusion began on the day of the surgery and continued for 4 weeks.

**General Behavioral Assessments**

The open field task was used to evaluate general activity levels. Each animal was placed in an open field chamber (27 x 27 cm) for 15 minutes in standard room lighting conditions. All activity in the open field was monitored with 16 photoreceptor beams on each side of the
chamber and analyzed with a computer-operated animal activity system (Med Associates, Inc). The elevated plus maze (EPM) consisted of two enclosed arms (30 x 5 x 15 cm) facing each other and two open arms (30 x 5 cm) also facing each other. Each arm is attached to a center platform (5 x 5 cm) and elevated 40 cm off the floor. Two of the runways were well lit and open and the other two were closed, providing protection. Testing was conducted under standard light conditions for 5 minutes. Contextual fear conditioning was conducted to assess fear-based learning and memory. Mice were trained in a chamber (25 x 25 cm) with wire grid flooring inside a sound attenuation chamber with white noise. Mice explored for 3 minutes before presented with the conditioned stimulus (CS- 90 dB tone) for 30 seconds. From 28-30 seconds, the unconditioned stimulus was given (US- 0.5 mA foot shock). Two CS-US pairings were given 90 seconds apart. Mice were placed in the conditioning chamber for 3 minutes at 1 hour, 24 hours, and 72 hours after training. Freezing was defined as the absence of movement for 2 consecutive seconds and measure objectively by a motion monitoring program (Med Associates, Inc). Nociception was recorded by placing the mice in a chamber with wire grid floor and administering mild foot shocks starting at 0.05 mA to 0.5 mA in 0.05 mA increments, separated by 30 seconds. Video monitoring allowed for reaction to the shocks (flinch, jump, vocalize). Testing concluded after. Sensorimotor gaiting was tested by acoustic startle and prepulse inhibition. Mice were placed inside a mouse restrainer located inside a startle response chamber (SD Instruments) with white noise (60 dB). After a 5 min acclimation, mice were presented with 6 different trials in pseudorandom order (no stimulation, 120 dB pulse, 70 dB prepulse, 76 dB prepulse, 82 dB prepulse, and 88 dB prepulse). Pulse was a 40 ms, 120 dB, 8 kHz tone; prepulses were presented for 20 ms with 100 ms interstimulus interval before presentation of 120 dB pulse. 10 presentations of each trial were averaged to analyze inhibition.
**Mixed Cortical/Hippocampal Primary Cell Cultures**

Mixed embryonic cortical/hippocampal cultures were prepared from pregnant C57/BL6 mice at embryonic day (E) 16 using standard procedures [169]. The brain of each embryonic pup was dissected, the meninges removed, and the hippocampi and cortical lobes isolated. The hippocampi and cortical lobes were transferred to phosphate buffered saline (PBS; pH 7.4) and mechanically dissociated with a Pasteur pipette. Cells were pelleted by centrifugation (600 x g) and plated on poly-L-lysine coated 6 well plates in neurobasal medium with B27.

**RAP and Corticosterone Exposure**

The mixed cortical/hippocampal primary cell cultures were cultured for 14 days, and treated with RAP (Enzo Life Sciences), CORT (Sigma) or a vehicle solution (0.01% DMSO). RAP was added at a concentration of 50 µg/mL 60 min prior to CORT exposure [170] and CORT was exposed to cell cultures (in 0.01% DMSO) at a final concentration of 100 nM. Mixed cortical/hippocampal cultures were chronically exposed to both RAP and CORT for 9 days. Both CORT and RAP exposures were replenished every 2 days.

**Western blotting**

Proteins from the total cell lysate were separated by SDS-PAGE using 10% SDS page-polyacrylamide gels. All proteins were transferred to PVDF membranes (Immobilon-P, Millipore), and were then incubated in antibodies against Glur1, Glur2/3 (Abcam) or Dab-1 (Santa Cruz Biotechnology) in 5% nonfat milk in 1X Tris-Buffered Saline-Tween (TBS-T) overnight at 4°C. The membranes were given three 15 min washes in TBS-T and were incubated for 2 hours at room temperature in HRP- conjugated secondary antibodies (Promega) and developed with enhanced chemiluminescence (1:500-1:000 dilution). The optical density of protein bands was quantified by densitometry using Image J software (Bethesda, Maryland).
**Data Analysis**

For the statistical analysis, data were represented by mean ± SEM. An unpaired Student’s t-test or one-way ANOVA with post-hoc Dunnett’s multiple comparison test. Significance was assigned for all tests at p < 0.05.

**Results**

RAP can bind *in vivo* to the known family of lipoprotein receptors, including non-Reelin receptors. We initially sought to determine the specificity of RAP on Reelin receptors, specifically ApoER2, which appears to be involved in the lion’s share of Reelin’s role in the initiation and maintenance of synaptic plasticity in the hippocampus [12,55,171,172]. We have shown previously that the application of Fc-RAP (two molecules of RAP connected by the Fc region of IgG) can increase synaptic plasticity through dimerization of lipoprotein receptors [32,173]. We performed a similar experiment using wild-type, ApoER2 heterozygous- and ApoER2 homozygous-deficient mice (ApoER2 +/-, ApoER2 -/-). Figure 2-1A shows that Fc-RAP application onto wild-type hippocampi increases area CA1 synaptic plasticity with 2 trains of 100Hz stimulation. ApoER2 +/- mice show no increase in synaptic plasticity with Fc-RAP application (Fig 1B). This result is consistent with ApoER2 -/- mice, which show reduced LTP with Fc-RAP (Fig 2-1C).

To ensure that the fusion protein Fc-RAP is functional RAP, we performed a concentration-dependency experiment. We hypothesized that the 5 nM Fc-RAP was at a low enough concentration to bind to two receptors or more to cause clustering or, at a minimum, receptor dimerization and initiate signaling involved in the enhancement of LTP. Thus, increasing concentrations of Fc-RAP should eventually overwhelm its ability to dimerize and instead block ApoER2 signaling identical to the situation observed with GST-RAP application only. We find
that increasing Fc-RAP from a concentration of 5 nM to 50 nM effectively blocks LTP induction in wild-type slices (Fig 2-1A). These data suggest that the reduced or absent ApoER2 expression is sufficient to disrupt the Fc-RAP-induced receptor dimerization and indicates that the Fc-RAP-induced increase in synaptic plasticity in wild-type mice relies on RAP-receptor binding and the positive actions of Fc-RAP is primarily dependent on ApoER2.

We next sought to determine the concentration of RAP necessary to elicit a 50% reduction in Dab-1 phosphorylation, the downstream adaptor protein essential for Reelin signaling through ApoER2. This would be reflective of the 50% reduction in Reelin signaling measured in HRM. The results of these tests would determine the approximate concentration of \textit{in vivo} RAP application. We incubated primary neuronal cultures with increasing GST-RAP concentrations for 48 hours to simulate chronic lipoprotein receptor inhibition. Concentrations were calculated for the entire volume of the culture media. We found that a 7 nM concentration of extended GST-RAP application results in an approximate 50% reduction in Dab-1 phosphorylation compared to GST control (Fig 2-2).

To replicate chronic RAP application \textit{in vivo}, we used an implantable osmotic mini-pump that would allow 2 weeks of continual GST-RAP or GST perfusion at 0.11 µL/hr. The mini-pump was connected to implanted cannulas to allow bilateral GST-RAP, GST, or ACSF application to the ventricles of adult 12-14 week old wild-type mice. To ensure that behavioral testing occurred under conditions of reduced Dab-1 activity, we isolated hippocampi, prefrontal and parietal cortex from wild-type animals perfused with 7nM GST-RAP or GST for 10-12 days. We found that Dab-1 phosphorylation was significantly reduced compared to vehicle treated animals (Fig 2-3A). Interestingly, when probed for ApoER2 receptor concentration, we found a significant reduction in hippocampal ApoER2 expression in GST-RAP perfused animals.
compared to controls. (Fig 2-3B). To determine if reduced ApoER2 receptor expression was due to reduced signaling, we also probed GST-RAP-treated primary cultures as well as HRM tissue and found a similar ApoER2 reduction compared to control treatment or animals (Fig 2-3B).

Previous studies have shown that adult HRM have no differences in overall activity or anxiety when compared to wild-type littermate controls [159]. Wild-type mice perfused with GST-RAP, GST, or ACSF were allowed to explore a novel context for 15 minutes and total activity, center to total ratio and rearings were determined. Sensory perception and baseline behavioral testing show that GST-RAP perfusion does not have an effect on activity (Fig 2-4A). To determine if GST-RAP perfusion alters somatosensory input and anxiety levels, we placed mice on the EPM and found no change in anxiety behavior compared to GST and wild-type control groups (Fig 2-4B). These tests show that GST-RAP application does not affect overall activity, exploratory behavior, mobility or anxiety in these mice. Furthermore, any differences of GST-RAP treated mice measured in subsequent associative learning and memory testing would not be due to altered anxiety, hyper- or hypo-activity.

The HRM shows severe learning and memory defects, particularly in hippocampal-dependent tasks, such as associative fear conditioned learning [20,160,174]. We have previously shown that these deficits in the HRM can be reversed with a single injection of purified Reelin [24]. It is unclear how a single Reelin application is sufficient to reverse these major phenotypes in the absence of a prolonged Reelin signal; however, this may be due to the reduction in ApoER2 receptor. This hypothesis is supported by recent research showing that differential splicing and altered glycosylation results in increased ApoER2 expression and enhanced synaptic plasticity [175]. This raises the question of whether the associative learning defects in the HRM are due to reduced chronic Reelin signaling, or another type of developmental defect that is overcome
with the single Reelin treatment. We next tested GST-RAP, GST, and ACSF treated mice to a standard two-shock fear conditioning learning task. All three groups of animals exhibit freezing behavior to the tone and shock during training (Fig 2-5A), showing that equal freezing behavior is possible in the GST-RAP treated mice. All groups show equivalent freezing behavior 1 hour following training, but significant differences in freezing develop in GST-RAP treated mice at both 24 hours and 72 hours after training (Fig 2-5C-E). To ensure that GST-RAP infusion did not disrupt nociceptive ability, we performed a shock threshold test by applying increasing amounts of foot shock intensity (0.05-0.5 mA, in 0.05 mA increments) and measuring flinch, jump or vocalization behaviors. No differences between groups were detected (Fig 2-5B), suggesting equal perception of shock during fear conditioned training. These data suggest that RAP-dependent reduction in lipoprotein receptor signaling is sufficient to disrupt long-term associative memory formation.

The disruption in a hippocampal-dependent learning and memory task prompted the examination of hippocampal synaptic function in all animal groups. Transverse hippocampal slices were made from GST-RAP, GST and wild-type untreated animals. Baseline synaptic transmission and input-output analysis show no differences in any of the animal groups indicating normal synaptic communication and normal presynaptic and postsynaptic responses to stimulation (Fig 2-6A). In addition, no changes were seen in stimulation pairing creating a facilitated second stimulation response (paired-pulse facilitation) between groups (Fig 2-6B). However, using a moderate 2 train 100 Hz stimulation shows a significant reduction of LTP in GST-RAP treated mice compared with controls. These results indicate that GST-RAP application is sufficient to alter the mechanism involved in hfs-mediated synaptic plasticity, without disruption in baseline synaptic
transmission. Moreover, the defect in LTP is supportive of the disrupted hippocampal-dependent learning and memory defect in GST-RAP treated animals.

Reduced Reelin signaling in the HRM and in schizophrenia has been associated with reduced sensory motor gating [24,176]. To test whether GST-RAP infusion results in alterations of sensory motor gating, we performed a pre-pulse inhibition (PPI) test using a 120 dB acoustic startle coupled with a 70, 76, 82 and 88 dB pre-pulse stimulation. All groups show similar startle to a 120 dB white noise stimulation (Fig 2-7A), suggesting RAP perfusion does not alter auditory processing. We also find that GST-RAP infusion can significantly alter PPI to an extent equal to that previously established in the HRM (Fig 2-7B) [159]. GST treated controls show similar PPI to wild-type control mice (Fig 2-7B) [159]. These data suggest that the sensory motor gating defect seen in the HRM is due to reduced Reelin signaling and not due to subtle developmental abnormalities.

Using an in vitro model, we next sought to determine how a decrease in Reelin signaling induced by GST-RAP, along with chronic physiological stress via CORT application, affected the surface expression of AMPA receptor subunits. Using antibodies against AMPAR-associated subunits Glur1 and Glur2/3, we used Western blot analysis to detect protein expression following a chronic 9-day exposure of RAP and CORT to mixed cortical/hippocampal primary neuronal cultures.

In these cortical/hippocampal primary neurons, CORT treatment alone significantly decreased Glur1 expression compared with the vehicle control (Fig 2-8A). RAP treatment alone significantly decreased Glur1 expression compared with the vehicle control. Decrease in the expression of Glur1 in cultures treated with CORT alone was not significantly different than
those treated with RAP alone. Cultures treated with CORT and RAP showed a significant decrease in Glur1 compared with vehicle control, CORT alone, and RAP alone.

We discovered that CORT treatment alone significantly decreased Glur2/3 expression compared with the vehicle control (Fig. 2-8B). RAP treatment alone did not significantly decrease Glur2/3 expression compared with the vehicle control. Glur2/3 expression in neurons treated CORT alone was decreased compared with neurons treated with RAP alone. Cultures treated with both CORT and RAP showed a significant decline in Glur2/3 levels compared with CORT alone and vehicle control, but interestingly not when compared to RAP alone.

**Discussion**

There is a clear relationship between Reelin signaling and normal mammalian synaptic plasticity and memory formation. Decreased Reelin protein expression, disruption of Reelin receptors ApoER2 and VLDLR, or reduced association with the intracellular adapter protein Dab-1 all lead to alterations in cognitive function [150,153,163,177]. This central role in synaptic function, coupled with its developmental role in specific neuronal positioning is indicative of its overall biologic importance and may in part underlie the lack of human disorders associated with Reelin signaling. However, there are reports of alterations in the Reelin signaling system with specific human disease. For example, downstream interruption of Dab-1 signaling results in Lissencephaly [178], and specific mutations in the Reelin gene has recently been associated with Autism development [161]. There are other interesting correlative relationships between human disease and Reelin levels in the post mortem brain of patients with schizophrenia and Angelman syndrome [10]. The primary goal of the current study was two-fold: first, determine if GST-RAP application could be used to replicate the major phenotypes of the HRM; second, establish a
model for Reelin deficiency that could be validated \textit{in vivo} for use with multiple factors for \textit{in vitro} experimentation.

We have previously shown that the HRM has specific deficits in learning and memory, hippocampal synaptic plasticity and PPI. These phenotypes correlate to the severe cognitive disruption and sensory motor gating symptoms in human schizophrenia. What remains unclear is the timing of the observed alteration in Reelin in the schizophrenic brain. The Reelin-dependent role in neuronal migration would suggest that CNS developmental loss of Reelin would result in significant altered neuronal positioning and subsequent changes in synaptic connectivity. Thus, other genetic or environmental insults coupled with hormonal changes may result in the development of schizophrenia. However, no significant alterations in neuronal positioning are observed in the hippocampus, cerebellum or cerebral cortex in the schizophrenia postmortem brain. This would suggest that Reelin protein reduction occurs in the postnatal schizophrenic brain, contributing to the overall phenotype. Furthermore, it raises the question of what extent the HRM phenotype is due to Reelin haploinsufficiency during CNS development versus reduced Reelin signaling in the adult brain.

Our results show that GST-RAP perfusion results in reduced constitutive Dab-1 phosphorylation in multiple brain regions. This is shown to correlate with defects in associative fear conditioned learning and memory without alterations in overall activity, sensory perception or nociception. Hippocampal synaptic plasticity is also significantly disrupted and may underlie the hippocampal-dependent fear-conditioning memory defect. Importantly, we find a deficit in pre-pulse inhibition, indicative of a sensory motor gating defect. Taken together, these results suggest that the postnatal disruption of the Reelin signaling system recapitulates the major biochemical, physiologic, and behavioral phenotypes of the Reelin haploinsufficient mouse
A caveat to this experimental design is the promiscuous nature of RAP binding to the family of lipoprotein receptors. In particular the LRP receptor is shown to interact with and alter MNDA receptor function [179]. The contributions of these receptors on the observed phenotypes in our RAP perfused mice are exceedingly difficult to assess especially when considering how reduced ApoER2 expression coupled with reduced receptor function can effect overall neuronal function. Moreover, these results also indicate the usefulness of GST-RAP in culture, in particular primary cell culture, where ApoER2 expression is predominate compared to other lipoprotein receptors that may be effected with GST-RAP application.
**Figure 2-1.** *Perfusion with Fc-RAP alters hippocampal LTP induction.* A. Percent increase of pEPSP slopes of WT hippocampi when perfused with Fc-RAP (5nM or 50nM) or Fc. (WT Fc: n=6; WT Fc-RAP (5nM): n=6; WT Fc-RAP (50nM): n=5, p<0.0001, one-way ANOVA). B. Percent increase of pEPSP slopes of ApoER2 -/+ Fc-RAP and ApoER2 -/- Fc hippocampi before application of LTP-inducing hfs. C. Percent increase of pEPSP slopes of ApoER2 -/- Fc-RAP and ApoER2 -/- Fc before application of LTP-inducing hfs to hippocampal slices. D. Average potentiation of last 20 minutes of recording. All results are graphed as the percentage of the slope pEPSP to the baseline recording. Horizontal solid line represents perfusion of Fc-RAP or Fc. Dashed lines represent the 100% mark of baseline synaptic responses. All data represent mean ± SEM.
**Figure 2-2. Inhibition of Dab-1 by GST-RAP.** A. *In vitro* measurement of Dab-1 intensity, which is presented as percent of vehicle. GST-RAP at increasing concentrations (0-1000 nM) inhibits Dab-1 activity. B. Lack of effect of GST-RAP on total Dab-1 activity and decreased p-Dab-1 activity induced by GST-RAP application. C. Data presented as the percent change of Dab-1 in the presence of GST-RAP compared with vehicle. Decreased p-Dab-1 activity was induced by GST-RAP application. No significant change in total Dab-1 activity was observed from GST-RAP application.
Figure 2-3. Effect of RAP on Reelin and ApoER2 expression. A. Decreased ApoER2 expression is linked with GST-RAP application in the hippocampus, prefrontal cortex, and the parietal cortex. B. Effect of GST-RAP on ApoER2 expression in the hippocampus, prefrontal cortex, and parietal cortex. ApoER2 expression can be blocked by GST-RAP in the prefrontal cortex and hippocampus.
Figure 2-4. Treatment with GST-RAP or GST does not alter activity or anxiety. A. Open field test was used to assess the general activity levels of the animals, by measuring the total distance traveled during a 15-minute test session in an open box in a lighted room. B. The elevated plus maze was used to evaluate anxiety-like behavior in GST-RAP, GST, or ACSF treated mice.
Figure 2-5. *GST-RAP inhibits contextual fear conditioning*. A. GST-RAP animals show normal freezing in response to the combination of a mild foot shock and an auditory cue. B. The shock threshold test was used to evaluate the sensitivity of each animal to footshock by measuring flinching, jumping, and vocalization to increasing footshock intensities. C. GST-RAP injected mice exhibited significantly reduced freezing responses when returned to the fear conditioning context at 24 hours and 72 hours after training. Data presented as mean ± SEM.

You may also want to discuss the Wasser paper (2014) in this context. There are major effects that are dose-dependent upon Apoer2 and which form is expressed.
Figure 2-6. **GST-RAP treatment in WT animals decreases synaptic plasticity.** A. Baseline synaptic transmission and input-output analysis show no differences in GST-RAP, GST, or ACSF treated animal groups. B. No changes were observed in stimulation-pairing producing a paired-pulse facilitation. C. A significant decrease in LTP is observed in GST-RAP mice following hfs. Dashed lines represent the 100% mark of baseline synaptic responses. All data represent mean ± SEM.
Figure 2-7. **GST-RAP treatment alters sensorimotor gaiting.** A. There were no significant differences in the acoustic startle reflex for GST-RAP and GST mice given a 120 dB pulse. B. GST-RAP decreased the percent inhibition to 70, 76, 82, and 88 dB pre-pulse compared with GST treated mice. All data represent mean ± SEM.
**Figure 2-8. CORT and GST-RAP can alter Glur1 and Glur2/3 expression.** A. Cultures treated with RAP + CORT showed a significant decline in Glur1 compared with RAP or CORT alone (n =5, * p< 0.05, one-way ANOVA). B. Neurons treated with RAP + CORT showed a significant decline in Glur2/3 compared with CORT alone, but not RAP alone (n=5, **p<0.01, one-way ANOVA).
CHAPTER THREE

ANALYSIS OF IPSC-DERIVED DOPAMINERGIC NEURON SUSCEPTIBILITY TO INFLUENZA AND EXCITOTOXICITY IN NON-AFFECTIVE PSYCHOSIS

Introduction

Schizophrenia is a devastating illness characterized by positive symptoms, such as psychosis, hallucinations, delusions, paranoia and negative symptoms, such as emotional apathy and loss of motivation [180-182]. There is a significant genetic element to schizophrenia [183,184]. The percentages of heritability for schizophrenia range from 2% to 48%, with incidence of the development increasing with genetic correspondence to an affected relative [185]. In addition to genetic significance, environmental factors, such as prenatal viral infection, are considered to be very vital to the development of schizophrenia. It is therefore postulated that an individual with a genetic predisposition to schizophrenia has a greater probability of developing the disorder when exposed to certain environmental agents, such as prenatal viral infection, when compared with an individual from the general population [186-188]. The dopamine hypothesis suggests that the positive symptoms of psychosis associated with schizophrenia are attributed to a hyperactive signal transduction within the dopaminergic system [189-191]. Maternal immune activation (MIA) in mice has been shown to result in offspring with deficits in behavior, histology, and gene expression that are observed in schizophrenia as well as other neurological disorders [192-194].
This chapter will give an overview of several animal models of prenatal infection for schizophrenia, and how these differing methods of infection affect the dopaminergic system. Induced pluripotent stem cells (iPSCs) will also be discussed as a potentially useful form of therapy futuristically for dopaminergic system malfunction from prenatal infection. The methods section for this chapter will describe how iPSCs and dopaminergic neurons were generated from heterozygous reeler mice (HRM), which come from an established animal model for schizophrenia. The results section will display the iPSCs and dopaminergic neurons generated from these methods, and the discussion section will discuss the potential usefulness of these cells as therapeutic agents in future scientific endeavors.

**Maternal Infection is an environmental risk factor linked with schizophrenia**

Maternal influenza infection has shown to be an environmental risk factor linked with schizophrenia development [195-197]. In addition to several studies being conducted by Mednick and colleagues beginning in 1988 [198], several studies have reported schizophrenia development following maternal infection of influenza. Similar relationships have been observed with Herpes Simplex Virus (HSV), rubella, and Toxoplasma gondii [199-203]. These early studies were successful at highlighting a correlation between maternal infection and schizophrenia. A study conducted by Brown and colleagues [195] involved the assaying of reserved maternal serum for influenza antibodies in pregnancies that resulted in offspring with schizophrenia. This study concluded that the probability of schizophrenia development increased 7-fold following influenza infection during the first trimester of pregnancy. No increased risk was found during the second and third trimesters in this study. Another study conducted by [204] showed that influenza infection during the second trimester of pregnancy was reported by 30.8% of the mothers of individuals with schizophrenia. Both of these studies support the claim that
there is a clear correlation between maternal influenza infection and schizophrenia development in offspring. In addition to these associations, several studies have also observed several cognitive deficits that are observed in schizophrenia, including declines in working memory, verbal memory, and fine-motor control, verbal memory, sensorimotor gating, and social interaction in association with pre-natal viral infection [205-209] The link between maternal infection and schizophrenia is not limited to only influenza infection. There are similar studies linking HCV, HSV, and Toxoplasma Ghondii to schizophrenia development. Several studies have also shown maternal infection to be linked with other neurological disorders, such as autism and bipolar disorder [210-212]. For example, maternal cytomegalovirus infection has been shown to be linked with both autism and bipolar disorder and developing offspring [213,214]. The commonality between the development of these neurological disorders from these varying methods of infection is the activation of the immune response within the mother [201].

Animal models of maternal influenza infection in the dopaminergic system

Being that maternal influenza infection has been shown to be linked with schizophrenia development in human offspring, animal models have the potential to be useful in the investigation of the mechanisms underlying this correlation. In a study conducted by Shi and colleagues [215], pregnant mice were exposed to a respiratory infection of a mouse-adapted human strain of the influenza virus on day 9 of pregnancy. The offspring of these pregnant mice were shown to exhibit reduction in the size of the hippocampus and neocortical thickness, reduced immunoreactivity of Reelin, decreased nitric oxide synthase (nNOS) expression, and decreased synaptosome-associated protein (SNAP-25), which are all seen in schizophrenia. The offspring also displayed abnormalities in open field and novel-object test.
In another study conducted by Fatemi and colleagues [216], pregnant mice were infected with the influenza A/NWS/33 (H1N1) virus or vehicle on day 9 of their pregnancy. The pregnant mice were allowed to deliver pups, and the brains of these pups were removed and examined by microarray analysis for gene expression. The microarray analysis from these studies revealed a significant upregulation of 21 genes and the down-regulation of 18 genes. These genes include HSC-70-interacting protein, the alpha subunit of the protein kinase inhibitor, and the synaptosomal complex protein SC65, which have all shown to be altered in various patients with schizophrenia.

The influenza virus is a single-stranded, negative-sense RNA virus that is in the Orthomyxoviridae family [217,218]. Viruses in the Orthomyxoviridae family are divided into five classes: Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, and Thogovirus [219]. Viruses in the Influenzavirus A class are transmitted to humans, mammals and birds [220]. Viruses in the Influenzavirus B and C classes infect humans and seals, and pigs [221]. The Influenza A H1N1 virus is spherical enveloped virus with an outer layer of lipid membrane [218]. This virus has hemagglutinin (HA) and neuraminidase (NA) glycoprotein spikes on the surface of the lipid membrane [222]. The matrix protein (M), which is beneath the lipid membrane, gives the lipid envelope a strong structure [223]. There are eight negative-sense RNA segments within the interior of each Influenzavirus A virion [224]. Each RNA segment consists of RNA combined with RNA polymerase proteins (PB1, PB2, PA) and nucleocapsid proteins [225]. Within the interior of the Influenzavirus A virion is also the nuclear export protein (NEP) [226].

In a study conducted by De Erausquin and colleagues [227], pregnant BALB-C mice were intranasally inoculated with 90 PFU/mL of influenza virus between days 9 and 11 of pregnancy.
The pups from these pregnancies were sacrificed and 15 um brain sections were obtained from these pups and analyzed using stereological quantification of dopaminergic neurons in the substantia nigra stained with tyrosine hydroxylase (TH). Two sets of sample groups of pregnant mice were infected with two strains of the influenza virus, A/WSN/33 and A/NC-L/99. Infection with both of these strains resulted in significant dopaminergic neuron loss in the mesencephalon of the adult offspring (post-natal day 90) of mothers infected with the virus. In addition to an in vivo study, an in vitro study was also conducted by this group. In this study, primary neurons from the rat mesencephalon were inoculated with 150 PFU/well of influenza. The cells in the mesencephalon were observed after a 24, 48, and 72 hour period. During the 72-hour period, the neurons appear to be the most damaged with observed dendritic loss and cytosol vacuolation. A high affinity of H1N1 InfV was also observed for in vitro dopaminergic neurons, leading to the activation of nuclear factor B and apoptosis.

**Animal models of maternal Poly I:C Exposure in the dopaminergic system**

In addition to using maternal influenza infection as an animal model for prenatal infection for schizophrenia, an inflammatory response is also induced in mice using Polyriboinosinic-Polyribocytidilic acid (Poly I:C) [215,228,229]. In animal models, an alternative to infecting mice with influenza is to induce an inflammatory response with poly I:C, which is composed from synthetic dsRNA [230,231]. Poly I:C is used to mimic viral infection, because it produces immune responses that are similar to viral infection, which include the release of pro inflammatory cytokines [229,232]. Upon injection, poly I:C executes its actions via toll-like receptor (TLR)3 [233].

The injection of Poly I:C has been shown to produce behavioral and cognitive deficits that are displayed in the offspring mice from mothers infected with influenza [215,229]. In a study
conducted by Ozawa and colleagues, pregnant mice were intraperitoneally (IP) with Poly I:C once every 6 days [234]. After 3 weeks, the offspring were taken from their mothers and tested for pre pulse inhibition (PPI) and novel object recognition. The cognitive deficits of these mice were also observed after giving them antipsychotic drugs. These mice were administered clozapine or haloperidol for 2 weeks. The results of this study indicated that poly I:C injection resulted in PPI deficits and cognitive impairment. The mice suffering from cognitive deficits were given clozapine and haloperidol. The cognitive deficits were shown to be reversed by the administration of clozapine, but not haloperidol.

Another study conducted by Zuckerman and colleagues induced immune activation by injecting the pregnant dams of mice with poly I:C [235]. The results show that juvenile offspring from Poly I:C mice show no difference in control mice in latent inhibition (LI). However, adult offspring from Poly I:C mice displayed LI disruption. This disruption in LI was reversed by both haloperidol and clozapine. In addition to deficits in PPI and LI, Poly I:C in animal models of MIA have also been shown to be linked with striatal dopamine release, which is similar to the condition of the mesolimbic system in patients with schizophrenia.

Animal models of maternal LPS Exposure in the dopaminergic system

Lipopolysaccharide (LPS) is used in animal prenatal models of schizophrenia to mimic bacterial infection inducing maternal immune activation (MIA) [236-238]. LPS acts through toll-like receptor (TLR)4 [239]. When pregnant mice were injected IP with LPS, many behavioral deficits were observed in offspring that are seen in offspring from mothers infected with influenza or poly I:C [229,234,237]. These behavioral deficits in offspring include PPI deficits, increased anxiety, deficits in social interaction, learning defects, and increased locomotion from amphetamine[236,240,241].
The maternal immune response has been shown to adversely affect fetal brain development by inducing the elevation of pro-inflammatory cytokine interleukin-6 (IL-6)[236,242,243]. IL-6 injection alone has been shown to produce classical behavioral abnormalities seen in many animal models of maternal immune activation in studies linked with schizophrenia[238,244]. Experiments that block IL-6 have been shown to prevent these behavioral abnormalities from manifesting in animal models [229]. The injection of poly (I:C) in pregnant rodent models has been shown to induce increased IL-6 protein in the placenta. IL-6 activates Janus Kinase (JAK) and signal transducer and activator of transcription (STAT) proteins [245].

In a study conducted by Ling and colleagues [246], LPS was infused into 7-month-old rats that were offspring from mothers who were also exposed to LPS. LPS was also infused into 7-month-old rats born from mothers who were not exposed to LPS. In this study, DA neuron loss only occurred mice born from mothers who experienced prenatal LPS exposure.

**Induced Pluripotent Stem Cells (iPSCs) as potentially useful therapeutic agent**

In 2006, Yamanaka and his colleagues were able to successfully reprogram fibroblasts into induced pluripotent stem cells (iPSCs)[247]. Since this breakthrough in the area of stem-cell science, a lot of progress has been made in this field in a very short period of time. The concept of iPSC-reprogramming is advantageous because it eliminates many of the controversies associated with stem cell research, such as human embryo use and immune-rejection of laboratory-generated tissues and organs. The use of this scientific technology gives scientists the ability to obtain fibroblasts from patients with Schizophrenia, as well as other neurological disorders, and create dopaminergic neurons from iPSCs that share the exact genetic make-up of each patient that the fibroblasts were obtained from. Using this technology, we have the ability to take iPSC-derived dopaminergic neurons that possess the different genetic risk-variants associated with each Schizophrenia patient and directly infect these neurons with the influenza
virus in-vitro. When this method is perfected, it will allow the study of biological pathways affected by influenza in an innovative fashion.

Induced pluripotent stem cells (iPSCs) are somatic cells that have been reprogrammed back to a pluripotent-state with ESC-like properties that express genetic markers that are characteristic of ESCs [248]. iPSCs also exhibit morphology and growth properties that are characteristic of ESCs [249]. Takahashi and Yamanaka showed that introducing the sox-2, oct-4, klf-4, and c-myc transcription factors into somatic cells resulted in the formation of induced pluripotent stem cells (iPSCs) [247]. They hypothesized that transcription factors that maintain embryonic stem cell (ESC) pluripotency also had a role in the induction of iPSCs. They developed an assay that would be able to detect pluripotency. β-galactosidase and neomycin resistance genes were fused to create a β-geo cassette. Homologous recombination was used to insert the β-geo cassette into the mouse Fbx15 gene, which is a marker of undifferentiated cells. The knock-in of the β-geo cassette would cause cell colonies that express Fbx15 to become stained. Fbx15, is expressed during the simultaneous expression of sox-2, oct-4, klf-4, and c-myc. Twenty-four genes that regulated pluripotency were chosen and introduced into mouse embryonic fibroblasts (MEFs) by retroviral transduction. After identifying iPSC colonies through G418 resistance, they begin to remove one reprogramming factor at a time from the pool of twenty-four genes to identify the key candidates responsible for inducing pluripotency. The remaining four transcription factors that produced pluripotency were sox-2, oct-4, klf-4, and c-myc.
Materials and Methods

Animals and Fibroblast Isolation

All animal experiments were performed according to the standards approved by the Institutional Animal Care Use Committee at the University of South Florida. HRM, homozygous reeler mice, and WT/ C57 mice were purchased from Jackson laboratories for the purposes of fibroblast isolation. Skin fragments from 6-week old C57/BL-6 mice were excised from the regions of the stomach, under-arm, and tail. The skin pieces were incubated in 1000 U/mL collagenase and 0.05% Trypsin EDTA, and stored in DMEM media (with FBS, Penicillin-Streptomycin, and Non-essential amino acids) at 37oC to allow the fibroblasts to exit the dermis of the tissue for one-week.

iPSC Reprogramming

Murine fibroblasts were plated and grown to a cell density of 1 x 105 cells in a 6- well plate in DMEM (with Glutamax©, ESC-qualified FBS, Penicillin-Streptomycin, β-Mercaptoethanol, and nonessential amino acids) at 37oC. The fibroblasts were transduced with 3 x 107 cell infectious units/mL (CIU/mL) of the oct-4, sox-2, klf-4, and c-myc transcription factors using 6 ug/mL of polybrene on a retroviral vector. The cells were grown on mouse embryonic feeder (MEF) layers in iPSC medium (Knockout© /F12-DMEM, ESC qualified FBS, non-essential amino acids, β-Mercaptoethanol, leukemia inhibitory factor (LIF), and penicillin-streptomycin) until day 30.

iPSC Colony Picking

On day 30, colonies with iPSC-characteristic morphology were picked and grown on MEF layers in iPSC medium. Colonies that are iPSCs typically have round colonies with enlarged-nuclei.
iPSC Characterization Tests

Picked colonies were tested with the alkaline phosphatase (AP) assay for pluripotency. The positive staining of the colonies with the Fast Red TR reagent indicates that the colonies were pluripotent. The colonies were also fixed for immunocytochemistry and stained with oct-4, sox-2, nanog, and ssea-1 anti-bodies. A positive staining for these four anti-bodies is shown and is further support that the colonies were indeed pluripotent. A successful reprogramming and characterization of murine iPSCs shows that this is a feasible technique that can readily be performed from human fibroblasts.

Dopaminergic Neuron Differentiation

Dopaminergic Neuron Differentiation. After the iPSCs were characterized with the alkaline phosphatase (AP) test and immunocytochemistry, the iPSCs were differentiated into DA neurons by using ventral mescencephalon floorplate progenitors, including sonic hedgehog (SHH) and fibroblast growth factor-8 (FGF-8).

Results

Figure 3-1 displays the results from the fibroblast isolation, immunocytochemistry, iPSC reprogramming, and iPSC characterization experiments. Panel A verifies the presence of vimentin and therefore is an indication of a successful fibroblast isolation from the murine skin excision experiments. Panels C and D display morphology characteristic of murine induced pluripotent stem cells, which are round in shape with colonies and enlarged nuclei. Panels F-H show a positive expression of nanog and sox-2, which are positively expressed in iPSCs (Fig. 3-1). In Panel A of figure 3-2, the iPSCs stain positive for alkaline phosphatase (AP). Panels E and F of Figure 3-2 show immunocytochemistry results from dopaminergic neurons positively
stained with tyrosine hydroxylase (TH), which is a marker and is positively expressed in
dopaminergic neurons.

**Discussion**

The exact mechanism that causes Schizophrenia development is unknown. Multiple studies show
that the risk for developing Schizophrenia is higher for individuals who were exposed to
influenza during fetal development during the second-trimester of their mother’s pregnancy.
Previous studies have demonstrated functional and structural abnormalities of mDA neurons in
the offspring from mothers who are intra-nasally infected with influenza during the pre-natal
phase. An abnormal behavioral phenotype was also observed in these offspring. The data from
these studies demonstrates the feasibility of reprogramming fibroblasts into iPSCs and
successfully characterizing them. We hypothesize that iPSC DA neurons obtained from
Schizophrenia patients will be more susceptible to influenza infection than those obtained from
health individuals. We also believe that analyzing the genetic and epigenetic variations that result
from this infection in the experimental group will provide more information to the biological
target of this disorder. We will reprogram fibroblasts from both the experimental and control
groups into iPSCs, and then differentiate these cells into DA neurons. These neurons will be
infected with influenza. Their susceptibility to the virus, and genetic and epigenetic variations
will be observed and analyzed. We highly anticipate that the results from this study will provide
more information about the causal mechanism of Schizophrenia.

Induced pluripotent stem cells have the potential to be very useful therapeutic agents in the field
of schizophrenia research, in addition to many other disorders. Successfully generating human
iPSCs for therapeutic purposes would ultimately benefit people suffering from
neurodegenerative disorders. Patients receiving this new form of treatment would not have to
suffer from immunorejection or the ethical issues associated with stem-cell use, which would be beneficial to the patient.

**Figure 3-1.** *iPSC Reprogramming and Characterization through immunocytochemistry.* A. Positive expression of vimentin fibroblast presence. C. iPSC morphology successfully represented after picking, round cells with enlarged nuclei. F-H. Successful iPSC reprogramming shown in positive expression of nanog and sox-2.
**Figure 3-2.** Dopaminergic Neuron Differentiation and Characterization through TH stain. A positive expression of tyrosine hydroxylase (TH) indicates the presence of DA neurons and is indicative of a successful DA neuron differentiation.
References

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CHAPTER FOUR

CONCLUSION

It is presently known that functional disruptions and structural reorganization can occur within the nervous system from environmental pressures, alterations in neurological signaling pathways, and functional disturbances. Several minimal changes within the nervous system can collectively lead to significant functional changes within the brain and notable shifts in behavioral manifestations. The ‘two hit’ hypothesis for schizophrenia suggests that genetic or environmental factors disrupt early central nervous system (CNS) developmental. These initial disruptions occurring early during development create an increased risk to encountering a ‘second hit’, and ultimately to the manifestation of schizophrenia.

A 50% reduction in Reelin protein and mRNA have been observed in the post-mortem brains of patients. Because Reelin plays a vital role in the regulation of early brain development, abnormalities in Reelin function during development have the potential to be identified as a potential ‘first hit’ in schizophrenia. However, because of Reelin’s role in the regulation of synaptic plasticity and neuronal function in the adult brain, Reelin also has the potential to pose as a ‘second hit’ within the brain. In addition to schizophrenia, chronic stress has also been shown to be linked with several other neuropsychiatric disorders, including bipolar disorder, and depression.

The RAP protein is an intracellular ER protein that functions as a molecular chaperone for LDL receptors. RAP functions as a non-competitive antagonist of Reelin for the ApoER2 and the
VLDL receptors, thereby blocking Reelin from binding to its receptors and activating the downstream effects of the Reelin signaling pathway.

In this dissertation we sought to determine if a decline in Reelin signaling with the use of RAP produces a similar phenotype to the HRM model, which is an established mouse model for schizophrenia. In our efforts to investigate this question, we determined that the perfusion of GST-RAP causes a reduction in Dab-1 phosphorylation in multiple areas of the brain. These results correlate with defects in associative fear conditioned learning and memory, without showing any alterations in sensory perception, nociception. We also show that GST-RAP perfusion is linked with deficits in prepulse inhibition, which is linked with a sensory motor gating defect. These results collectively suggest that the disruption of Reelin signaling postnatally replicates many of the major behavioral biochemical and physiological phenotypes of the heterozygous reeler mouse, which is an established mouse model for schizophrenia.

A disadvantage to our experimental design is the promiscuous binding ability of RAP to the family of lipoprotein receptors. In addition, these results also indicate the usefulness of RAP for in vitro models in which ApoER2 expression is predominate in comparison to other lipoprotein receptors have the potential to be affected with GST-RAP application.

In this study we also used an in vitro model with RAP in primary neuronal cell culture. This model was used to determine how decreased Reelin signaling, combined with a model of chronic stress, affected the insertion of the Glur1 and the Glur2/3 AMPAR subunits. The difference seen in these subunits following these exposures is believed to occur through differing mechanisms of insertion. Glur1 has been shown to be inserted through the Reelin activation of PI3K. Glur2/3, however, has not been suggested to be inserted through this mechanism. Future studies will need
to be conducted with the use of PI3K inhibitors to determine how Glur1 and Glur2/3 are affected by PI3K inhibition.