A REVERSE TRANSLATION MOUSE MODEL FOR SCHIZOPHRENIC PSYCHOSIS: CONTRIBUTION OF HIPPOCAMPAL SUBFIELD PATHOLOGY

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DEDICATION

I would like to thank my family who has supported me and pushed me when I wanted to give up. I also want to thank the many friends I have met along this journey, for listening to the joys and the pains of this endeavor. I especially want to thank my children Manaia, Grady, Kiley and Dagny for being there every step of the way, being so patient, and being my number one fans.

A REVERSE TRANSLATION MOUSE MODEL FOR SCHIZOPHRENIC PSYCHOSIS: CONTRIBUTION OF HIPPOCAMPAL SUBFIELD PATHOLOGY

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A REVERSE TRANSLATION APPROACH TO A MOUSE PREPARATION FOR SCHIZOPHRENIC PSYCHOSIS BASED ON HUMAN HIPPOCAMPAL SUBFIELD PATHOLOGY

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Schizophrenia is a serious and lifelong psychotic illness that affects all aspects of cognitive and affective function and whose etiology and brain mechanisms remain elusive. Schizophrenia affects not only those who express the condition, but also their family members, friends, and society as a whole. There is a worldwide prevalence of 1%, and the illness in 2012 alone, cost the USA an estimated $62.7 billion in medical care cost and lost wages. Schizophrenia is an extremely complex disease with a heterogeneous mixture of symptoms, including cognitive dysfunction, mood dysfunction, negative symptoms, and the defining symptom set, positive psychotic symptoms. The antipsychotic effects of dopamine receptor antagonists led people to hypothesize that schizophrenia is a disorder of dopamine hyperfunction, but considerable research has generated no strong evidence to support such a simple mechanistic hypothesis. Most recently, the glutamate system has become an etiologic focus in schizophrenia research and its research is proving more promising. We have studied the molecular basis of psychosis in human post mortem hippocampus in schizophrenia, and its related proteins important for learning and memory, especially the *n*-methyl-*d*-aspartate (NMDA) glutamate receptor system. Based on our findings we have developed a testable hypothesis of psychosis, formulated as a learning and memory disorder. In order to fully test this hypothesis we first needed to create a dynamic animal model based on our tissue findings that could be manipulated and probed. We found that knocking out the obligate subunit (GluN1) of the NMDA receptor selectively in the

dentate gyrus paradoxically led to an increase in neuronal activity in the CA_3 and several behavioral changes parallel to those we observe in schizophrenia. Furthermore we combined a pharmacological risk factor (phencyclidine) and a genetic risk factor (*DISC1*) with the knockout mouse that we believed would have the highest probability of interacting in a manner reminiscent of schizophrenia. These particular combinations did not exacerbate the symptoms of the dentate gyrus-specific GluN1 knockout mouse. Now we plan to use this dynamic mouse preparation to study the mechanisms whereby the reduction in GluN1 protein in dentate gyrus sensitizes and stimulates neuronal activity downstream within the hippocampus to better understand psychosis processes.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATIONS

SZ - schizophrenia

GluN1-ionotrophic glutamate receptor subunit zeta-1

PCP- phencyclidine

AP – antipsychotics

DG- dentate gyrus

PSD-95- post synaptic density protein 95

NMDA- *n*-methyl-*d*-aspartate

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CA_3 – *cornu ammonus* 3

CA_1 - *cornu ammonus* 1

MWM – Morris water maze

FC – fear conditioning

HC – hippocampus

KO – knockout

MTL - medial temporal lobe

ACSF-artificial cerebral spinal fluid

EPSC- excitatory post synaptic current

GABA- γ-aminobutyric acid

PFC-pre-frontal cortex

AMG-amygdala

CHAPTER ONE
Introduction

Schizophrenia

Schizophrenia is a chronic psychotic illness that exhibits complex manifestations of cognitive dysfunction, depression, and negative symptoms in addition to the phenomenon of psychosis (Carpenter and Buchanan, 1994; Tamminga and Holcomb, 2004). An initiative to understand the specific and independent targets within this complex disease—the MATRIC initiative—conceptualized the illness as a syndrome with distinguishable symptom dimensions. (Green *et al.,* 2004; Nuechterlein *et al.,* 2005; Stover *et al.,* 2007). MATRICS formulated these symptom dimensions (speed of processing, attention/vigilance, working memory, verbal learning and memory, visual learning and memory, and reasoning and problem solving) as independent from each other with respect to etiology, course, and biology, based on the symptom's course, specific content, and pharmacology of each of these symptom dimensions.

Our understanding of schizophrenia is based predominantly on observations of disease phenomenology; the phenomenological characteristics (ie auditory and visual hallucinations) are extensive and have been honed for many decades, even millennia (Tamminga, 1994). Nonetheless, confirmed molecular markers of diagnosis, much less pathophysiology, are simply not available (Nestler and Hyman, 2010). Psychiatric disease definitions as currently available were created to organize

phenomenology, not with an eye to discovering neural mechanisms, but primarily to generate clinical consistency (Keller *et al.,* 2011). Now is the time, while we pursue disease and dimension definitions, to keep a focus on how to organize disease manifestations based on hypotheses of neural organization (Hyman and Fenton, 2003; O'Tuathaigh and Waddington, 2010; Cuthbert and Insel, 2010). To do this, one of the greatest needs in the field of functional brain disease is to identify the pathophysiology of our psychiatric behavioral dimensions; this will provide a molecular basis for disease understanding, diagnosis, and treatment development and guide us toward finding direct and spontaneous measures of psychosis or cognitive dysfunction—in a much more predictive manner.

Hippocampus

The hippocampus (HC) lies within the medial temporal lobe (MTL). As a limbic system structure, the HC is known to be critical for learning, memory, consolidation and spatial navigation. The MTL includes the HC proper: dentate gyrus (DG) and cornu ammonis (CA) regions, pararhinal/parahippocampal and entorhinal cortex (EC), and subiculum (Figure 1-1). With the perirhinal and parahippocampal cortices, the hippocampal formation is functionally specialized to contribute to declarative memory (memory of facts and events) processing using glutamate-mediated signaling, a function demonstrated to be dysfunctional in SZ (Tamminga, Southcott *et al.,* 2012). The HC proper is comprised of several subfields with distinct memory

functions that receive input from and send out signals to several areas of the brain. In rodents the HC is functionally differentiated along its dorsoventral axis. The dorsal HC in rodents corresponds to the posterior HC in primates and is used primarily for cognitive functions, memory, precise spatial navigation or spatial relational information. The ventral HC of rodents corresponds to the anterior HC in primates and this region relates to stress, emotion and affect, and anxiety related behaviors (Fanselow and Dong, 2010; Clark and Squire, 2013; Nadel *et al.*, 2013; Satpute *et al.,* 2012; Strange *et al.* 2014).

The neural microcircuit of the HC is largely one-way and excitatory. This microcircuit is known as the trisynaptic pathway and it is mediated by excitatory glutamatergic mechanisms and is distinguished by its cheifly one-way information flow. This flow of excitation runs from 1) EC to DG granule cells (perforant path), to 2) CA_3 pyramidal cells (mossy fiber path), to 3) CA_1 pyramidal cells (Schaffer collaterals), and onto the subiculum (Figure 1-2), a microcircuit that plays a distinctive role in specialized declarative memory functions. Although the HC has other connections within (ie EC to CA_1 and the CA_3 recurrent collaterals, see Figure 1-2), the trisynaptic pathway described above is our focus. This pathway (Anderson, 1975) is functionally important for pattern separation, and pattern completion (CA_3) (Kesner *et al.*, 2004; Tamminga, Southcott *et al.,* 2012). The DG is important for pattern separation and keeps memories distinct and resistant to confusion, it allows for discrimination among similar experiences, and renders stored memory patterns

distinct from one another, especially the temporal and spatial relationships comprising events. The CA_3 is important for pattern completion, or the association between spatial location and object and completing a memory in recall. Pattern completion can also be thought of as the recovery of a full or more complete memory from a partial clue.

Due to many replicable changes (decreased volume, loss of interneurons, abnormal architecture, etc.) observed in the HC, and HC-based behaviors in SZ that correlate with psychosis, the HC structure has become the focus in many laboratories.

Learning and Memory

Over the years, scientists have segmented learning and memory into different functions such as motor memory, habit memory, and declarative memory. A type of memory that is supported by the hippocampus is declarative memory. Declarative memory is that of facts and events and their relationships to each other and they are to inform decisions and perspectives in the future. We have garnered much of our understanding about declarative memory from a patient known as H.M. (Dossani *et al.,* 2015). Henry Molaison underwent surgery for a bilateral MTL resection and removal of the hippocampus in order to cure intractable seizures. Upon completion and recovery from surgery it was discovered that H.M. had developed anterograde amnesia and could no longer form nor recall new declarative memories. H.M. was able to develop new motor skills and habits therefore observers concluded that

different types of memories are created and consolidated in different areas of the brain.

The molecular mechanisms of learning and memory involve dynamic alterations of synapses and resultant postsynaptic molecular and cellular changes, believed to encode and store memory. The current understanding of what underlies learning and memory are modifications in the strength of previously existing synaptic connections between numerous excitatory and inhibitory neurons, which create patterns of new cell firing presumably tied to specific memories. These modifications occur through regulation of the properties of different membrane channels, such as the ionic glutamatergic receptor channels. The opening of the NMDA receptor (the most well characterized glutamatergic receptor implicated in learning and memory processes) after a sensitizing signal [(100-200 pulses, 1-2 Hz) with a depolarizing voltage-clamp pulse (1-3 min duration)] causes the subsequent release of neurotransmitter into the synaptic cleft, which upon binding to the receptors on the post synaptic membrane increases the amount of calcium that enters the post synaptic density (Bliss and Collingridge, 1993; Eccles, 1983). This process increases the number of receptors available at the surface of the post synaptic membrane and leads to the modulation of additional neuronal membrane channels, increasing the excitability of the neuron and enhancing the number of action potentials elicited by a stimulus and magnifying its impact on neighboring cells (Bliss and Collingridge, 1993). This also leads to structural alterations, such as increases

in the number or size of spines on the dendrites, representing an increased number of excitatory synapses per neuron (Moser *et al.,* 1994; Van Reempts *et al.,* 1992; O'Malley *et al.,* 2000; Geinisman *et al.,* 2001). Learning also involves secondary messenger systems, phosphorylation of intracellular proteins, changes in protein synthesis, changes in gene regulation, and structural modifications of dendrites (Nguyen and Kandel, 1996, Rubio *et al.,* 2012, Hou et al. 2013, Salter and Kalia, 2004, Lattal and Wood, 2013).

Hebbian and Metaplasticity

Learning and memory can be understood in the context of plasticity, changes of the excitatory properties of the cells in response to changes in environment and modifications by experience. In 1949 Dr. Hebb introduced a pivotal concept in his book *The Organization of Behavior.* Hebbian theory concerns the form and function of cell assemblies. Dr. Hebb postulated how neurons might connect themselves together to become neural networks. Hebbian plasticity describes a basic mechanism for synaptic change due to activity dependent signaling and attempts to explain associative learning, in which simultaneous activation of cells, presynaptic activity that correlates with postsynaptic firing, leads to pronounced increases in synaptic strength and connection between them (Bear, 2003). It describes an explanation for the adaptation of neurons during learning, which was used in the laboratory of the Nobel Prize winning Dr. Kandel using siphon withdrawl reflex of

Aplysia californica (a marine gastropod) (Bailey *et al.*, 1979). Simply stated, Hebbian plasticity is the concept that explains the weighted increase between two neurons that activate simultaneously and will tend to become associated, and facilitative. It also explains the weight reduction if they are activated separately. Experienced based, lasting cellular changes that add to the stability of the system are the basis of synaptic plasticity and long term potentiation (LTP) that are needed for learning and memory (Citri and Malenka, 2008; Huginair and Nicoll, 2013; Vitureira and Goda, 2013; Nabavi *et al.,* 2014; Fernandes and Carvalho, 2016).

Principles of Hebbian plasticity govern synaptic encoding of memory signals. These precise connections are thought to be fundamental for memory storage. An increase in synaptic efficacy arises from the presynaptic cell's persistent stimulation of the postsynaptic cell that leads to some growth process or metabolic changes in both cells and strengthens the synapse. The presynaptic cell must respond to action potentials and influx of calcium, with the release of vesicles filled with neurotransmitters. Hebbian plasticity governs that the probability of this vesicle release will depend on the previous activation of this cell. The amount of neurotransmitter released affects the postsynaptic cell based on how many receptors they activate, which in turn changes the membrane potential. Retrograde signals can also be sent by the postsynaptic cell to the presynaptic cell in order to modulate the synapse. Once the synapse is potentiated by this event the cell can then be further activated with greater ease, leaving it prone to hyper-excitability

(Citra and Malenka, 2008; Vitureira and Goda, 2013; Nabavi *et al.,* 2014). Evidence of deficits in neuronal plasticity can be seen in psychiatric diseases such as SZ (Haracz, 1985; Steven *et al.,* 2006; Daskalakis *et al.,* 2008; Stephan *et al.,* 2009; Voineskos *et al.,* 2013).

Two mechanisms of synaptic plasticity, thought to contribute to the activity-dependent refinement of neural circuitry during development and with adult learning, are LTP and long-term depression (LTD). Both of these mechanisms depend on the amount of calcium influx as a result of an action potential, the number of vesicles in the presynaptic cell, and the detection of activity through NMDARs (Bear, 2003). Rapid adjustments in the strengths of individual synapses are made in response to specific patterns of correlated pre- and postsynaptic activity. LTP is associated with synaptic strengthening due to a prolonged period of coordinated activity between the pre- and post-synaptic neurons (Purves *et al.,* 2001 and Bear, 2003). LTD is induced based on a lack of depolarization coincidence. Much of the work done to elucidate the learning and memory mechanism, LTP and LTD, has been done in the hippocampus. The Schaffer collateral are easily visualized and stimulated, and the generated excitatory post-synaptic potentials are easily recorded from the CA_1 pyramidal cells. In mice, lesions of the hippocampus have prevented memories from forming. As well, the human hippocampus can be imaged during memory tasks and activation can be measured.

Modulating synaptic plasticity in response to the previous activity state of the

synapse, while preserving the Hebbian-directed memory signals, can be explained by various homeostatic plasticity mechanisms, like synaptic scaling, intrinsic plasticity, and metaplasticity.

In 1996, Abraham and Bear described the plasticity of synaptic plasticity. They dubbed this plasticity metaplasiticity and based it on bidirectional synaptic plasticity and the sliding modification threshold for the post synaptic cell's ability to elicit LTP or LTD. This threshold for the ability to elicit potentiation or depression they found varies with the cells history of activity (Abraham and Bear, 1996 and Abraham and Bear, 2003). Metaplasticity is this dynamic process that modulates the sensitivity of a population of synapses. This modulation is in response to afferent stimulation and relies on changes in NMDA and AMPA receptors and their trafficking to effect synaptic sensitivity changes.

Metaplasticity refers to activity dependent tuning of neuronal function that modulates subsequent synaptic plasticity. In other words a synapse's previous history of activity determines its current and future plasticity. The mechanism of metaplasticity depends of also by the extrinsic influences, i.e. neurotransmitters, neurotrophic factors and hormones. Because Hebbian plasticity in theory, could lead to positive feed-forward excitation that would result in hyperactivation of neurons, metaplasticity ensures the overall stability of the neuronal circuits, via homeostatic mechanisms that are still being uncovered. Disruptions in plasticity mechanisms could lead to altered neuronal activation patterns throughout the brain (Abraham and

Bear, 2009; Yger and Gilson, 2015; Sweatt, 2016) and in turn lead to mental illnesses, such as SZ.

Glutamate and the synapse

Glutamate is the most abundant excitatory neurotransmitter in the brain. Glutamate signaling impacts a majority of neurons in mammalian brain, supporting activity-dependent signaling, neuronal growth, and synaptic plasticity. The glutamate system in brain is highly complex and includes multiple interacting receptors, modulating co-transmitters and multisystem synapses. Presynaptic, postsynaptic, and astrocytic mechanisms are important to overall excitatory signaling and to unique plasticity mechanisms which are associated with glutamate transmission; the 3 compartments (pre-, postsynaptic, and astrocytic) are all candidate systems for pathology in glutamate-related brain diseases (Tamminga, Southcott *et al.,* 2012).

Glutamate is stored pre-synaptically in vesicles that are released upon depolarization of the cell due to an action potential reaching, and surpassing, threshold. Once released glutamate then binds to its post-synaptic ionotropic and metabotropic receptors causing activation and galvanizing signaling mechanisms (Collingridge *et al.,* 1992; Kandel, *et al.,* 2000).

Metabotropic receptors do not characteristically have a channel that opens but are linked to cytoplasmic G-proteins that, upon conformational change of the receptor due to binding with a ligand, will be released and functions as a secondary

messenger. A much faster mechanism of action upon the cell is mediated by the ionotropic receptors. These receptors are permeable to Na^+ (flow into the cell) and K^+ (flow out of the cell), but upon binding of ligands these receptors then open their channels to let ions (Ca^{2+}, Na^+, K^+, or Cl^-) pass (Zarei and Dani, 1994; Alberts *et al.,* 2002; Purves *et al.,* 2008).

This action of glutamate on NMDA and- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors is especially important in learning and memory (Konrida and Heckers, 2003, Ctri and Malenka, *2003;* Mitsushima *et al,* 2011; Sanders *et al.,* 2008; Wang and Peng, 2016). It is the ratio of the different receptor types and the number of receptors at the surface of post-synaptic cells and the composition of the subunits of each receptor that determines the effectiveness and action of the transmitter on the cell. The number of receptors and the ratio of these receptors are determined by the prior action upon the cell (Collingridge *et al.,* 1992; Cull-Candy *et al.,* 2001; Abraham and Bear, 2009; Savtchouk *et al.,* 2016).

AMPA receptors mediate the fast synaptic transmission and flow of Na+ and Ca^{2+} into the postsynaptic cells. They generate the large early part of the evoked post-synaptic potential (EPSP). Which scaffolding protein the tetramer, that is the complete receptor, interacts with depends on the combination of the different subunits: GluA1, GluA2, GluA3, and/or GluA4 (Bennett *et al.,* 1996). Phosphorylation of the receptor at different amino acids regulates localization,

channel conductance and ion permeable pore open probability (Malenka and Nicoll, 1993; Konradi and Heckers, 2003; Wang *et al.,* 2005; Wyllie *et al.,* 2013).

The receptor of great importance for the study of learning and memory is the NMDA receptor (Li and Tsien, 2009). These glutamate receptors are slower acting compared to AMPA receptors and have Mg^{2+} ions in the channel pore, blocking the flow of most ions at resting membrane potential. NMDA receptors are considered voltage gated channels because they require a higher voltage to dislodge this Mg^{2+} ion and enable the channel to open. The NMDA receptors are unique in that they need a co-agonist, glycine (Wood, 1995) or D-serine (Mothet *et al.,* 2000; Shleper *et al.,* 2005; D'Ascenzo *et al.,* 2014). Once open, these receptors allow Ca^{2+} ions to flow in. The Ca^{2+} that enters the cell through the receptors then acts as a secondary messenger to increase the AMPA receptor permeability (those that are at the membrane surface) and triggers the trafficking of AMPA receptors to the membrane (Henley, 2013; Anggono and Huganir, 2012; Malinow *et al.* 2002) as well as dendritic spine plasticity (Chen *et al.,* 2015; Hunt and Castillo, 2012) in order to change functional organization so that the circuitry is more efficient.

These NMDA receptors are heterotetramers made of different combinations of subunits: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, and GluN3B. These subunits give the receptor its properties, form and function (Monyer *et al.,* 1994; Cull-Candy *et al.,* 2001; Konradi and Heckers, 2003; Paoletti *et al.,* 2013;).

The subunit composition of the NMDA receptor determines the permeability of Ca^{2+} (Evans *et al.,* 2012; Siegler Retchless *et al.* 2012; Paoletti *et al.,* 2013).

Of the different NMDA receptor subunits GluN1, GluN2A and GluN2B are the most widely expressed in brain and extensively studied for their role in the induction and maintenance of LTP. They are furthermore the predominant subunits in the CA_1 and CA_3 pyramidal cells (Shipton and Paulsen, 2013).

GluN1 is the obligate subunit of the NMDA receptor and is ubiquitously expressed throughout the brain (Cull-Candy *et al.,* 2001; Wenzel *et al.,* 1997; Law *et al.,* 2003). Without the GluN1 subunit the NMDA receptor does not function. The binding site for glycine is contained in the GluN1 subunit. The combination of different subunits with GluN1 leads to variability in function and pharmacological properties (Nakanishi, 1992), such as affinity for and binding kinetics of glutamate, channel open probability and deactivation rates, and interactions with intracellular proteins (Sanders *et al.,* 2013). For instance, NMDA receptors that form with GluN2A subunits have a higher probability of opening compared to those composed with the GluN2B subunit. NMDA receptors containing GluN2A are important for rapid information processing and allow for LTP to be induced more easily due to their fast activation and deactivation (Paoletti *et al.,* 2013; Monyer *et al.,* 1994). In contrast when the predominant subunit in the NMDA receptor is GluN2B LTP in the HC is more difficult to induce. However, when LTP is induced by the NMDA receptors with GluN2B it is larger in magnitude because these receptors carry more Ca^{2+} per unit of current (Sobczyk *et al.,* 2005).

As well, GluN2B is important because it interacts with and anchors the calcium/calmodulin-dependent protein kinase II (CaMKII) at the postsynaptic density. The anchoring of CaMKII enables activation of downstream signaling cascades that mediate synaptic strength. Thusly, NMDA receptors with GluN2B subunits may be important for learning and memory when information needs retention for a delayed time or there is incremental task acquisition over days (Shipton and Paulsen, 2014). GluN2B has higher calcium influx compared to GluN2A, it has a lower open probability and peak current, as well as a slower deactivation supporting twice as much charge transfer, slower rise and slower decay. It also has a lower Mg^{2+} sensitivity and lower conductance (Shipton and Paulson, 2014).

These subunits also differ in abundance during development of the brain. GluN2B is considered the immature subunit of NMDA receptors, as it is the predominant subunit in early development present prior to birth and decreases in abundance until adulthood, while the opposite is true for the GluN2A subunit that becomes the predominant subunit in mature brain (Williams *et al.,* 1993; Wenzel *et al.,* 1997; Cull-Candy *et al.,* 2001; Law *et al.,* 2003; Shipton and Paulson, 2014) and is likely the reason for changes in activity-dependent synaptic plasticity late in development. The insertion of AMPA into the post-synaptic membrane is facilitated with this developmental switch as well (Hall *et al.,* 2007; Elias *et al.,* 2008; Adesnik *et al.,* 2008) and LTP is elicited at lower frequencies (Dumas, 2012; Tse *et al.,* 2012; Araki *et al.,* 2015).

Glutamate in Schizophrenia

In the 1950s when antipsychotics were first discovered many hypotheses in the field of SZ research focused on a dysfunctional dopamine system because antipsychotics are dopamine receptor antagonists (Lieberman *et al.,* 1987; Seeman *et al.,* 1990; Davis *et al.,* 1991; Lieberman, 2004; Ramachandraiah *et al.,* 2009). Since then this idea has been worked on extensively with little evidence so far that dopamine dysfunction alone causes psychosis (Mailmon *et al.,* 1981; Meltzer, 1987; Martinot *et al.,* 1990; Javitt and Zukin, 1991; Jentsch and Roth, 1999; Goff and Coyle, 2001; Patil *et al.,* 2007; Seeman, 2009). Perhaps the involvement of the dopamine system in SZ is a secondary perturbance due to a primary dysfunction in another related cerebral system, such as the glutamate system in the HC (Javitt, 2007).

Synaptic glutamate signaling in brain is highly complex and includes multiple interacting receptors, modulating co-transmitters and distinct regional dynamics. It was when the NMDA receptor antagonist phencyclidine (PCP), a potent psychotomimetic, was tested as an anesthetic that the focus of SZ research changed to the glutamate system (Tamminga, 1998). When PCP was tested as an anesthetic, volunteers reported hallucinations and delusions. The NMDA receptor is the site of action of psychotomimetics such as PCP and related anesthetics, which can mimic schizophrenia symptomology (Tamminga, 1998; Coyle, 2012): cognitive, negative symptoms and positive symptoms. Furthermore, PCP taken by SZ patients

exacerbates their symptoms (Malhotra *et al.,* 1997; Lahti *et al.,* 1995). Another line of evidence that points toward the glutamate dysfunction hypothesis is agents that stimulate NMDA receptor-mediated neurotransmission (ie. glycine-site agonists and glycine transport inhibitors) have shown antipsychotic efficacy. Encouraging results have been also been observed with metabotropic 2/3 agonists that alter resting glutamate levels (Kantrowitz and Javitt, 2012).

Regional characteristics of synapse architecture, local circuitry, and specialized signaling mechanisms suggest that important aspects of glutamate regulation are local and dynamic. Even within anatomic regions, specificity of function occurs on a microcircuit level with the same glutamatergic architecture serving distinct functions. It is already known that hippocampus and the surrounding MTL cortex is dependent on glutamate signaling to a greater extent than other neocortical tissue, a feature that underlies its learning and memory functions (Tamminga, Southcott *et al.,* 2012).

MTL memory structures receive excitatory inputs from neocortical sensory and associational projections: afferents from neocortex pass to parahippocampal cortex, then to layers II/III of entorhinal cortex, and then onto hippocampal subfields. Principles of Hebbian plasticity govern synaptic encoding of memory signals, and homeostatic plasticity processes influence the activity of the memory system as a whole. Moreover, HC is one of the brain regions whose function is altered in SZ (Heckers *et al.,* 1998; Medoff *et al.,* 2001; Schobel *et al.,* 2013; Mathew *et al.,* 2014;

Samudra *et al.,* 2015; Lui *et al.,* 2015; Stan *et al.,* 2015), suggesting the potential relevance of glutamate transmission in this structure to psychosis pathophysiology. The role of altered glutamate signaling in schizophrenia (i.e. decreased glutamate activity in limbic brain structures, changes in subunit expression and binding sites in schizophrenia appear to be specific to limbic, cortical and hippocampal brain regions, and an increase of NMDA receptor binding was in the posterior cingulate cortex) and the clinical and pharmacological basis (PCP and ketamine studies demonstrating the ability of NMDA receptor antagonists to induce positive, negative and cognitive symptoms in controls, as well as exacerbate psychosis in those with SZ) for this formulation have already been amply reviewed (Konradi and Heckers, 2003; Tamminga, 2010; Tamminga, Southcott *et al.,* 2012; Poels *et al.,* 2014).

Hippocampal imaging studies in schizophrenia identified 2 alterations in MTL (1) increases in baseline blood perfusion and (2) decreases in task-related activation. These observations along with converging postsynaptic hippocampal protein changes (detailed in next section) suggest that homeostatic plasticity mechanisms might be altered in schizophrenia HC. For instance, if hippocampal pattern separation is diminished due to partial DG failure (resulting in ‘spurious associations') and also if pattern completion is accelerated and increasingly inaccurate due to increased CA_3 associational activity, then it is conceivable that associations could be false and, especially if driven by anxiety or stress, could generate psychotic content, with the mistaken associations being laid down in

memory, despite their psychotic content, especially delusions and thought disorder (Tamminga, Southcott, *et al*., 2012).

Evidence for SZ as a Learning and Memory Disorder

In disorders with psychotic manifestations, the details, ideas, content and stability of psychotic phenomenon resemble human memories. When asked how he could think his hallucinations are real, John Nash, the famous Nobel Prize winner, has been quoted saying, “Because the ideas I had about supernatural beings came to me the same way my mathematical ideas did” (Nasar, 1998). The content of chronic psychosis is personal and enduring. Other evidence that psychosis is a deficit of learning and memory is declarative memory perturbations (Stone and His, 2011; Preston *et al.,* 2006); decreased capacity for associational memory (Tamminga and Zukin, 2015). Many studies also show a reduction in HC activation with memory task performance. Specifically, Das *et al.,* (2014) showed that performance of a pattern separation task, thought to be mediated by the DG, was decreased compared to normal controls. Another form of evidence that points towards a dysfunctional DG in SZ is a decrease in both protein and mRNA levels of the necessary subunit of the NMDA receptor, GluN1 here (Figure 1-3)(Gao *et al.,* 2000; Stan *et al.,* 2015).

There are some behavioral deficits observed in humans with SZ that can be directly translated and studied in animal models that are directly related to

hippocampal dysfunction. One example of this is spatial learning and memory deficits seen in patients after the first episode of SZ, which was studied using a virtual reality task (Fajnerová *et al.,* 2014). In this task patients used reference memory to find a stable goal position. This task was designed to test spatial learning, much like the Morris water maze task in mice. The Morris water maze has been demonstrated in mice to be a HC mediated task (Morris, 1981; Morris *et al.*, 1982; Morris, 1984, Morris *et al.*, 1986). First episode SZ patients showed impaired pointing to goal and flawed navigational accuracy after being trained to use cues to find a target. In relation to this, NMDA receptors have been shown to be essential for this type of memory. In the CA_3 they play a role in the rapid acquisition and associative retrieval of spatial information. In mice, when NMDA receptors were blocked it resulted in impairment in finding the hidden platform (Nakazawa *et al.,* 2004).

In the CA_3 our lab has shown that there is an increase in GluN2B containing NMDA receptors (Figure 1-3)(Li *et al,* 2015). While AMPA receptor activation leads to depolarization via sodium influx, (fast and non voltage dependent), NMDA receptor activation leads to depolarization via calcium and sodium influx. GluN2B is critical for the functional maturation of glutamatergic synapses. GluN2B is also extremely important as it mediates the anchoring of the synaptic proteasome responsible for fine-tuning AMPA receptors (Halt *et al.,* 2012 and Shipton and Paulsen, 2014).

Another interesting finding in the CA_3 of post mortem brain tissue in SZ is an increase in brain derived neurotrophic factor (BDNF) mRNA (Figure 1-3) (Tamminga *et al.,* 2012). BDNF is a neurotrophic factor important for regulating and supporting neuronal survival, neuronal differentiation and growth during brain development. BDNF also has effects on neurogenesis and neuroplasticity. Importantly for our studies, BDNF has activity-dependent effects of on neuronal transmission in HC. BDNF can modulate NMDA receptors, by controlling the development and activity of neurotransmitter systems and primarily through phosphorylation and activation of the GluN1 subunit, particularly the PKC Ser-897 site. Importantly, this neurotrophic factor has been implicated in SZ. A few studies show altered BDNF mRNA in PFC of SZ (Weickert *et al.,* 2003; Wong *et al.,* 2010; Ray *et al.,* 2014), another finds that blood levels are reduced in drug-naïve and medicated SZ samples and difference increases with age (Green *et al.,* 2010), yet another finds C-281A polymorphism in paranoid SZ (Suchanek *et al.,* 2011). The most compelling and most widely studied BDNF link to SZ is the Val66Met polymorphism in psychiatric disorders. In independent studies and meta-analysis this single nucleotide polymorphism linked this polymorphism to brain morphology, cognitive function, age of onset and psychiatric symptoms in schizophrenia (Chao *et al,* 2008*;* Gratacos, *et al,* 2007; Zhang *et al.,* 2013; Rosa *et al.,* 2006) and may even have a link to HC volume (Szeszko *et al.,* 2005).

An additional significant finding that we suggest points toward hyperactivity of the CA_3 is elevated levels of PSD-95 in SZ compared with matched normal controls (Figure 1-3). Post-synaptic density protein 95 is a membrane associated guanylate kinase (MAGUK). This MAGUK is a major scaffolding protein in the excitatory postsynaptic density and a potent regulator of synaptic strength (Chen *et al.,* 2011). It regulates synapse strength by spatially localizing aggregates, or clusters, of receptors by direct interactions with its PDZ domain (Gomperts, 1996; Ehlers *et al.,* 1996;O'Brien *et al.,* 1998). PSD-95 anchors receptors to the cytoskeleton so that they are in the correct location for precise and maximum utilization. PSD-95 is also important for helping to maintain the balance between excitation and inhibition in neuronal circuits via synaptic scaling, modulating synapse morphology, and AMPA receptor trafficking (Keith and El-Husseini, 2008). Importantly, PSD-95 has been implicated in SZ, in studies that show mRNA to be reduced in the PFC of those with SZ, gene polymorphisms, altered levels in HC and thalamus in SZ, and its modulation by antipsychotics (Ohnuma *et al.,* 2000; Lasevoli *et al.,* 2014; de Bartolomeis *et al.,* 2014).

In our lab, Golgi staining was used to examine the morphologic correlates of molecular changes on the pyramidal cell architecture. Both the proximal apical and basal dendrites corresponding to the CA_3 stratum radiatum and CA_3 stratum oriens substrata, respectively were examined. There is a clear increase in spine density limited to stratum radiatum, at the apical trunk of pyramidal CA_3 neurons (Figure 1-

3)(Li *et al.,* 2015). An increase was detected in the number of mossy fiber receptive sites, the thorny excrescences, as well (data not shown). The presence of greater spine density is compatible with findings of elevated PSD-95, as overexpression of this protein has been shown to elevate spine density in hippocampal cultures (El-Husseini *et al.,* 2000). Increased spine number is regularly observed following LTP-mediated increases in synaptic strength at excitatory synapses (Maletic-Savatic *et al.,* 1999; Engert and Bonhoeffer, 1999). The reduced input from the MF could lead to increased excitability and strength of the CA_3 apical dendrites through metaplasticity mechanisms.

This increase in spine density in CA_3 could also represent the morphologic manifestation of increased GluN2B-containing NMDA receptors in CA_3 in SZ, particularly as the GluN2B subunit advantages long term potentiation (Barria and Malinow, 2005; Zhao and Constantine-Paton 2007). This increase in GluN2B containing NMDA receptors could be an increase in silent synapses within CA_3, this being a condition which would prime the synapse for an increase in experience-dependent plasticity with synaptic 'un-silencing'/input of AMPA receptors into the postsynaptic membrane (Konrida and Huang *et al.,* 2009). Silent synapses are those that contain only NMDA receptors and, at resting potential, lack electrophysiological responses (Konrida and Hekers, 2003). This un-silencing could be interpreted as consistent with the presentation of psychosis in humans, in that most SZ individuals with schizophrenic psychosis retain a psychosis propensity after an initial psychotic

episode and many show a cyclic recurrence of psychotic manifestations. The alteration in CA_3 plasticity conditions with an initial psychotic episode could create a vulnerability to new excitatory inputs after a florid psychosis, which could generate 'run away' activity within the recurrent collateral that would diminish prediction error mechanisms, increase associations and allow false memory formation with psychotic content. The CA_3 subfield changes may also provide the molecular and cellular substrate supporting hippocampal hyperactivity *in vivo,* hyperactivity represented by increased hippocampal cerebral blood flow (Medoff *et al.,* 2001; Heckers *et al.,* 2002) and blood volume (Schobel *et al.*, 2013) well described in schizophrenia patients (Li *et al,* 2015).

Animal Models

Psychosis is described as a loss of contact with reality, marked by hallucinations or delusional beliefs with an aberrant perception or evaluation of the salience of environmental stimuli. The multifactorial, polygenetic and pleomorphic characteristics of schizophrenia have made this disease extremely difficult to study. Cellular and molecular mechanisms of SZ are still a mystery, although clues exist. Postmortem studies have allowed for a level of molecular analysis not yet available with imaging research, nonetheless confounding variables (alcohol, illicit drug and tobacco use, treatment with different combinations of neuroleptics over various amounts of time, hospitalization, etc.) hamper crucial discoveries. Human studies assessing SZ brain are limited to *in vivo* imaging and post mortem pathology. These

in vivo studies are complementary nonetheless they are still insufficient to determine pathways and key players in the emergence of psychosis (Harrison, 2000; Perlman *et al.*, 2004).

Achieving animal models of schizophrenia that are representative of clear pathology in the illness is critical to understanding full pathophysiology and developing novel treatments for the complex syndrome. However, as Nestler and Hyman (2010) point out, even this line of study (study of animals models) has its caveats such as weak validation and poor predictive power for drug efficacy. As well, the phenomenology used to diagnose psychiatric illnesses is uniquely human (i.e. hallucinations and delusions verbally expressed to clinicians). Discoveries of the mechanisms underlying SZ have come largely from animal models based on risk genes and from studying the action of psychotomimetic drug. Now it is becoming possible to develop animal models through reverse translation, mimicking the biology of the human condition, in addition to genetic candidates, and pharmacological manipulations. Animal models for psychosis have been inadequate because the cellular and molecular characteristics of the condition itself have been obscure (Yanagi, Southcott *et al.,* 2012, Flores *et al.,* 2016). As well, the group of psychotic disorders is characterized by considerable heterogeneity and a complex clinical course that reflects many factors that cannot be reproduced readily in animals. Symptomatic manifestations reflect temporally and spatially integrated outputs from dysfunctional circuits and complex genetic architecture. Any previously

established model is unlikely to reflect the full extent of this neuropsychiatric disorder. It is for this reason that we must focus on specific symptoms, or dimensions, rather than syndromes, or diagnosis. Using experimentally manipulated animals, or animal models, we can apply the invasive experimental designs that are invaluable for investigating and establish underlying pathophysiology and psychological processes that bring about the behavior observed in humans (Geyer and Moghaddam, 2002; Nestler and Hyman, 2010; Fernando and Robbins, 2011).

There are three symptom domains which scientists seek to replicate in animals models: negative, cognitive, and positive. With each domain we look to model behavioral correlates. The focus of negative symptom studies is largely on decrease sociability and this overlaps with depression and autism symptoms. Cognitive symptoms, such as decreased executive function, also largely overlap with autism and bipolar disorder. The most challenging domain to replicate in animals is that of positive symptoms. Primate models of mental processes, particularly in the domains of higher cognitive thinking, auditory and visual image processing have proved invaluable in the study of psychiatry (Simen *et al.,* 2009). These models further the understanding of complex neurocognitive processes due to the similar complexities of the adult human and monkey brains. Primates have the advantage of expanded associative cortices, and increase complexity of PFC neurons compared to rodents (Simen, 2010). The most prominent modality among the non-human primate models are psychostimulant-induced psychosis and amphetamine sensitization paradigms

(Castner and Williams, 2007). The primates exhibit positive symptoms of SZ and persistent cognitive deficits (i.e. altered working memory and social deficits). Similarly, the model successfully predicts the therapeutic efficacies of conventional and novel antipsychotic treatment (Nelson and Winslow, 2009). However, primates are expensive to recruit, care for, and house. Another caveat of working with primates is the difficulty to handle them safely. Primates are highly labor intensive and when dealing with neurodevelopment, primates are not ideal as they have prolonged period of maturation.

It is the relatively short maturation time and established behavior paradigms with similar circuitry of rats and mice that make rodents the most established mammalian model. As well, rats and mice give birth to multiple offspring at one time giving to large numbers to study. Rats and mice are relatively inexpensive to maintain colonies and they are easy to manipulate both molecularly and genetically (National Institute of Health).

There is even a possibility to use zebrafish and *Drosophila* to study SZ (Sawa, 2009). No matter the chosen animal used to model disease, they are meant to be validated on the basis of how well their performance in a given test that would predict the performance of humans with SZ and on whether the model provides a sound theoretical rationale; models should display predictive and construct validity (Marcotte *et al.,* 2001; Nestler and Hyman, 2010).

The Schizophrenia Research Forum (SRF) has listed 150 putative rodent

models for aspects of schizophrenia (www.schizophreniaforum.org). These models can be broken down into 5 main categories: developmental, genetic, pharmacological, lesion-based and environmental. However, these appear to fall short; gene manipulations are not 100% penetrant and are only weakly associated with the illness and we are unsure of when their maximal impact occurs (during development, or in adulthood); pharmacological models do not address the mechanism, disrupt the whole neuronal system, and have off target affects; lesion models do not have construct validity; we don't know the mechanism of developmental models such as Methyl-azoxy-methanol (MAM) or maternal infection resulting in developmental abnormalities in offspring, and environmental models have only weak results. Informative animal models/preparations that allow us to understand SZ (the most severe of the psychosis) are needed in order to further its study. New models have to be categorically different. We need putative mechanistic approaches that are more proximal to disease pathology to foster novel drug discovery.

Hypothesis

Using correlative evidence that our lab has collected from human post mortem tissue (Figure 1-3), we have formulated a testable hypothesis of psychosis as a learning and memory disorder with the HC as harboring the core pathology. We hypothesize that a reduction of the obligate subunit, GluN1, in the DG leads to

increased activity of the CA_3 seen in post mortem tissue as an increase in post-synaptic plasticity markers, such as increased GluN2B-containing NMDA receptors, PSD-95 and spines on apical dendrites of pyramidal neurons (Figure 1-3). We suggest that the decreased activity in the DG is manifested as poor pattern separation and that this decrease is associated with down stream hyperactivity in CA_3 manifested as exaggerated pattern completion or mistakes of memory, which combined would result in disorganized declarative memory (Figure 1-3) as observed clinically in people with SZ. In order to test this hypothesis we will knockout the GluN1 subunit in the DG of mice to mimic the human biology and test the behavioral outcomes as well as observe protein or morphological changes in CA_3. Our objective is to develop a relevant animal model of the HC disease pathophysiology of SZ psychosis in order to study its development and its impact on other regions of the brain and in order to eventually develop new and novel antipsychotic drug targets.

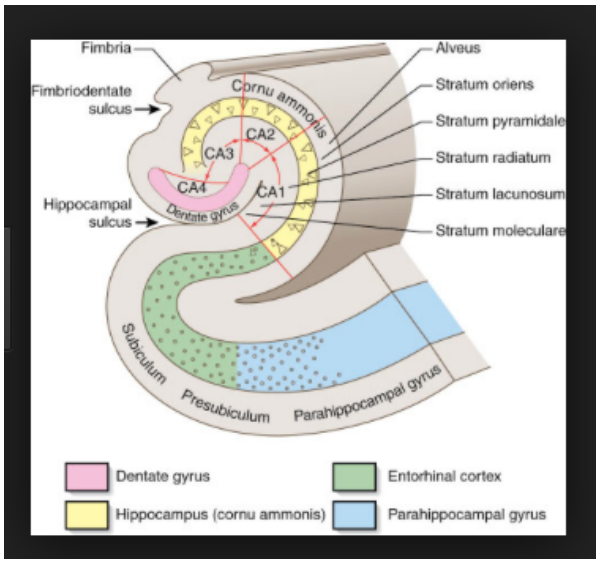


Figure 1-1. Hippocampus. Cartoon of the hippocampus proper. The dentate gyrus, the CA_3, CA_2, CA_1, subiculum, entorhinal cortex, and the parahippocampal gyrus. http://medicine.academic.ru/3923/Hippocampus

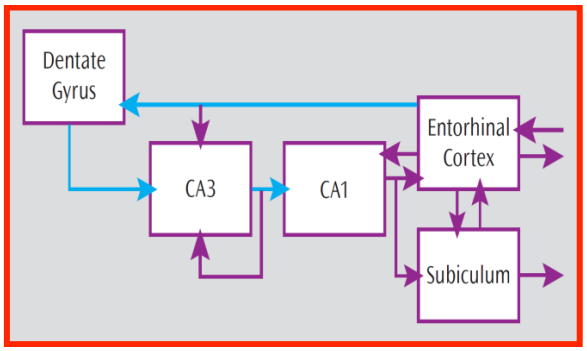


Figure 1-2 Trisynaptic pathway. Cartoon of the trisynaptic pathwa. The excitatory pathway from the entorhinal cortex, to the dentate gyrus, to the CA_3, to the CA_1 to the entorhinal cortex or subiculum. Blue arrows represent the tri-synaptic pathway. Purple arrows are other excitatory connections.

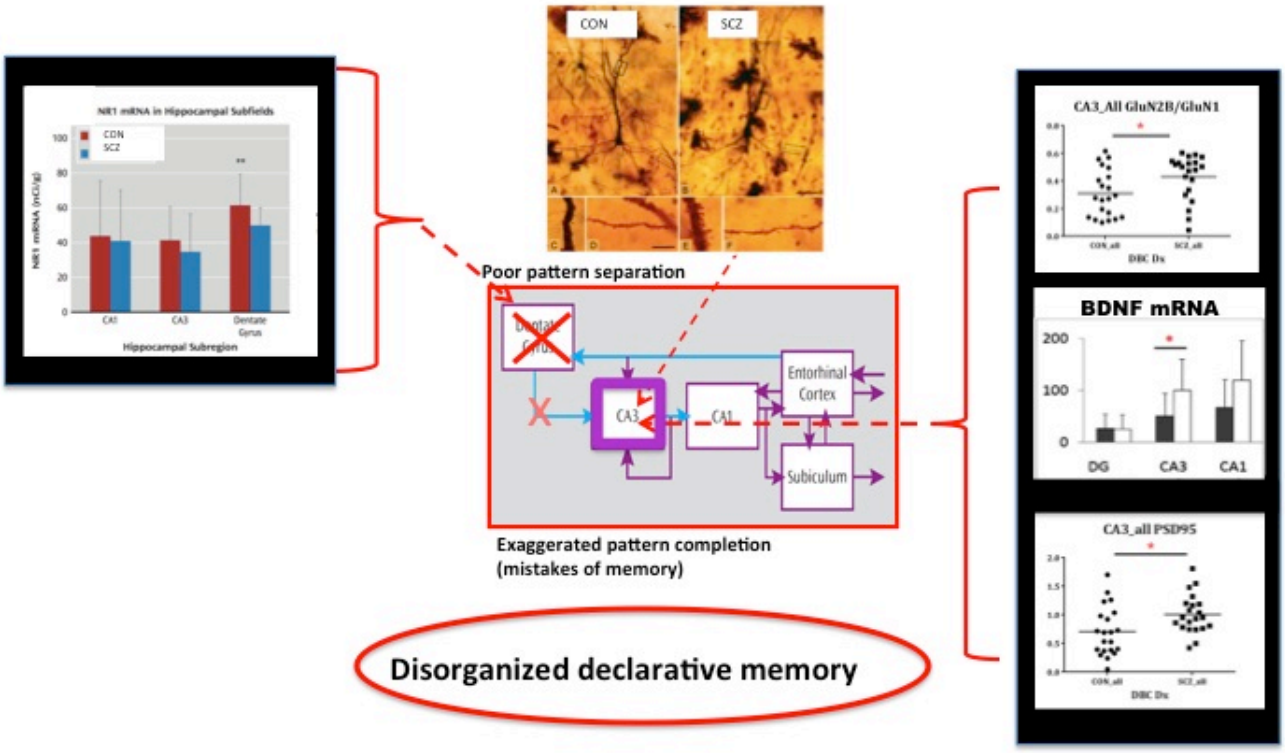


Figure 1-3. Model of learning and memory dysfunction in SZ psychosis. Reduced GluN1 in the dentate gyrus could result in poor pattern separation. This decrease in activity in the dentate gyrus could lead to a compensatory upregulation of excitatory mechanisms (increased GluN2B/GluN1, increased BDNF, increased PSD-95 and increased spines) in the CA_3 that could result in exaggerated pattern completion. The combination of the decrease activity in dentate gyrus and the increase in activity of the CA_3 could then lead to disorganized declarative memo

CHAPTER TWO
Reduced GluN1 in mouse dentate gyrus is associated with CA3 hyperactivity and psychosis behaviors

Our lab has extensively studied high quality, human, post mortem HC tissue. This tissue is dissected by subfield and examined for proteins related to excitatory and inhibitory signaling as well as synaptic plasticity. In the DG tissue we discovered decreased GluN1. In the CA_3 we found increased BDNF mRNA, increased GluN2B-containing NMDA receptors, increased PSD-95 protein and increased numbers and size of dendritic spines on apical dendrites. The increases in GluN2B-containing receptors, the early developmental variant of the NMDA receptor, and in PSD-95 protein in SZ, occur without indication of AMPA receptor subunit alterations in SZ. The GluN2B/GluN1 increase in CA_3 was present in the entire SZ cohort, without any difference between cases on- and off-antipsychotic medication, indicating that this is a disease effect and not a chronic medication effect (Li *et al.,* 2015).

In clinical studies of psychosis in schizophrenia, we show whole hippocampal hyperactivity and hyperperfusion of Ca_3/CA_1, GluN1 protein reduction, particularly in dentate gyrus (DG) and synaptic strengthening at the NMDA receptor in CA_3 along with alterations in relational memory capacity in the illness (Tamminga, et al., 2010 and 2012; Li, et al., 2012); these are the molecular and behavioral targets we modeled in the animal preparation of psychosis.

Introduction

Observations from human brain imaging and postmortem tissue analysis in schizophrenia implicate the hippocampal formation in psychosis along with alterations in declarative memory functions, as previously presented. Whereas the prefrontal cortex (PFC) (commonly targeted in schizophrenia) is thought to be critically involved in cognitive dysfunction in the illness, the hippocampus is thought to be involved in psychotic manifestations and in declarative memory dysfunction. Observations from *in vivo* imaging studies in schizophrenia show increased perfusion in HC (Medoff *et al.,* 2001; Heckers *et al.,* 1998; Schobel *et al.,* 2009) and observations from postmortem schizophrenia tissue studies show *decreased* GluN1 mRNA and protein in hippocampal subfields, particularly in DG (Gao *et al.,* 2000; Deicken *et al.,* 1999). These are seemingly paradoxical observations, which, however, converge to suggest homeostatic plasticity alterations in schizophrenia hippocampal CA_3 subfield as a mechanism for psychosis. The preliminary increases in homeostatic plasticity markers that we have already identified within CA_3 underscore the feasibility of this formulation and testing.

It is the functional effect that this reduced glutamate signaling in DG has on CA_3 that could be of interest for psychosis. CA_3 is the projection target of DG in the trisynaptic pathway through the mossy fiber projection. Currently, the human construct suggests that the decrease in activity-dependent signaling within DG

projected through the mossy fiber pathway sensitizes its target tissue to incoming stimuli and through metaplastic mechanisms, generates a lower LTP threshold and increased cellular sensitivity (Abraham and Bear, 1996; Malenka and Bear, 2004), thus generating higher levels of neuronal activity in CA_3 (Tamminga *et al.,* 2010; Medoff *et al.,* 2001; Gur 1978; Malaspina *et al.,* 2004; Honea *et al.,* 2005). The literature supports this model, with findings by Kolomeets *et al.* (2005) showing a reduction in mossy fiber synapses in CA_3 and a Reif study (Reif *et al.,* 2006) showing reduced neurogenesis in DG, each in SZ tissue. This would also lead to increased associational function, especially within the system of CA_3 recurrent collateral synapses, a unique feature of CA_3 innervation (Amaral and Insausti, 1990; Suzuki and Amaral, 2003; Lavenex *et al.,* 2007). The mossy fiber innervations in CA_3 contact both the excitatory pyramidal neurons at thorny excrescences and inhibitory interneurons by way of *en passant* ('passing through') synapses, and these innervations have opposing effects on CA_3 excitation (McBain, 2008). Thus, strengthened transmission at the pyramidal cell and reduced inhibitory control onto local interneurons occur together, augmenting CA_3 pyramidal cell excitation from two sites, advantaging feed-forward excitation (Lawrence and McBain, 2003). Uncontrolled feed-forward excitation might be the cerebral process that fuels hyper-association, false memories and psychotic mental events.

Based on this glutamate-mediated metaplasticity model (Tamminga *et al.,* 2010) we predict an increase in markers of plasticity in CA_3, such as an increase in

NR2B-containing NMDA receptors (augmenting the sensitivity of NMDA receptors), increases in PSD-95 (indicating increased synapse strength and possibly number) and increases in BDNF activity in CA_3 in SZ. It is a speculation, albeit with increasing support (Hoffman *et al.,* 2011), that increases in CA_3 associational function could generate false associations some with psychotic content, which would then be laid down in memory as psychotic thoughts and memories. This idea could explain both the observed reduction in glutamate signaling in DG (represented by GluN1 protein reduction) and the increase in HC perfusion that we detect overall in HC with rCBF measures (Tamminga, *et al.,* 2010) and our finding that BDNF is increased regionally in CA_3 in SZ tissue (Ghose *et al.,* 2006).

While GluN1-null mice do not survive (Forrest *et al.,* 1994; Li *et al.,* 1994), genetically engineered mice with reduced but not absent (5–10% of normal) GluN1 protein can survive to adulthood (Young *et al.,* 2010). These GluN1 knockdown mice can show PCP-like behavioral changes mimicking SZ, including increased locomotor activity, stereotypy, and impaired social interaction, each reversible with antipsychotic medications (Gao *et al.,* 2009). As well, a selective knockout of GluN1 in DG already exists in a mouse; a POMC-Cre recombinase driver mouse is crossed with a GluN1-LoxP mouse to create a mutant mouse with DG-specific GluN1 protein reduction and with behavioral and anatomic features reminiscent of SZ. In the POMC-Cre recombinase line, Cre expression was strong and dense within the granule cells of the DG, while expression was less dense in the hypothalamus

(arcuate nucleus) and the habenula. The DG-specific GluN1 knockout mouse, displays a decrease in GluN1 mRNA expression in DG granule cells starting at postnatal days 10–21 and progresses to completion by 16 weeks, with normal hippocampal architecture. McHugh *et al.* showed that the DG NR1 knockout mouse has impaired pattern separation which they demonstrated using a fear conditioning paradigm with two similar, but different contexts. They were also able to demonstrate an alteration in CA_3 place cell firing in these different contexts, but with normal CA_1 LTP (McHugh *et al.,* 2007).

Materials and Methods

Mice

All experiments and procedures in this study were carried out in accordance with institutional guidelines, conformed to the Guide for the Care and Use of Laboratory Animals and were approved by The Institutional Animal Care and Use Committee at UTSW Medical Center. Mice were kept in the animal vivarium accredited by the Association for Assessment and Accreditation of Laboratory and Animal Care at UT Southwestern Medical Center. Mice were group housed in temperature-controlled rooms on a constant 12 h light/dark cycle with *ad libitum* access to food and water. Experiments were conducted at approximately the same time each day. All experiments were conducted during the light cycle. Animals between the ages of 16 and 24 wks were used for all experiments, as this is when

GluN1 RNA is shown to be at its lowest levels in this mouse preparation (McHugh *et al.,* 2007). For behavioral testing, separate cohorts of mice were used in each experimental task such that animals were not retested in a behavioral paradigm. Scientists who were blind to the genotypes of the animals conducted all behavioral tests. Behavioural equipment was wiped with disinfectant (NPD) and dried between each use.

Breeding and Genotyping

In order to generate the DG granule cell layer specific-GluN1 knockout mice we crossed a mouse that restricted Cre-recombinase expression to pro-opiomelanocortin (POMC) cells to mice with loxP sites flanking about 12 kb of *NMDAR1* gene exon 19 that corresponds to the trans-membrane domain and the C-terminal region (Tsein, *et al.,* 1996). The POMC-Cre mice originally developed by Dr. Joel Elmquist were kindly given to us by Dr. Jeffrey Zigman (UT Southwestern Medical Center). The floxed GluN1 mice were originally developed in the Tonegawa lab (MIT) and were purchased from Jackson Laboratory (http://jaxmice.jax.org /strain/005246.html). McHugh (2007) previously established this method of generating tissue specific KO of GluN1 protein in the DG.

The genotyping of Cre detection was performed as in the published paper McHugh *et al.* (2007), and the protocol from the Jackson Laboratory was used for genotyping floxed-GluN1. The offspring were genotyped for both the POMC-Cre and

floxed-P site gene via PCR amplification followed by separation and visualization on an agarose gel (1.5%). Mice homozygous for LoxP-GluN1 and heterozygous for POMC-Cre were crossed with mice homozygous for LoxP-GluN1. Resultant offspring were all homozygous for LoxP-GluN1, while approximately half were homozygous for POMC-Cre (KO) and half did not contain the POMC-Cre allele (cnt).

We used REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich) to extract and amplify DNA from a small snippet of each mouse's tail. For amplification we mixed 10 μL REDExtract –N-Amp PCR reaction mix, 0.5-1 μL forward oligonucleotide (supplemental Table 1) and 0.5-1 μL reverse oligoneucleotide (supplemental Table 1), 6 μL water and 2 μL DNA extract.

Behavioral Experiments

Locomotion

Individual mice were placed into clean home cages with a small amount of fresh bedding. Locomotor activity was collected in 5-min bins by horizontal photocell beams linked to computer data acquisition (San Diego Instruments, San Diego, CA, USA) for 120 min period.

Pre-Pulse Inhibition (PPI)

Startle response was measured using a San Diego Instruments SR-Lab Startle Response System (San Diego, CA). Following a 5-minute acclimation period

testing of mice consisted of 40-startle stimuli preceded (100 msec) by a pseudorandom order of pre-pulse stimulus (20 msec). Pre-pulse intensities were 0, 2, 4, 8 or 12 dB above the background noise and presented with an average inter-stimulus interval of 15 sec (range 7-23 sec). Outcomes were analyzed using two-way ANOVA and represented as a percentage of the startle response. N=20 (cnt) and 18 (KO). PPI is expressed as percentage for each prepulse intensity: %PPI=100x[(startle stimulus reactivity-startle stimulus reactivity in the presence of prepulse)/(startle stimulus reactivity)].

Passive Avoidance (PA)

KO and cnt mice were trained in a computerized one-trial-passive avoidance apparatus (Med Associates, Inc, St. Albans, VT). The apparatus consists of two chambers separated by a motorized door and a grid floor. One chamber is brightly lit, while the other is completely dark. The latency to enter the dark chamber from the lit was measured once the door between them was lifted. Once in the dark chamber the door lowered. On the training day the animals received two 1 s, 0.5 mA foot-shocks in the dark chamber. After 24 hrs retention of the memory of being shocked in the dark chamber was measured as the increased latency to enter this compartment upon the raising of the door that separated the 2 chambers. Outcomes were test using unpaired t-test.

Fear Conditioning (FC)

Fear Conditioning was measured in boxes equipped with a metal grid floor connected to a scrambled shock generator (Med Associates, St. Albans, VT, USA) as previously reported (Ruediger S, 2011). We employed a paradigm from Pattwell *et al.* (2012). In short, after 2 min of acclimation, mice received five presentations of 80 dB white noise for 10 s with the last 1 s of each tone terminating with a 1 s 0.5 mA foot shock. The inter-stimulus interval was 30 seconds. Measuring freezing with mice in the same chamber 24 hours later without tones and foot shock tested contextual fear. Cued fear conditioning was measured 48 hours after training, with mice in the chamber but with modified environmental stimuli (vanilla scent, decreased light level, white plastic floor to cover the bars, and black plastic tent structure). Tones were presented without shock and freezing measured. Med Associates software scored the freezing behavior.

Morris Water Maze (MWM)

Mice were trained to find a fixed submerged platform in a pool of opaque water (144 cm, diameter) with 4 training trials/day (inter-trial interval of 30–45 min) for 12 days as previously described (Petrik, 2012). In all trials mice were released from pseudo-random assigned start locations and were allowed to swim until they stopped for 2 sec on the platform or 60 sec elapsed. They were manually guided to the platform in the case of failure. To assess spatial learning ability, distinct distal

cues were placed on the surrounding walls. On days 7 and 14, a probe test was conducted in the morning with the platform removed. The mice were allowed to swim for 60 sec.

The percentage of time in each quadrant and number of times crossing a particular area were measured (along with distance moved, thigmotaxis, swim speed and path) with Ethovision software (Noldus Information Technology, Leesburg VA). For each group of mice, relative radial-quadrant occupancy time (%) was compared to that in all other quadrants; and, the platform crossings in the target quadrant were compared to other quadrants.

Molecular and Cellular Experiments

Immunoblotting

The hippocampus from both hemispheres of the mouse brain were removed and chopped into 500 μm slices via a McIlwain Tissue Chopper. Once chopped the slices were micro-dissected under low magnification into three sections: DG, CA3 and CA1. We mixed right and left hippocampus, but separated the anterior and posterior (down the middle for an equal number of slices for each region) for separate analysis. For this experiment we only analyzed the dorsal region.

Tissue was then flash frozen in tubes on dry ice and placed into -80^{o}C freezer until use. Western blots were carried out on micro-dissected hippocampal subfield tissue to analyze subfield specific molecular markers in mice.

Tissue was homogenized in radioimmunopreciation assay (RIPA) buffer containing: 1:100 dilution PMSF/ml buffer, Sodium Orthovanadate/ml buffer and 1:50 dulution protease Inhibitor/mL buffer (Santa Cruz biotechnology, Inc). Immediately prior to use 1:100 diluted Protease cocktail A and Protease cocktail B (Millipore) were added. Total protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce). Samples were resolved by SDS-PAGE, transferred onto nitrocellulose, and probed with commercially obtained antibodies: GluN2A and GluN2B (Millipore, CA), GluN1 (R&D systems, MN), GluA1, GluA1 p-831 and GluA1 p-845 (Thermoscientific, IL) and PSD-95 (CA_3 tissue only, Cellular Signaling Technology, Inc., MA). β-Tubulin (Thermoscientific, IL) immunoreactivity was used as a loading control. After washing with TBST, membranes were incubated with peroxidase-conjugated secondary antibody (1:10, 000) and were visualized by enhanced chemiluminescence (Amersham NJ). Film-based images of immunoreactive bands were captured using Color Video Camera 3CCD Exwave HAD (SONY) and band intensities were analyzed by densitometry using Scion image software (version 1.62c). All the measurements were done in replicate (2 gels with samples in the same order).

Immunohistochemistry

To perform c-Fos immunohistochemistry, fresh perfused (4% PFA) mouse brain was collected, cryoprotected in 30% sucrose in 0.1M PBS, and sectioned (40

μm) on a freezing microtome (Leica SM2000R). Every 9th 40-μm coronal section of hippocampus was mounted on superfrost plus slides, dried at room temperature overnight, subjected to microwave antigen retrieval (citra solution, BioGenex; 95°C for 10 min), quenched free of endogenous peroxidases in 0.3% H_2O_2, and blocked in 3% normal donkey serum, prior to overnight incubation of rabbit anti-c-Fos polyclonal antibody (Thermo Scientific, MA). Primary antibody was detected by sequential incubation with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, PA) and avidin-biotin complex (Vector Laboratories, CA). Diaminobenzidine chromogen was used to detect the immunoperoxidase signal.

Quantification of c-Fos immunoreactive cells was performed with an Olympus CH30 microscope. c-Fos positive cell nuclei were counted (blind to mouse genotype) in hippocampal subregions bilaterally, at each stained coronal section from rostral (dorsal)-to-caudal (ventral) (-1.46 mm to -2.92 mm from bregma). The total number of c-Fos positive nuclei was calculated by (9x) the sum of c-Fos positive nuclei on all stained coronal sections.

Electrophyisiology

Transverse hippocampal slices (350 µm) from cnt and DG GluN1 KO mice (4-5 months old) were obtained by cutting tangentially to the longitudinal axis of the HC. Slices recovered in a holding chamber for at least 1 h before use. During slicing (0-2°C) and recordings (at 24.5-25.5°C), slices were superfused with artificial cerebral

spinal fluid (ACSF) saturated with 95% O2/5% CO2 and containing (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 4 $MgSO_4$, 4 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose. High Ca^{2+} and Mg^{2+} concentrations (4 mM) for extracellular solution was used to reduce cellular excitability and thus to inhibit the epileptiform activity to which the CA_3 region is especially prone. It also allows us to better isolate the MF-CA_3 excitatory post-synaptic current (EPSC) from recurrent associational/commissural (i.e., polysynaptic) EPSCs. For most of the experiments, LY-354740 1μM, a highly selective and potent agonist of group II mGlu (metabotropic glutamate) receptors, was also applied at the end of the experiments to verify that evoked EPSCs were mediated by glutamate release from mossy fibers. Kynurenic acid (2 mM) was used to block glutamatergic transmission during slicing, and thereby limit excitotoxic processes. Pyramidal cells in the CA_3 field were visualized using infrared-differential interference contrast optics. Synaptically evoked EPSCs were measured a holding potential of -70 mV for AMPAR-mediated transmission and at +40 mV for NMDAR-mediated transmission using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA). During recordings, ACSF containing picrotoxin (100 μM) was used to block *γ*-aminobutyric acid A ($GABA_{A)}$ receptor-mediated inhibitory postsynaptic potentials and CNQX (10 μM) to block AMPARs when recording NMDAR-mediated transmission. Recording electrodes (3–5 MΩ) contained (in mM): 120 Cs-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, and 0.3 NaGTP. Afferents were stimulated at 0.05 Hz by a glass monopolar microelectrode filled with ACSF that was

always positioned in the granular cell layer of the DG or in the DG hilus. Data were filtered at 2 kHz, digitized at 10 kHz, and collected and analyzed using Clampex 10.3 software (Clampex 10.3.0.2, Molecular Devices). Membrane potentials of CA_1 neurons ranged between -70 and -55 mV. Series resistances ranged from 10 to 20 MΩ and input resistances (Ri) were monitored on-line with a 40 pA/150 msec current injection given before every stimulus. Only cells with a stable Rs ($\Delta < 10\%$) for the duration of the recording were kept for analysis (Segev *et al.,* submitted).

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software (San Diego, CA, USA). Significance was set as $p<0.05$ for all experiments. Outcomes for locomotion were tested using a two-way ANOVA (genotype x time) and an unpaired t-test. Two-way ANOVAs were also used to assess differences in PPI (decibel x genotype), FC (situation x genotype), c-Fos positive nuclei along the sequential coronal sections (Bregma co-ordinants x genotype), AMPA and NMDA receptor EPSCs (stimulus intensity x amplitude), and paired pulse ratio (PPR X interstimulus interval). Either an un-corrected Fisher least significant difference, Bonferroni's multiple comparison or Sidak's multiple comparisons *post hoc* test were performed when significance was found with ANOVAs. An unpaired t-test was used to test group difference (PA, MWM average vs target platform crossing, western blots, total c-Fos, and the 30 ms time point of the PPR). In the probe MWM test, one-way

ANOVA followed by Dunnett's *post hoc* test (comparing the means of the other areas to the target/control mean) was used to assess target area recognition. One-way ANOVAs were also used for electrophysiological analysis. Full statistical outcomes are included with the figure legends.

Results

DG GluN1 knockout mice display normal Locomotor activity

Locomotor activity differences between GluN1 KO mice and the cnt littermates could affect the results of subsequent behavior tests. Therefore it was important that we observed and measure the spontaneous activity of these mice for 120 mins. The KO mice did not display any differences in locomotor activity compared to the cnt mice (Figure 2-1).

DG GluN1 knockout mice display reduced PPI

Pre-pulse inhibition (PPI) is considered a measure of sensorimotor gating and it appears be disrupted in SZ (Neal *et al.,* 2006; Braff *et al.,* 1978). This physical reaction to a startle can be measured as well as the normal reduction in a startle response that occurs when the startling stimulus is preceded by a weak lead stimulus. When DG GLUN1 mice were compared to their cnt littermates we found a significant interaction between decibel level and genotype. The GluN1 mouse has reduced sensory motor gating (Figure 2-2).

DG GluN1 knockout mice display increased fear memory

Contextual fear conditioning is known to involve the hippocampus while cued fear conditioning is thought to rely more on the amygdala, but still involves the hippocampus (Anagnostaras *et al.* 2001; Phillips and LeDoux, 1992). The behavioral response of the mouse to fear is freezing. When tested in both a contextual and cued paradigm (before and after tone), GluN1 mice showed a significant increase in freezing behavior compared to their cnt littermates (Figure 2-3). Another test to confirm this increase fear phenotype was the passive avoidance paradigm. DG GluN1 knockout mice display increased latency to enter the dark compartment 24 hrs after training indicating that they had a stronger memory of the shock they received in the dark compartment the previous day (Figure 2-4).

DG GluN1 knockout mice display a deficit in visual spatial memory

The Morris water maze is a paradigm used to assess spatial memory and relies on the HC and NMDA receptor function (Vorhees and Williams, 2006). The mice must use distal cues surrounding a pool of water (Figure 2-5a) in order to find a hidden platform after many days of training. Both the KO mice and their cnt littermates spent significantly more time in the target quadrant in the water maze than in other quadrants on the final test day (Day 14-Figure 2-5b). In this probe test, cnt mice crossed the platform area significantly more times than the parallel areas in other quadrants, while the KO mice did not show a significant increase in crossing at the platform area (Figure 2-5c).

DG GluN1 knockout mice show reduced levels of NMDA receptor subunits in DG only.

Using western blot techniques employing subfield micro-dissected bilateral hippocampi a decrease in GluN1 protein in the KO mouse compared with its littermate cnt was confirmed. This protein reduction was confined to DG and not present in CA_3 (nor the CA_1, data not shown) (Figure 2-6 and 2-7). In DG, reductions in related NMDA receptor subunits (GluN2A and GluN2B) were also found, (Figure 2-6), but not the AMPA receptor GluA1, nor two of its phosphorylation sites important for receptor trafficking (p-831 and p-845). There were no observable significant differences in protein concentrations shown between the cnt and the GluN1 KO mice CA_3 hippocampal tissue (Firgure 2-7). Other proteins examined include CREB, p-CREB, VCP, GAD-65 and 67 and found no differences (data not shown).

DG GluN1 knockout mice have decreased activity in the DG and increased activity in the CA_3 compared to their littermate controls. Performed by Dr. Chunfeng Tan.

To examine overall cellular activity in DG and CA_3, we examined c-Fos positive nuclei in hippocampal pyramidal neurons using immunohistochemistry. We counted c-Fos positive nuclei, a marker of cellular activation/neuronal depolarization (Cruz *et al.,* 2013; Bullitt, 1990; Dragunow and Faull, 1989) throughout the subfields along the anterior/posterior axis in the KO and cnt animals. We did not find a change in c-Fos containing granule cells in DG but we did find a significant increase in the

number of c-Fos positive pyramidal neurons in CA_3 in the KO compared with the cnt mouse, as well as an elevated number of c-Fos positive nuclei in pyramidal neurons in mouse KO CA_1, albeit quantitatively lower in CA_1 than in CA_3 (Figure 2-8). We examined the number of c-Fos positive nuclei in pyramidal neurons along the anterior-posterior axis in hippocampus and find a significant increase posteriorly located in ventral CA_3 and in CA_1. The human anterior HC corresponds to the dorsal HC in mice and the posterior human HC corresponds to the mouse ventral HC.

DG GluN1 knockout mice have decreased NMDA/AMPA receptor ratio in DG granular cells at 2 and 4 months, but CA_3 is not changed. Performed by Dr. Amir Segev.

Using whole-cell patch clamp recording of granule cells in freshly dissected hippocampal slices is a stringent assay for assessing synaptic transmission with high neuroanatomical resolution. Specifically, the NMDAR/AMPAR ratio (NAR) was measured in DG granular cells at two time points during developmental, 80-90 and >120 days of age. Both biophysical and pharmacological (Figure 2-9a) approaches show that NMDAR-mediated current in granule cells of the DG-GluN1 KO mouse decreases during the first few months of development and is totally eliminated by four months of age. These observations are consistent with the loss of GluN1 mRNA and protein in the DG granule cell. We find that DG granule cells in GluN1 DG-KO mice do not exhibit any NMDAR-mediated current (Figure 2-9a). The NMDA/AMPA ratio (NAR) decrease observed at 80 d is driven by 3/8 cells that were

not exhibiting any NMDAR-mediated current. Using a pharmacological approach, D-APV at 50 µM did not have any effect on evoked EPSC in DG-GluN1 KO and CNQX at 10 µM almost eliminated evoked EPSC. DG-GluN1 KO EPSC is mainly mediated by AMPAR. We find that DG granule cells in GluN1 DG-KO mice do not exhibit any NMDAR-mediated current.

Using whole-cell patch-clamp recordings and focusing within CA_3, we assessed whether excitatory glutamate transmission is augmented in CA_3, a finding that would support overall hippocampal hyperactivity in the KO mouse. Using both biophysical and pharmacological approaches, we find no change in NAR in DG-GluN1 KO mice when compared to cnt (data not shown); however, when both receptor-mediated currents were assessed separately, a significant increase in both AMPAR- and NMDA receptor-mediated EPSCs (Figure 2-9b) was unveiled (Segev *et al.,* submitted).

DG GluN1 knockout mice have an enhanced paired-pulse ratio and release probability. Performed by Dr. Amir Segev.

To determine whether this genetic manipulation altered the capability for granule cells to convey information, we measured the responses to paired-pulse stimulation, a standard paradigm to test for changes in glutamate release probability (p_r). We find that granule cells from DG-GluN1 KO exhibit an enhanced PPR at short inter-stimulus intervals (50 ms) (Figure 2-10a), indicating a decrease in p_r. PPR is also increased at 30 ms ISI (Figure 2-10b) (Segev *et al.,* submitted).

Discussion

In these experiments, we examined the characteristics of a mouse with a DG specific GluN1 KO as a model for maladaptive, metaplastic, neuro-modifications that are observed in human brain tissue from SZ. Whole-cell recording of granule cells, an approach that provides high neuronal specificity, show that NMDA receptor-mediated current is totally eliminated in recorded DG cells. Consistent with the abatement of the GluN1 subunit, the essential subunit of the NMDA receptor, we find that GluN2A and GluN2B are reduced in the DG as well. The NMDA receptor functions as a heteromeric complex with an essential GluN1 subunit (Kutsuwada *et al.,* 1992; Monyer *et al.,* 1992), it is therefore more than conceivable that the depletion of GluN1 attenuates NMDA receptor formation, and, consequently, is associated with a decreased efferent excitatory activity from DG. This genetic manipulation is also associated with enhanced AMPA receptor- and NMDA receptor-mediated currents in CA_3. This enhanced postsynaptic excitability at the mossy fibers- CA_3 synapses translated into increases pyramidal cell activity in CA_3 and CA_1 (data not shown) as shown by the increase in the number of nuclear protooncogene metabolic marker, c-Fos, positive pyramidal cell nuclei in the KO mouse and are associated with increased glutamatergic synaptic strength at the mossy fibers- CA_3 synapses. c-Fos is greatly influenced by past history of activation and in order for c-Fos to be induced the neuron must be strongly activated (ie bursting of DG granule

cells), so we can be assured that this immunohistological marker does indeed represent the increased activation we hypothesize to occur in the CA_3.

These characteristics parallel and extend the findings reported from human SZ tissue, suggesting DG granule cell activity reduction induces changes in CA_3 pyramidal cell activation in SZ. The speculative link between hippocampal hyperactivity [consistently reported in schizophrenic psychosis (Medoff *et al.,* 2001; Schobel *et al.,* 2013; Talati *et al.,* 2015; McHugo *et al.,* 2015)] and psychosis manifestations, is the plausible scenario that hippocampal hyperactivity causes mistakes of association, false memories and memories with psychotic content, accounting for positive symptoms in schizophrenia (Tamminga *et al.,* 2010) and behavioral changes, such as increased fear and impaired PPI, in the DG GluN1 mice. The cellular mechanisms through which GluN1 KO in the DG lead to enhanced CA_3/CA_1 activity is unclear, however, one might speculate that decreased granular cell activity triggers a homeostatic adaptation in CA_3 pyramidal cells that aim to normalize mossy fibers-CA_3 excitatory transmission (reviewed in Tamminga *et al.,* 2010). Interestingly, DG granular cells in DG-GluN1 KO mouse exhibit a decreased p_r. Whether this led to maladaptive CA_3 pyramidal neuron hyperexcitability, *i.e.*, a network-wide homeostatic adaptation that aims to normalize transmission is yet to be tested. However, these findings are reminiscent of what has been observed in SZ psychosis patients.

In order to demonstrate translational relevance mouse behaviors that are

abnormal in humans with psychosis (PPI, MWM) were assessed in the KO vs cnt mouse as well as behaviors known to index direct learning and memory performance in mice (FC and PA)(Wickelgren, 1962; Winocur, 1985; Anagnostaras *et al.,* 2001; Kim and Jung, 2006; Isaacson and Broussard *et al.,* 2016). All of the paradigms used behaviors with varying degrees of involvement of hippocampally-mediated processes. The behavioral characteristics established here for this DG-specific GluN1 KO mouse, previously shown to have reduced pattern separation performance (McHugh *et al.,* 2007), extend and focus the behavioral profile onto psychosis and CA_3 hyperfunction. The impaired PPI and MWM performance are both characteristics of mouse models of psychosis reflecting cognitive impairments also found in human SZ (Grillon *et al.,* 1992; Karper *et al.,* 1996; de Hoz *et al.,* 2003; Bast and Feldon, 2003; Ludewig *et al.,* 2002; Fajnerová *et al.,* 2014; Wong and Josselyn, 2016) . In addition the animals show an increase in fear conditioning and passive avoidance behavior, with locomotor activity remaining intact, both seemingly reflecting CA_3 neuronal hyperactivity/hyperassociation.

The literature already shows that CA_3-lesioned rats have reduced contextual and cued fear conditioning (Hunsaker and Kesner, 2008; Hunsaker *et al.*, 2009). Moreover, the CA_3-specific GluN1 KO mouse shows reduced freezing in contextual fear conditioning (McHugh *et al.,* 2007) and impaired rapid associative memory in a modified passive avoidance test (Cravens *et al.,* 2006).

Although less well tested, the increase in fear learning suggests that in critical areas of cognition, where learning is driven by fear and anxiety, a more persistent or concrete kind of memory is obtained and focused in SZ psychosis and may manifest itself as hallucinations and delusions with paranoia. These findings are consistent with the overall model of psychosis as a learning and memory disorder with deficient DG function and increased CA_3 associative function (Tamminga *et al.,* 2010; Tamminga, Southcott *et al.,* 2012; Hunsaker and Kesner, 2008; Hunsaker *et al.*, 2009).

Combined with previous reports that CA_3 is a critical region in the formation of associative memory, these results are consistent with increased/overly-increased CA_3-mediated associative memory function in the KO. Furthermore, mouse studies indicating that modulating mossy fiber pathway transmission compromises contextual fear memory (Ceccom *et al.,* 2013; Daumas *et al.,* 2004; Ceccom *et al.,* 2014; Yanagi, Southcott *et al.,* 2012) implicate abnormal fear memory with mossy fiber pathway pathology.

In consideration of the evidence that DG is a principal region in pattern separation function (Schmidt *et al.,* 2012; Kesner, 2007; Kesner, 2013) we interpret the lack of memory accuracy seen in MWM and the impaired pattern separation seen in the modified fear conditioning paradigm (freezing in both the trained and untrained context) as secondary to GluN1 depletion in DG in the KO mice.

These outcomes establish the association of reduced excitatory pathology in the DG and increased pyramidal cell activity in CA_3 as a potential explanation for overall hippocampal and CA_1 hyperactivity, which has become established in schizophrenic psychosis (Tregellas *et al.,* 2014). An argument for this being a causal association between reduced excitatory drive in DG and an increase in CA_3 pyramidal cell activity will be dependent on future studies, testing causality. Moreover, in order to show more direct relevance to human psychosis, the action of known antipsychotic drugs on these molecular, cellular and electrophysiological outcomes will become essential. This would include tests of first (i.e. Haldol) and second (i.e. clozapine) generation antipsychotics. Whether known risk factors for psychosis, like exogenous psychotogens (i.e. Phencyclidine or amphetamine) or risk genes (i.e. *DISC1* or *NRG1*) enhance these neural deficits will also be important to consider. If demonstrated, the availability of an animal preparation which would be informative for the behavior of a human neural microcircuit in hippocampus believed to be important in a brain disorder like SZ (Nestler *et al.,* 2010; Jones *et al.,* 2011) will be a significant advantage for studying psychosis in schizophrenia. These findings support the further testing of the DG-GluN1 KO as an animal preparation informative about psychosis in schizophrenia.

PPI of the acoustic startle response is used to measure sensorimotor gating mechanisms protecting neuronal information processing, in other words precognitive processing. It has been show many times over that PPI is reduced in SZ, and is the

most translatable and easily demonstrable task when it comes to animal models (Swerdlow *et al.,* 2006; reviewed in Swerdlow and Geyer, 1998). People with SZ may have reduced startle response due to constant stimulation. We also know that the HC is involved with PPI. Chemical stimulation of the ventral hippocampus reduces PPI. The same effect occurs with ventral or dorsal infusions of $GABA_A$ agonist, muscimol. Furthermore, this affect is also produced when a Na^{2+} channel blocker, TTX is applied to either of these regions. The decrease in PPI in people with SZ is thought to be a reflection of constant stimulation and the inability to distinguish salient stimuli. PPI may be mediated by HC projections to the amygdala or the bed nucleus of the stria terminalis which both have access the brain stem startle circuit. The decrease in activity induced either by muscimol, TTX, or our GluN1 decrease in the DG, may change the neuronal activity in the AMG, PFC or NucAC, which all have strong HC projections (Zhang *et al.,* 2002).

Perturbance in associative learning in drug-naïve first episode SZ was demonstrated using a within-subject learned irrelevance paradigm (Orosz *et al.,* 2008). A retardation of associative learning occurred in this experiment, if the conditioned stimulus and the unconditioned stimulus are pre-exposed in an unpaired manner prior to the establishment of the association and is not restored over time. In other words people with SZ demonstrate a deficit in learning associations. It is thought that this also demonstrates an inability to filter out irrelevant environmental clues or an inability to ignore inconsequential stimuli, as in the PPI paradigm. This

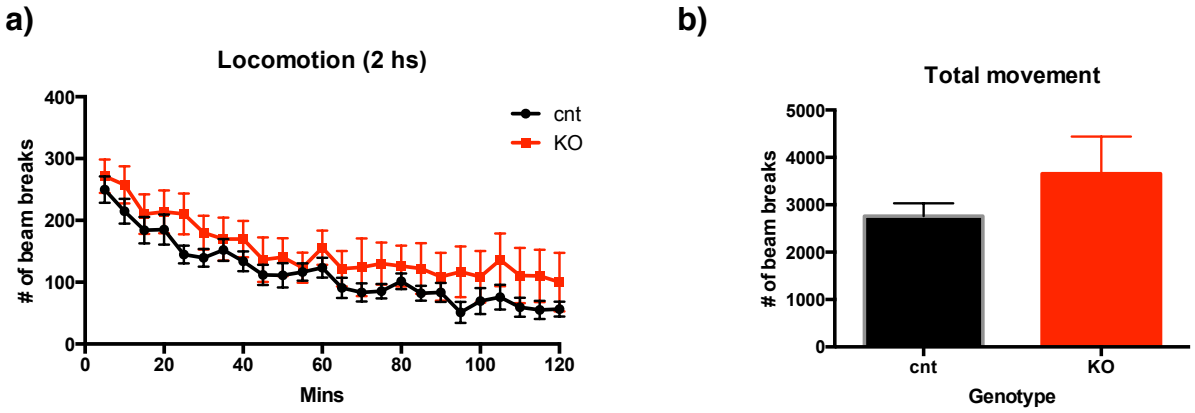
may explain the findings of the GluN1 KO mice in the FC paradigm both in our hands where these mice freeze more in both the contextual and cued test compared to their cnt littermates, and in the Tonegawa lab (McHugh *et al*, 2007) where they freeze to a similar level in similar contexts. Maybe the increase in activity in the CA_3 increases generalization or decreases association, and therefore the result in the mice displaying increased fear behavior (freezing) in both the same and a different context, as well as with and without a tone after training. FC is a memory task that involves the MTL that encompasses elements of the HC and the AMG. It is quite possible that the increased freezing behavior in the cued condition is a result of increased activity in the AMG, due to compensatory mechanisms (Bremner, 2006), such as in models of post-tramatic stress disorder (Bremmer *et al.,* 1995; Rouch *et al.,* 1998; Rouch *et al.,* 2006; Bremner, 2006; Bremmer *et al.,* 2008). This increase in activity in the AMG would also project back to the HC (Pikkarainen and Pitkänen, 2001; Felix-Ortiz *et al.,* 2013; Beyeler *et al.,* 2016) adding to the increase in activity in the CA_3. Indeed, we have observed increased c-Fos activation in AMG in the KO animals (data not shown). The generalized fear behavior displayed in the GluN1 KO mice could also be a demonstration of the dysfunctional threat perception in SZ (Henry *et al.,* 2010), or a parallel to paranoia often associated with SZ.

With a different task involving the HC, the MWM, we found decreases in accuracy to find the target platform. This can again be attributed to over generalization, or lack of association between the distal cues and the target. This

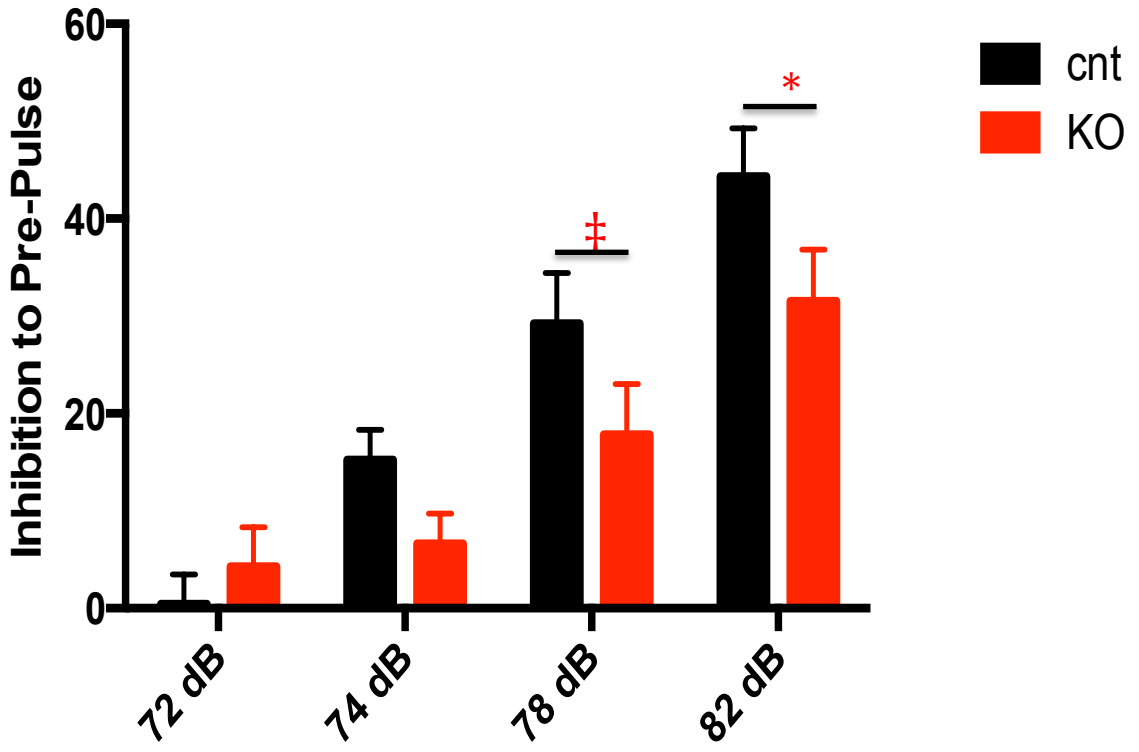
task is also more complicated and taxing as the learning occurs over many days, and involves physical exertion (swimming).

We observed behavioral changes that mimic behavioral attributes of SZ, so we next looked molecularly at the DG and CA_3. Using immunohistochemisty techniques we found significant increases of the immediate early gene c-Fos positive nuclei in the CA_3 of KO mice. This finding indicates that these c-Fos positive neurons were recently activated and the fact that they are increased more so in the KO mice denotes that the CA_3 has an increase in baseline neuronal activity in this subfield. A more physiologically relevant way to look at this is to use electrophysiology to discover electrical property changes and functions. By stimulating the granular cells of the DG and looking upstream at the effect on the CA_3 pyramidal neurons we showed that there is an increase in EPSCs for both the AMPA and NMDA receptors. This leads us to the conclusion that there is an increase of glutamate transmission at the mossy fiber to CA_3 synapses. Whether this is due to a presynaptic phenomenon (increased number of vesicles releasing or an increase in size of presynaptic vesicles) or a postsynaptic modulation or refinement (increased number of glutamate receptors or increased sensitivity) is yet to be determined. The lack of changes in postsynaptic proteins in the CA_3 (Figure 2-6) and the differences in PPR indicate to us that this is a presynaptic event and gives us some insight into the mechanism of hyperactivity in the CA_3.

Figures

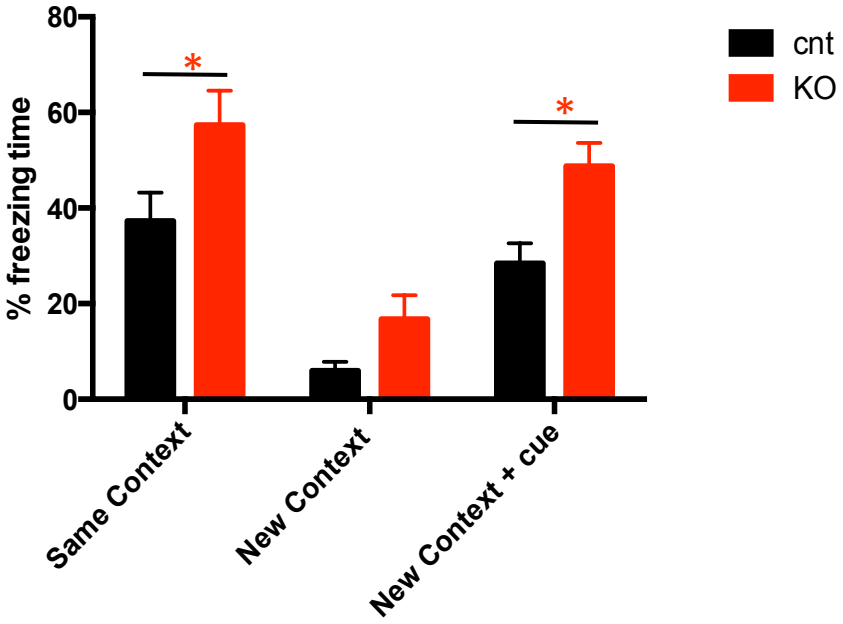


Figures 2-1 GluN1 KO mice display no differences in locomotor behavior. GluN1 KO mice and the cnt littermates were tested for differences in locomotion over 2 hrs. N=7-9. a) There is no interaction nor is there a significant difference in genotype when assessing locomotion over 2 hrs (two-way ANOVA genotype x time, Interaction: $F(23, 322) = 0.5589$, $p=0.9517$; Time: $F(23, 322) = 24.8$, $p<0.0001$; Genotype: $F(1, 14) = 1.41$, $p=0.2547$ b) There are no differences in total movement over 2 hours (t-test $t=0.64$, $df_{1,36}$, $p=0.53$).

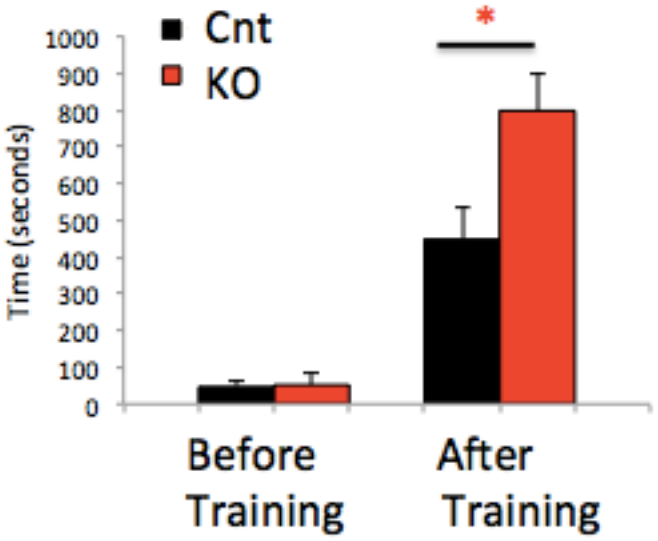


Figures 2-2 GluN1 KO mice display reduced sensory motor gating. Both GluN1 KO and the cnt littermates were tested for response to a startle. Two-way ANOVA (genotype x dB level): Interaction: F (3,108)=3.299, * p=0.0232; Decibel level: F (3, 108)=55.15, p<0.0001; Genotype: F (3,108) 2.127, p=0.1534. *post hoc* 78 dB *p=0.039, and a trend towards significance at 76 dB ‡ p=0.066

a)

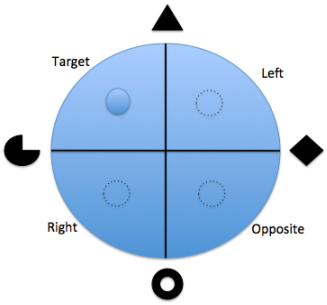

80
60
40
20
0
% freezing time
*
*
cnt
KO
Same Context
New Context
New Context + cue

b)

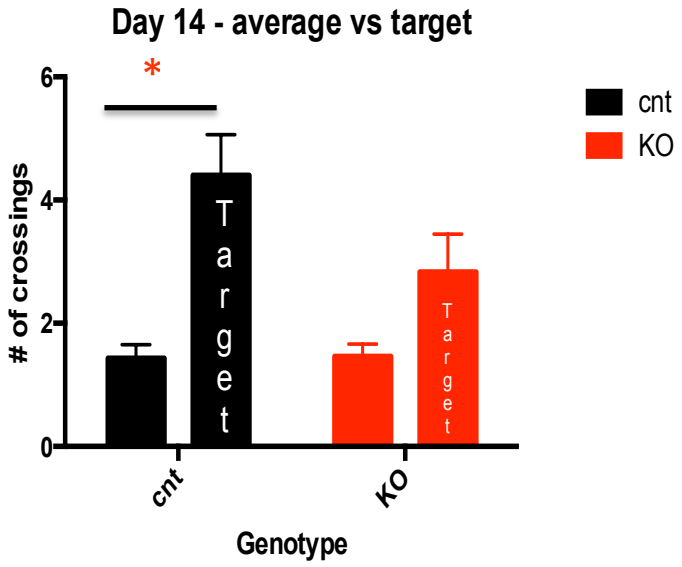

1000
900
800
700
600
500
400
300
200
100
0
Time (seconds)
Cnt
KO
*
Before Training
After Training

Figures 2-3 GluN1 display increased fear memory. GluN1 and the cnt littermates were tested for contextual memory retention as well as cued memory. a) Two-way ANOVA (genotype x situation): Interaction: F(2,28)=0.4838, p=0.6215, Situation: (2,28)=34.43, ****p<0.0001, Genotype: F(1,14)=20, *p=0.0005, [*post hoc* contextual test (*p=0.0160) and the cue test (*p=0.0147)], but no significant difference in fear memory in the new context (p=0.1792). After training in the PA paradigm b) the GluN1 KO mice also displayed an increased latency to enter the dark compartment where they had previously been shocked. N=19/group t-test before training p=0.44, after training *p=0.01.

a)



b)



c)

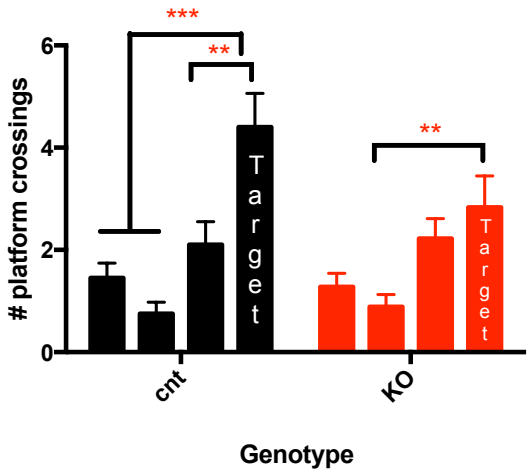


Figure 2-4 GluN1 KO mice display a deficit in precise spatial memory.
a) A cartoon schematic of the MWM pool with surrounding distal cues. N= 18-20 b) After 13 days of training the control mice were able to remember where the platform had been, whereas the KO mice did not. The average of the other quadrant crossings and number of crossings of the target, t-test cnt: t=3.554 df=19 **p=0.0021, KO: t=1.808 df=17, p=0.0883 c) The exact number of crossings of the target to parallel places in the other quadrants. One-way ANOVA cnt: F(3,76)=7.342 ****p<0.0001, *post hoc* comparison: Target vs right p=****0.0001, Target vs Opposite ****0.0001, Target vs left **p=0.034; KO: F(3,68)=**0.0027, *post hoc* comparison: Target vs right p=0.0566, Target vs opposite **p=0.0032, Target vs left p=0.9595.

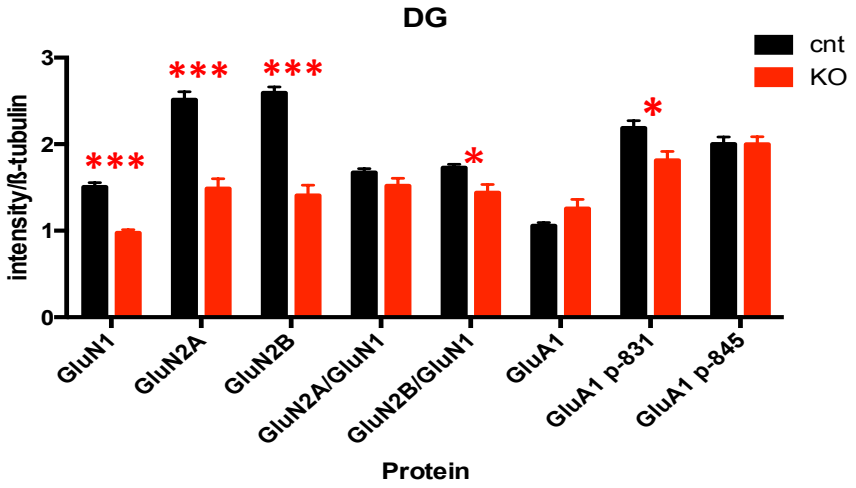


Figure 2-5 GluN1 mice have decreased GluN1, GluN2B containing NMDA receptors in the DG and phosphoGluA1-831, but not GluA1, phosphoGluA1-845 nor GluN2A containing receptors. Western blots were performed utilizing micro-dissected DG tissue from cnt mice and GluN1 KOs. Densitometric analysis of GluN1, GluN2B, GluN2A, GluA1 and 2 specific phosphorylated sites on GluA1 that are important for receptor trafficking (normalized to β-Tubulin). N= 7. Unpaired t-test, GluN1: ($t=0.06$, $df_{1,12}$,) ***$p<0.0001$; GluN2A: ($t=0.15$, $df_{1,12}$,) *** $p<0.0001$; GluN2B: ($t=0.14$, $df_{1,12}$,) *** $p<0.0001$; GluN2A/GluN1: ($t=0.10$, $df_{1,12}$,) $p=0.1401$; GluN2B/GluN1: ($t=0.10$, $df_{1,12}$,) *$p=0.0162$; GluA1: ($t=0.11$, $df_{1,12}$,) $p=0.1087$; GluA1 p-831:($t=0.13$, $df_{1,12}$,) *$p=0.0154$; GluA1 p-485: ($t=0.12$, $df_{1,12}$,) $p=0>0.9975$.

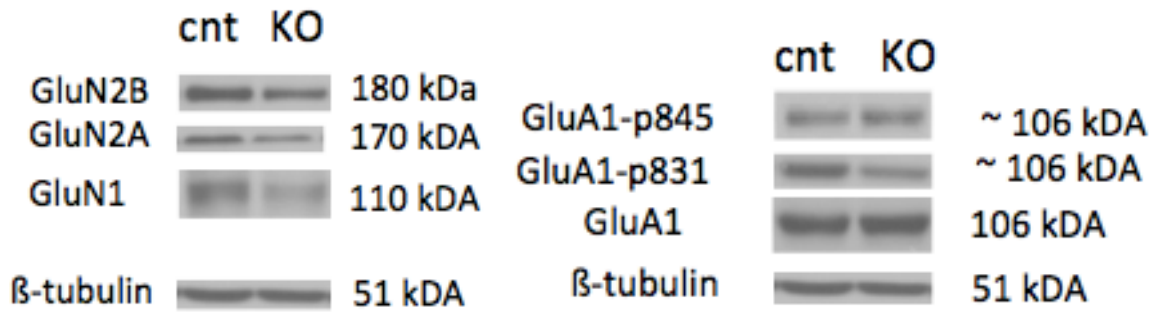
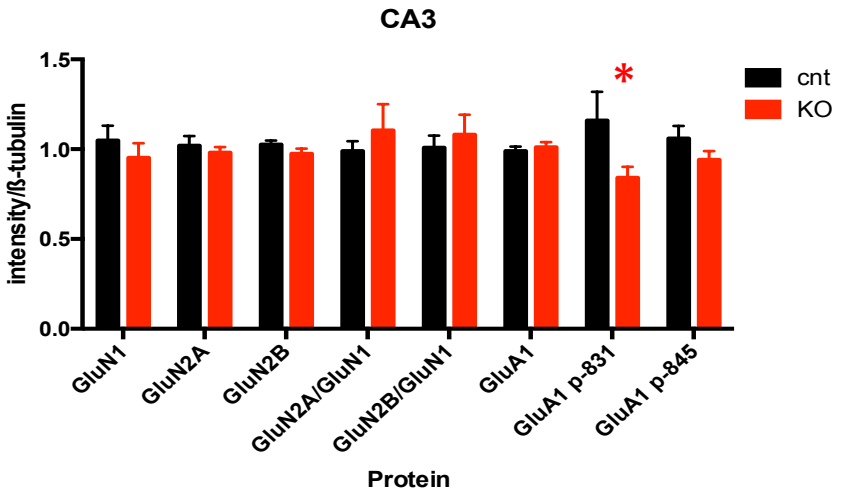


Figure 2-6 Representative Western Blot Figures from DG. Cnt and KO mice GluN proteins and GluA1 proteins. ß-tubulin is used as the loading control.



Figures 2-7 GluN1 mice have no changes in post synaptic proteins in the CA_3. Western blots were performed utilizing micro-dissected CA_3 tissue from cnt mice and GluN1 KOs. We probed for GluN1, GluN2B, GluN2A, GluA1, 2 specific phosphorylated sites on GluA1 and PSD-95 since we had seen an increase in this subregion in SZ. Densitometric analysis of these proteins resulted in no significant differences. Unpaired t-test, GluN1: ($t=0.84$, $df_{1,12}$,) $p=0.4175$,; GluN2A: ($t=0.62$, $df_{1,12}$,) $p=0.5461$; GluN2B: ($t=1.41$, $df_{1,12}$,) $p=0.1813$; GluN2A/GluN1: ($t=0.73$, $df_{1,12}$,) $p= 0.4773$; GluN2B/GluN1: ($t=0.55$, $df_{1,12}$,) $p=0.5908$; GluA1: ($t=0.54$, $df_{1,12}$,) $p=0.6018$; GluA1 p-831: ($t=1.86$, $df_{1,12}$,) $p=0.0877$; GluA1 p-845: ($t=1.38$, $df_{1,12}$,) $p=0.1935$; PSD-95: ($t=0.16$, $df_{1,12}$,) $p=0.8743$.

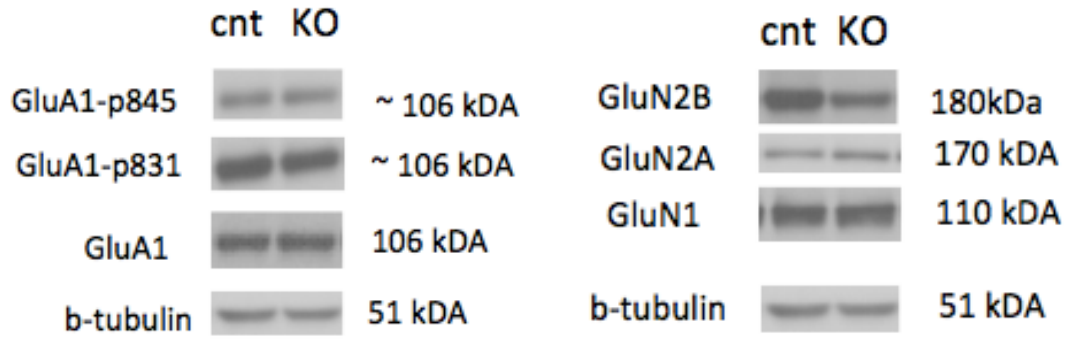
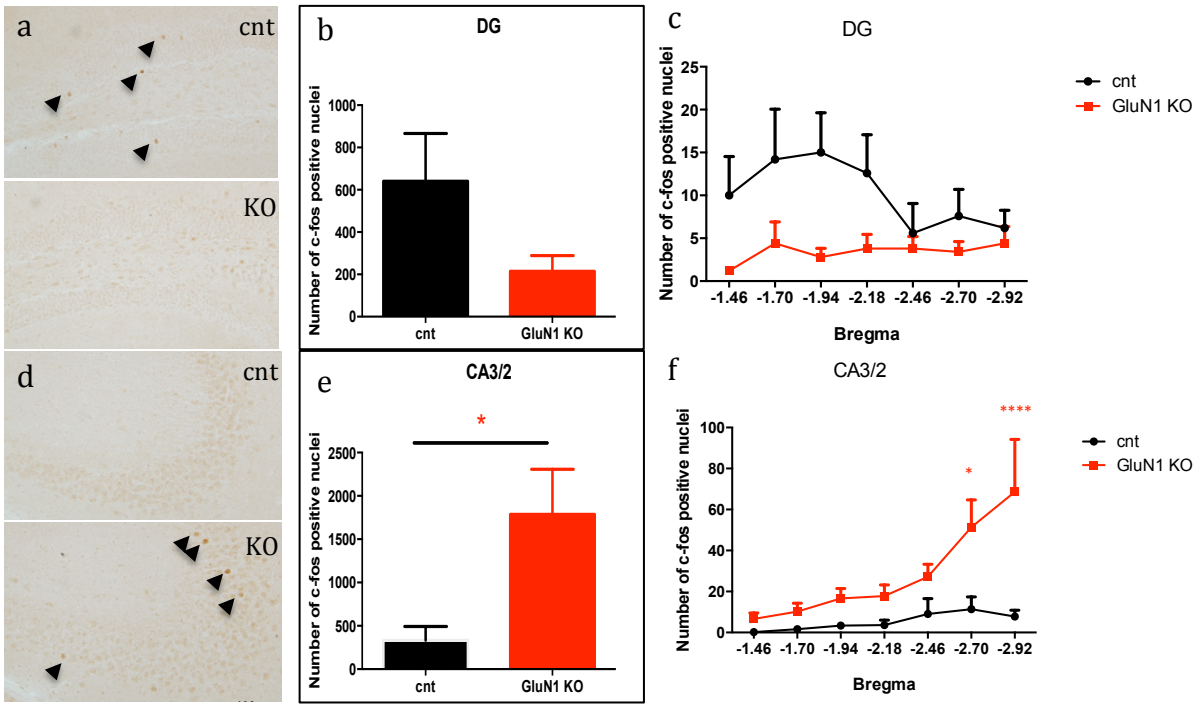
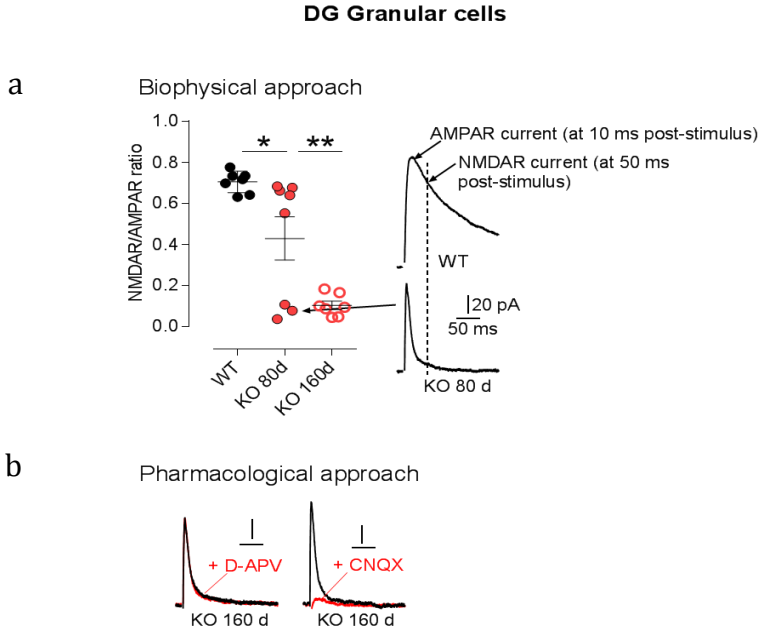


Figure 2-8. Western Blot Figures from CA3. Cnt and KO mice

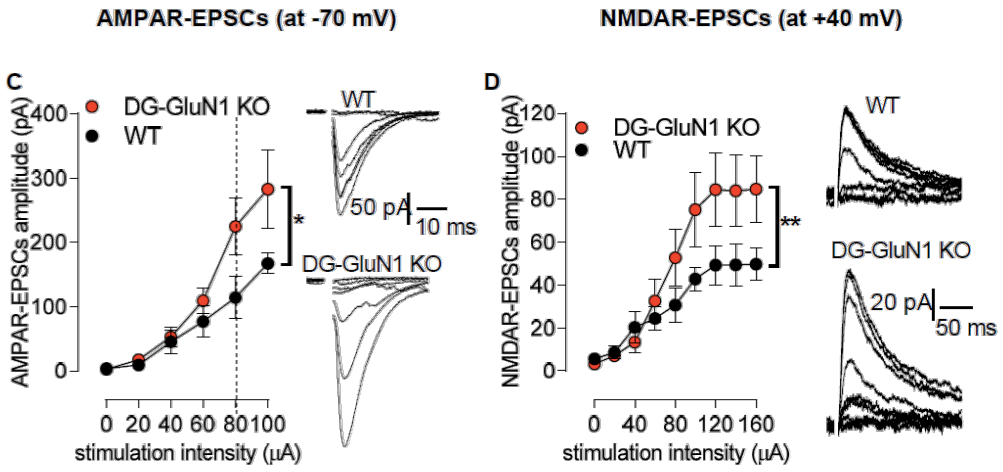


Figures 2-9 GluN1 mice have decreased levels of c-Fos activated neurons on the DG and increased c-Fos activated neurons in the CA3. The number of c-Fos positive neuronal nuclei is decreased in hippocampal DG (a-c). N=5 a) Representative images of c-Fos immunohistochemistry illustrate decreased c-Fos positive nuclei in DG (upper image) in the GluN1 KO mouse tissue. b) The total

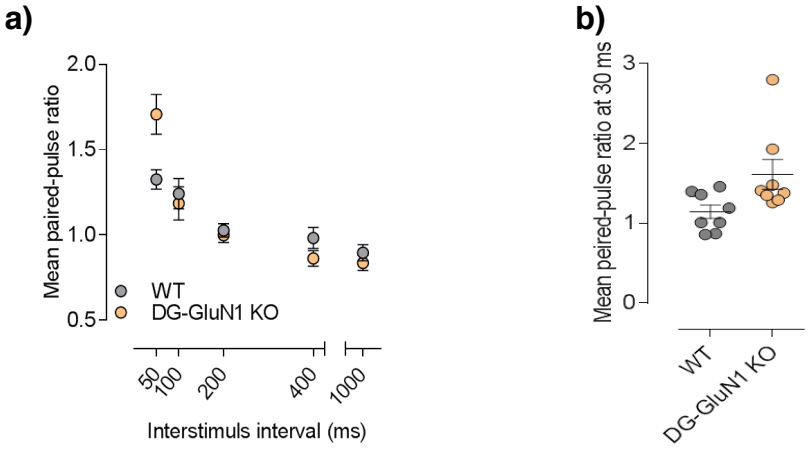
number of c-Fos positive nuclei was not significantly decreased in DG (t-test: t(8), p=0.1099) c) The number of c-Fos positive nuclei along the dorsal-to-ventral hippocampus distance from bregma. Two-way ANOVA statistical analyses show no significant differences with genotype x rostral-caudal axis: Interaction: F(6, 56)=0.8588, p=0.5308; Genotype: F(6, 56)=0.748, p=0.6135; Hippocampal rostral-caudal axis: F(1, 56)=16.07, ***p=0.0002. The number of c-Fos positive neuronal nuclei is increased in hippocampal CA_3 (d-f). d) Representative images of c-Fos immunohistochemistry illustrate increased c-Fos-positive nuclei in CA_3 (lower image) in the GluN1 KO mouse. e) The total number of c-Fos positive nuclei was significantly increased in CA_3 (t-test: t(8)=2.665, *p=0.02). The numbers of c-Fos positive nuclei over the rostral (dorsal) –caudal (ventral) axis (-1.46 to -2.92 mm bregma) were compared between cnt and KO. Two-way ANOVA statistical analyses show significant interaction between the factors, genotype, and hippocampal rostral-caudal axis interaction in CA3; Interaction: F(6, 56)=2.634, * p=0.0254; Genotype: F(6, 56)=4.635, *** p=0.0007; Rostral-caudal axis: F(1, 56)=24.53, **** p<0.0001 f) A significant increase in the number of c-Fos positive nuclei was detected in the caudal (ventral) CA_3 *post hoc* *p=0.01 at -2.70mm from bregma, ****p<0.0001 at -2.92mm from bregma).



Mossy Fiber to CA_3



Figures 2-10 In DG GluN1 KO mice have reduced NMDAR/AMPAR ratio at 80d and 160 day due to the elimination of NMDA receptor EPSC. a) NMDAR/AMPAR ratio (NAR) in cnt (N = 7 cells, 5 mice) and DG-GluN1 KO (N= 8 cells, 6 mice). AMPAR- and NMDAR-EPSC amplitudes are extracted from the dual component obtained at +40 mV, at 10 and 50 ms post-stimulus respectively. Biophysical analysis of the dual component at +40 mV showed that NMDAR-mediated current in DG granular cells (dash line) is absent in DG-GluN1 KO (4-5 months-old). Note that the NAR decrease observed at 80-90 days is driven by 3/8 cells that were not exhibiting any NMDAR-mediated current (measured at 50 ms, dash line). Hash marks indicate group means ± SEM. (One-way ANOVA: F (2,19) = 18.41: p**** < 0.0001; *post hoc* test: *p < 0.05; **p < 0.01). b) Pharmacological approach: D-APV at 50 µM did not have any effect on evoked EPSC in DG-GluN1 KO. CNQX at 10 µM almost eliminated evoked EPSC. AMPAR- and NMDAR-mediated transmission at MF-CA3 synapses is increased. AMPAR-mediated transmission was assessed at -70 mV. c)AMPAR- mediated transmission at MF-CA_3 synapses are increased (assessed at -70 mV) d) NMDAR-mediated transmission at MF-CA_3 synapses are increased (elicited at +40 mV). *Right panels* for c and d: Example of AMPAR- (Calibration: 50 pA, 10 ms) and NMDAR-EPSCs traces (Calibration: 20 pA, 50 ms) from a cnt and a DG-GluN1 KO neuron over the stimulus range 0, 20, 40, 60, 80, and 100 µA. cnt, n = 10 cells, 4 mice; DG-GluN1 KO, n = 16 cells, 4 mice. Two-way ANOVA statistical analyses showed significant genotype and genotype x stimulation interaction effects for both AMPAR- (Genotype effect: F(1, 24) = 4.312, *p = 0.048, and interaction effect: F(4, 96) = 3.850, **p = 0.006) and NMDAR-EPSCs (Genotype effect: F(1, 17) = 5.336, *p = 0.033, and interaction effect: F(8,136) = 6.160, ***p < 0.0001). Data are represented as means ± SEM.



Figures 2-11 Paired Pulse Ratio and Probability of release in DG GluN1 KO mice. The responses to paired-pulse stimulation was measured. a) Granule cells from DG-GluN1 KO exhibit an enhanced paired-pulse ratio (PPR) at short interstimulus intervals (50 ms) (Two-way ANOVA PPR X interstimulus interval: Interaction: $F(4,120) = 6.328$; ***$p = 0.0001$; *post hoc* test at 30 ms: ***$p < 0.01$). b) PPR is also increased at 30 ms interstimulus interval (t-test: $t(14)=2.294$, *$p < 0.05$). Error bars represent SEM.

CHAPTER THREE

Chronic phencyclidine administration alters, but does not enhance, behavioral deficits of the DG-GluN1 KO mouse psychosis preparation

Several pharmacological drugs have psychotomimetic actions, some more potent than others. These are drugs that cause psychotic symptoms in humans, the most reliable ones often induce SZ-like symptoms and tend to exacerbate the psychotic manifestations in people with SZ; PCP appears the most genuine psychotomimetic in this regard. An understanding of the mechanism(s) of these psychosis-inducing actions might contribute to our fundamental understanding of the illness. These drugs have been administered to animals both for disease pathophysiology studies, but even more importantly, in order to model pharmacological antagonist approaches as treatments (Yanagi, Southcott *et al.,* 2012). When PCP was administered to the DG GluN1 KO mouse over the course of one month, we found that PCP reduces sensory motor gating at lower decibels. However, this combination of psychotomimetic and KO of GluN1 in the DG did not alter other behaviors that were tested, nor the post-synaptic proteins that were probed in the CA_3.

Introduction

Phencyclidine (PCP) is a reversible, noncompetitive antagonist that has high affinity for the open state of the voltage-gated NMDA ionophore. On its own, PCP

acts to decrease glutamate signaling by blocking the NMDA receptor channel (Monaghan and Jane, 2009). This NMDA receptor antagonist was originally developed as a safe anesthetic agent; however, its use was discontinued because of serious psychotomimetic side effects, despite its other safety characteristics. Inevitably, PCP causes mental status changes in normal humans, which are SZ-like and resemble the cognitive, positive and negative symptoms of the illness (Javitt and Zukin, 1991; Lahti *et al.,* 1995; Krystal *et al.,* 2002; Moghaddam and Krystal, 2012). The acute use of PCP leads to hallucinations, delusions and mania. Users may feel estranged from their surroundings, or dissociation from reality. Auditory hallucinations, image distortion, severe mood disorders, and amnesia may also occur. It has also been shown to cause anxiety in some and in others, paranoia and violent hostility (Luisada, 1978), indistinguishable from SZ.

Based on the observations that PCP generates SZ-like psychosis in humans, the PCP-exposed animal has been one of the most widely used models for studying SZ and has been repeatedly examined. Acute PCP-treated animals show an increase in locomotor activity and stereotyped movements, a decrease in social interaction, alterations in memory and spatial function, and impairments in PPI, mimicking this same change seen in SZ (Perrin *et al.,* 2010).

The psychotomimetic behavioral manifestations of PCP were known before PCP was identified as an NMDA receptor antagonist. Therefore, when PCP was identified as an NMDA receptor antagonist (Aanosen and Wilcox, 1986; Snell and

Johnson, 1986; Coan and Collingridge, 1987), this observation implicated glutamate system dysfunction in SZ; then, the glutamate hypofunction hypothesis of SZ was articulated and has been pursued, thereafter, generating evidence of glutamate dysfunction in brain tissue from individuals with SZ (Tamminga, 1998; Coyle, 2006). This sequence of discovery has suggested both NMDA insufficiency in SZ and a therapeutic action of NMDA receptor enhancement for psychosis and cognition. Therefore, PCP has been pursued as a model of SZ, both for hypothesis-driven reasons and phenomenological ones. The idea that glutamate dysfunction might be regional and not necessarily present at all glutamatergic synapses has been more recently understood (Tamminga *et al.,* 2010). However, the clear observations of glutamate hypofunction in schizophrenia itself have made this modeling approach a direction with strong construct validity. These lines of evidence suggest the NMDA antagonists model—administration of PCP, ketamine, or MK801 (all direct antagonists of the NMDAR and congeners of PCP)—as a solidly supported construct to translate to animals with behavioral assessment (Yanagi, Southcott *et al.,* 2012).

Although acute injections of PCP have been used in research to mimic behaviors and to understand the pathophysiology of SZ we know that SZ is a chronic condition and that it develops and manifests symptoms over time. It is for this reason that long term, chronic or subchronic PCP administration mimicking a glutamate transmission dysfunction, may be a superior dosing paradigm compared with acute administration (Balla *et al.,* 2001; Morris *et al.,* 2005; Cochran *et al.,* 2003; Jentsch

and Roth, 1999; Guidali *et al.,* 2011). Disinhibitory activation on pyramidal cells can be produced by PCP's inhibition of tonic, fast spiking, GABA inputs which contain open NMDARs (Greene, 2001). This would have a faster and greater effect than the slow spiking, pyramidal cells which respond mostly to AMPA agonists. This change in activation can have long lasting effects (Suzuki et al, 2002) and may lead to plasticty changes as we have hypothesised occurs in the HC in SZ. As well, the chronic administration of PCP is important because this induces changes over time, which would be necessary to resemble the developmental aspects of schizophrenia, and to induce changes that are stable over time (Jones *et al.,* 2011).

With our DG-GluN1 mouse preparation we found behaviors that are reflective of SZ, but failed to find the relevant protein changes we were pursuing in the CA_3. With the solid evidence that PCP produces pertinent characteristic changes both in humans and mice, as well as exacerbating symptoms in SZ, we hypothesized that this pharmacological agent would do the same with our DG-GluN1 KO mouse. We speculated that PCP would drive the HC system further, amplifying the CA_3 hyperactivity and producing the activity marker changes we see in post-mortem tissue.

Materials and Methods

Breeding and Genotyping

All experiments in this study were carried out in accordance with institutional guidelines, and were approved by The Institutional Animal Care and Use Committee at UTSW Medical Center. We utilized DG-GluN1 KO mice and their cnt littermates that were previously generated in our lab (see Chapter 2). In brief, POMC-Cre mice were crossed with floxed-GluN1 mice following the method previously established (McHugh TJ *et al.,* 2007). Mice were housed at UTSW in a vivarium accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were group housed in temperature-controlled rooms on a constant 12 h light/dark cycle. Animals had food and water available *ad libitum*. Experiments were conducted during the light cycle. Animals between the ages of 16 and 18 wks were used for all experiments, as this is when the GluN1 protein is maximally depleted (no mRNA) in DG (McHugh *et al.,* 2007).

PCP administration

Phencyclidine (PCP) was dissolved in a 0.9% saline solution to make a 0.3mg/ml solution and kept at 4^0C. Intraperitoneal (i.p.) injections of 3mg/kg PCP solution or 0.9% saline solution were administered every other day, 3 days a week. On days when behavior testing occurred, the injections were administered at least 2

hrs after the behavioral testing in order to ensure that drug effects represented the chronic administration of treatment, and not an acute drug effect which typically occurs within one hour post injection (Sturgeon *et al.,* 1979; Matsuzaki and Dowling, 1985; Bay and Patel, 2007).

Behavioral Experiments

Each mouse was tested in all behavior paradigms. The testing was conducted in a fixed order from least to most stressful paradigm (Figure 3-1).

Elevated Plus Maze

A plus shaped apparatus with four arms (two open and two enclosed with open tops) was used to test anxiety. This test relies upon rodents' proclivity toward dark, enclosed spaces and aversion of heights and open spaces. The maze was elevated 99 cm from the floor. Time spent in the open arms was compared among genotype and treatment. Mice were placed in the intersection of the four arms and their behavior is recorded for 5 min. during a single testing session (Walf and Frye, 2007). In a dim room these sessions were recorded with Ethovision software (Noldus Information Technology, Leesburg VA) and a digital video camera mounted overhead.

Locomotor activity

See Chapter One.

PPI

See Chapter One.

FC

Mice were trained and tested for fear conditioning in boxes equipped with a metal grid floor connected to a scrambled shock generator (Med Associates, St. Albans, VT, USA). For training, mice were individually placed in the chamber to freely explore for 2 min; then each received 3 presentations of conditioned stimulus (80 dB white noise for 30 sec) and the last 1 s of each tone was paired with the unconditioned stimulus (foot shock, 0.5 mA) with an inter-tone interval of 60 seconds. To test for contextual fear conditioning 24 hours after training, mice were placed into the same chamber for 5 min without a tone played and without receiving any foot shocks. 48 hours after training, mice were placed in the training chamber with modified environmental stimuli (eg. vanilla scent, white plastic floor over the grid bars, and a black triangle roof). Mice explored for 3min, followed by 3 min tone presentations to test for cued fear conditioning. Med Associates software automatically scored freezing behavior (every 0.5 sec) during all conditions.

Molecular Experiments

Immunoblotting

See Chapter Two

GluN2A, GluN2B, GluN1, GAD-67, and PSD-95 were used, and only for CA3.

β-Tubulin (Thermoscientific, IL) immunoreactivity was used as a loading control.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software (San Diego, CA, USA). Significance was set as $p<0.05$ for all experiments. Outcomes for locomotion were tested using a two-way ANOVA (genotype x time) and an unpaired t-test. Two-way ANOVAs were also used to assess differences in EPM (genotype x treatment), PPI (decibel x genotype), FC (situation x genotype) and western blots (genotype x treatment). An un-corrected Fisher least significant difference *post hoc* test was performed when significant differences were found. We hypothesized *a priori* that the best comparison for this model would be between the cnt+saline and KO+PCP, therefore we preformed *post hoc* tests even when significance wasn't apparent from ANOVAs and reported significant findings. Full statistical outcomes are included with the figure legends.

Results

DG GluN1 knockout with and without chronic PCP mice do not display anxiety

EPM is a test designed to use mice's innate fear of open and bright spaces to test for anxiety like phenotypes. In the EPM there is no significant differences of time spent in the open arm or in the dark arm (Figure 3-2). This indicates that the GluN1 KO mice do not have an anxiety phenotype, which would have been evident by them

spending more time in the dark arms than their cnt littermates. We show also, that chronic PCP is not anxiogenic.

DG GluN1 knockout mice with and without PCP do not display hyperlocomotion

The number of beam breaks in 5 minute bins collected for 2 hours. Over the 2 hr time period all mice decreased their movement at about the same rate. All of the mice started at a similar level of movement. There was no significant difference in locomotion between the Cnt and GluN1 KO animals. When looking at individual time points over 2 hrs we only saw a difference at 90 minutes, and the difference was between cnt+saline and GluN1 KO + PCP (Figure 3-3a). The difference in locomotion at this time did not affect the overall difference in motion (Figure 3-3b) based on the evaluation of the accumulative number of beam breaks, which represents total movement, over the period of 2 hrs.

Chronic PCP treatment reduces sensory motor gating at low decibels

Inhibition of startle response to a pre-pulse was observed at 72, 74, 78 and 82 dB (Figure 3-4). There was a significant difference of treatment and a trend for a difference of genotype, but an interaction at 72 dB was not observed. The difference was specifically seen between cnt+saline vs KO+PCP. At 74 dB there was a strong trend toward a difference of treatment, but there was neither difference for genotype nor an interaction. At 78 dB the only difference that was found was between

cnt+saline vs KO+PCP. As well, there was no interaction seen at 82 dB, nor an effect of genotype, nor treatment.

DG GluN1 knockout mice with and without chronic PCP do not display fear behavior

Mice were tested for both contextual and cued fear conditioning. We found no significant difference in freezing in any paradigm (Figure 3-5).

DG GluN1 knockout mice with chronic PCP do not change the protein profile of the CA3 in hippocampus

Hippocampal subfield dissections were performed and run on an SDS-PAGE gel in order to look for differences in protein concentrations. We tested 5 proteins: 3 NMDA receptor subunits (GluN1, GluN2A, and GluN2B), 1 postsynaptic marker (PSD-95) and 1 interneuronal marker (GAD-67). Proteins that were measured were normalized to ß-tubulin (Figure 3-6). No effect of PCP on GluN1 KO and cnt mice was demonstrated with any of the proteins we measured.

Discussion

Disruption of the glutamate system is a major detriment to the human brain. In schizophrenia the disruptions in the glutamate system lead to multiple symptoms of the disease. In particular disruption of the glutamate system in the hippocampus can lead to multiple behaviors that resemble psychosis. We have previously shown that by eliminating the obligate subunit of the NMDA receptor, GluN1, in the DG a

phenotype of reduced PPI, increased freezing in fear conditioning and increased avoidance of a safe environment, and reduced spatial memory will emerge (Segev *et al.,* submitted).

PCP is the most widely used drug employed to mimic schizophrenia in rodents and we chose to use chronic PCP with the hypothesis that this drug would have a high probability of interaction with the DG dysfunction/deficit and would therefore enhance the DG GluN1 deficits we saw previously, and galvanize the feed forward excitation in the CA3, therefore eliciting changes in the CA3 that would be reminiscent of SZ post mortem tissue findings.

Interestingly we found a very minimal divergence of behavior observed with chronic administration of PCP. The PPI paradigm was the only test for psychosis-relatable behavior where we saw significant difference between groups. The differences solicited at the lower decibel levels, 72 and 74, however were due to an effect of the PCP, and not a significant augmentation of the DG GluN1 phenotype.

There were no differences seen in the EPM. We tested this to determine whether our previous finding of high freezing in FC and PA was due to an anxiety phenotype.

Neither the cnt mice with PCP, nor the KO mice with and without PCP showed any significant differences in the contextual and cued fear conditioning paradigm. Previously we have found that the DG-GluN1 KO mice have an increased fear response (Segev *et al.,* submitted). There are several possible explanations for

this discrepancy, a finding that could reflect the increase in CA3 neuronal activity in the KO mice (see Charpter 2). First, the KO mice may not show a difference in freezing because we used a different paradigm (5 tones paired with shock previously, and three tones paired with shock here). They also may not display a significance difference in freezing due to an increase in the freezing of our cnt animals. Our control animals froze at an unusually high rate. Normally, wild type animals freeze about 40% in the same context after training, 0% in the new context, and 20% with the cue (personal communication, Dr. Birnbaum). Our cnt animals have a higher baseline-freezing-rate and now it is difficult to see a robust change in freezing rate of the KO from this. The effect, or lack there of, may also be due to the stress of handling and injections (whether saline or PCP) over the one month chronic treatment period (Ben-Menachem-Zidon *et al.,* 2008; Yun *et al.,* 2010). Stress has been demonstrated to regulate NMDA receptor function (Tse *et al.,* 2012). Lastly, the lack of differences may also come from desensitization (Gadek-Michalska and Bugajski, 2003). To elucidate whether this change in behavior is due to stress, desensitization, or a change in paradigm, FC could be repeated but with different variables. First we would use mice that are not handled parallel with those that are and if this would tell us if it was the paradigm change from 5 to 3 shocks that produced the differences in freezing. Next we could look at stress markers such as corticosterone levels to elucidate the effect of handling and injections over time, and whether this is influencing the differences in behavioral outcomes. McHugh *et al.,*

(2007) reported that KO mice performed normally in the same context in which they were trained, using a one shock and tone pairing. When comparing the DG-GluN1 KO mice and the littermates, at a cellular level, differences were seen in place cells (cells that fire in certain locations and are thought to provide a cognitive map) in the CA_3 of the HC. We may choose to do this, because it is difficult to assess these place cells, cellular level changes or lack thereof, in a behavioral paradigm. We may choose to do this, because it is difficult to assess these place cells, cellular level changes or lack thereof, in a behavioral paradigm.

As well, the protein data for CA3 showed no significant differences in GAD-67, GluN1, GluN2A, GluN2B or PSD-95 when the cnt mice with and without PCP were compared to the KO mice with and without PCP. Perhaps the PCP binds to PV interneurons as suggested by Greene (2001) allowing for disinhibition, and therefore increased DG activity through the AMPA receptors, looking like amelioration of the DG lesion, and hyperactivity of the CA_3 as seen in SZ. Because of the lack of the DG GluN1 receptor subunits we are eliminating any direct effect of PCP. As well, we know that PCP is not a selective inhibitor or NMDA receptors alone. Therefore it is not above speculation that PCP produces a direct and/or indirect multisystem neurochemical perturbation (Kapur and Seeman, 2002). Jodo (2013) suggests that PCP's action may be through the ventral hippocampus to the medial prefrontal cortex (mPFC). Therefore if we render the hippocampus unable to function properly due to the GluN1 knockout in the DG of the hippocampus, the signal may not be able

to be sent to the PFC to influence the behavioural changes. Studies of the actions of PCP also suggest that NMDA dysfunction may lead to secondary dopaminergic dysregulation in striatal and prefrontal brain regions (Javitt, 2007 and Poels *et al.,* 2014). This “dirty drug” can increase DA efflux, as well as increases in extracellular concentrations of other major neurotransmistters (i.e. acetylcholine and seretonin) in several brain regions, which can be blocked by administration of antipsychotics (DA receptor antagonists). Also, Benneyworth *et al.,* (2010) found that mice with altered NMDA receptor function had discordant behavioral effects with PCP; specifically, PPI disruption by PCP was unaffected in mice with either serine racemase underexpression and in mice with reduced glycine modulatory site activity. In other words we may have off target actions, PCPs actions may be blocked by a lack of NMDA receptors in the DG, or we may not be able to further perturb an already disrupted system.

Figures and Tables

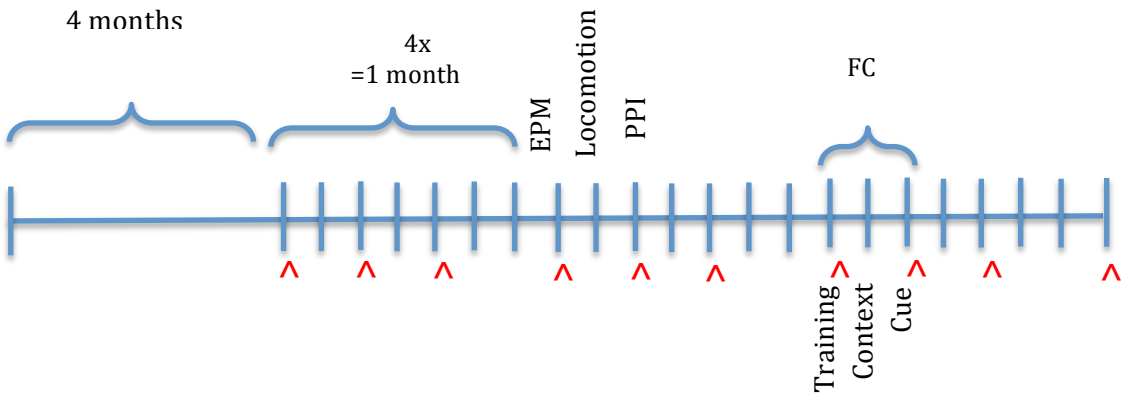


Figure 3-1 Timeline of experiments. Red carrots represent injections (either saline or PCP). The first vertical line represents birth. The last vertical line represents the day we performed dissections. The time between two vertical lines represent a day (24hrs).

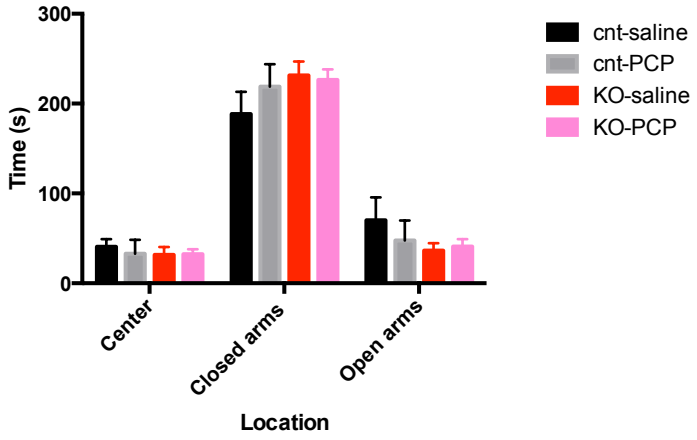


Figure 3-2 GluN1 KO mice do not display anxiety in EPM
Time spent in the center, the closed arms and the open arms. Two-way ANOVA genotype x treatment: Open arm [Interaction: $F(1,48)=1.096$, $p=0.3005$; Genotype: $F(1,48)=1.445$, $p=0.2352$; Treatment: $F(1,48)=1.097$, $p=0.3002$], closed arm [Interaction: $F(1,48=0.778$, $p=0.3823$; Genotype $F(1,48)=1.568$, $p=0.2166$; Treatment: $F(1,48)=0.404$, $p=0.5282$], and center [Interaction: $F(1,48)=0.1895$, $p=0.6653$; Genotype: $F(1,48)=0.2358$, $p=0.6295$; Treatment: $F(1,48)=0.1267$, $p=0.7234$).

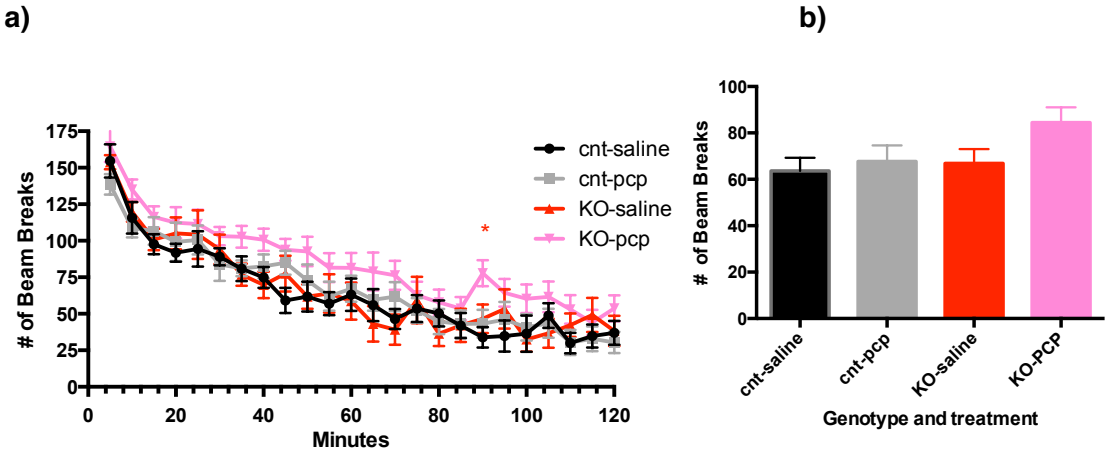


Figure 3-3 GluN1 KO mice display no differences in locomotor behavior. GluN1 KO mice and the cnt littermates were tested for differences in locomotion, both with saline and with PCP. a) Over the 2 hr time period all mice decreased their movement at a similar rate. All of the mice started at a similar level of movement. Only the cnt mice given saline and the KO mice given PCP differed in movement, and only at the 90 min time point, *p=0.0170. The filled black circle represents cnt mice given saline, the filled grey square represents cnt mice given PCP injections, the red filled triangle represents the GluN1 KO mice given saline, and the upside down filled pink triangle represents the GluN1 KO mice given PCP injections. There are no significant differences in locomotion over 2 hrs [Two-way ANOVA Genotype+treatment x time: Interaction: $F(69, 1104) = 0.7555$, $p=0.9307$; Time: $F(23, 1104)=62.12$, $p<0.0001$; Genotype+treatment: $F(3,48)=2.605$, $p=0.0626$] b) The accumulative number of beam breaks over 2 hrs [Two-way ANOVA genotype x treatment: Interaction: $F(1,48)=1.355$, $p=0.2502$; Genotype: $F(1,48)=3.082$, $p=0.0.0855$; Treatment $F(1,48)=2.831$, $p= 0.0990$]

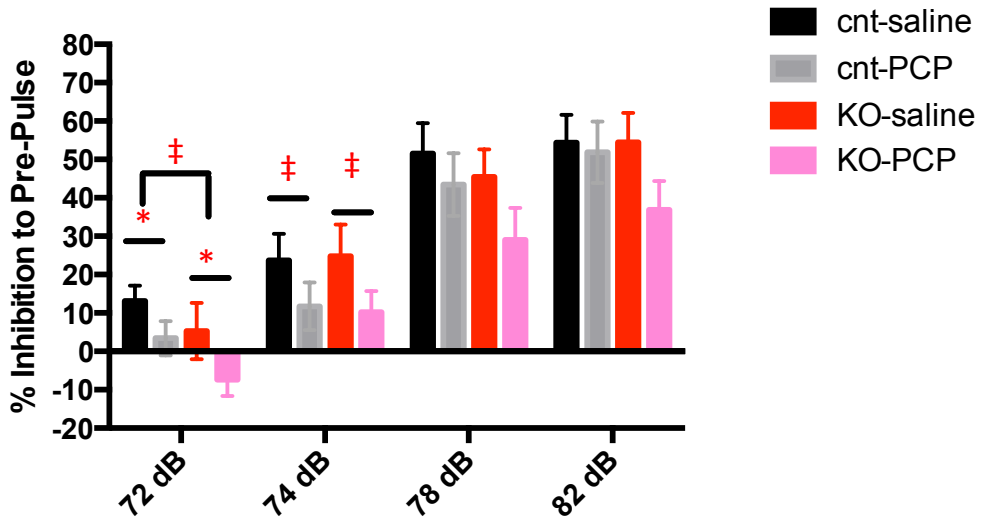


Figure 3-4 PPI. There was a significant difference of treatment (Two-way ANOVA: F(1,48)=5.147,*p=0.0278), and a trend for a difference of genotype (F(1,48)=3.559, †p=0.0653), but an interaction (F(1,48)=0.096, p=0.7581) at 72 dB was not observed. The difference was specifically seen between cnt-saline vs KO-PCP (*post hoc*, *p=0.018). At 74 dB there was a strong trend toward a difference of treatment (F(1,48)=3.936, †p=0.0530), but there was neither a difference for genotype (F(1,48)=0.0012, p=0.9722) nor an interaction (F(1,48)=0.0416, p=0.8392). At 78 dB the only difference that was found was between wt+saline vs KO+PCP (*post hoc*, *p=0.0411, Interaction: F=(1,48)=0.267, p=0.6077; Genotype: F(1,48)=1.626, p=0.2085; Treatment: F(1,48)=2.3, p=0.1359). As well, there was no interaction seen at 82 dB, (F(1,48)=0.9707, p=0.3294), nor an effect of genotype (F(1,48)=0.944, p=0.3361), nor of treatment (F(1,48)=1.714, p=0.1968).

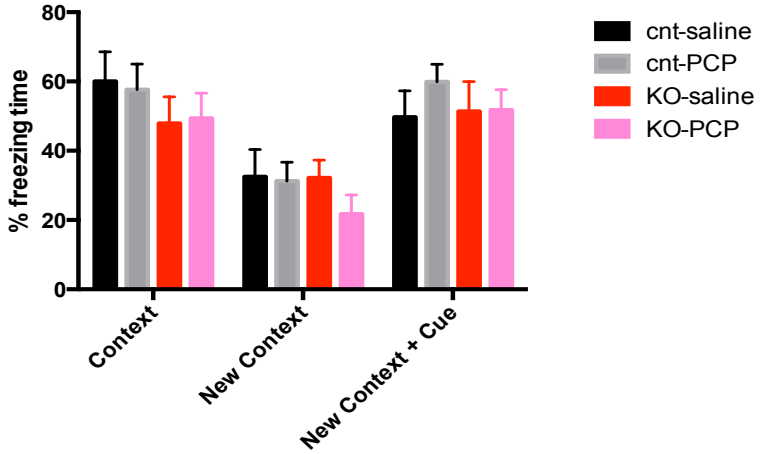


Figure 3-5 FC. No significant difference in freezing, neither due to genotype nor treatment (Two-way ANOVA: interaction F(1,43)=0.0613, p=0.8055, genotype F(1,48)=1.692. F(1,48)=0.0036, p=0.1995, treatment p=0.9524). When the mice were placed in a new context, we also found no significant differences in freezing behavior (interaction F(1,48)=0.5222, p=0.4735, genotype F(1,48)=0.5920, p=0.0.4455, treatment F(1,48)=0.8320, p=0.3663, cue). As well, when the tone that was paired with the shock during training was played in this new context, the mice all froze at similar levels, and there was no significant difference found in freezing rate (interaction F(1,48)=0.5136, p=0.4771, genotype F(1,48)=0.2234, p=0.4455, treatment F(1,48)=0.5905, p=0.3663).

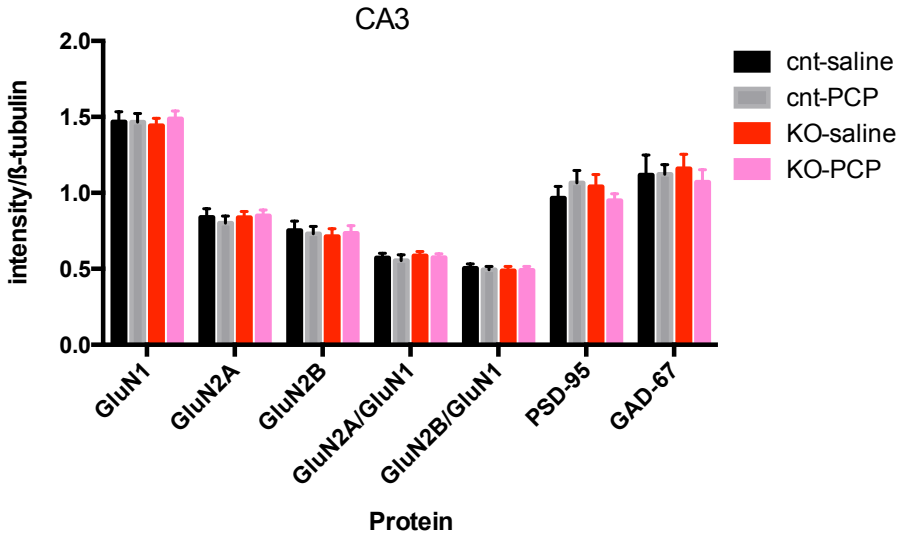


Figure 3-6 Immunoblotting of CA_3 post synaptic proteins.
A two-way testing effect of PCP on GluN1 KO and cnt mice showed no significant difference with any of the proteins we measured in CA_3.

Table 1.

	GluN1	GluN2A	GluN2B	PSD-95	GAD-67
Interaction	F(1,47) =0.18, p = 0.6720	F(1,47) =0.29, p = 0.5902	F(1,47) =0.15, p = 0.6975	F(1,47) =1.82, p = 0.1837	F(1,47) = 2.1, p = 0.1532
Genotype	F(1,47) = 0.0004, p = 0.9842	F(1,47) = 0.27, p = 0.6087	F(1,47) =0.12, p = 0.7398	F(1,47) =0.09, p = 0.7643	F(1,47) =0.02, p = 0.8889
Treatment	F(1,47) = 0.14, p = 0.7099	F(1,47) = 0.08, p = 0.7722	F(1,47) =0.0002, p = 0.9897	F(1,47) =0.004, p = 0.9485	F(1,47) =0.03, p = 0.8582

Table 2.

	GluN2A/GluN1	GluN2B/GluN1
Interaction	F(1,47) = 0.02, p = 0.8877	F(1,47) = 0.05, p = 0.8217
Genotype	F(1,47) = 0.30, p = 0.5844	F(1,47) = 0.14, p = 0.7125
Treatment	F(1,47) = 0.24, p = 0.6265	F(1,47) = 0.02, p = 0.8953

CHAPTER FOUR

Schizophrenia risk gene *DISC1* does not enhance the psychosis mouse preparation DG-GluN1 KO

The Disrupted-in-Schizophrenia-1 (*DISC1*) gene was discovered in a large Scottish family, by its segregation with the diagnoses of SZ, depression, and mania. This gene is disrupted in a t(1;11)(q42.1;q14.3) translocation. Alternate transcriptional splice variants, encoding different isoforms, have also been characterized. Since its discovery in the 1970s it has also been found in a Finnish, a Chinese Han, and a European American population (Callicot *et al.,* 2005).

The DISC1 protein is predicted to contain multiple blocks of coiled-coil motifs and two leucine zippers at the C-terminal domains, domains that may mediate critical protein–protein interactions (Taylor *et al.,* 2003; Callicot *et al.,* 2005).). The N-terminal protein contains a globular domain and two putative nuclear localization signals. Disruption of the protein-coding gene on chromosome 1 is believed to lead to the C-terminal truncation of the *DISC1* gene and is predicted to result in a dominant negative form of its translated protein throughout the brain and renders those with this translocation or single nucleotide polymorphisms (SNPs) in this gene vulnerable to several psychiatric disorders.

Introduction

Endogenous DISC1 is preferentially expressed in the pyramidal neurons of the cortex and HC (Hikida et al., 2007) and is present in the brain throughout development (Jaaro-Peled H, 2009). When properly translated this protein can be found in the cytoplasm, the mitochondria and the nucleus of the neuron. Mitochondria are the primary site of DISC1 expression, but it has also been found to locate to the centrosome, axons, and synapses. DISC1's role is highly diverse and its functions seem to depend on its cellular location. DISC1 in mitochondria have a role in oxidative phosphorylation, calcium homeostasis, and apoptosis, which may be relevant for the neuronal mechanisms believed to be involved in SZ. In addition, DISC1 is associated with cytoskeleton protein ie NUDEL) and microtubule function, and with the membrane trafficking of receptors (Millar *et al.,* 2003). The DISC1 protein regulates morphogenesis, maturation, migration, and synaptic integration of neurons. It also regulates the proliferation of progenitor cells, specifically the rate of neurogenesis at the point where progenitor cells mature into neurons. Additionally, DISC1 interacts with membrane associated and signal transduction proteins (Callicott*, et al.,* 2005). For example, DISC1 participates in neurite outgrowth when it interacts with its binding partner NUDEL. However, there is an isoform that results in DISC1 truncation at the open reading frame and prevents this interaction with NUDEL. DISC1 (as well as interactions with ATF4 and 5) overall levels are not

decreased, but this shorter version's location is highly nuclear (Sawamura, *et al.,* 2005). Also, there are several single nucleotide polymorphisms (SNPs) identified and these variations, particularly Ser704Cys, have been shown to be important for the structure and therefore function of the HC, even in those without schizophrenia; normal controls with Ser/Ser have decreased HC grey matter volume (Callicott*, et al.,* 2005).

Many studies have shown correlations between DISC1 and quantitative traits in psychiatric disorders, including SZ, including abnormalities in HC structure and functions (Johnstone *et al.,* 2011; Opmeer *et al.,* 2014). Seven different transgenic mouse strains of the DISC1 gene have been characterized (Jones *et al.,* 2011). These mice exhibit enlarged lateral ventricles and reduced cortical thickness and brain volume, mimicking some characteristics of SZ. Some mutant mice have reduced parvalbumin in the PFC and HC. Other mutants display reductions in hippocampal dendritic complexity, structure, and density. Some DISC1 mice have PPI deficits that are attenuated with antipsychotics (haloperidol and clozapine). Other behavioral abnormalities seen in some mutants are hyperactivity, reduced sociability, working memory, and executive function impairments, all which can be seen in the human illness. Talbot *et al.* (2004) has suggested that an abnormality in the DISC1 gene could negatively impact cognition and working memory processes, possibly through altering the PFC and HC molecular environment during development. DISC1 polymorphisms are associated with reduced hippocampal grey

matter volume and the N-acetylaspartic acid signal and abnormal engagement of the HC during several cognitive tasks. The mechanisms may therefore involve HC development and ensuing function (Harrison, 2004). While the use of DISC1 mice has proven useful as an animal model of mental illness to identify a range of potential cellular mechanisms related to several psychiatric disorders, such as depression and SZ, the use of DISC1 mutants has not contributed to an identification of affected systems and proteins which define a psychosis mechanism.

The 93.6 kDa protein is moderately conserved across species. In 2002, Ma, *et al.,* cloned the mouse ortholog of this SZ risk gene, facilitating the study of DISC1's role in the brain and how its malfunction participates in mental illness. *In situ* hybridization displayed an expression pattern in the HC, with highest levels being found in the dentate gyrus (DG) and this is one of the reasons we have become interested in this particular genetic risk gene. Genetic models with DISC1 have diverse phenotypes, many of which resemble characteristics of human SZ (ie. decreased PPI, enlarged lateral and 3rd ventricles, reduced PV staining in PFC, etc) (Johnstone *et al.,* 2011). However, it is known that not everyone who carries this translocation develops SZ/psychosis, or even major mental illness. Therefore genetic deficiency may need to be expressed in the context of another risk factor in order to manifest mental illness/psychosis. As stated before, we found relevant behavioral changes in our DG-GluN1 KO mouse preparation for psychosis, but fell short of producing protein changes in the CA3 that are reminiscent of those reported

in post mortem SZ tissue. Ergo, we combined the DG-GluN1 mouse preparation with the mouse the expresses the human translocation *DISC1* (DISC1^{TG}) (Hikida *et al.,* 20007) and hypothesize that this genetic burden will affect the vulnerable HC system further, amplifying the CA_3 hyperactivity and producing the activity marker changes we see in post-mortem tissue and intensify the behavioral phenotypes seen in either of these SZ mouse preparations alone. By assessing sensory motor gating and hippocampally mediate tasks we will test whether the DISC1 translocation enhances hippocampal pathology in the DG-GluN1 KO mouse and assess how this translates into the clinical manifestations of the disorder.

Materials and Methods

Mice breeding and genotyping

In order to generate the DG granule cell layer specific-GluN1 knockout mice we crossed POMC-Cre mice with floxed GluN1 mice (see chapter 2) using a technique previously described by McHugh *et al.* (2007). We next crossed these DG specific GluN1 KO mice with DISC1^{TG} mice that were kindly given to us from the Sawa lab at John Hopkins University.

The genotyping of Cre detection was performed as in the published paper McHugh *et al.* (2007), and the protocol from the Sawa lab was used for genotyping DISC1^{TG} (Appendix A and B). The offspring were genotyped for both the POMC-Cre

and DISC1^{TG} via PCR amplification followed by separation and visualization on an agarose gel (1.5%). All male mice were used in behavior experiments. The groups that were compared were cnt (no DISC1^{TG} and no Cre detected), KO (Cre positive, DISC1^{TG} negative), Disc 1^{TG} (DISC1^{TG} positive, but Cre negative), and double mutant DISC1^{TG}GluN1 KO (both Cre positive and DISC1^{TG} positive).

We used REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich) to extract and amplify DNA from a small snippet of each mouse's tail. For amplification we mixed 10 μL REDExtract –N-Amp PCR reaction mix, 1 μL forward oligonucleotide (supplemental Table 1) and 1 μL reverse oligoneucleotide (supplemental Table 1) 6 μL water and 2 μL DNA extract.

Behavioral Experiments

Each mouse was tested in all behavior paradigms. The testing was conducted in a fixed order from least to most stressful paradigm (Figure 4-1). These experiments were always evaluated with all four groups, then cnt and the double mutant were pulled and tested separately against each other since we hypothesize this to be the preparation for psychosis.

EPM

See Chapter Three.

Locomotor activity

See Chapter Two.

PPI

See Chapter Two.

MWM

FC

See Chapter Three.

Molecular Experiments

See Chapter Two. All subfields were collected, but only CA_3 protein levels were assessed. For this experiment only GluN2B, GluN1, GAD-67 and PSD-95 were used and β-Tubulin was used as a loading control.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software (San Diego, CA, USA). Significance was set as $p<0.05$ for all experiments. Outcomes for locomotion were tested using a two-way ANOVA (genotype x time) and a one-way ANOVA comparing the results from the different genotypes. One-way ANOVAs were also used to assess differences in EPM, average movement over 2 hrs, PPI, and FC. We used a two-way ANOVA to assess differences in western blots. Tukey's multiple comparison *post hoc* test was performed when significant differences were found. One-way ANOVA was also used to assess differences in MWM, and Dunnette's multiple comparison *post hoc* test was used so that we could compare the means to the target mean. We hypothesized *a priori* that the best comparison for

this model would be between the cnt and the double mutant (DISC1^{TG} x GluN1 KO), therefore we preformed *post hoc* tests even when significance wasn't apparent from ANOVAs. Full statistical outcomes are included with the figure legends.

Results

DISC1^{TG}GluN1 knockout mice do not display anxiety

EPM is a test designed to use mice's innate fear of open and bright spaces to test for anxiety like phenotypes. In this behavioral paradigm DISC1^{TG}GluN1 mice do not display significant differences of time spent in the open arm or in the dark arm compared to their littermates (Figure 4-2).

DISC1^{TG}GluN1 knockout mice do not display hyperlocomotion

The number of beam breaks in 5 minute bins collected for 2 hours. Over the 2 hr time period all mice decreased their movement at about the same rate. All of the mice started at a similar level of movement. There was no significant difference in locomotion between any of the 4 genotypes we tested (Figure 4-3a). Overall difference in motion based on the evaluation of the accumulative number of beam breaks, which represents total movement, over the period of 2 hrs was not significantly different (Figure 4-3b).

DISC1^{TG}GluN1 knockout mice do not show sensory motor gating deficits.

Inhibition of startle response to a pre-pulse was observed at 72, 74, 78 and 82 dB (Figure 4-4). There were no significant differences among or between the 4 different genotypes that were tested at any pre-pulse decibel level. All groups had similar reactivity.

DISC1^{TG}GluN1 knockout display a deficit in visual spatial memory

Using distal cues surrounding a pool of water in order to find a hidden platform after many days of training is known as the Morris water maze. This paradigm is used to assess spatial memory and relies on the hippocampus and NMDA receptor function (Vorhees and Williams, 2006). On test day, when the platform was removed, cnt and DISC1^{TG} crossed the platform area significantly more times than the parallel areas in other quadrants; while the DISC1^{TG}GluN1 KO and GluN1 KO mice did not show a significant increase in crossing at the platform area (Figure 4-5).

DISC1^{TG}GluN1 knockout mice display a deficit in contextual fear memory

DISC1^{TG}GluN1 KO mice and their littermates were tested for contextual memory retention as well as cued memory. DISC1^{TG}GluN1 KO mice freeze significantly less in the contextual test, but showed no differences in freezing in the new context nor with the cue presented (Figure 4-6).

DISC1^{TG}GluN1 knockout mice do not change the protein profile of the CA3 in hippocampus

Hippocampal subfield dissections were performed and run on an SDS-PAGE gel in order to look for differences in protein concentrations. We tested 4 proteins: 2 NMDA receptor subunits (GluN1, and GluN2B), 1 postsynaptic marker (PSD-95) and 1 interneuronal marker (GAD-67). Proteins that were measured were normalized to ß-tubulin (Figure 4-7). There was no effect of DISC1^{TG} on GluN1 KO and cnt mice demonstrated with any of the proteins we measured.

Discussion

A significant impact of this study is that it took a promising genetic lead that we hypothesized would have a high probability of interaction and provided insight into its function in the subfields of the hippocampus. Although *DISC1* has statistical relevance to SZ, its psychiatric vulnerability has encouraged its non-selectivity to be overlooked. This study emphasizes the pleiotropy of genetic models and demonstrates the variety of phenotypes that are expressed when this protein is truncated.

A genetic risk factor for SZ, DISC1 translocation, has been revealed to be important in hippocampal development, structure and function (Austin *et al.* 2004; Callicot *et al.,* 2005; Pletnikov *et al.,* 2008*;* Brandon *et al.,* 2009). It is for these reasons that we decided to combine this genetic risk model with our GluN1 knockout psychosis mouse preparation. In this study we compared littermates; mice that had GluN1 knockout out, the human DISC1 transgene, the combination of the two, and neither (cnt). We initiated this study by observing locomotor behavior as performed

previously. We found no differences in locomotion over two hours. With this information we can say with confidence that the outcome of behavioral differences we found were not affected by hyper or hypo-locomotion. Although we found no evidence of an anxiety phenotype with the GluN1 cohorts given saline or PCP (see chapter 3), we chose to evaluate the behavior of the mice in the elevated plus maze. There were no significant differences in anxiety levels among the 4 genotypes in this task. Next we looked at PPI and found no significant differences at any of the decibel levels tested. We found diminished PPI in the GluN1 KO mouse (Figure 2-2) and diminished PPI at low decibels due to chronic PCP administration (Figure 3-4). The DISC1 transgenic mouse was tested (Hikida *et al.,* 2007) and only had a reduction of PPI at 74 dB, so it was not surprising to not find a difference between this mouse and their control littermates. It is possible that the $DISC1^{TG}$ x GluN1 KO mouse also did not show a significant reduction in PPI due to a disruption of the whole hippocampus, which was show to have no effect on PPI (Zhang *et al.,* 2002). It is curious though why we did not see a reduction in PPI with the GluN1 KO alone compared to their littermates.

We next assessed fear conditioning memory. We found a significant decrease in context specific memory in the $DISC1^{TG}$ x GluN1 KO mouse. Because we found no difference between the other genotypes, this would indicate that the burden of the DG specific GluN1 KO in the $DISC1^{TG}$ mouse severely affects contextual memory, but not $DISC1^{TG}$ alone, nor the DG specific KO itself. This is important, as it shows that a combination of factors (proteomic and genetic) is

needed to elicit a phenotypic change in a SZ mouse model, and may be so in humans. There were no significant differences in behavior in the tone fear conditioning test for amygdala based cue memory.

We then moved to a spatial memory task and asked if we could observe any differences in learning or memory in the Morris Water Maze task. In this hippocampally mediated task the double mutant mice did not show a significant difference in platform crossings in all areas of the pool, indicating that these mice were unable to remember where the target was based on the surrounding cues that they were trained with. However, this difference was not altered any more so than the GluN1 knockout mice. Time to platform did not significantly differ between genotype over the training days (Supplemental Fig 3). This lack of difference indicates that the different genotypes learn the Morris water maze task at the same rate, demonstrates that the deficit that is seen is in memory. This indicates that GluN1 is necessary in the DG in order to mediate spatial recall.

An explanation for the lack of reduction in PPI in the GluN1 KO mice could be simply that the Cre-recombinase failed to reduce the levels of GluN1 mRNA in the DG of these mice. However, this is unlikely since we did see a combinatorial effect in the elimination of contextual fear behavior and the retention of the spatial memory deficit in the MWM.

We emphasize the GluN1 KO mouse as a preparation to mimic the hyperactivity of the $CA_{3,}$ but a preparation that may require an additional insult to truly represent psychosis tissue changes. Therefore with this mouse preparation we

next looked at post-synaptic protein changes there in CA_3. Here we compared protein levels of GluN1, GluN2B, PSD-95 and GAD-67. When we dissected out the CA_3 region of the hippocampus and ran the tissue out on a SDS-PAGE gel we found an increase in the scaffolding protein PSD-95 in the double knockout when we did a two-way ANOVA with Cre+ and DISC1 as the 2 factors, when GluN1 is knocked out of the DG in the $DISC1^{TG}$ mouse. This increase in PSD-95 in the CA_3 is one of the signature protein changes seen in humans that we are attempting to replicate in our mouse preparation.

The diversity of the findings in these studies only emphasizes the complexity and heterogeneity of schizophrenia and its manifestations.

Figures and Tables

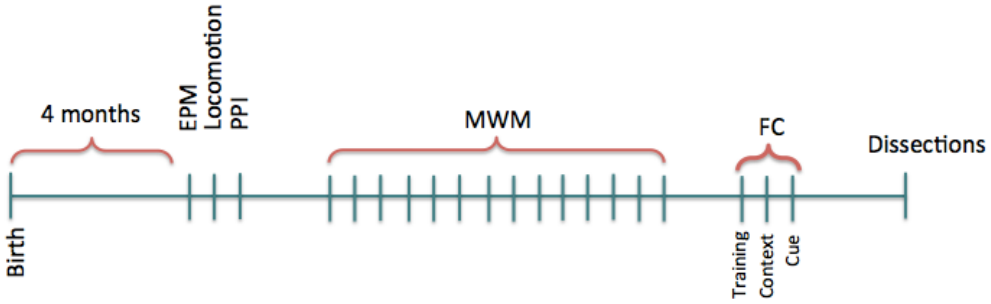


Figure 4-1 Timeline of experiments. The first vertical line represents birth. 4 months occurred before experiments began. The time between two vertical lines represent a day (24hrs). The last vertical line represents the day we bilaterally dissected HC for western blots.

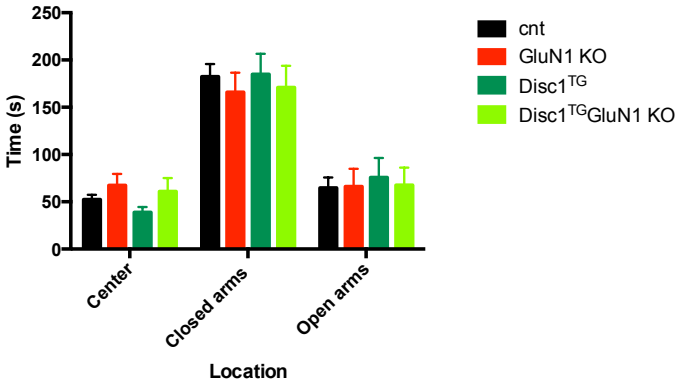


Figure 4-2 DISC1^{TG}GluN1 KO mice do not display anxiety behavior. There were no differences found between genotypes for time spent in open arm (one-way ANOVA F(3,62)=0.0824, p=0.9694), the closed (one-way ANOVA F(3,62)=0.2118, p=0.8879), nor the center (one-way F(3,62)=1.608, p=0.1752).

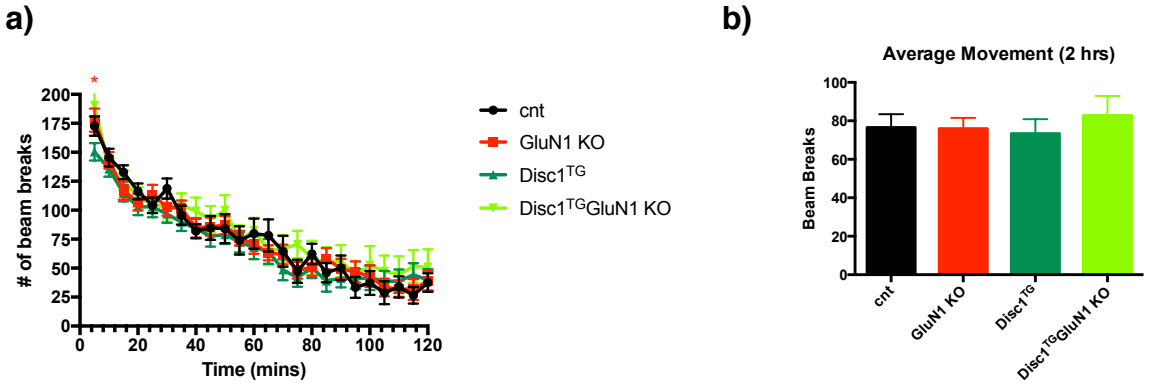


Figure 4-3 DISC1^{TG}GluN1 KO mice display no differences in locomotor behavior. Over the 2 hr time period all four groups of mice decreased their movement at about the same rate. a) The filled black circle represents the cnt littermates, the filled pink square represents GluN1 KO mice, the dark green filled triangle represents the DISC1^{TG} mice, and the upside down light green triangle represents the DISC1^{TG}GluN1 KO mice. There are some differences in locomotion. Two-way ANOVA, time x genotype: Interaction: F(69, 1488)=0.4446, p>0.9999; Time: F(23,1488)=54.62, ***p<0.0001; Genotype: F(3, 1488)=5.865, ***p=0.0006, *post hoc* test showed a significant difference at 5 mins between DISC1^{TG} and DISC1^{TG}x KO, *p=0.0200. b) The average cumulative number of beam breaks over 120 mins (2hrs) (One-way ANOVA: F(3,62)=0.2633, p=0.8515).

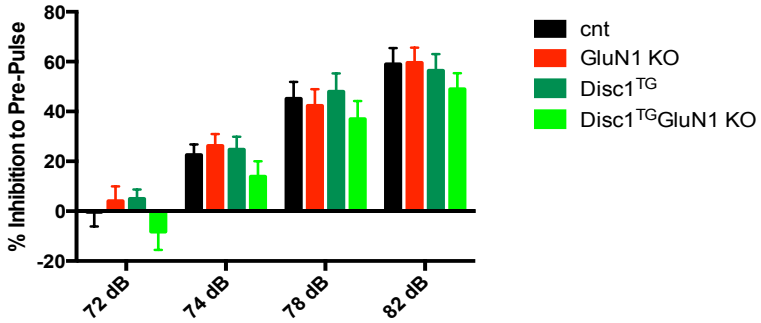
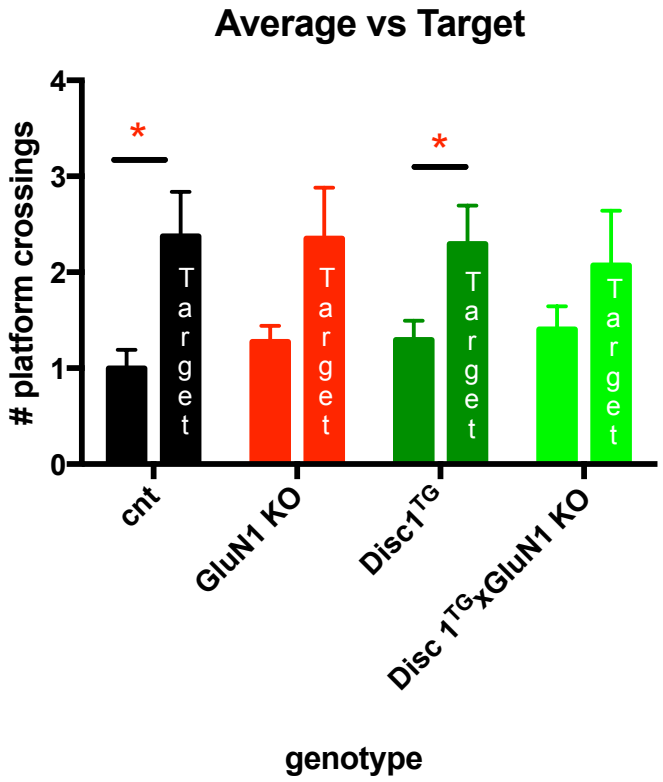


Figure 4-4 DISC1^{TG}GluN1 KO mice do not have reduced PPI. There were no significant differences among or between the 4 different genotypes that were tested. (One-way ANOVA 72dB: F(3,62)=1.172, p=0.3274; 74 dB: F(3,62)=1.224, p=0.3085; 78 dB: F(3,62)= 0.4241, p=0.7364; 82 dB: F(3,62)=0.6770, p=0.5694).

a)



b)

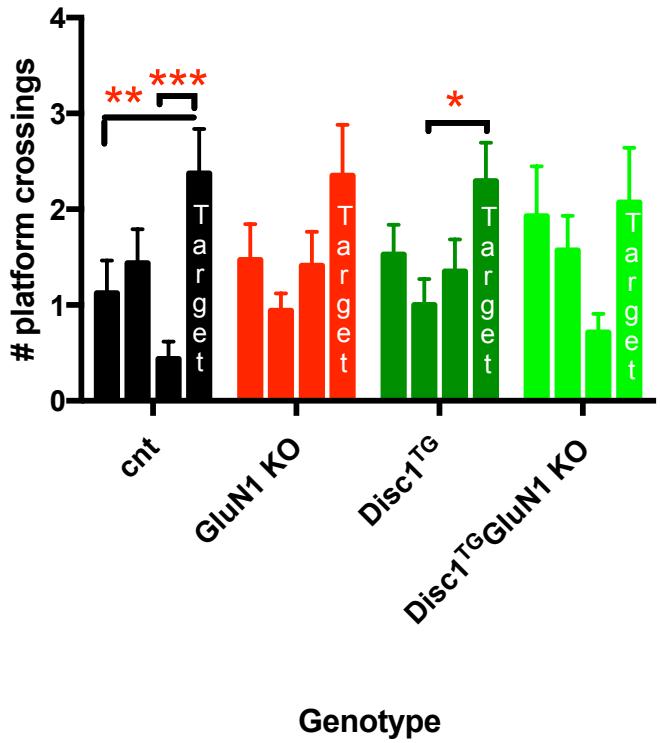


Figure 4-5 DISC1^{TG}GluN1 KO reduced spatial memory, but similar to GluN1 KO alone. The control and DISC1^{TG} were able to find the target platform when the platform was removed. a) t-test between each average crossings of the other 3 areas that correspond to where the target would be if it was in those quadrants vs the area corresponding to the target area where the mice were trained to go. Cnt: t=3 df=15 **p=0.0090, GluN1 KO: t=1.902 df=16 p=0.0753, DISC1^{TG}: t=2.231 df=16 *p=0.0404, DISC1^{TG}xGluN1 KO: t=1.084 df=13 p=0.2982. b) One-way ANOVA to compare the individual quadrant platform crossings, and Dunnette's multiple comparison *post hoc* tests: cnt: F=2.983(3,60), **p=0.0026, 1 vs target *p=0.0376 and 3 vs target ***p=0.0007; KO: F=4.274 (3,64), p=0.0759; DISC1: F=0.6928(3,64), *p=0.0449, 2 vs target *p=0.0179; DISC1^{TG}xGluN1 KO: F=1.507 (3,52), p=0.1324.

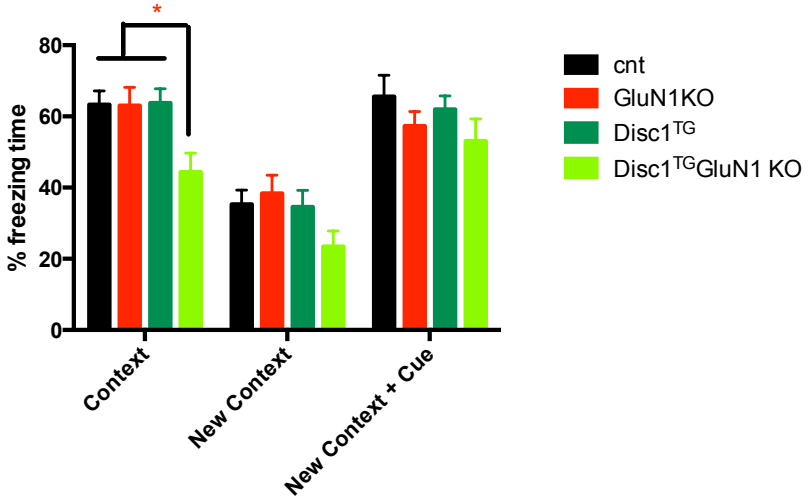


Figure 4-6 DISC1^{TG}GluN1 KO reduced contextual memory. One day after fear conditioning training a significant decrease was found in freezing in the context in which the mice were trained (one-way ANOVA F(3,60)=4.082, *p=0.0105). DISC1^{TG} GluN1 KO mice froze significantly less than cnt, the GluN1 KO, and DISC1^{TG} (Tukey's multiple comparison *post hoc* *p=0.0327, *p=0.0317, *p=0.0196). There were no significant freezing differences in the new context, nor with the cue (one-way ANOVA new context: F(3,60)=1.889, p=0.1407; new context+cue: F(3,60)=1.131, p=0.3507).

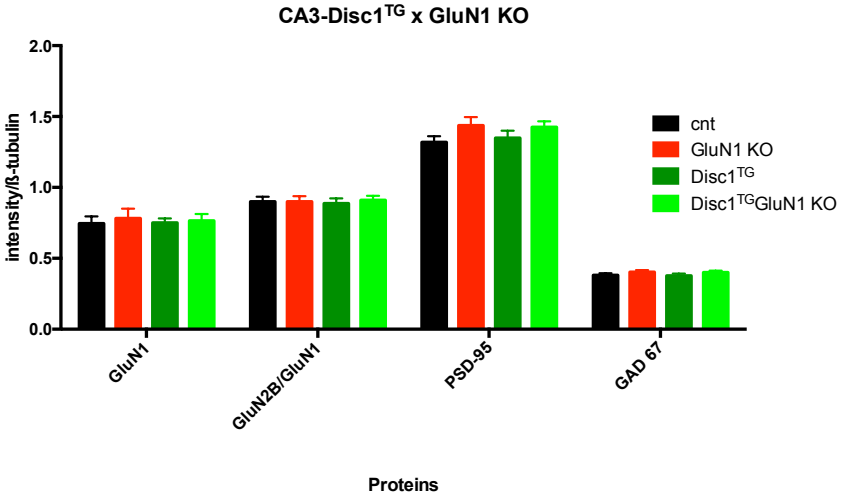


Figure 4-7 DISC1^{TG}GluN1 KO trends toward increased PSD-95 protein content. Two-way ANOVAs for each protein with GluN1KO x DISC1^{TG} as the factors. See table 3 for statistical analysis.

Table 3.

	GluN1	GluN2B/GluN1	PSD-95	GAD-67
Interaction	F(1,56) =0.0049, p = 0.9442	F(1,58) = 0.1034, p = 0.7489	F(1,54) =0.2117, p = 0.6473	F(1,58) = 0.0014, p = 0.9706
GluN1KO	F(1,56) = 0.1633, p = 0.6877	F(1,58) = 0.1055, p = 0.7465	F(1,54) =5.154, *p = 0.0272	F(1,58) =2.574, p = 0.1140
Disc1^{TG}	F(1,56) = 0.8308, p = 0.3660	F(1,58) = 0.0015, p = 0.9697	F(1,54) =0.0159, p = 0.9002	F(1,58) =0.0748, p = 0.7854

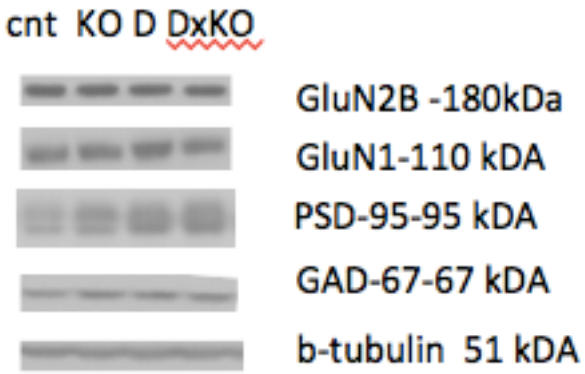


Figure 4-8. Representative Western Blot Figures from CA3. Cnt, KO, DISC1^{TG} (D) and the double mutant (DxKO). GluN1 and 2B, PSD-95 and GAD-67 protine. ß-tubulin is used as the loading control.

CHAPTER FIVE

Conclusions and Future Directions

The previous chapters in this dissertation describe the progress in generating a reverse-translation animal preparation for schizophrenic psychosis that, once verified, will be pivotal in contributing to our formulation of the molecular, cellular and plasticity-based elements of human psychotic illnesses. The preceding chapters emphasize the crucial need within the field of SZ research for a molecular understanding of psychosis. This being said, fundamental molecular, genetic, anatomic, and/or functional elements of SZ pathophysiology need to be identified in affected human brain systems and replicated in the animal for dynamic studies and understanding. Here I describe a novel, testable animal created in a reverse-translational manner, based on human SZ HC subfield specific tissue pathology, compelling *in vivo* imaging data of increased HC activity, as well as clinical findings of declarative memory deficits in psychosis. We have provided evidence that a novel molecular animal preparation generated to concretely parallel these human data in a reverse-translation direction will be available for forward translation.

In the experiments put forth, DG human tissue molecular findings from contrasting normal and schizophrenia cases were reproduced in a mouse. Based on molecular and anatomic markers of synaptic plasticity within the CA_3 HC subfield in humans with SZ we hypothesized that we would show evidence of altered plasticity in this subfield in the mouse preparation. This rational was based on findings of

increased GluN2B-containing NMDA receptors, increased PSD-95 protein and an increase in spine density in apical dendrites (Li *et al.,* 2015). The interpretation we made of these tissue findings is a dynamic one and not a formulation that can be tested primarily in postmortem tissue. Therefore, we moved to create an animal that would be useful in answering questions regarding psychosis pathology.

Using available human and animal experimental approaches this animal preparation emerged as a speculative and testable construct of psychosis pathology and behavior. Moreover, the translation of the human construct to an animal preparation is direct and demonstrable, with the possibility of high construct validity.

Notwithstanding these data, the mechanism that propagates the increase in activity in the CA_3, seen as an increase in activated c-Fos positive neurons and increased AMPA and NMDA receptor EPSCs, remains to be explored. The compelling data leads us to determine that the decreased activity of the DG causes the increase in activity in the $CA_{3,}$ but we need to demonstrate this in a more direct manner. We fell short of finding an increase in GluN2B containing NMDA receptors in conjunction with PSD-95 increase, both findings which would further substantiate the metaplastic change we hypothesized to occur in the HC of SZ psychosis. We postulated that the reduction of GluN1 in the DG would cause an increase in neuronal activity markers, but failed to see these results with our chosen methods. It is plausible that our methods were insufficient to detect functional molecular changes. It is also conceivable that these findings in these different subfields are

unrelated. We then speculated that we needed an additional risk factor for SZ to augment the deficits we observed and to bring about the CA_3 protein changes. Neither the specific pharmacological agent, nor a genetic risk, was able to produce the full range of changes we see in SZ human tissue.

In the first phase of developing a useful animal, we adopted the DG-specific GluN1 KO mouse already developed in the Tonegawa laboratory. This was a logical place to start because that mouse mimics as least one of the actual pathological elements of the human schizophrenia tissue; low GluN1 limited in hippocampus to dentate gyrus. Findings by McHugh *et al.,* (2007), who were able to spatially restrict the loss of the requisite subunit of the NMDA receptor, GluN1, to the granule cells of the DG and demonstrated that the DG is important for rapid discrimination between two similar contexts with limited experience, inspired us to replicate the spatial restriction loss of this subunit and ask if behavior and molecular changes would parallel those of psychosis. McHugh *et al* (2007) report that in behaviors known to involve the HC, MWM and contextual FC there are no significant differences between DG-GluN1 KO and their control littermates (MWM target quadrant memory and one shock FC contextual memory). They do however go on to establish that when testing contextual memory in a similar context, the GluN1 mice (those that retained the GluN1 in DG) froze significantly more than their GluN1 KO littermates. Multi-tetrode recordings of place cells in CA_3 and CA_1, an *in vivo* measurement, was used to demonstrate this lack of discrimination when mice were allowed to explore

two different contexts. In these two different contexts ensemble activity of firing neurons was measured, first in a familiar/trained context and next in a new context. When placed in a new situation the rate of remapping that place cells undergo is measured. The new firing pattern is believed to represent the encoding of the new context. They then compared the average firing rates in each context. An increase in firing rate correlates with the formation of a new and distinct memory, suggesting the representation of a different context. GluN1 KO mice display larger place cell fields (% of sampled space) in CA_1 and showed similar rate differences in CA_3 and CA_1, unlike the littermates, where CA_3 had larger rate differences than CA_1. As well, the rate difference was significantly lower in the CA_3 of GluN1 KO mice compared to their littermates. The Sawa group speculated that the loss of input from the DG to the CA_3 may have led to an increase of input from the entorhinal cortex, therefore reducing the ability of the CA_3 to encode (detect, amplify and reflect subtle differences). With this evidence they conclude that GluN1 is important in the DG for pattern separation.

Initially we tested the mice in several behavior paradigms that were known to involve hippocampus to some degree. We tested PPI, FC, PA and MWM. Secondarily, we looked for tissue markers of increase plasticity/activity in the CA_3, by western blot and immunohistochemistry. Lastly, we looked at a functional readout of activity, electrophysiology.

We propose that our findings strengthen the notion of subfield-specific contribution to memory and learning. We demonstrated that with five-shock training, contextual and cued fear conditioning was increased in the DG-GluN1 KO mice. We also attest to the memory for target quadrant (general area) in the MWM by both GluN1 KO mice and their littermates, but further demonstrate that the removal of GluN1 protein in the DG inhibits mice from remembering the specific location of the target. With this animal we were able to go even further to show that the activity of pyramidal cells in the CA_3 is increased, demonstrating associated activity-dependent changes downstream in CA_3 and in CA_1 with specific molecular, cellular and electrophysiological characteristics, most consistent with the findings not only in postmortem tissue but also SZ characteristics found in *in vivo* imaging (eg, hyper-activity in hippocampus); we demonstrate this both by immediate early gene (c-Fos) detection as well as the functional demonstration of increased EPSCs.

A caveat of this mouse preparation is the lack of understanding of the cellular mechanisms through which removal of GluN1 in the DG leads to enhanced CA_3/CA_1 activity. One might speculate that decreased granular cells activity triggers a homeostatic adaptation in CA_3 pyramidal cells that aim to renormalize mossy fibers-CA_3 excitatory transmission, but the mechanism remains elusive at this point. Interestingly, DG granular cells in DG-GluN1 KO mouse exhibit a decreased paired pulse ratio. Whether this led to maladaptive CA_3 pyramidal neuron hyperexcitability, *i.e.*, a network-wide homeostatic adaptation that aim to re-normalize transmission is

yet to be tested.

One of the major reasons we need an animal preparation of the psychosis disease model is because the brain is dynamic and involves alterations in activity-dependent signaling within the hippocampal trisynaptic circuit. Demonstration of the feasibility of the disease model must include careful study of the response of each hippocampal subfield to changes in DG activity and to the timing of those changes. The relationships between CA_3 -CA_1 firing has been extensively studied in defining metaplasticity principles (Bear and Malenka, 1994). The relationship between DG and CA3 firing has been less extensively studied, but detailed by McBain, *et al* (2008) to show the primary excitability of CA_3 in response to alterations in DG afferent signaling. Moreover, all of these principles are developmentally and genetically influenced.

When we not detected no protein changes in the CA_3 that would point towards increased activity (GluN2B and PSD-95) we proposed that in order to induce hippocampal changes after these first experiments, we needed to test the hypotheses that adding risk factors for psychotic illness, both a pharmacological (PCP) and a genetic risk factor (human *DISC1* translocation), would produce the tissue changes of the GluN1 KO mouse to replicate those seen in human SZ.

We began by combining a pharmacological risk factor that is established as a model for SZ, PCP, with the GluN1 KO mice. With this combination we again looked at behaviors relevant to SZ and a deficit in HC learning and memory. We did not see

any differences in anxiety-induced behavior, which we reasoned might explain the increased freezing of the GluN1 KO mice in both the contextual and cued FC paradigms. Surprisingly, we also did not observe any significant differences in FC. In the PPI paradigm we found significant differences, but the effect was due to PCP only and it wasn't a combined effect. It was not surprising, however, after these observations, that we did not find any differences in post-synaptic proteins in the CA_3. These findings are compelling evidence that the psychosis mechanism is complex and multifaceted.

One caveat of this study is that the methodology of chronic PCP administration is not widely established. It is important to establish the dose of PCP given, the method of delivery, and the time line. Early studies of PCP as a model for SZ focused on acute injections, and only recently have groups initiated chronic administration as more ideal because it likely reflects the chronic changes in SZ brain. Because PCP works in multiple brain areas and we were focused on the hippocampus perhaps there is a compensatory mechanism that rectifies the deficits we saw previously. There is evidence, at least in the pre-frontal cortex, that PCP-induced abnormalities of acute systemic injection, such as hyperlocomotion and stereotypies, are ameliorated by prior administration of the dissociative drug (Jodo, 2013). As well, there is the possibility that we failed to see an intensification of deficits observed in the GluN1 KO mouse because we have already perturbed the system to maximum capacity. We hypothesize that we may have seen a rectification

or normalization of behavior in the GluN1 KO animals with and without PCP because of the amount of handling that they experienced, desensitizing fear behavior. Perhaps the animals became accustomed to having repeated stressful experiences (i.p. injections) over the course of a month. Conceivably, the animals had become desensitized. In the future in order to see if this is the case we would want to implant an infusion pump so that the animals are not being handled as much as in our protocol. We could also infuse or inject PCP straight into the HC to get a regional, localized effect that would be more relevant to our model of the HC being the locus of psychosis.

There is compelling evidence that SZ is a polygenetic disorder and highly heritable. A child with a parent who has SZ is 10 times more likely to develop the disorder than those without. Based on meta-data analysis and twin studies the heritability has been reported at around 80% (Gejman *et al.,* 2010; Cardno and Gottesman, 2000; Sullivan *et al.,* 2003). Thus, we asked if a well-known genetic risk factor for SZ, the human *DISC1* translocation, in combination with our GluN1 KO mouse preparation would perhaps enhance the psychosis features that were already observed in these two animal models alone and would elicit the protein changes in the CA_3 that are reminiscent of SZ post mortem tissue findings. Here we failed to augment behavioral deficits seen with the GluN1 KO mouse alone. PPI was not significantly different for any genotype, at any decibel level so in fact we normalized the PPI deficits previously seen. The double mutant mice displayed spatial memory

loss in the MWM task, although it was not to an extent greater than the GluN1 KO alone. Importantly we found that GluN1 and functional DISC1 interact and this interaction is needed in the DG for contextual learning; in the absence of both GluN1 protein in DG and the dysfunction of DISC1 we found the opposite behavioral findings in contextual fear conditioning; a lack of freezing as oppose to an increase in freezing. This loss of contextual fear memory in the double mutant is convincing evidence that the interaction of GluN1 and DISC1 are required in the DG for contextual fear memory. With the inhibition of endogenous DISC1 protein and the loss of the GluN1 subunit of the NMDA receptor in the DG (the GluN1 loss being spatially restricted) the mice were less able to form a memory based on their surroundings and therefore had reduced freezing in the test chamber compared to their littermates.

The limitation of this experiment is the whole brain expression of the DISC1^{TG} protein. Although relevant because in SZ the expression of dysfunctional DISC1 isn't limited to a particular brain region (Millar *et al.,* 2003; Austin *et al.,* 2004, Shen *et al.,* 2008), when we postulate that psychosis is a learning and memory disorder of the HC, having DISC1^{TG} expression throughout the brain as a whole makes the changes difficult to pin point effects that are part of the psychosis mechanism and the effects from other areas of the brain. Perhaps, again, we have compensatory mechanisms that mask the effect on psychosis behaviors and protein changes. The area we focus on and hypothesize to be the mechanism of psychosis is that of the pathways

and circuits in the HC. To explore this, in the future, we would need to limit the DISC1^{TG} expression to the HC, or even individual subfields of the HC. Perhaps we could use an AAV to deliver the transgene to the DG and look at the effects on behavior and tissue changes that result. As well, maybe *DISC1* is not the strongest gene candidate for this preparation; perhaps 22q11 deletion mouse would be a better combination to elicit the changes we see in SZ. Patients with this genetic risk factor for SZ display changes in the HC [volume reduction (Debbané *et al.,* 2006 and Flanhault *et al.,* 2011) and malrotation (Andrade *et al.,* 2013)], a decrease in PPI (Sobin *et al.,* 2005; Paylor and Lindsay, 2006) and there is an extremely high risk of development of SZ (Murphy *et al.,* 1999; Bassett *et al.,* 2003; Zinkstok and van Amelsvoort, 2005; Gothelf *et al.,* 2005 and 2007; Paylor and Lindsay, 2006; Piskorowski *et al.,* 2016; Ellegood *et al.,* 2014; Ouchi *et al.,* 2013; Drew *et al.,* 2011). This mouse model may also be a better candidate to match with the GluN1 DG-specific KO because it already shows a significant increase in volume of the 3rd ventricle [the most common brain structure abnormality found in SZ (Ellegood *et al.,* 2014)] an age dependent increase in hippocampal LTP (Earls *et al.,* 2012), decreased PPI (Gogos *et al.,* 1999; Paylor *et al.,* 2001; Paylor and Linsay, 2006), and decreased contextual fear conditioning (Paylor *et al.,* 2001; Paylor and Lindsay, 2006).

A different risk factor we can use that is both pharmacological, believed to be genetically directed and whose action is developmentally restricted, is adolescent

cannabis use; as cannabis use risk for psychosis is limited to early-mid adolescent use (Semple *et al.,* 2005 and French *et al., 2015*). This may be better to combine with the DG specific KO mouse. The early adolescent cannabis-use risk is limited to one of the COMT polymorphisms: Val158Met (Caspi *et al.,* 2005; Henquet *et al.,* 2006, Zammit *et al.,* 2007; Gutiérrez *et al.,* 2009; O'Tuathaigh *et al.,* 2010; Nieman *et al.,* 2016). The interplay of psychosis risk factors for brain diseases like psychosis is complex, anatomically, molecularly and developmentally; exacerbating this illness pathology will not be simple, albeit data regarding risk factors are informative, whether they are positive or negative.

There is also the confound of epigenetics and epigenetic mechanisms (Grayson *et al.,* 2009; Peter and Akbarian, 2011) that are important in psychiatric disorders and these changes can be based on parental experience, individual experience and even environmental factors. Epigenetic alterations, such as histone methylation, regulate gene expression and transcriptional regulation. This could also explain the lack of consensus in neuropathology in SZ as well as explain the heterogeneity of brain pathology see in this complex disorder. Dysregulated BDNF gene transcription in general, rather than a certain gene, would compromise neuronal circuitry and/or neurotransmission. The question of why neither risk factor worked to exacerbate the behavioral phenotype seen in the GluN1 KO mice, nor appreciably extend or expand the hippocampal tissue pathology which we are trying to establish to model psychosis in schizophrenia is a puzzle and suggests

complexity of brain mechanisms for psychosis and that risks for SZ may not be simple and perhaps there might be multiple neural circuits that support psychosis in SZ (Johnstone *et al.,* 2011; Pletnikov *et al.,* 2008; Hikida *et al*., 2007; Shen *et al.,* 2008).

HC is involved in the mechanistic action of PCP, PCP is likely to generate psychosis across different anatomic regions [ie PFC (Guidali *et al.,* 2010 and Jodo, 2013), thalamus (Celada *et al.* 2013 and Troyano-Rodriguez *et al.,* 2014), anterior cingulate (Tamminga *et al*., 2003), as has been demonstrated by previous pharmacological studies of PCP as a model for psychosis, rather than precisely within these hippocampal subfields that we are examining. And the knowledge that the *DISC1* translocation disturbs hippocampal anatomy and function might affect some but not all of the pathology defined from human psychosis tissue. This latter is not inconsistent with what we know of the DISC1 phenotype, since only a fraction of the human DISC1 translocation carriers actually have schizophrenic psychosis *per se*.

We understand and support the human data suggesting that it is the interaction among primary neural circuits, especially circuits within the limbic cortex, not an isolated regional alteration, which is the most likely basis for manifestations of such a pervasive cognitive disturbance like psychosis. Our current formulation is that the HC is the part of the limbic circuit that generates the psychotic 'memory' (analogous to the way that declarative memories themselves are generated), but is

not the only brain region involved in its consolidation, storage, saliency or many other characteristics. Moreover, memory itself is broader than declarative memory. Therefore, this implicates the whole limbic psychosis circuit, including but not limited to HC.

In order to directly inhibit or stimulate neurons in specific subfields we have chosen to use Designer Receptor Activated by a Designer Drug (DREADD) technology. We will use this DREADD technology in order to alter firing in DG and in CA_3, to test the outcomes in an animal preparation of these kinds of dynamic subfield changes on overall hippocampal activity and CA_3 firing. In the future, the use of an inhibitory DREADD in DG granule cells (ie. dorsal or ventral or both) of C57Bl/6 mice with an examination of cellular activity in CA_3 will suggest whether a simple change in excitatory transmission in the mossy fiber pathway can generate hyperactivity in CA_3 [measured electrophysiologically and analyzing c-Fos activated neurons, and possibly *in vivo* electrodes (Ferguson and Neumaier, 2012)] and whether anything additional is necessary for inducing psychosis behaviors. Then, also an excitatory DREADD in CA_3 when activated should show overall hippocampal hyperactivity and be associated with behaviors of psychosis in the mouse. This DREADD technology will remove the developmental aspect of the Cre recombinase system. This technology is also more direct and will allow us to spatially localized the excitation or inhibition of neurons. We could also use this technology to look at different developmental timelines to see if said activity is important at a specific time

of maturation. We could also experiment with different protocols to observe the differences between acute and chronic activation/inhibition of HC neurons.

We are in the process of gathering and analyzing specific data from the human subfield transcriptomes in the schizophrenia vs healthy cases. Once this is achieved additional genetically manipulated mice will be considered. Of the important research to carry out in the future is a characterization of the pharmacology of these animal preparations, including not only dopamine antagonists, but also muscarinic agonists (Jones *et al.,* 2012; Digby *et al.,* 2012; Byun, *et al.,* 2014; Gibbons and Dean, 2016), all thought to influence neuronal activity in HC and shown to alleviate psychotic symptoms.

We do not yet have an irrefutable understanding of how psychotic symptoms are generated and what the complexity of dysfunctional gene, or combination of genes, [i.e. their level of expression, location of expression (or lack there of), etc.] is in relation to these psychotic symptoms and their manifestations. We also do not know the possible drug (if not PCP, maybe ketamine), its dose and the duration of use and at which developmental point to use it for maximal stimulation of the HC with this animal preparation. It is possible that our beginning hypotheses were extensively naïve. Nonetheless, this animal preparation can now be further tested in combination with SZ risk factors such as maternal deprivation, environmental stress factors and genetic anomalies that potentially increase the risk for the development of SZ. The availability of an animal preparation which will be informative for the

behavior of a human neural microcircuit in HC believed to be important in a brain disorder like SZ with psychosis will be a significant advantage for studying psychosis.

APPENDIX A
Primers

Cre – M260 5'-GGCCAGCTAAACATGCTTCATCGT-3'

M265 5'-TATCTTTAACCCTGATCCTGGCAA-3'

Floxed p - oIMR3742 5'-GTGAGCTGCACTTCCAGAAG-3'

oIMR3743 5'-GACTTTCGGCATGTGAAATG-3'

oIMR6916 5'-CTTGGGTGGAGAGGCTATTC-3'

oIMR6917 5'-AGGTGAGATGACAGGAGATC-3'

DISC1-DN-DISC1^{TG} sense 1354-1373 5'-GAA TGG AGC CGA GGC TGT TG-3'

Anti-sense alpha CaMKII-R 5'-CCC AGC CTA GAC CAC AAT GC-3'

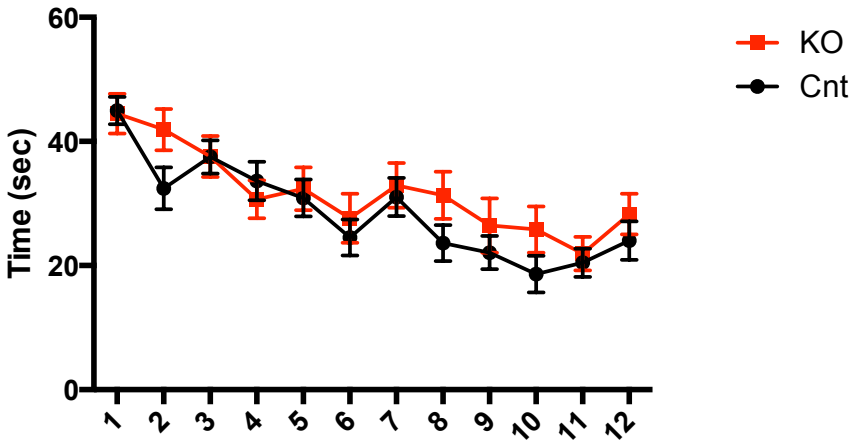
APPENDIX B
Cycling Parameters for PCR

Cre:	1 cycle	94°C 4 min.
	35 cycles	94°C 30 sec.
		55°C 30 sec.
		72°C 1 min.
	1 cycle	72°C 10 min.
	Hold	4°C

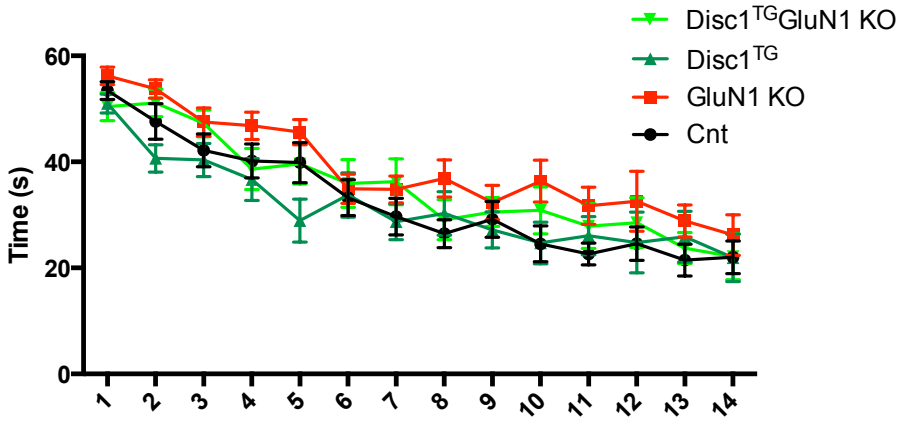
Flox p:	1 cycle	94°C 4 min.
	40 cycles	94°C 45 sec.
		61°C 45 sec.
		72°C 1 min.
	1 cycle	72°C 7 min.
	Hold	4°C

DISC1:	1 cycle	94°C 5 min.
	40 cycles	94°C 45 sec.
		58°C 45 sec.
		72°C 1 min.
	1 cycle	72°C 7 min.
	Hold	4°C

SUPPPLEMENTAL MATERIAL



Supplemental Figure 1. MWM learning pattern. Cnt and KO mice learned to find the target platform at similar rates over 12 days.



Supplemental Figure 2. MWM learning pattern. Cnt, KO, DISC1^{TG} and the double mutant learned to find the target platform at similar rates over 14 days.

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