A Deficit in Parvalbumin-Expressing Interneurons in the Hippocampus Leads to Physiological and Behavioral Phenotypes Relevant to Schizophrenia in a Genetic Mouse Model

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ABSTRACT

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Deficits in hippocampal interneurons, that secrete *gamma*-aminobutyric acid (GABAergic), are implicated in the pathophysiology of schizophrenia. Postmortem histological analyses show alterations in the number and/or function of parvalbumin-expressing (PV+) GABAergic interneurons in the cerebral cortex of these patients. A parallel line of research using functional imaging of cerebral blood flow or volume has shown that hyperactivity of the hippocampus may contribute to psychotic symptoms as well as cognitive deficits in schizophrenia. It is not known if changes in GABA transmission, particularly in the number and function of PV+ interneurons, are causally related to hippocampal hyperactivity and expression of behavioral and cognitive abnormalities in schizophrenia. To help answer this question, we used genetic mouse models with deficits in cortical GABAergic interneuron development to test the hypothesis that a selective deficit in PV+ interneurons in the hippocampus can lead to schizophrenia relevant phenotypes, such as hippocampal hyperactivity, dysregulation of the mesolimbic dopamine system, enhanced psychomotor responsiveness to amphetamine, and disruption of hippocampus dependent cognition.

Here I describe my studies primarily on a mouse model with a deletion of the cell-cycle gene cyclin D2 (cD2 null). This mutation disrupts interneuron development in the medial ganglionic eminence (MGE), leading to a partial and selective deficit in PV+ interneurons in the neocortex and the hippocampus. I show that the cD2 null mouse

shows regionally heterogeneous, persistent structural and functional deficits in PV+ interneurons, with a relatively larger and more functional deficit in the hippocampus. The GABAergic deficit in the hippocampus is associated with signs of disinhibition, such as increased cerebral blood volume, as found by functional magnetic resonance imaging (fMRI).

Upon establishing the evidence for hippocampal disinhibition in the cyclin D2 null mouse, I examined the relationship between this disinhibition and two areas of neural function know to be altered in psychosis and schizophrenia: mesostriatal DA system function and hippocampus-mediated cognition. I found that the cD2 null mice showed increased dopamine population activity in the ventral tegmental area and enhanced psychomotor response to amphetamine. The latter was eliminated by a partial lesion of the ventral hippocampus, indicating hippocampal disinhibition as the driver of DA neuron dysregulation. In addition, cD2 null mice showed deficits in cognitive functions that recruit and depend on the hippocampus, such as contextual and cued fear conditioning. Lastly, to test for a causal relationship between the PV+ interneuron deficit in the hippocampus, and the abnormalities in hippocampal metabolism, imaging phenotype, the mesolimbic dopamine dysfunction and contextual learning and memory, I examined the effects of replacing GABAergic interneurons in the hippocampus. I used transplantation of GABAergic interneuron precursors derived from the medial ganglionic eminence (MGE) into the adult hippocampus of cyclin D2 null mutants. MGE-derived progenitor cells developed into structurally and functionally mature PV+ and other GABAergic cells, and normalized hippocampal hypermetabolism. In addition, the MGE

transplants normalized ventral tegmental area (VTA) dopamine cell activity, normalized amphetamine sensitivity and improved hippocampus-dependent learning and memory.

Taken together, these studies establish the plausibility of a causal relationship between hippocampal PV+ interneuron pathology and psychosis-relevant pathophysiological and cognitive phenotypes. Moreover, they provide a rationale for limbic cortical GABAergic-interneuron-targeted treatment strategies in psychotic disorders.

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Section I: General Introduction and Methods

Chapter 1: General Introduction

1.1   A General Introduction to Schizophrenia

Prevalence and Burden:

Schizophrenia is a chronic disabling psychiatric condition. The lifetime prevalence of schizophrenia is about 0.7%, with relatively little variation across the world. Due to its high incidence and chronicity, schizophrenia causes significant morbidity. The economic burden of schizophrenia has been estimated to be over $30 billion annually, in the United States alone. Schizophrenia usually emerges in late adolescence and early adulthood, however, behavioral and cognitive disturbance can be identified in retrospect in some patients (Sadock et al., 2007) (Jarskog et al., 2007)

Symptoms and Course of Illness:

Schizophrenia is a heterogeneous syndrome characterized by perturbations of perception, thinking, language, affect and volition. These symptoms have been divided into *positive* symptoms (additional features that are not present in unaffected individuals), *negative* symptoms (features present in unaffected individuals but reduced in patients with schizophrenia) and *cognitive* symptoms. Positive symptoms include hallucinations (mostly auditory) and delusions. Negative symptoms include flat affect, poor attention, lack of motivation and deficits in social function. Cognitive disturbances include difficulties with attention, concentration and memory.

Like other psychiatric disorders, the diagnosis of schizophrenia is based on symptomatology. No biological diagnostic marker is available at the moment.

Schizophrenia is usually first recognized and diagnosed on the basis in late adolescence or early adulthood in males, early-mid adulthood in females, usually in the context of an episode of psychosis (Sadock et al., 2007). However, there is evidence that behavioral deficits that resemble negative symptoms, as well as cognitive deficits, precede psychotic symptoms and may be predictive of the first psychotic break (Corcoran et al., 2007). Moreover, positive symptoms appear to take a different course than negative symptoms and cognitive deficits. Positive symptoms wax and wane over time, and generally become less prominent with age. Negative symptoms and cognitive deficits, on the other hand, are more persistent, usually stable over time, and are refractory to treatment. Importantly, cognitive deficits also predict long-term functional outcome in patients with schizophrenia (Sadock et al., 2007).

Treatment:

Since the serendipitous discovery of antipsychotic action of chlorpromazine in the early1950s, several antipsychotics have been developed (Delay and Denicker 1952, reviewed in Ban 2007). Many of the antipsychotic compounds have been selected based on animal screens such as the drug's ability to block apomorphine induced vomiting in dogs or amphetamine induced locomotion in rodents. Later, it was found that these screens select for chemical compounds that block the dopamine D2 receptor. A correlation was subsequently found between D2 receptor affinity and the clinical potency of antipsychotic drugs.

Current pharmacotherapy for schizophrenia is far from ideal. Firstly, antipsychotic drugs relieve positive symptoms but do not adequately treat negative or cognitive symptoms. Secondly, many

of these medications have unwanted side effects, most notable of which are movement difficulties and prolactinemia (which are associated with traditional antipsychotic agents) and metabolic derangements and obesity (associated with the newer/atypical agents). Finally, up to a third of the patient population is treatment-refractory. Therefore, better treatment modalities are desparately needed for the management of schizophrenia.

Genetic and Environmental Risk Factors:

Adoption, twin and family studies provide compelling evidence for the heritability of schizophrenia. The risk of developing schizophrenia is proportional to the degree of genetic relatedness to a patient. While the life time risk of schizophrenia in the general population is about 1%, it reaches close to 50% in identical twins, if one twin has schizophrenia (Cardno et al., 2001; Sadock et al., 2007). The concordance rate for a fraternal twin or a non-twin sibling is close to 12%. Absence of complete concordance indicates interplay of environmental factors, and/ or epigenetic factors in the disease**.**

Epidemiological studies of schizophrenia risk factors suggest a neurodevelopmental etiology for schizophrenia. Many of the environmental factors strongly associated with increased risk of schizophrenia (such as maternal influenza infection during gestation and maternal starvation, discussed below), occur during the prenatal or perinatal periods of life, implicating a possible disruption of a developmental process. Two examples of such prenatal risk factors are maternal infection and maternal malnutrition. Second-trimester maternal influenza or rubella infection has been associated with increased risk for schizophrenia in the offspring (see meta analysis by Khandaker et al., 2013). Similarly, individuals conceived in famine conditions, including at the

height of the Dutch Hunger Winter of 1944–1945 and during the Chinese famine of 1959-1961, showed an increased risk for schizophrenia (McClellan et al., 2006).

In addition to early life environmental risk factors, clinical genetic studies have identified a number of alleles that increase the risk of schizophrenia (Harrison and Owen, 2003; Rodriguez-Murillo et al., 2012). Recent data provides a possible point of convergence between these two lines of research and suggests that several of the schizophrenia risk alleles may influence the development and plasticity of brain circuits (for example see Flames et al., 2004; Harrison and Weinberger, 2005; Niwa et al., 2010).

Importantly, unlike disorders easily recognized as "developmental", there is a lag of many years between the presumed initial insult, which presumably begins to operate in-utero, and the onset of behavioral symptoms upon which we diagnose the disease, usually emerge in early adulthood. To explain this, Weinberger (Weinberger, 1987) proposed that a neurodevelopmental brain lesion early in life may sit clinically silent until it is revealed by, or interacts with developmental processes that occur in adolescence or early adulthood, just before the disease manifests behaviorally. An elaboration of this is the "two-hit" hypothesis, which, in its several versions (discussed later) suggests that abnormal development of the brain during early life (prenatal or postnatal), presumably due to genetic or/and environmental factors, may lead to susceptibility to schizophrenia. In a subset of those exposed to these risk factors, abnormal maturational processes, adverse effects of drugs, or stress, may further exacerbate the pathological process, ultimately leading to overt disease.

Neural Circuit Pathology in Schizophrenia

1.2 Overview of Neuropathology and Neural Circuit Abnormalities in Schizophrenia

Schizophrenia remains a symptom complex and not a discrete biological disease entity. Heterogeneity observed in clinical features (such as presentation, symptoms, response to treatment and prognosis) raises the possibility that schizophrenia may constitute a constellation of discrete disease entities each with its own unique pathology. As such, multiple neural mechanisms have been implicated in its pathogenesis. Different etiologies may be differentially involved in each patient, and this may be reflected in the heterogeneity of behavioral symptoms as mentioned above. Common features across individuals diagnosed with schizophrenia, however, suggest a relatively common set of pathophysiological mechanisms that may underlie specific symptom clusters. Identifying and understanding these mechanisms is fundamental for finding treatments for patients refractory to currently available treatment modalities. Towards this end, animal models have been developed to test ideas about the causal relationships between pathophysiology in specific neural systems and the psychopathology of schizophrenia.

As background for the animal models used in this thesis, I present a brief review of the neuro-histopathology of schizophrenia, the evidence for involvement of the hippocampus, and the evidence for dopamine dysregulation.

Neuropathological Findings in Schizophrenia

In order to understand the biological underpinnings of schizophrenia, post-mortem studies of the brains of individuals with psychosis have been conducted for more than 100 years. Earlier studies showed that there is no gross brain damage, florid degeneration, or hydrocephalus in schizophrenia. Starting in the 1980s, studies using more detailed analyses increasingly reported subtle alterations, which are significantly more frequent in schizophrenia patients than in healthy controls or controls with other mental illnesses. In general, these alterations are not robust enough to serve as diagnostic markers; however, these can be useful to generate insights and hypotheses about pathogenic or pathophysiological mechanisms, that may, in turn, lead to novel targets for pharmacological interventions (Harrison et al., 2011). There is an ongoing debate about the authenticity and specificity of post-mortem histopathological findings in schizophrenia, and how they relate to structural imaging studies of this disorder. With that caveat in mind, the following are some of the most robust findings:

*Absence of widespread gliosis in schizophrenia***:** Gliosis is the process of proliferation and hypertrophy of astrocytes. It is one of the most robust indicators of a recent or active injury. Most postmortem studies in schizophrenia have failed to find evidence for gliosis in schizophrenia (Benes et al., 1991). It has recently been suggested the autopsy studies of gliosis in schizophrenia have generally been poorly controlled and underpowered to satisfactorily address this question (Schnieder and Dwork, 2011), emphasizing the need for further research in this area.

Neurodegeneration versus abnormal neuronal development and synaptic maintenance:
Florid neural degeneration, of the extent present in classic neurodegenerative diseases such

as Alzheimer's disease, is absent in schizophrenia. However more sensitive analytical techniques have revealed subtle changes in brain volume, as well as increases in cell density in certain regions, which together suggest that the neuropil is decreased in schizophrenia. In support of this idea, reductions in spine density in various cortical regions such as the subiculum (Rosoklija et al., 2000) and the auditory cortex (Sweet et al., 2008), have been reported. Other reported neuropathological alterations consistent with a disruption of neurodevelopment and/or neural activity include decreases in somal size of cortical projection neurons and disorganization in the distribution and orientation of these neurons. These latter abnormalities have been observed in projection neurons of the hippocampus (Arnold, 1999).

Brain regions implicated in structural imaging studies.

In vivo brain imaging studies have also revealed macroscopic changes in many regions of the brain in schizophrenia including temporal lobe structures, prefrontal cortex and the thalamus. There have been reports of reduced overall brain volume and length (around 3-4 % (Wright et al., 2000; Harrison et al., 2003)) , enlargement of lateral and third ventricles, and whole-brain-corrected reduction in gray matter of specific regions, most notably, the hippocampus. Studies have found structural changes in un-medicated patients, and at or near time of the first psychotic episode, indicating that volume reduction is not an effect of medication exposure. It is not clear whether these changes are progressive or stable over time, or how they are affected by treatment. Recent longitudinal studies, however, show a reduction in hippocampal volume occurring over the course of the first episode of psychosis (Schobel et al., 2013). Other cross-sectional studies have provided some support for progressive hippocampal and frontal lobe volume loss as a

function of multiple psychotic episodes and refractoriness to antipsychotic treatment (Jarskog et al., 2007).

1.3 Alterations in GABA Interneuron Function in the Hippocampus and Other Cortical Regions in Schizophrenia

Alterations in markers of GABAergic neurotransmission have been found in various regions of the brain in schizophrenia. Two regions that have been studied most extensively, and show the most robust changes, are the prefrontal cortex and the hippocampal formation.

Abnormalities in GABAergic markers in the hippocampus.

The hippocampus is one of the most severely affected regions in schizophrenia. Alterations in many markers of the GABAergic system have been reported in the hippocampus and the associated temporolimbic cortex in patients with schizophrenia. A brief overview of the reported alterations is given below.

> *Alterations in GAD:* The rate-limiting enzyme in GABA synthesis is the glutamic acid decarboxylase (GAD). GAD is present in two isoforms, which are coded by two separate genes: GAD1 - which codes for GAD67 isoform, and GAD2 - which codes for GAD65. In situ hybridization for GAD 65 and GAD 67 mRNA in the hippocampus showed deficits in bipolar disorder but not in schizophrenia (Heckers et al., 2002).

> *Alterations in GABAergic Interneurons:* Benes and colleagues provided some of the first evidence for alterations in hippocampal interneurons in schizophrenia (Benes et al., 1998). Using morphological criteria, these researchers distinguished pyramidal cells from non-pyramidal cells (presumably interneurons, glia were distinguished from neuronal cells) in the hippocampus. They reported reductions in density of non-pyramidal cells in the CA2 subfield of the hippocampus in patients with schizophrenia as well as those with

'manic depression' (Benes et al., 1998). No differences were found in the density of pyramidal cells in either group (Benes et al., 1998). Since then, a number of studies have quantified interneurons, as identified by immunological markers of peptides expressed by specific interneuron subpopulations. Zhang and Reynolds (Zhang and Reynolds, 2002) quantified Calretinin or Parvalbumin immunoreactive neurons and found a reduction in PV+ IN in all sub-regions of the hippocampus and in the dentate gyrus in schizophrenia subjects. In the same study, subjects with bipolar disorder showed reduction in the CA1 region. No alterations were found in the subjects with major depression and no alterations were found in CR cell density in either group. These findings have been replicated, by the same group, in an independent sample of patients with schizophrenia (Zhang and Reynolds, 2002).

In an independent collection of brains (Stanley Brain consortium) PV interneuron in CA2 were found to be reduced in schizophrenia and bipolar disorder (Knable et al., 2004). Subsequently in a larger study (described below), parvalbumin deficit was found in all regions of the hippocampus and the dentate gyrus (Torrey et al., 2005). In a recent larger study, Konradi et al employed more controlled quantitative methods, and found a reduction in PV+ and somatostatin positive (SST+) cells in the hippocampus (Konradi et al., 2011). This study used immuno-labeling and real time quantitative PCR (to measure mRNA levels), and found a reduction in the expression of somatostatin, parvalbumin and GAD 67. The reduction in these markers was found in various subfields of the hippocampus, but was most consistently decreased in the CA2/3 region.

Over all, there is converging evidence for the number of PV+ and possibly SST+ interneurons in the hippocampus. It should however be pointed out that many of the

subtypes of GABAergic interneurons in the hippocampus (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008, see below) have yet to be explored by post mortem histological studies of schizophrenia.

Alterations in GABA agonist binding: Initial studies reported an increase in $GABA_A$ receptor binding activity in several key corticolimbic regions, including the hippocampal formation in postmortem schizophrenic brains. These studies showed increased $GABA_A$ receptor binding activity throughout the hippocampal formation including the DG, CA fields, subiculum, and presubiculum of the schizophrenic group (Benes et al., 1996). These changes were thought to be compensatory to a decrease in presynaptic GABA, raising the question, whether the number or function of GABAergic cells is reduced in these brain regions in the disease. In the hippocampus this change was more pronounced in the CA3/4 regions, with relative sparing of the CA1 region. The marked increase of the $GABA_A$ receptor in CA1 was found primarily on pyramidal. The same group later reported reduction in perisomatic GABAergic synaptic inputs to pyramidal cells as shown by GAD-65 +ve puncta on pyramidal cell bodies (Todtenkopf and Benes, 1998). These changes were also reported to be marked in CA3 but subtle in the CA1 region.

Which subregion within the hippocampus is most affected? Of all the subfields of the hippocampus, CA2/3 has been shown consistently to have alterations in interneurons (Benes et al., 1998; Todtenkopf and Benes, 1998; Zhang and Reynolds, 2002; Konradi et al., 2011). Deficits in other regions, including the CA1, have also been reported (Zhang and Reynolds, 2002; Konradi et al., 2011).

GABAergic deficits in the other cortical regions: Alterations in GABAergic system in psychotic disorders are not restricted to the hippocampus, and have also been reported in

other temporolimbic structures, such as the entorhinal cortex (Reynolds et al., 2002), and in the prefrontal cortex (Lewis et al., 2005; 2012).

Alterations in various markers of the GABAergic system have been reported in the prefrontal cortex in schizophrenia, particularly in the dorsolateral PFC (Lewis et al., 2005; 2012). There is a general consensus that the PV expressing basket cells are most severely affected of all GABAergic cells, and their function but not density is reduced. Most widely reported changes are: presynaptic changes such as reduction in the expression of Parvalbumin, GAD 67 and GABA transporter (GAT-1); and post synaptic changes, such as an increase in α2 subunit containing $GABA_A$ receptor. Lewis et al have hypothesized that these alterations result in a reduction in GABAergic neurotransmission in the PFC, and these may represent a homeostatic compensatory response by the cortical circuit in response to reduced excitation of pyramidal cells (Lewis et al., 2012).

1.4 Imaging and Cognitive Studies of Hippocampal Dysfunction in Schizophrenia

Hippocampal volume is reduced in schizophrenia.

The hippocampus is one of the most affected regions in schizophrenia. Imaging studies have consistently shown a decrease in hippocampal volume in schizophrenia (Wright et al., 2000; Jarskog et al., 2007; Heckers and Konradi, 2010). The timing of this reduction is unclear. Some studies suggest that volume reduction is present by the time of the first episode (for review see (Heckers and Konradi, 2010)).

Hippocampal volume change in schizophrenia is subtle. While reduction in mean hippocampal volume is consistently associated with schizophrenia, wide variations are reported even in unaffected subjects (for review see (Heckers and Konradi, 2010)). Volumetric measurements of the temporal lobe, particularly the hippocampal formation, have revealed reductions in these areas in subjects with schizophrenia as compared to matched healthy controls (Shenton et al., 1992). Although reduction in hippocampal volume is a robust finding in schizophrenia, the source of this volume reduction is unclear. Earlier studies found evidence for cell loss (for example, Falkai and Bogerts, 1986), while many recent studies have failed to replicate this deficit in either the pyramidal cell layer (Heckers et al., 1991b) or in the extra-pyramidal-cell layers (Konradi et al., 2011).

Brain imaging techniques estimate a resting or basal hypermetabolic state in the hippocampus in schizophrenia

A large number of studies over the years have shown that cerebral blood flow (CBF) and cerebral blood volume (CBV) are correlates of brain function and metabolism (Small et al., 2011). Brain metabolism can be estimated using functional imaging by using various techniques including: 1) Cerebral Blood Flow and glucose uptake (PET and SPECT exogenous tracer techniques as well as by Magnetic Resonance Imaging), 2) endogenous tracer techniques (the oxyhemoglobin to deoxyhemoglobin BOLD fMRI signal), and 3) cerebral blood volume (CBV, relying on exogenous contrast agent fMRI using paramagnetic contrast agents including gadolinium). CBV provides a sub-millimeter resolution functional maps, hence it is preferred for the analysis of hippocampal sub regions. Acute as well as chronic changes in brain metabolism can be measured with these techniques. Multiple studies, using these imaging techniques, have reported increased baseline hippocampal blood flow in schizophrenia (Benes et al., 1998; Jarskog et al., 2007; Schobel et al., 2009; Heckers and Konradi, 2010; Schobel et al., 2013).

Abnormal hippocampal function during cognitive testing in schizophrenia: Patients with schizophrenia show cognitive deficits consistent with abnormal hippocampal circuit function in schizophrenia. In particular, there is clear evidence for deficits in verbal recall and episodic memory (see meta-analysis by (Aleman et al., 1999)). Functional imaging studies have shown that patients with schizophrenia show a loss of dynamic regulation of the hippocampus by cognitive demands (as evidenced by induced increases in CBF) (Heckers and Konradi, 2010). As an example, in a study by Heckers and colleagues (Heckers et al., 1998), patients with schizophrenia showed increased baseline cerebral blood flow (CBF). Hippocampal activity was then measured while the patients performed a word stem completion task, which recruits the

hippocampal in normal subjects. The patients performed poorly on the task as compared to the controls. More importantly, they failed to mount an increase in blood flow during the performance of the task. This and other pieces of evidence suggest that a hyperactive hippocampal network may be functionally deficient.

Recent evidence from clinical studies of amnestic mild cognitive impairment (aMCI) - a high risk state for Alzheimer's dementia- has shown evidence for increased baseline hippocampal hyperactivity, reducing which leads to improvement of hippocampus dependent memory (Bakker et al., 2012).

Dopaminergic Dysfunction in Schizophrenia

Several lines of evidence, from behavioral pharmacology to brain imaging studies, implicate dopamine (DA) dysfunction in psychosis. Below, I present a brief introduction of the dopamine systems in the brain and review the evidence linking dopamine dysfunction with schizophrenia.

A brief introduction to DA systems innervating the forebrain: A majority of dopaminergic neurons in the brain are concentrated in the mesencephalon (Mendez, 2005). Traditionally, mesencephalic dopaminergic regions has been divided into two distinct areas (Mendez, 2005):

1) The substantia nigra pars compacta (SNC)

2) The ventral tegmental area (VTA)

The VTA and substantia nigra pars compacta dopamine cells are present in close proximity to each other and have similar histological structure and cell compositions, however various differences occur in the input and output connections of these regions. It has been suggested

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that there is a dorsal-ventral divide in the DA neurons that stretch across the dorsal substantia nigra pars compacta and contiguous ventral tegmental area (for review see (Bentivoglio and Morelli, 2005; Björklund and Dunnett, 2007; Haber et al., 2012)). The dorsal tier includes a band of neurons stretching across the dorsal substantia nigra pars compacta and contiguous ventral tegmental area. The ventral tier consists of cells of the ventral substantia nigra pars compacta and a corresponding ventral group of ventral tegmental area neurons.

Fibers from these dopamine neurons form three major projection pathways:

1) Nigrostriatal dopamine system: Dopaminergic fibers in this pathway project to the caudate nucleus and the putamen, also referred to as the dorsal or neostriatum. Traditionally, this pathway has been assigned a motor function - due to its association with Parkinson's disease. More recently, the nigrostriatal DA system has also been implicated in associative learning and habit formation.

2) Mesolimbic dopamine system: This pathway projects to cortical and subcortical regions involved in emotional regulation and reward (traditionally referred to as the *limbic areas*). The output regions of the mesolimbic dopamine pathway include: the septum, the hippocampus and the ventral striatum – which includes the nucleus accumbens, the medial and ventral portions of the caudate and putamen, the striatal cells of the olfactory tubercle and the anterior performated substance. This pathway is believed to play a role in motivation and reinforcement learning.

3) Mesocortical dopamine system: This pathway projects to the neocortical mantle with the highest projections to the frontal cortex. This pathway is believed to support cognitive functions.

Previously, it was thought that dopaminergic neurons from the midbrain that project to the

striatum along the nigrostriatal pathway come almost exclusively from the substantia nigra,

while those that innervate the cortex and the ventral striatum come from the VTA (for review see

(Björklund and Dunnett, 2007; Haber et al., 2012)). Recent studies show that the cells of origin

of the three major dopaminergic pathways (discussed above), are intermixed across the

substantia nigra – VTA complex and that this intermixing is especially prominent in primates

(for review, see (Björklund and Dunnett, 2007)). For example, the Substantia nigra contains not

only the neurons projecting to the striatum, but also the neurons that innervate the cortical and

limbic areas. Similarly, the DA neurons of the VTA project to the caudate and putamen (for

review, see (Bjorklund 2007)).
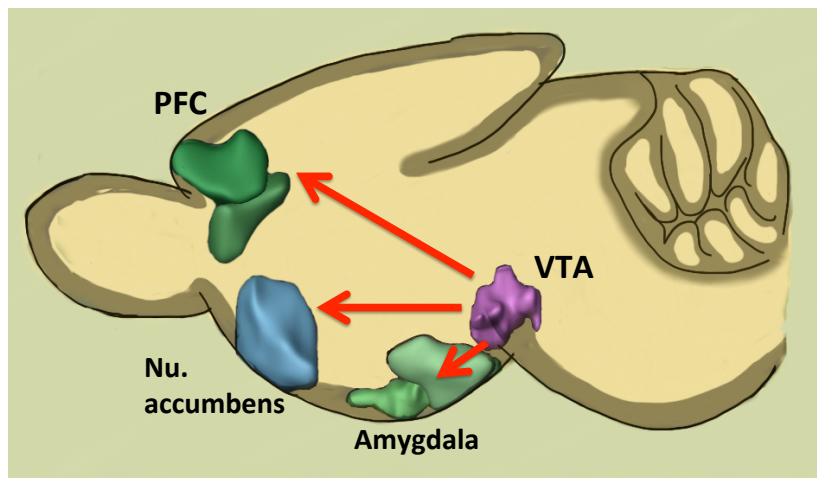
Figure 1-1: Mesolimbic and mesocortical dopaminergic pathways in the mouse brain.
Schematic representation of a coronal section of the mouse brain. Mesocortical and mesolimbic
dopamine pathways are shown. Adapted from (Hyman, 2007). Redrawn using Allen Mouse Brain
Atlas (©2013 Allen Institute for Brain Science).

The original formulation of the DA hypothesis of schizophrenia: excess [striatal] DA in schizophrenia

The original dopamine hypothesis of schizophrenia posited that excessive dopamine activity leads to schizophrenia. The theory is based on two key observations: 1) the relationship between DA D2 receptor blocking properties and the efficacy of drugs such as the antipsychotics; and 2) the pharmacology of amphetamines.

Antipsychotic drugs are antagonists at the dopamine receptors: The first indication of the role of dopamine in schizophrenia came from the serendipitous discovery of antipsychotic property of chlorpromazine, initially designed as an antihistamine (Delay and Denicker 1952, reviewed in Ban 2007). Later, Carlson and Lindquest proposed that hyperactivity of DA transmission was responsible for the positive symptoms in schizophrenia (Carlsson and Lindqvist, 1963). It was found that the antipsychotic action of these medications correlated with the extent of dopamine receptor activity (Seeman and Lee, 1975). Although many of the antipsychotic compounds affect neurotransmitters other than dopamine, D2 receptor antagonism is the unifying property that is shared across all anti-psychotic medications.

Psychostimulants induce dopamine efflux: The second indication that dopamine dysfunction may be involved in psychosis came from the finding that drugs that induce large increases in dopamine efflux, such as amphetamine and cocaine, can produce psychosis in normal subjects, and exacerbate psychotic symptoms in patients with schizophrenia. In addition, patients with schizophrenia are more sensitive to the psychotogenic effects of many DA-enhancing drugs, and show psychotic symptoms at lower doses (Lieberman et al., 1987). While hallucinations and delusions are not usually fully manifested with a single amphetamine administration, repeated high dose exposure in non-schizophrenic subjects may

induce a paranoid psychosis, quite similar to that in schizophrenia (Connell 1958) (for review see (Guillin et al., 2007)).

Evidence for striatal DA abnormalities from brain imaging studies

The DA hypothesis has been supported and expanded by studies using positron emission tomography (PET) imaging methods that are sensitive to amphetamine-induced dopamine efflux in the striatum (Boileau et al., 2006). These studies have used the displacement of radio-labeled [11C]Raclopride to estimate dopamine release. This ligand has high specificity but low affinity for the Dopamine D2 receptor, and is easily displaced by dopamine. Accordingly, amphetamine leads to decreases in [11C]raclopride or [123I]IBZM binding that is proportional to the concomitant increase in extracellular DA, and thus presumably due to DA release (Laruelle, 2000). Using this paradigm, it has been shown that patients with schizophrenia (including drug-naive) show a greater amphetamine-induced DA efflux in the striatum (Abi-Dargham et al., 1998; Laruelle, 2000). This finding has, in turn, been hypothesized to reflect increased phasic activity in the DAergic projections from the midbrain to the striatum (Abi-Dargham et al., 2000). Schizophrenia patients may also have higher tonic (baseline) levels of DA in the striatum. In experiments in which endogenous dopamine was depleted using administration of the tyrosine hydroxylase inhibitor alpha- methyl-para-tyrosine (AMPT), Abi-Dargham and others have found higher baseline occupancy of striatal D2 receptors by endogenous DA in schizophrenia. Consistent with the above data are multiple studies using [18F] or [11C] DOPA showing increased rates of DOPA uptake, a correlate of synthesis in the DA terminal, in the striatum of schizophrenia patients (for review see (Guillin et al., 2007)). Generally, these changes are thought to represent an increased capacity or level of DA release in the striatum, and not an

increase in DAergic innervation of this region since dopamine transporter (DAT) density, which correlates closely with the density of dopaminergic terminals in the striatum, has not been found to be changed in histological and *in vivo* PET imaging studies (for review (Guillin et al., 2007)) (Laruelle et al., 2000).

In addition to presynaptic alterations generally considered to indicate an increase in DA release, there is also *in vivo* imaging and post-mortem evidence for modest but reliable increases in DA D2 receptors in the striatum (Guillin et al., 2007). This effect was initially attributed to the effect of chronic antipsychotic use (Burt et al., 1977; Guillin et al., 2007). Later, PET and SPECT imaging studies found a small (12% in a meta-analysis by Guillin et al (Burt et al., 1977; Guillin et al., 2007)) but significant elevation of striatal D2 receptors in untreated patients with schizophrenia.

The modified dopamine hypothesis: mesocortical hypo-dopaminergia /mesolimbic hyper-dopaminergia

The resistance of both cognitive and negative symptoms to antipsychotic treatment suggested that increased dopamine might not be involved in the development of these symptoms. Functional imaging studies implicated frontal and temporomedial cortex in these symptoms (Guillin et al., 2007). These, and other findings led to the modification of the dopamine hypothesis, which in its current formulation states that the DA systems in schizophrenia are characterized by an imbalance between subcortical and cortical DA systems: subcortical mesolimbic DA projections might be hyperactive resulting in hyperstimulation of D2 receptors and positive symptoms. Additionally, mesocortical DA projections to the PFC might be deficient

(resulting in hypostimulation of D1 receptors), a condition contributing to negative symptoms

and cognitive impairment (for review see Guillin et al., 2007)

1.5 Limitations of Clinical Studies and a Need for Animal Models

Limitations of neuropathological and imaging studies in schizophrenia

Although clinical imaging studies and post-mortem neuropathological studies are valuable in elucidating the neurobiological underpinnings of schizophrenia, their role is limited for various reasons described below:

Clinical findings are correlational: By their very nature, findings of most clinical studies are correlational. It is not possible to find out if a certain alteration found in any one group of patients is pathological and contributes to the disease process, or has nothing to do with the disease process. Any given finding could represent one of 4 possible scenarios (Lewis and Gonzalez-Burgos, 2008):

1.   Cause: An upstream factor related to the pathogenesis of the illness.

2.   Consequence: A deleterious effect of a cause.

3.   Compensation: A response to either a cause or consequence that helps restore homeostasis(partially or completely) in the brain

4.   Confound: A product of factors frequently associated with, but not a part of the disease process (or an artifact of the approach used to obtain the measure of interest).

Effects of confounding factors: It is often not possible to control the various environmental and drug exposures in a particular patient. Furthermore, observations may be made after a

life long disease process. Hence, the possible effect of confounding factors such as drug treatment, co-morbid disease conditions, impoverished lifestyle, poor diet and illicit drug exposures – are often difficult to control in clinical studies.

*Wide variance among samples***:** Wide variations are usually found across different studies, and even within the same study, across different subjects. Although the cause of this variance is not entirely clear, it is possible that the current diagnostic criteria for schizophrenia encompasses several biologically distinct disease entities, resulting in noisy findings.

Limitations of post-mortem studies of GABAergic deficits:

It is evident from the above-mentioned studies that there are various reports of dysfunction in the GABA system in the hippocampus. These findings warrant caution due to various reasons. Firstly, several studies have employed different techniques, ranging from ligand binding, immunohistochemistry and expression analysis employing PCR, microarrays and in situ hybridizations. Secondly, different sub fields of the hippocampus have been reported to have deficits in GABAergic transmission. Various discrepancies exist in the available literature on the subject. Several studies have failed to replicate earlier reports. Reynolds and colleagues reported deficits in PV+ cells all over the hippocampal formation (Zhang and Reynolds, 2002), Benes and colleagues have repeatedly found deficits predominantly in the CA2/CA3 regions, with relative sparing of the CA1 (Benes et al., 1998; Todtenkopf and Benes, 1998). Thirdly, post-mortem studies are conducted in patients with different etiologies underlying their disease. The etiology of reduced PV+ interneuron density in the hippocampus in patients with schizophrenia , is

unclear at the moment, and it remains unknown whether this alteration represents developmental hypoplasia or an atrophic process.

Clinical studies generate hypotheses that require animal models to be fully tested.

In general, neuropathological and brain-imaging findings in schizophrenia lack the strength and consistency to serve as diagnostic markers (perhaps due to the limitations discussed above). However, these findings have led to a number of conceptual models of the pathophysiology of schizophrenia that have been useful in guiding research in animal models. For example, alterations in PV+ interneuron subpopulation in schizophrenia has led to the idea that reduction in the function of these neurons may be involved in the pathophysiology of psychosis (Lisman et al., 2008; Lodge and Grace, 2011). This specific hypothesis can be tested in animal models by selectively targeting PV+ interneurons in selected regions of the brain and then observing the physiological and behavioral effects of this manipulation.

1.6   A Conceptual Model of Abnormal Hippocampal Activity and Dysregulation of Striatal Dopamine in Psychosis

Hippocampal regulation of the VTA dopaminergic system:

There is little evidence for structural or histopathological abnormalities in the dopamine system in schizophrenia (discussed in section 1.4). This has given rise to the idea that the primary dysfunction may lie upstream of the dopamine pathway. One candidate 'dysregulator' of the DA system, particularly the mesostriatal system, is the anterior (ventral in rodents) hippocampus. Evidence for this is summarized below.

> ***Increased locomotor response to amphetamine***: The response to amphetamine (AMPH) is exaggerated in the presence of a hyperactive/ disinhibited ventral hippocampus (vHIPP) (White et al., 2006; Lodge and Grace, 2007). Excitotoxic lesion or inactivation of the vHIPP through local injection of lidocaine leads to reduction in amphetamine response while acute pharmacological excitation of the vHIPP by local injection of *N*-Methyl-D-aspartic acid (NMDA) leads to an increase in locomotor response to amphetamine (White et al., 2006). These data are consistent with the idea that glutamate input from the vHIPP modulates locomotion via the nucleus accumbens (NAc).

> ***Increased VTA DA cell activity***: NMDA mediated activation of the vHIPP leads to increased DA cell population activity (Floresco et al., 2001; 2003). Increased DA population activity has also been observed in animal models that are expected to have hippocampal disinhibition, such as the MAM E17 rat model (Lodge and Grace, 2007), a

developmental rodent model that recapitulates many of the neuropathological features of schizophrenia (Moore et al., 2006). Moreover, vHIPP inactivation reverses elevated DA neuron population activity in the MAM E17 model (Lodge and Grace, 2007).

Increased DA efflux in the nucleus accumbens: Electrical stimulation or NMDA mediated activation of the vHIPP leads to increased DA efflux in the nucleus accumbens (Legault and Wise, 1999; Legault et al., 2000) (discussed below).

Chemical stimulation of the ventral striatum increases dopamine concentration in the nucleus accumbens (NAc) (Legault and Wise, 1999; Legault et al., 2000). Legault et al (Legault et al., 2000) showed that chemical stimulation of the ventral subiculum (vSub) increased NAc DA concentration and that this effect relied on cell activity in the VTA, as inactivation through Tetrodotoxin (TTX) injection eliminated this effect.

Activation of the ventral Subiculum did not increase burst firing of dopamine neurons, however, inactivation of the vSub attenuated bursting. In the study by Todd and Grace (Todd and Grace, 1999) and Floresco et al (Floresco et al., 2001) chemical stimulation of the ventral subiculum (vSub) /Entorhinal cortex (EC) did not affect DA cell firing rate, however blockade of vSub/ EC cell firing by TTX injection decreased the firing rate of DA neurons. Legault et al (2000) however found that half of the VTA DA neurons showed increased DA cell firing by NMDA injection (Legault et al., 2000).

Floresco et al (2001) suggest that vSub exerts its effect on the activity of DA neurons in the VTA via glutamatergic mechanisms localized within the NAc. Glutamatergic receptor blockade in the NAc attenuated the effect of vSub stimulation on DA cell activity (Floresco et al., 2001).

These data suggest that the increase in meso-accumbens DA release after chemical stimulation of

the vSub is mediated in part by increased firing of VTA DA neurons. This idea is supported by previous data that basal striatal DA efflux correlates strongly with DA neuron population activity (Moore et al., 1998; Floresco et al., 2003) but not with either the average firing rate or burst firing level of individual nigrostriatal DA neurons (Moore et al., 1998).

Other studies have shown that vSub stimulation increased the number of spontaneously active VTA dopaminergic neurons (Todd and Grace, 1999; Floresco et al., 2001).

Candidate circuits mediating hippocampal regulation of midbrain DA neuron activity ventromedial striatal DA release?

Various studies have tried to elucidate the neuroanatomical circuit mediating hippocampal regulation of the dopamine neurons. Various elements of the proposed circuits involved in hippocampal regulation of VTA DA cells are described below.

> ***Direct projection of the Hippocampal formation to the nucleus accumbens:*** The CA1 as well as the subiculum projects directly to the nucleus accumbens (Raisman et al., 1966; Kelley and Domesick, 1982) through the fornix. Studies in the rat, using the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHA-L) showed that the subiculum projects not only to the entire nucleus accumbens - which constitutes a major part of the so-called ventral striatum, but also projects to the medial, ventral, rostral and caudal parts of the caudate-putamen complex. The projections to the ventral and caudal parts of the caudate-putamen are prominently derived from the ventral subiculum, whereas the projections to the rostral part of the caudate-putamen are derived from the dorsal subiculum (Groenewegen et al., 1987).

> ***Direct projection of NAc to the VTA***: Various studies have found evidence for direct

NAc projections to the VTA. Earlier work showed that stimulation of the NAc evokes short-latency inhibitory responses in VTA neurons (Maeda and Mogenson, 1980), suggesting the presence of a direct NAc GABAergic projection to the VTA neurons. More recently, a study has found that a significant number of NAc medium spiny neurons (MSNs) directly project to the VTA (Xia et al., 2011). Xia et al expressed the light-sensitive channel rhodopsin-2 in the rat NAc and made electrophysiological recordings from VTA neurons ex vivo (Xia et al., 2011). This study showed that the NAc neurons directly target non-dopaminergic VTA neurons acting thorough the $GABA_A$ receptors. These VTA neurons include non-DA neurons including those that project back to the nucleus accumbens.

Two scenarios could be imagined regarding indirect effect on DA neurons. First, this feedback could indirectly inhibit VTA dopamine neurons if the NAc MSNs target VTA glutamate neurons, which make local collaterals onto dopamine neurons (Dobi et al., 2010). Alternatively, if NAc afferents affect the GABAergic neurons - that make local contacts with dopamine neurons (Omelchenko and Sesack, 2009) - activation of this projection could increase dopamine neuron firing. Such a GABA–GABA–DA loop has been demonstrated in the substantia nigra (Grace and Bunney, 1985; Chuhma et al., 2011).

Nucleus accumbens to VP pathway: The Ventral pallidum receives a dense GABAergic projection from the NAc (Zahm and Heimer, 1990) and in turn provides a direct GABAergic projection to the VTA (Swanson et al., 1984; Zahm and Heimer, 1990) (Swanson et al., 1984; Zahm and Heimer, 1990). Unlike NAc medium spiny neurons, the ventral pallidal neurons show high rates of tonic activity and therefore supply a potent

GABAergic inhibitory influence over DA neuron firing (Yang and Mogenson, 1985; Zahm and Heimer, 1990; Floresco et al., 2003). Stimulation of either the NAc or its glutamatergic afferents (including the vSub) can inhibit VP neuronal firing (Yang and Mogenson, 1985). The decrease in VP activity would then be expected to cause a reduction of the GABAergic inhibition over the VTA, thereby leading to VTA DA cell activity (Yang and Mogenson, 1985; Floresco et al., 2003).

Ventral Pallidum to pedunculopontine tegmental nucleus pathway: The VP sends projections to the pedunculopontine tegmental nucleus (PPTg) (Swanson et al., 1984; Zahm and Heimer, 1990). PPTg sends glutamatergic and cholinergic inputs to the VTA DA neurons, activation of which leads to increased burst firing of VTA DA neuron (Floresco et al., 2003).

VTA to Nucleus accumbens non-DA cell pathway: The VTA consists of DA cells, glutamatergic cells that make local collaterals onto the dopaminergic cells (Dobi et al., 2010) and GABAergic cells that make local contacts with the DA cells (Omelchenko and Sesack, 2009). In addition, GABAergic neurons of the VTA can form long range projections (Carr and Sesack, 2000a; 2000b); a significant proportion of NAc-projecting VTA neurons are GABAergic and stain positive for GAD67 (Carr and Sesack, 2000a; 2000b).

Dorsal CA3 - Lateral septum - VTA pathway: Luo and colleagues, using track tracing and in vivo electrophysiological methods, have identified a novel pathway that mediates hippocampal regulation of VTA dopamine neurons (Luo et al., 2011). In contrast to the pathway from the ventral subiculum, CA3 cells from the dorsal hippocampus project to

the VTA through the lateral septum as a relay. Luo and colleagues showed that stimulation of the CA3 had an excitatory effect on the VTA dopamine (DA) neurons, and inhibited non-DA neurons. (Luo et al., 2011). DA neuron excitation was likely mediated by disinhibition because local antagonism of γ-aminobutyric acid receptors blocked responses to CA3 stimulation. Although retrograde tracers injected into the VTA labelled the dorsal, intermediate and the ventral hippocampus - only those from the dorsal hippocampus seemed to use the lateral septal relay - suggesting that the ventral hippocampus projections to the VTA dont pass through the septum.
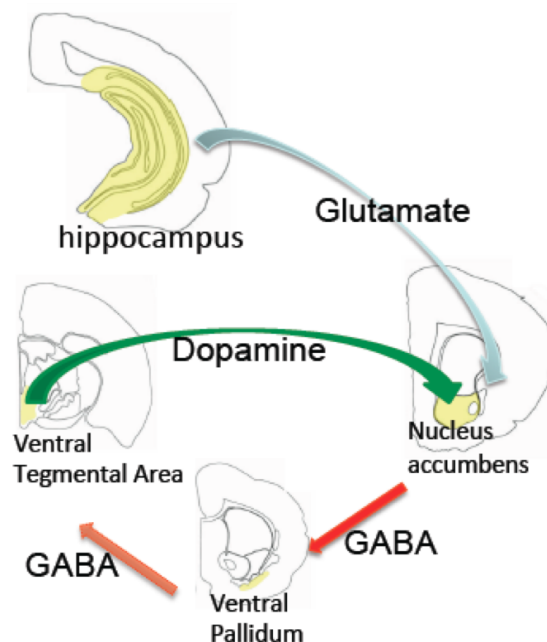
Figure 1-2: Hippocampal regulation of the mesolimbic dopamine system:
Schematic representation of one of the proposed circuits presumed to underlie hippocampal regulation of VTA Dopamine activity. For additional proposed circuits see text.

Regional differences in Dopamine Receptor dysfunction in schizophrenia

Older imaging studies lacked the spatial resolution to differentiate between the various sub-regions of the striatum. Hence it was not clear if regional differences existed in dopamine receptor dysfunction in schizophrenia. The most prominent theoretical model generated from rodent models was that psychotic symptoms were driven by increased dopamine neurotransmission in the 'mesolimbic system' projecting to the ventromedial striatum and that antipsychotic efficacy was achieved through the blockade of ventromedial striatal D2-like receptors. On the other hand, blockade of D2 receptors in the dorsal striatum (dorsal caudate and putamen) was thought to lead to Parkinsonian-like extra-pyramidal side effects of antipsychotic drugs. More recent studies suggest that the striatal subregion in which excess DA release is most tightly correlated to psychotic symptoms is the rostral caudate, part of the 'associative striatum', so named due to inputs from frontal cortical regions involved in cognitive processing more so than movement generation (Joel and Weiner, 2000; Kegeles et al., 2010). Thus, it is likely that there is a DA subsystem that may be differentially important for psychosis. This may be due to unique properties of the neurons in the midbrain that project to the rostral caudate in the primate (e.g. the balance between intrinsic firing patterns and indirect regulation by the hippocampus and limbic basal ganglia) and/or due to local regulation of DA at the terminal region in the rostral caudate. In any case, determining the rodent homologue of this dopamine system will be important for furthering research in this field.

We hypothesize that rostromedial striatum in the rodent, including the medial caudate, has some homology with the rostral caudate of the primate. We further hypothesize that this region is innervated by DA neurons of the VTA and medial substantia nigra (SN) that are, in turn, regulated by hippocampal output through the circuits shown in figure 1-2. A combination of

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tract-tracing methods (with cell-specific markers) and in vivo electrophysiology will be needed to test this hypothesis.

Linking hippocampal interneuron pathology to DA dysregulation in schizophrenia.

The evidence presented above strongly implicates hippocampal hyperactivity in the pathogenesis of psychosis in schizophrenia. Interestingly, evidence from neuropathological literature, presented in the previous section would suggest that PV+ interneuron deficit could produce disinhibition in the hippocampus and could potentially explain the hypermetabolic phenotype seen in schizophrenia patients, as well as the relation of hippocampal over activation producing excess striatal DA and related behavioral abnormalities in rodent models. However, so far there has been no evidence linking GABA interneuron pathology and chronic hippocampal disinhibition to DA system disinhibition. One major aim of this thesis has been to test the plausibility of causal links among GABA interneuron pathology (determined post-mortem), hippocampal disinhibition and related cognitive deficits (assessed by functional imaging and cognitive neuroscience studies), and excess striatal DA and related behavioral abnormalities (assessed in neurochemical PET and pharmacological studies). In the next section, I will briefly describe the necessary background on the important functional features of the GABAergic interneurons and their development and then introduce the mouse models of interneuron deficit used in this study

1.7 Parvalbumin-expressing Interneurons of the Cerebral Cortex

GABAergic interneurons of the cortex

Cortical GABAergic interneurons are neurons that use GABA for fast synaptic neurotransmission.

GABAergic regulation of the level and pattern of activity in cortical projection neurons.

GABAergic interneurons exert a number of effects on their target neurons and cortical network activity. At synapses, GABA is released in an action-dependent manner from the terminals, e.g. of PV+ interneurons synapsing on the soma of a hippocampal projection neuron. GABA at the $GABA_A$ receptor gates a chloride conductance with a rapid onset and a rise time of few hundred microseconds. In most neurons, this chloride conductance hyperpolarizes the membrane or stabilizes it at a resting potential or -60 to -70 mV, the reversal potential of the chloride conductance in neurons. As a result, Cl⁻ flux through the channel can be either depolarizing or hyperpolarizing. In either case, it can act as a shunt for strong hyperpolarizing (inhibitory) *or* strong depolarizing (excitatory) conductances. There are some notable cases in which GABA may depolarize the membrane enough to excite a neuron (Ben-Ari, 2002; Cossart et al., 2005). One example of this is the axo-axonic synapse formed by the PV+ interneuron contact with the pyramidal neuron. In other cells, GABA mediated hyperpolarization may de-inactivate voltage-gated sodium channels, hence leading to renewed excitability of the cell. Overall, however, the common effect of $GABA_A$ –mediated chloride conductances is to stabilize the membrane in a

particular state that in most cases shunts excitatory inputs and makes the neuron resistant to spike firing.

The effect of the GABA-mediated chloride conductance may be thought of as holding the postsynaptic cell 'at the ready', such that when the chloride conductance ceases in the context of active excitatory inputs, the cell fires immediately and robustly. In this way, GABA tone, and intermittent interruptions thereof, can serve to gate inputs to the cell. Information between cortical areas is transmitted via axonal projections of glutamatergic pyramidal cells, but they alone may not produce the required high degree of temporal precision between brain regions. Together with other state-modulating inputs (e.g. monoaminergic inputs from brainstem, midbrain and basal forebrain), GABAergic inputs can reset neurons so they respond more reliably and precisely to a given glutamatergic input. This property of GABA interneuron-mediated inhibition also allows for synchronization of activity across cortical projection neurons (Klausberger and Somogyi, 2008). GABA may also modulate which inputs to a neuron are processed. For example, in hippocampal and other cortical projection neurons, activity of interneurons innervating the distal dendrites can functionally "eliminate" a dendritic segment while leaving excitatory inputs to other portions of the cells unshunted and free to propagate to the soma (Miles et al., 1996; Klausberger and Somogyi, 2008).

Perisomatic- and distal dendrite-targeting inhibitory interneurons play different roles in cortical circuit

Multiple subtypes of GABAergic interneurons exist, which differ in their expression of calcium buffer proteins or neuropeptides, dendritic arborization, firing characteristics, shape and position of the soma and pattern of axonal projections (Freund and Buzsaki, 1996) (Markram et al.,

2004). Broadly speaking, GABAergic interneurons can be divided into two large groups (Miles et al., 1996). These two types of GABAergic cells affect the postsynaptic cell differently and play different roles in the cortical circuit.

> ***Distal dendrite targeting cells:*** These neurons innervate the distal dendrites of pyramidal cells and control the inputs of principal neurons and calcium currents. These cells modulate glutamatergic inputs to pyramidal cells. The activity of these cells can help the cell to control reception of inputs from various sources. (Miles et al., 1996; Buzsaki, 2006).

> ***Perisomatic/ Axon hillock and proximal dendrite targeting cells:*** Neurons that innervate the cell body of pyramidal cells, thereby controlling sodium spikes and the output of the cells. The perisomatic synapses can effectively control the probability as well as timing of action potential spike firing of pyramidal cells.

PV+ cells are fast-spiking, perisomatic-targeting interneurons

An important class of GABAergic neurons express the calcium-binding protein parvalbumin. These cells can be further distinguished from other types of interneurons by properties including morphological and electrophysiological properties (Kawaguchi and Kubota, 1997). PV+ interneurons constitute about a quarter of all GABAergic cells in the cortex (Freund and Buzsaki, 1996).

Morphology and synaptology. Morphologically, PV+ cells can be classified into two major groups based upon their axonal targeting patterns:

1.   **Basket cells:** These cells innervate the perisomatic or axon initial segment of pyramidal cells. This innervation can appear as a 'basket' of PV+ fibers around a pyramidal neuron soma (figure 1-3).

2.   **Chandelier or axo-axonal cells:** These cells innervate the axon initial segment of pyramidal cells. Their axons and terminal formations resemble chandeliers with low-power microscopy.

Electrophysiological properties

Despite morphological differences, the two classes of PV+ interneurons have almost identical intrinsic electronic properties. PV+ Interneurons are distinguished by their ability to exhibit a very high non-adapting spike firing pattern, which along with the perisomatic location of the outputs of PV+ cells ensure a strong inhibition of their target pyramidal cells. Parvalbumin - a calcium binding protein- plays a role in conferring the unique electrophysiological properties to these cells. Parvalbumin accelerates the initial decay of the post-impulse Ca^{2+} transient in the interneurons, without having a major effect on the intracellular Ca^{2+} concentrations at rest or the peak concentration during activation. Reducing or knocking out parvalbumin affects the kinetics of the Ca^{2+} transient and leads to abnormalities in the firing of PV+ interneurons, increased seizure susceptibility and other deficits related to decreased GABAergic function (For review, see (Freund and Buzsaki, 1996; Schwaller, 2010).

Distribution and inputs

PV+ interneurons have a non-uniform distribution across and within cerebral cortical regions. In the hippocampus, more than 80% of all PV+ interneurons are present in the stratum pyramidale and stratum oriens. PV+ Basket cell dendrites receive inputs from all major input pathways such

as mossy fibers, Schaffer collaterals, commissural and entorhinal afferents. In addition, the

recurrent collaterals of glutamatergic projection neurons also synapse on the PV+ interneurons.
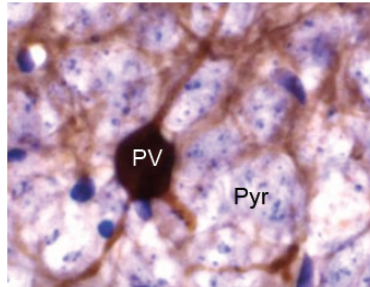
Figure 1-3: Photomicrograph showing PV+ cell body and "baskets" in hippocampus:
A PV-immuostained section of the hippocampus showing a PV-immunoreactive cell body and
PV+neurites surrounding a PV-ve soma (Pyr) presumably a pyramidal cell. The section is counterstained
with cresyl violet

PV+ cells are widely connected in the cortical circuit. Around 25-40 basket cells converge on a

single pyramidal cell in the hippocampus (Buhl et al., 1994). The divergence for axo-axonic cell

is also enormous, with a single PV cell projecting to as many as 1,200 pyramidal cells) (Soltesz,

2006). PV+ interneurons have a large number of asymmetrical (~15,000), symmetrical (~1000)

and electrical synapses (gap junctions) (Freund and Buzsaki, 1996), which may put them in a

position to act as coincidence detectors and 'synchronizers' of cortical activity (see below).

Importance of PV+ cells in cortical function

Several structural features of PV cells make them excellent candidates for their proposed roles as

potent inhibitor and synchronizer of pyramidal cells. Being perisomatic, GABAergic synapses

made by PV cells can mediate a much stronger inhibitory effect on a pyramidal cell's membrane

potential as compared to a distally placed synapse. Similarly, extensive diverging and converging

connections, frequent gap junctions with other PV cells, a fast firing rate and fast rise and decay

kinetics of PV cell IPSCs would be ideal for a role in role in coincidence detection, generation of

network oscillations and network synchronization (Freund, 2003). In the hippocampus, a single chandelier neuron can synchronize the firing of multiple pyramidal neurons (Cobb et al., 1995), suggesting that chandelier neurons may help coordinate and entrain pyramidal neuron firing.

PV+ interneurons are also thought to be critical nodes in the generation of electrical-field oscillations in the cortex. Cortical oscillations represent synchronous activity of a large numbers of neurons. Neural networks in the mammalian forebrain demonstrate several oscillatory bands covering a wide frequency range. Notable of these are: theta (3-6 Hz), beta (15 -30 Hz) and gamma (>30 Hz). PV+ basket cells are ideal candidates for generation of high frequency network oscillations particularly in the gamma band. These cells have a high firing rate, are one of the most abundant GABAergic neuron sub class in the cortex, are highly interconnected by electrical (gap junctions) as well as GABAergic synapses, and are seen to be highly active during gamma band oscillations (Buzsáki and Draguhn, 2004). Computational models have suggested that networks of interneurons particularly the PV+ interneuron subclass, interconnected by GABAergic synapses as well as electrical gap junctions, can generate coherent rhythmic activity and synchronize spiking of pyramidal cells, thereby generating gamma oscillations (Wang and Buzsaki, 1996). This is supported by recent experiments showing that selective stimulation of PV+ interneurons amplifies local field gamma band oscillations selectively in the gamma band, while stimulation of pyramidal cells increases power in lower bands (Cardin et al., 2009; Sohal et al., 2009). Conversely computational models (Volman et al., 2011) and experimental work (Manseau et al., 2010) both suggest that reduced PV expression in interneurons may lead to disruption of gamma band activity.

These findings support the idea that fast-spiking PV+ interneuron activity is critically involved in the generation of gamma oscillations

1.8   Origins of Cerebral Cortical GABAergic Interneurons

Majority of GABAergic neurons are born in the Ganglionic Eminences:

The developing telencephalon can be divided into the pallium (or roof) and the sub-pallium (the floor) regions (figure 1-4). While most cortical projections neurons derive from the ventricular region of the pallium- migrating radially to the cortical mantle, most cortical interneurons originate in the subpallium- migrating tangentially to reach their final position in the cortex and the hippocampus. The developing sub-pallium can be divided into medial, lateral and caudal ganglionic eminences (Anderson et al., 2001). Together the ganglionic eminences give rise to a vast majority of GABAergic interneurons of the cortex. A smaller contributions comes from the adjacent preoptic area -which produces about 10% of the interneurons (Gelman et al., 2011). Production of GABAergic neurons in the ganglionic eminences has been found in rodents such as mice and ferrets as well as in humans.
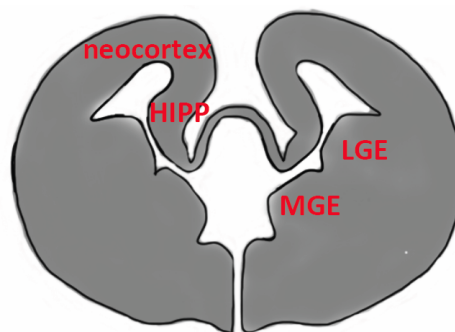
Figure 1-4: A schematic of a coronal section of the embryonic mouse brain showing the ganglionic eminence:
Notice the developing neocortex and hippocampus (HIPP) and the medial (MGE) and lateral (LGE) parts of the ganglionic eminence.

Ventral-dorsal difference in signaling molecule concentration controls neural patterning:

Development of interneurons in the forebrain follows the general rules of neural development, first established in the spinal cord and the hindbrain. This paradigm states that speciation-or development of unique identity of neurons- results from the effect of signaling molecules from certain embryonic structures (such as the notochord), which surround the developing neural tube.

The notochord secretes inducers, such as Sonic hedgehog (Shh), retinoic acid (RA), noggin and chordin, which then diffuse to the neural tube. These chemicals form a concentration gradient along the dorso-ventral axis of the tube. Under the effect of this concentration gradient, different sets of genes are expressed by different parts of the neural tube. The expression of these genes starts a cascade of gene expression ultimately leading to differentiation along distinct paths. (Jessell, 2000). The ventral part of the tube under the effect of Shh assumes a motor fate, while the dorsal part differentiates into sensory neurons (Purves, 2013).

Temporal and spatial factors control interneuron development:

Although many of the details of GABAergic interneuron speciation are not clear, it is generally believed that different subtypes of GABAergic interneurons arise from distinct sets of progenitors within the ganglionic eminence. As in other regions of the developing brain, the initial positional identity of cells in the ganglionic eminence plays a major role in determining the final subtype of interneurons. Each progenitor features a unique set of transcriptional regulators that sets into motion a certain transcriptional blueprint thereby leading to the expression of a particular set of genes-such as ion channels, neurotransmitter receptors,

neuropeptides and calcium binding proteins, etc, that are ultimately responsible for the

molecular, histological and electrical features of an interneuron (Wonders and Anderson, 2006;

Flames et al., 2007; Batista-Brito and Fishell, 2009). In addition to spatial distinctions, temporal

distinctions have also been identified, that is: different classes of interneurons may be born from

the same or similar progenitors at different times (Butt et al., 2005).

The medial ganglionic eminence (MGE) gives rise to a majority of fast spiking PV+ cells and

the regular spiking SST+ cells (Butt et al., 2005). The caudal ganglionic eminence produces

bipolar interneurons that express vasoactive intestinal peptide (VIP) and/or the calcium-binding

protein calretinin (CR), and multipolar interneurons that contain neuropeptide Y (NPY) or reelin

(Xu et al., 2004; Butt et al., 2005). Within the MGE, there is a bias for SST+ interneurons to

originate from the dorsal MGE, and the PV+ interneurons to originate from the ventral MGE

(Flames et al., 2007; Wonders et al., 2008).The lateral ganglionic eminence gives rise to the

projecting medium spiny neurons in the striatum, nucleus accumbens and olfactory tubercle

(Wichterle et al., 2001).

Migration of interneurons:

After development in the GE, the interneurons leave the proliferative zones, migrating

tangentially along the intermediate and marginal zones, and finally radially toward the inner

cortical layers. Motogenic factors such as HGF/ SF (Hepatocyte growth factor/ Scatter factor),

stimulate the cells to leave the ganglionic eminence and begin migration. Disruption of these

signals leads to aberrant migration. For example disruption of uPAR enzyme function, which

controls the activation of HGF/SF, leads to reduction in the number of PV IN in the cortex.

Many factors are thought to guide the interneurons on their way to their final destination, one of

which is Neuregulin/ErbB4 signaling. ErbB4 is expressed by certain populations of developing interneurons while the migratory route expresses its receptor Neuregulin-1 (Flames et al., 2004).

GABAergic system may be particularly susceptible to risk factors:

GABAergic cells follow a protracted course of development. GABAergic interneurons migrate long distance to reach their final target in the cortex. Moreover, the migration of IN, development of synaptic contacts and their subsequent pruning and maturation takes place later than for most other types of neurons. Parvalbumin protein is not expressed completely till later 2nd/ early 3rd week in mice. Similarly, studies in macaques have shown that pruning of GABAergic synapses continues well into adolescence (2-4 years after birth).

This may make the GABAergic system particularly vulnerable to a wide variety of environmental insults during perinatal period (maternal infection, obstetric complications, maternal starvation), through childhood and during adolescence (stress, drug exposure such as cannabis, etc). These factors may influence the development or migration of interneurons, as well as the development or pruning of GABAergic synapses. These explanations are consistent with a neurodevelopmental view of schizophrenia (Weinberger, 1987).

1.9 Introduction to the Cyclin D2 Null Mouse Model of GABAergic Interneuron Deficits

In the following section, I briefly review the rationale behind using an animal model to investigate human disease. I then present a brief introduction to the cyclin D2 null mouse model, which I have used in my research.

Characterization of "cortical PV interneuron deficit" models, particularly with regard to disinhibition of the hippocampus and its impact on behavior, is important for increasing our understanding of the potential significance of cortical PV interneuron deficits in schizophrenia.

A number of manipulations in animals designed to model aspects of the etiology or pathophysiology of schizophrenia affect PV+ interneuron number or function (Penschuck et al., 2006; Harte et al., 2007; Berretta et al., 2009; Schobel et al., 2013). However, one important limitation in understanding the significance of PV+ interneuron deficits in schizophrenia has been the lack of animal models of a selective and partial PV+ interneuron deficit.

Role of cyclin D2 in GABAergic interneuron development

> ***Cell cyclins play a pivotal role in cell division***: Control of cell division plays an
> important role in brain development. One of the key regulators of cell division is the class
> of proteins called the cell cyclins, called so because of the cyclical nature of their
> production and degradation. The cyclins form activating subunits of the cyclin dependent
> kinases (Cdks). The cyclin- cylin dependent kinase complex phosphorylates important
> proteins in the cell, which in turn lead the cell forward through the cell cycle (Alberts et
> al., 2002). More than a dozen different cyclins have been discovered and classified into

multiple classes. Cyclins of type A, B1 and B2 control the advance through the G2 phase of the cell cycle. Cyclins D1, D2 and D3 control the progression through the mid G1 restriction point and cyclin of the E type regulate the G1/S transition. Although there is redundancy in each type of cyclin, the expression pattern of different cyclins does not always overlap, leading to subtle but definite deficits in knockout conditions (Alberts et al., 2002). The activity of Cdks is negatively regulated by cyclin dependent kinase inhibitors (CDKIs) such as p21, p27 and p57 (Alberts et al., 2002).

Cyclin D2 regulates cell division: Cyclins D proteins interact with cyclin dependent kinases (Cdks) 2, 4, 5, and 6. In proliferating cells, cyclin D-Cdk4/6 complex accumulation is of great importance for cell cycle progression. After formation, Cyclin D-Cdk4/6 complex partially phosphorylates the retinoblastoma protein (Rb), which is then able to induce expression of genes such as the E class cyclins, which are important for progression through the S phase of the cell cycle Alberts et al., 2002).

The role of cyclin D1 and D2 in GABAergic interneuron development: In the brain, control of cell proliferation and speciation plays a major role in brain size, architecture and function. The duration of cell cycle, and notably of G1 phase, determines the balance between expansion of progenitor pools and neuronal differentiation (Calegari, 2012). Cyclin D1 and cyclin D2 are two G_1-phase active cyclins, with non-overlapping patterns of expression in the MGE (Glickstein 2007). GABAergic neurons may arise from either the ventricular zone (VZ) or the subventricular zone (SVZ) of the ganglionic eminence.

Asymmetric neurogenic divisions occur in the Ventricular zone, this yields a neuronal daughter cell and a replenished progenitor cell. Symmetric neurogenesis occurs mainly in the SVZ, in which both daughters develop into neurons (Glickstein 2009).

CD2 is important for the expansion of intermediate progenitor pool: cD2 is expressed as the GABAergic progenitors in the MGE transition from radial glial cells (which stain positive for Pax6) in the VZ to the intermediate progenitors (which stain positive for Tbr2) in the SVZ. Here the intermediate progenitors undergo 'transit amplifying' divisions resulting in 2 daughter neurons. cD2 is important for the expansion of the intermediate progenitor cell pool through these transit amplifying divisions. Loss of cD2 is associated with reduced proliferation of both the radial glia like progenitors and the intermediate progenitors, as well as enhanced exit through the cell cycle (Glickstein 2007, 2009).

Partial depletion of the intermediate progenitors in the cD2 null shows that cD2 is not an absolute requirement for the formation of these cells, however in its absence there is depletion of these intermediate progenitors (Glickstein 2009). cD2 affects phosphorylated retinoblastoma protein (pRb) and p27, two key regulators of the cell cycle. p27 is increased, while pRb is decreased in the SVZ in cD2 -/-. The increased expression of p27 and decreased expression of pRb in cD2null is consistent with a slower progression through the G_1-phase in the absence of cD2 (Glickstein 2007, 2009).

Although the same progenitor cells are thought to give rise to both the PV+ as well as SSN+ neurons in the MGE, the cD2 null mutation leads to a selective reduction in PV+

neurons (see below), suggesting that the 'transit amplifying' cell divisions in the MGE

preferentially give birth to PV cells (Glickstein 2007).

Selective deficit in parvalbumin interneuron density in cD2 null mice

Using the cD2 null mouse, Glickstein and colleagues (Glickstein et al., 2007) showed that the there is a relatively selective reduction of PV+ interneuron density. The PV+ interneuron deficit is static and does not increase with age in this model (Glickstein et al., 2007). Stereological counting of other interneuron populations in immunostained sections did not show deficits in SST. No qualitative difference was seen in the density of interneurons positive for Neuropeptide-Y (NPY), Vasoactive Intestinal Peptide (VIP), Calretinin (CR) or Calbindin (CB) (Glickstein et al., 2007).

Reduction in cell bodies immunoreactive for Parvalbumin could arise from reduction of PV expression in PV cells. One possible solution to this problem is to test for GABA/PV co-immunolabeling. However, previous experience points out that the immunostaining of GABA is notoriously unreliable. GABA is a small neurotransmitter, which is not readily preserved in formaldehyde fixed tissue. Although GABA preservation is better in Glutaraldehyde fixed sections, it can still be unreliable and hence unfit to rule out small differences in cell densities across different groups of mice.

Glickstein et al (Glickstein et al., 2007) used a mouse strain that expresses GFP in a subset of PV+ interneurons (GAD67eGFP) (Ango et al., 2004) . The GAD67eGFP mouse expresses GFP under the effect of GAD 67. This mouse was bred with the cD2 line to generate cD2+/+::GAD67eGFP and cD2–/–::GAD67 eGFP mice. Stereological counting of GFP +ve cells showed a reduction in GFP+ cells in the cD2 null. Moreover, in PV/GFP co-immunostained sections, no increase was found in PV-ve - GFP+ cells. These data strongly argued that the reduction in PV+ cells in the cD2 null was due to a reduction in PV cell number and not loss of PV expression in a subset of PV+ cells (Glickstein et al., 2007).

Other effects of cD2 null mutation on brain structure and function

Deficient adult neurogenesis in DG and olfactory bulb: Various studies have reported an almost complete depletion of adult neurogenesis in the dentate gyrus (Kowalczyk et al., 2004; Jaholkowski et al., 2009, personal observation, data not shown). Kowalczyk et al. (Kowalczyk et al., 2004) showed that neurospheres grown from in vitro expansion of neuronal precursors from adult hippocampi express solely cyclin D2, whereas the neurospheres derived from 5 day old hippocampi express all three cyclins D. These findings suggest that cD2 is the only D-type cyclin (out of D1, D2, and D3) expressed in dividing cells derived from neuronal precursors present in the adult hippocampus, perhaps explaining the near absence of adult neurogenesis in the cD2 null but not in the cD1 null mice.

Microcephaly and cortical thinning: Overall brain size is reduced by about 20% in the cD2 null due primarily to cortical thinning (Glickstein et al., 2007). There is evidence for disproportionate effect of the mutation on certain brain regions, including the olfactory bulb, the hippocampus, cerebellum, amygdala and the entorhinal cortex (Kowalczyk et al., 2004). No difference in neuronal packing density was found in the somatosensory barrel field cortex (Glickstein et al., 2007; 2009). The superficial layers of the cortex were affected disproportionately as compared to the deeper layers - both in packing density as well as in PV cell density. Disproportionate effect of this mutation on superficial layers supports the idea that cD2 gene is required to maintain the progenitor pool and/or support later neurogenic events (Ross and Risken, 1994; Glickstein et al., 2007; 2009).

Hypomorphic cerebellum: A prominently affected region in the cD2 null is the cerebellum, which shows a disproportionate reduction in size in comparison to the overall size of the brain. Histological analysis of the cerebellum shows severe loss of granule cells and a near total ablation of stellate interneurons (Ross and Risken, 1994; Huard et al., 1999). The stellate cells are the last generated interneuron subtype in the cerebellum. In the mutant cerebella, young post mitotic interneurons undergo severe delay of their maturation and migration. The progenitor pool is precociously exhausted and the number of interneurons is significantly reduced. (Glickstein et al., 2007; 2009, Leto et al., 2011).

Role of cell cycle proteins in post-mitotic neurons

CD2 shows high level of expression in various regions in the developing brain. In addition to high level of expression in the MGE, cD2 is expressed in the cerebellum, dorsal mesencephalon, cerebral cortex and epithalamus (Ross and Risken, 1994; Allen Institute, 2013; Gensat-Project, 2013).

CD2 expression is much limited in the post-natal brain. Various studies have found that in the post natal brain, cD2 mRNA is largely limited to the cerebellum (Ross and Risken, 1994; Schmetsdorf et al., 2007; Allen Institute, 2013). Other studies have reported cD2 expression in the olfactory bulb, the sub-ventricular zones, the dentate gyrus and CA3 (Greene et al., 2007; Allen Institute, 2013).

While most neurons lose the ability to undergo cell division, the expression of many cell cycle genes continues. Schmetsdorf at al reported that the adult mouse brain continues to express various cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinase

inhibitors (Schmetsdorf et al., 2007). Furthermore, CDKs are properly complexed to their respective cyclins and exhibit kinase activity (Schmetsdorf et al., 2007). These findings suggest that in post mitotic cell, these proteins may have a cell cycle independent role. Several studies have suggested that cell cycle related proteins may be involved in memory formation and their dysregulation may be associated with cell death in degenerative diseases (Arendt et al., 2000; Greene et al., 2007).

With respect to cyclin D2, the major cdk partners are cdk4 and cdk 6 (Alberts 2002), although negative regulation of cdk5 by cyclin D2 has also been reported (Guidato et al., 1998).

Inappropriate expression of cdk4 has been seen to be involved in cell death during disease and trauma (Greene et al., 2007). Studies of Alzheimer's disease have shown over-expression of cyclin D, cdk4 and an increase in phosphorylated Retinoblastoma protein (pRb) (Arendt et al., 2000).

Similarly, cdk5 has been found to play important roles in post-mitotic neurons. Cdk5 has been implicated in memory formation and plasticity in hippocampal neurons (Angelo et al., 2006; Guan et al., 2011). Some of these effects may be explained by the findings that in post mitotic neurons, cdk5 phosphorylates neurofilaments and tau protein (Angelo et al., 2006). Loss of cdk5 function in the hippocampus leads to severe impairments in memory formation and retrieval, while gain of function mutations of cdk5 have been associated with increase synapse number and increased long term potentiation (Angelo et al., 2006).

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In light of these findings, the possible role of cD2 null mutation on post-mitotic neurons

needs to be studied in detail.

1.10 Main Hypotheses of the Study

The cD2 null mice with a relatively selective deficit in PV+ interneuron density - the magnitude of which is greatest in the hippocampus - provides a unique opportunity to test the hypothesis whether a loss of PV+ interneurons in the hippocampus leads to hippocampal disinhibition, increased excitation, and dysregulation of the mesostriatal dopamine system.

First, I show that the cD2 null mutant mouse exhibits reductions in PV+ interneurons and GABA-mediated synaptic inhibition (Chapter 3). I then describe a study utilizing imaging of cerebral blood flow, a measure frequently used in schizophrenia, to see whether reduction in PV+ interneuron density is associated with signs of hippocampal hyperactivity *in vivo* (Chapter 4). I then present experiments showing that hippocampal disinhibition can cause dysregulation of the VTA DA neuron activity and related behavioral effects (Chapter 5). Following this, I present the evidence that hippocampal disinhibition leads to deficits in hippocampus-mediated cognition in the cD2 null (Chapter 6). Lastly, I present experiments in which I used transplants of GABAergic precursors derived from the embryonic brain (Chapter 7), to study the effect of restoring PV+ cells in the hippocampus on the psychosis-relevant neurophysiological and behavioral phenotypes of the cyclin D2 null mutant mouse.

Chapter 2: General Materials and Methods

Animals and Treatments:

All experimental studies were approved by the New York State Psychiatric Institute Animal Care and Use Committee following USDA Regulations. Cyclin D2 knockout mice (cD2 null),in which exons I and II of the gene were disrupted (Sicinski et al., 1996), were maintained on a C57/BL/6 background under standard housing conditions. Heterozygous (cD2+/–) mice were bred to obtain wild type (CD2+/+) and homozygous null littermates (cD2–/–).

For the Six3Cre:Smofl/fl mouse model, transgenic mice expressing Cre recombinase under the control of a 9-kb genomic promoter fragment of the Six3 gene (Furuta et al., 2000) were mated with LoxP-smoothened mice (Dassule et al., 2000) to generate Six3Cre:Smofl/fl mice. Cre negative Six3Cre:Smofl/+ and Six3Cre-negative:Smofl/fl littermates were used as controls. Mice were maintained on a C57BL/6J background.

All animals were maintained on a 12 h light/dark cycle (lights on at 6:00 A.M.) and given ad libitum access to food.

Tissue processing and immunohistochemistry:

Mouse brains were perfused and post fixed for 1 hour in 4% Paraformaldehyde (PFA) in 0.1M Phosphate buffer (PB). These were then successively transferred to 10, 20 and 30% sucrose (in PB) over three days at 4o C.

For all animals, the brain was sectioned in its entirety according to the principles of systematic random sampling. Briefly, sectioning was initiated at a random starting point, 40 micron thick sections were then collected serially into 5 equal sets (i.e. with a sectioning interval of 5). Sets were processed with the stains described below.

For Diaminobenzidine (DAB) staining, brains were processed as follows: Sections were incubated in anti-parvalbumin antibody (α-PV-235, Swant, Switzerland; 1:1000) for 24 hours, followed by incubation in goat anti-mouse IgG (1:200 in PB containing 0.1% BSA and 0.25% Triton X-100) and avidin-biotin-peroxidase complex (Vectastain Elite Kit; 1:100 in PB; Vector Laboratories, Burlingame, CA). DAB was used as a chromogen. Immunohistochemical stains for all genotypes were processed in parallel to control for inter-experiment variability.

For fluorescent staining, primary antibodies included anti-mouse PV (Swant, 1:1000), anti-rat somatostatin (Chemicon/Millipore, Temecula, CA 1:200), anti-rabbit GABA (Sigma-Aldrich, St Louis, MO, 1:1000) and anti-rabbit or anti-chicken GFP (Molecular Probes/ Invitrogen, 1:1000). Secondary antibodies tagged with Rhodamine Red, DyLight 488, Dylight 405 and DyLight 647 were used (all from Jackson ImmunoResearch, West Grove, PA, used at 1:200 concentration).

Cell quantification:

For all experiments the PV+ interneuron numbers and density were obtained by two-dimensional modified stereologic counting methods, using a Zeiss Axioplan2 microscope (Oberkochen, Germany) interfaced with Stereoinvestigator (MicroBrightField, Williston, VT). Interneurons were counted in CA1 region of hippocampus, and the PFC, using previously defined boundaries (Paxinos and Franklin, 2001). Cells were counted in one every 5th stained section (one 40 µm

section per 200 μm) throughout the rostro-caudal extent of the hippocampus. Since PV+ interneurons have a non-random distribution in the hippocampus, grid and counting frame sizes were selected so as to cover most of the area of the region of interest. For each animal, cell counts were summed across all sections. Density was calculated by dividing the total cells counted by the average area of the region of interest as determined with the Cavalieri estimator. Density was compared between genotypes using Independent (Student's) T-tests.

In-vitro electrophysiology

cD2 −/− and sex matched cD2+/+ litter-mates, ages 3-6 weeks were anesthetized with ketamine/xylazine anesthesia (90/10 mg/kg), decapitated and immediately transferred to ice cold solution containing (in mM) 220 Sucrose, 10 D-glucose, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 2 MgCl2. Tissue block containing the brain region of interest (either the hippocampus or the PFC) was mounted on a vibratome (Leica VT1200, Leica Microsystems, Wetzlar, Germany), and 400 μm thick horizontal slices were made. Coronal sections were made for the PFC while horizontal slices were made for the hippocampus. Slices were immediately transferred to a holding chamber with oxygenated artificial cerebrospinal fluid containing (in mM) 124 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 1 CaCl2, 1 MgSO4 and 10 D-glucose. After at least 1 hour of incubation, each slice was transferred to the recording chamber, perfused with oxygenated aCSF at 2-3 ml/minute with a gravity-fed system. All incubations and recordings were done at room temperature (24ºC).
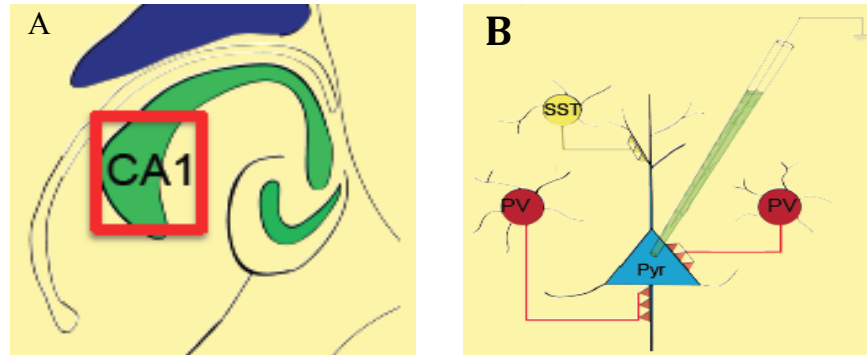
Figure 2-1: Basic scheme of the current clamp experiment:
 A) Schematic representation of a horizontal section of ventral hippocampus showing CA1 pyramidal cell layer. **B)** Schematic representation of a simplified pyramidal cell-GABAergic interneuron circuit in the CA1. Proximal targeting Parvalbumin expressing (PV) and distal dendrite targeting somatostatin expressing (SST) GABAergic interneurons are shown flanking the pyramidal cell. An electrolyte filled glass electrode connected to voltage/current clamp equipment is also shown.

Pyramidal cells were identified in the PFC or the CA1 region of hippocampus using an upright fixed-stage microscope (Zeiss Axioskop FS; Zeiss, Thornwood, NY) with Nomarski optics. Whole-cell voltage-clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA). Data were filtered at 5 kHz, digitized at 10 kHz, and stored directly to disk using a Labmaster TL-1 DMA acquisition board (Molecular Devices). Recording pipettes, pulled from borosilicate glass tube (1.2 mm external diameter), with 3-5 MΩ resistance, were filled with internal solution containing (in mM) 140 CsCl, 2 MgCl2, 0.1 CaCl2, 10 HEPES, 1 EGTA at pH 7.3 (adjusted with NaOH) and connected to Ag/AgCl electrode attached to headstage of AxoClamp 2B amplifier (Axon Instruments, Foster City, CA). The series resistance was estimated by applying voltage steps of 5 mV at the beginning and the end of each recording session. Access resistance was periodically tested, and the cell was discarded if the access resistance changed by more than 10% or becomes >20 MΩ. Series resistance was typically between 20 and 40 MΩ and were compensated offline to avoid adding excessive baseline noise.

Currents were measured using whole cell voltage clamp at -70mV, at which GABAergic currents were expected to be inward. GABAergic nature of current was confirmed by bath application of gabazine (20μM, SR 95531 hydrobromide) in a selected set of recordings. Data was recorded using custom written macros in Igor Pro (WaveMetrics, Lake Oswego, OR). Five second sweeps (100 in total) were collected and saved individually as Igor binary files. All sweeps were concatenated and converted to Axon Binary Files using ABF Utility software (Synaptosoft, Leonia, NJ). Analysis was done in MiniAnalysis software (Synaptosoft). LoPass Elliptic filter with a cutoff frequency of 1000Hz was applied before analysis to filter out high frequency noise. GABAA miniature inhibitory postsynaptic currents (mIPSCs) were isolated with the addition of tetrodotoxin (TTX, 1.0 μM), 2-amino-5-phosphonopentanoic acid (5-AP, 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 40 μM) in perfusion solution. Threshold amplitude for mIPSC detection was set to three times the root mean square of noise. Other criteria to detect peaks were inward direction of peak, suitable area threshold, a maximum limit for decay time, rise time shorter than the decay time, etc. Events picked up by the program were visually inspected to weed out aberrant events. Total average mIPSC frequency, amplitude, 10-90 rise time and decay time were calculated, frequency distributions were obtained. At least 100s of record was analyzed for each cell.

For miniature excitatory post synaptic current (mEPSC) measurement, Gabazine (10 μM) replaced AP5 and CNQX. The internal solution contained 140 mM potassium gluconate and a holding potential of -80mV was used.

Event frequency, amplitude and decay time for mIPSCs or mEPSCs were averaged across cells for each subject. Each characteristic was compared across genotypes using Independent

(Student's) T-tests. Multivariate analyses including frequency, amplitude and decay times as variables yielded results consistent with the T-tests.

In vivo imaging

Previously described (Moreno et al., 2006; Schobel et al., 2013) design and procedures of imaging were used. Briefly, four sets of axial T2-weighted images were acquired sequentially to generate high-resolution (86μm×86μm) cerebral blood volume (CBV) maps of the rodent brain. Each set consisted of 24 images, acquired over 16 min. The contrast agent Gadodiamide was injected (13 mmol/kg i.p.) after a pre-contrast set was acquired. As previously described (3), rCBV was mapped as changes in the transverse relaxation rate (R2) induced by the contrast agent. rCBV maps were measured from steady-state T2-weighted images as CBV R2 = ln(Spre/Spost)/TE, where TE is the effective echo time, Spre is the signal before the contrast administration, and Spost is the signal after the

Spost is the signal after the regions of interest used for rCBV measurement are outlined. HP, hippocampus, mPFC, medial prefrontal cortex, CBL, cerebellum.
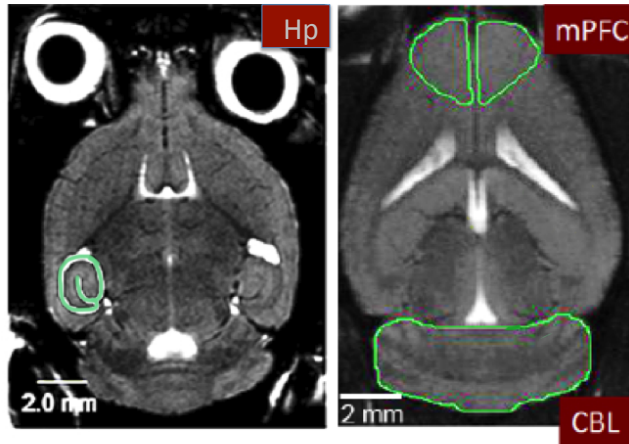
Figure 2-2: Structural MRI of the mouse brain.
Regions of interest: hippocampus (Hp), medial prefrontal cotex (mPFC) and cerebellum (CBL) are outlined.

contrast agent reaches steady-state. The derived maps were normalized to the maximum 4-pixel

signal value of the posterior cerebral artery. Visualized anatomical landmarks were used together

with standard atlases (Paxinos and Franklin, 2001) to define the region of interest. The

hippocampal region of interest included the CA fields, subiculum and dentate gyrus with the

ventral border approximated to be the dorsal border of the medial entorhinal cortex. The borders

of the PFC region of interest extended anteriorly and laterally from the genu of the corpus

callosum to the pial surface at about 1.5 mm lateral to the midline; slices at and dorsal to the

dorsal surface of the caudate were used. For the cerebellum, the entire structure was included.

Independent t-tests were used for comparisons of hippocampal, medial prefrontal and cerebellar

rCBV between genotypes. For comparisons between live- versus killed-cell MGE transplanted

mice, the 'cerebellar-corrected hippocampal rCBV' was defined as the ratio of

hippocampal:cerebellar rCBV values. This measure was compared across transplant groups with

independent (Student's) t-tests.

*In vivo single-unit recordings of dopamine neurons***:**

Stereotaxic surgery and single-unit extracellular recording and neuron sampling methods were adapted from Moore et al (Moore et al., 1998) for the mouse. Mice were anesthetized with chloral hydrate. Electrodes pulled from glass pipettes (tip diameter approximately 1 μm; impedance 4-10 MΩ) were filled with 2M NaCl and stereotaxically lowered into the ventral tegmental area (VTA) using the following target coordinates (relative to Bregma and dural surface: 3.3 mm posterior (AP), 0.8 mm lateral (L) and 3.6-4.2 mm ventral (DV). The electrode was lowered slowly through the midbrain to detect and characterize spontaneously active neurons. The signals from individual neurons were processed with previously-described recording methods (Moore et al., 1998). DA neurons were identified by the long-duration, triphasic waveform with the "somatodendritic notch" on the positive phase (Fig. 2D) and by their tonic irregular firing and intermittent bursts (Fig. S3B). Bursts were defined as per the criteria of Grace et al (Grace and Bunney, 1984a; 1984b) and described in (Mameli-Engvall et al., 2006). The onset of bursts was marked by an inter-spike interval (ISI) less than 80 msec; the first spike thereafter preceded by an ISI of greater than 160 msec was considered the last spike in the burst. Within bursts on average, spike amplitudes decreased as ISI lengthened (Fig. S3B). DA neurons were sampled in 4 recording tracks arranged in 0.15 mm-spaced grid within the VTA. A coronal section from a representative case, showing histological evidence of several tracks at approximately 3.5 mm posterior to Bregma (Fig. S3A). DA neurons were defined as spontaneously active if spiking was detected prior to the electrode moving proximal to the neuron and if the firing rate of the neuron was stable for at least 2 min after discrimination. The number of spontaneously active DA neurons per track and the average firing rate and proportion of spikes fired within bursts for each DA neuron were quantified. Average spontaneously-active

DA cells per track was compared between cD2 –/– mice and their cD2+/+ littermates with an independent (Student's) t-test.

Behavioral experiments:

cD2 –/– mice and sex matched CD2 +/+ littermates 2.5- 4 months of age were used for behavioral testing. Mice were habituated to handling but were otherwise behaviorally naïve for each experiment except for MGE transplant mice, for which contextual fear conditioning followed a few days after locomotor response.

Open field:

Locomotor activity was measured in a 17" x 17" open field box with clear walls and white floor, fitted with computer-interfaced infrared motion sensing system (Med Associates, St. Albans, VT). Mice were placed in open field for 30 minutes, after which, amphetamine (2 mg/kg dissolved in isotonic saline at 0.2 mg/ml) was injected i.p.) and activity was measured for another 60 minutes. Total activity for successive 5 min bins was analyzed. Two separate cohorts were tested many months apart and very similar results were obtained.

A mixed ANOVA design followed by planned comparisons was used, with genotype and drug as factors, and time (before or after injection) as a repeated measure. Planned comparisons used independent (student's) t-test. of genotypes within drug condition separately for baseline locomotion and locomotion after drug or saline injection.

Contextual fear conditioning:

Mice were acclimated to the testing room 1 hour prior to the training/testing session. Two plexiglass chambers with shock grid floors placed within a white melamine sound-attenuating chamber were used as training/testing apparatus. Each chamber featured a distinctive set of visuospatial, tactile and odor cues, which together defined the context. For the training session, mice were placed in a distinct context (Context A). Conditioned stimulus (CS) consisting of pure tones (85 dB, 20 sec duration, 4.5 kHz) were presented at 300, 470, 580, 670 and 840 seconds. During the last second of each tone, a 0.7 mA scrambled current was delivered through the floor grid. Animals were returned to their home cages 980s after the start of the experiment.
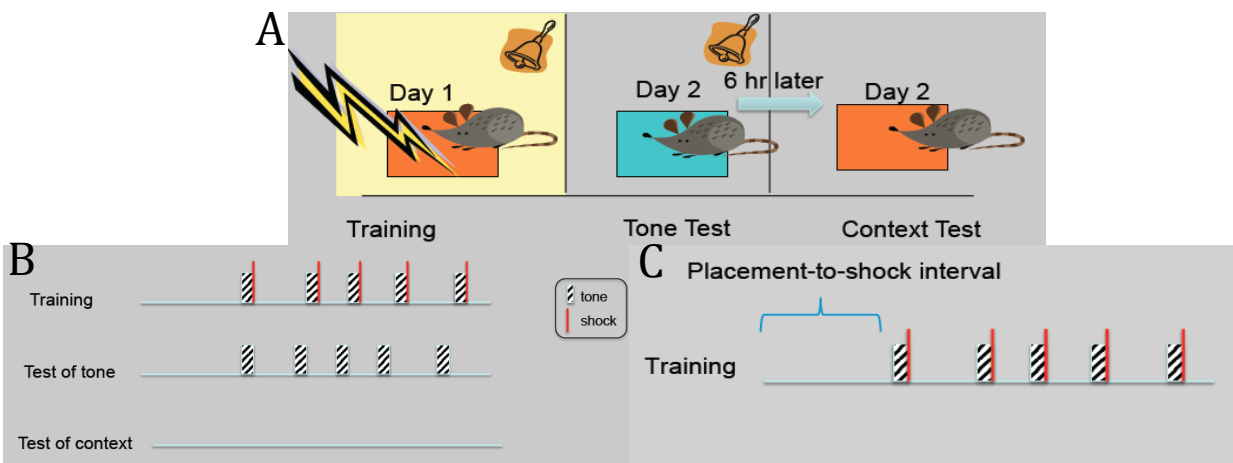
Figure 2-3: Summary of the overall design of the fear conditioning paradigm.
A, B) Schematic representation of the overall experiment, showing training, test of tone and test of context phases of the experiment. Each auditory tone was 20 sec in duration and co-terminated with a 1 sec electric shock. C) Fear conditioning experiment in which placement to shock interval was systematically varied during training

Twenty-four hours later, each mouse was placed in a novel context (Context B) that was constructed with visual, tactile and olfactory cues with a high level of contrast with Context A. In Context B, mice were exposed to the conditioned tone (CS) only (without shock) at 300, 410,

580, 670 and 830s. Six hour after being tested in the novel context B, mice were placed in the training context for 600s. During training and testing sessions, freezing was measured using an automated video monitoring system (Med Associates, St. Albans, VT). Freezing was defined as absence of movement except for respiration. Additional parameters for measuring freezing were bout duration of 0.25s and motion index threshold between 2 and 5.

An average conditioned freezing response (the CR) was calculated for three retrieval "phases": initial response to the tone CS+ (tone CR), freezing persisting from 40-100 sec following offset of the CS+ (post-tone CR) and freezing in response to the training context (context CR). For statistical analysis, a mixed ANOVA design was used with retrieval phase as the repeated measure and genotype as the between-subjects factor.

Partial lesion of the caudal hippocampus or parietal cortex:

Mice were anesthetized with isoflurane anesthesia and placed mounted on a stereotaxic frame. Following aseptic preparation of the skin and skull, small burr holes were drilled through the skull. The coordinates used to target ventrocaudal CA1 in wild-type mice were (relative to bregma and skull surface) AP -3.0, L +/- 3.5, DV -2.9. For the cD2 –/– mice, coordinates were modified to adjust for smaller brain size; the coordinates were AP -2.9, L +/- 3.3, DV -2.7. For parietal cortex cannulations, the coordinates used were AP -2.9, L +/- 3.5, DV -1.4 for CD2+/+ and AP -2.9, L +/- 3.3, DV -1.25 for cD2 –/–. To induce partial lesions to cortical regions, custom made steel cylindrical poles (outer diameter 0.64mm, BD, Franklin Lakes, NJ) were lowered to the right depth through the skull hole and tissue was mechanically disrupted by a cannula that was then held in place using dental cement. The cannulae were left in place for 1

week prior to behavioral testing. Pilot experiments showed that this manipulation led to a

consistent and selective lesion of the hippocampus. The lesion sites were confirmed

histologically.
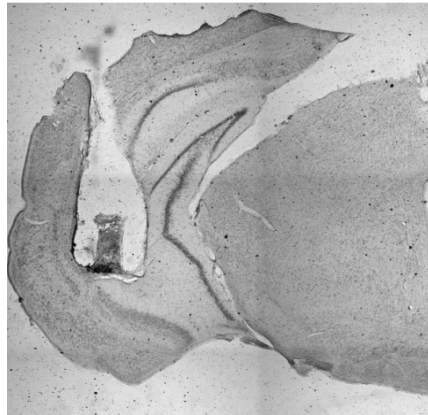
Figure 2-4: Coronal section of the hippocampus showing a representative example of hippocampus lesion.

Dissection, dissociation and transplantation of progenitor cells from the MGE

Transgenic mice expressing Green Fluorescent Protein (GFP) driven by chicken β-actin

promoter (FVB.Cg-Tg(CAG-EGFP)B5Nagy/J Stock Number 003516) (Hadjantonakis et al.,

1998), were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on a CD1

background. Breeding pairs, a homozygous pan-Green Fluorescent Protein expressing (GFP+)

male and a wild type female were placed in the mating cages at 17:00 and separated the next

morning, (9 AM designated as embryonic day 0.5, E 0.5). FDams were sacrificed on E 15.5 by

cervical dislocation. GFP+ pups, identified by fluorescence under 488nm light, were placed in

Hanks' balanced salt solution (Gibco/ Invitrogen, Grand Island, NY). The brains were removed

and embedded in 4% low melting point Agarose (Invitrogen) in PBS and sliced at 250 μm thick

coronal sections on a vibrating microtome (Thermo Scientific HM650V, Waltham MA). MGE

was identified visually and slabs of tissue corresponding to the ventral two thirds of the MGE

regions were dissociated using fine forceps (Fig. 4A). Samples obtained from both hemispheres

of two slices from two embryos were combined and considered an individual experiment for

statistical purposes. Donor cells were dissociated by trituration, centrifuged at 500g for 5 min,

resuspended in 15-30μl of NB/B27 medium (Gibco/ Invitrogen). A density of 6 x 103 live

cells/μl was obtained. For killed cell control transplants, cells (obtained as described above) were

killed by repeated freeze thaw cycle (-80o C, 1 minute x 3) immediately prior to transplantation.

Killed-cell or live-cell suspensions were injected into the brain using a glass pipette with a 50 μm

outer tip diameter connected to a nano-injector (Drummond Scientific, Broomall, PA).
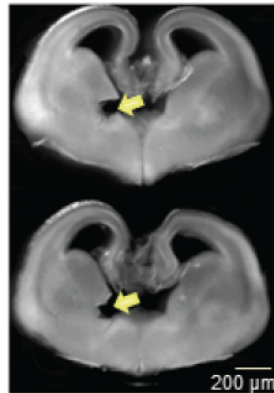
Figure 2-5: Photomicrograph showing MGE dissection:
Coronal section through the mouse brain at embryonic day 15.5. Representative dissections of the MGE
used for GABAergic progenitor isolation are shown by the arrow.

The internal surface of glass pipettes were first coated with mineral oil, following which,

suspensions containing the cells were forward filled. The glass pipette was then connected to the

injector mounted on a stereotaxic carrier. The suspended cells were transplanted bilaterally into

the hippocampi of mice aged 6-8 weeks. The surgical techniques and target coordinates for the

caudo-ventral CA1 described above were used. The electrode was lowered slowly into the brain,

taking several minutes to reach the target region, then a volume of 3.5 μl of the suspension was

ejected over 5 min in 70 nl steps, for a total deposit of 2.0 X 104 cells per hemisphere.

Section II: Studies in Genetic Mouse Models of Cerebral Cortical Parvalbumin+ Interneuron Deficits

Chapter 3: Anatomical and Electrophysiological Characterization of the cD2 Null Mouse: Evidence for Hippocampal Disinhibition

Introduction:

Stereological quantification of PV+ interneuron density in the somatosensory and motor cortices and the hippocampus and the DG by Glickstein et al showed a 30- 40 % reduction, respectively, in the cD2 null as compared to the WT mice (Glickstein et al., 2007). Although it was not within the scope of Glickstein et al (2007) to compare the size of the PV+ interneuron deficit across cortical regions, heterogeneity could be observed. Moreover, the medial prefrontal cortex, a region of particular importance for the cognitive dysfunction in schizophrenia (Lewis et al., 2012), had not been examined. I thus aimed to confirm and extend the findings of Glickstein et al (2007) by first quantifying PV+ interneuron density across the CA1 region of the hippocampus and the medial prefrontal cortex in the same brains. I then determined if reductions in PV+ interneurons predicted a decrease in GABA-mediated synaptic inhibition onto pyramidal cells in these regions and, ultimately, evidence for hippocampal disinhibition *in vivo*. For the last measure, we used measurements of cerebral blood volume as a proxy for basal metabolic activity and in order to test whether a deficit in synaptic inhibition can manifest as hyper-perfusion (and presumably hypermetabolism) of the affected brain region.

The following experiments were conducted in cyclin D2 null mutants and their age- and sex-matched littermates:

1.   Stereological quantification of PV+ interneurons in hippocampus and PFC

2.   Measurement of GABAergic and Glutamatergic synaptic inputs onto pyramidal cells

3.   Estimation of metabolic activity in the hippocampus by measurement of relative cerebral blood volume (rCBV) with high-spatial resolution functional magnetic resonance imaging (MRI).

Hypotheses:

Disruption of interneuron development in the MGE in the cyclin D2 will lead to loss of PV+ interneurons in the cortex including the hippocampus, and this will in turn be associated with loss of GABAergic inhibition onto pyramidal cells and signs of cortical disinhibition.

Anatomical characterization of the PV+ interneuron deficit in the cD2 null mouse

Brief Methods:

Coronal sections were cut from formalin fixed brains from cD2 null and WT mice. Forty micron thick sections were cut on a vibratome, and 1 in every 5 sections was stained for PV using anti-parvalbumin antibody (**figure 3-1**). Contours were drawn around the regions of interest (ROI). For the hippocampus ROI, CA1 region was encircled, using Paxinos atlas. Both dorsal as well as ventral hippocampus was included in the analysis. PV+ interneurons were counted in the entire

ROI. The density measure was obtained by factoring in the area of ROI - found using the

Cavelieri method. (See also Materials and Methods)

Results:

cD2 null mice show mild microcephaly: Consistent with previous findings (Kowalczyk et al.,

2004; Glickstein et al., 2009) (Glickstein et al., 2007), I found evidence for mild microcephaly in

the cD2 nulls. On visual inspection the cD2 null brain differed from the WT in having more

visible colliculi, as a result of reduction in cortical volume. The brain is narrower and shorter

than the WT. In slices, there was evidence for mild cortical thinning in most areas. On

systematically measuring the area of the hippocampus and the PFC (cingulate, Pre-limbic and

infra-limbic regions), we found significant reductions in the total area of these regions.
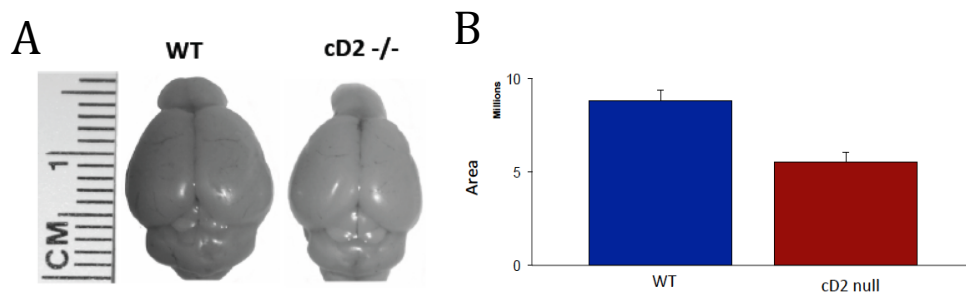
Figure 3-1: Comparison between a cD2 null and a WT brain.
A) cD2 null mice show mild microcephaly. Notice the more exposed colliculi in the cD2 null brain. **B)** Hippocampal CA1 area measured by the Cavalieri method is reduced by about 40% in the cD2 null. $P < 0.01$ independent (Student's) t-test.

Reduced PV+ interneuron density in the hippocampus but not in the PFC. We found that that the

cD2 null mice showed a reduction of around 45% in PV-IR cell density in the CA1 region

(figure 3-2 c). This is in line with the findings of Glickstein at al (Glickstein et al., 2007), who

reported a similar deficit when the entire hippocampus was considered as a whole. Although the

density of PV cells in other regions of the hippocampal formation, such as the CA3, DG and

Subiculum were not measured in this study; qualitatively the CA1 region appeared to show the most profound deficit. Qualitatively, a reduction was found in PV+ neurite density, which corresponded with the reduction in PV+ cell bodies.
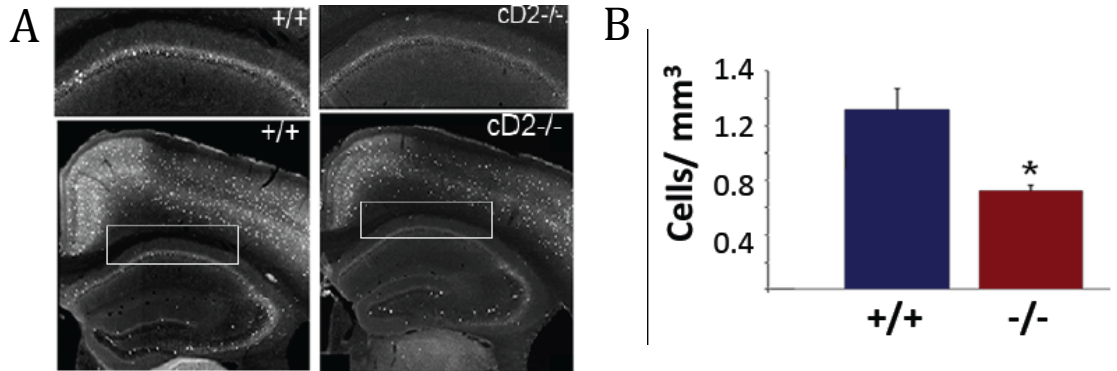
Figure 3-2: Reduced hippocampal PV+ interneuron density in the cD2 null.
A) Representative brain sections of rostral hippocampus, stained for parvalbumin. Right panels show higher-magnification image of PV+ cell density in CA1. **B)** PV+IN density in CA1, quantified by unbiased methods, is reduced in cD2–/– mice ($t_6 = 3.8$, $p < 0.01$; n=4).

In the PFC, an ROI was drawn to include the cingulate, Pre limbic and Infra-limbic regions. In contrast to the hippocampus, PV+ interneuron was not reduced in the PFC (**figure 3-3**).
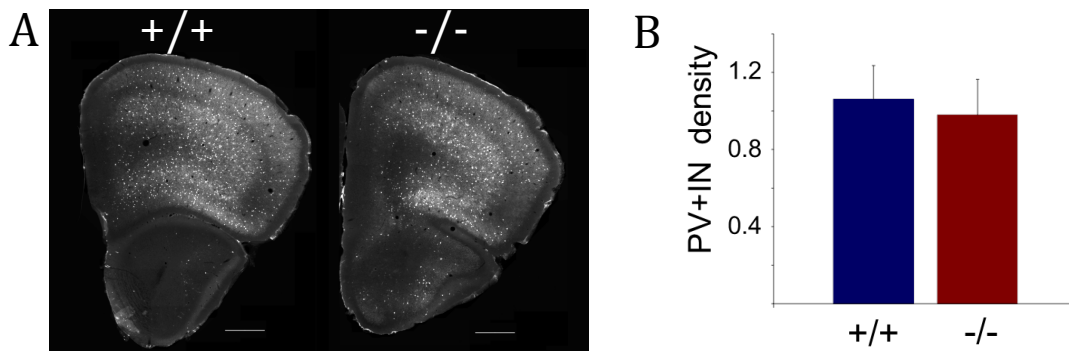
Figure 3-3: Prefrontal Cortical PV+IN density is not reduced in the cD2 null.
A) Representative coronal sections of the Prefrontal cortex, stained for Parvalbumin. **B)** PV+IN density in PFC, quantified by unbiased methods, is not affected in the cD2–/– mouse. Student's ttest ($p > 0.05$; n=5).

GABAergic synaptic neurotransmission in the cD2 null cortex

Brief Methods:

I measured miniature inhibitory post synaptic currents in pyramidal cells in the hippocampus and the PFC. A mIPSC represents a post synaptic response due to action potential independent, stochastic release of a single GABA vesicle. mIPSC frequency is a close approximation of the density of GABAergic synapse on pyramidal cells in organotypic cultures (Hartman et al., 2006; Swanwick et al., 2006). Whole cell voltage clamp recordings were used to measure miniature inhibitory post synaptic currents from CA1 pyramidal cells. A holding potential of -70mV was used. All recordings were conducted in the presence of TTX (to block fast Na channels), APV (to block NMDA) and CNQX (to block AMPA channels). The recording electrode contained Potassium gluconate or Cesium Chloride to block leak potassium channels. Resting membrane properties and action potential discharge properties of hippocampal pyramidal neurons were measured under current-clamp conditions. (See Materials and Methods section for details.)

Results:

CD2null mice show reduced GABAergic currents onto hippocampal CA1 pyramidal cells, but not in the PFC. Corresponding to the reduction in PV+ interneuron density, cD2 null mice showed a reduction in mIPSC frequency in CA1 pyramidal cells (t_{19} = 2.7, p < 0.05; n= 9-12) **(figure 3-4 b)**. However, there was no difference in mIPSC amplitude or kinetics across genotypes. As cD2 –/– cells did not differ significantly from cD2 +/+ cells in mean amplitude (t_{17} = 0.11, p > 0.05) **(figure 3-4 c)**, or decay time (t_{17}=0.15 p > 0.05) **(figure 3-4 d)** of mIPSC (n=9-10). GABAergic nature of mIPSCs was confirmed in a subset of recordings by bath infusion of

Bicuculline, a GABA$_A$R antagonist, which eliminated all events. The mIPSC events showed

reversal at around -65mV. No change was found in the series resistance, capacitance and other

recording parameters between the genotypes.
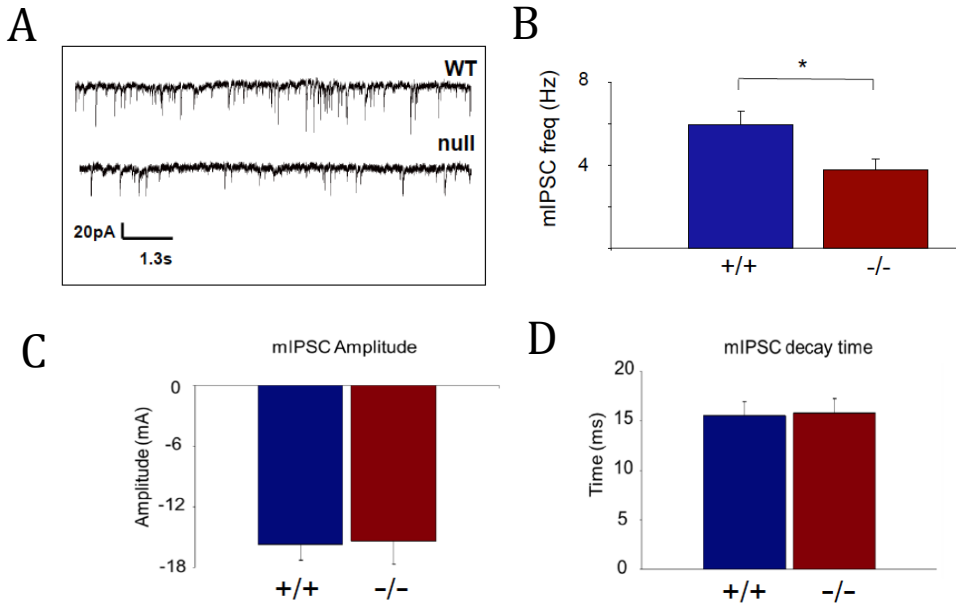
Figure 3-4: Reduced GABAergic synaptic neurotransmission in the hippocampus in the cD2 null.
A) Representative trace showing miniature IPSCs from the cD2 null and WT mice in the CA1 pyramidal cells. **B)** GABAergic mIPSC frequency in CA1 pyramidal cells is reduced in the cD2 null mice. **C, D)** mIPSC amplitude or decay time was not altered in the cD2 null.

Consistent with our finding that the cD2 null did not show a statistically significant reduction in

PV+ interneuron density in the prefrontal cortex, we did not find a statistically significant

reduction in mIPSC frequency in this region (t_{14}=1.05, p>0.05; n=8) **(figure 3-5)**
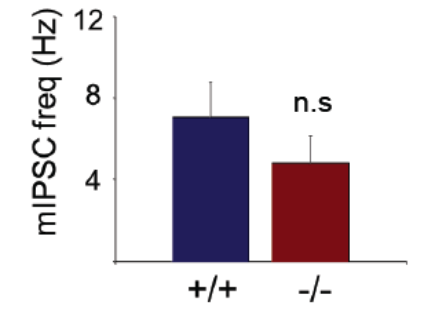
Figure 3-5: GABAergic currents are not reduced at pyramidal cells in the Prefrontal cortex in the cD2 null.
No difference in mIPSC frequency was found in PFC Pyramidal cells between cD2 null and WT mice

Glutamatergic currents at pyramidal cells are not different in the cD2 null.   To determine if the changes in synaptic transmission observed in the hippocampus were specific to GABA-mediated inhibitory events, we looked for alterations in excitatory synaptic neurotransmission in the hippocampus in the cD2 null mice. Pyramidal cells receive excitatory glutamatergic synaptic inputs from other cortical pyramidal neurons and from sub cortical structures. We reasoned that a developmental loss of inhibition may lead to homeostatic tuning to reduce excitatory glutamatergic neurotransmission in the hippocampus.

Action potential independent miniature excitatory post synaptic currents were measured from CA1 pyramidal cells using voltage clamp recordings. The glutamate dependency of these currents was shown by complete block of these currents by AMPA and NMDA blockers CNQX and APV, respectively. No differences were found in the frequency, amplitude ($t_{17} = 0.11$, p > 0.05) or decay kinetics ($t_{17}=0.15$ p > 0.05) of mIPSC (n=9-10) of miniature excitatory post synaptic currents onto CA1 pyramidal cells **(figure 3-6)**. This measurement likely includes both AMPA as well as NMDA Receptor currents.
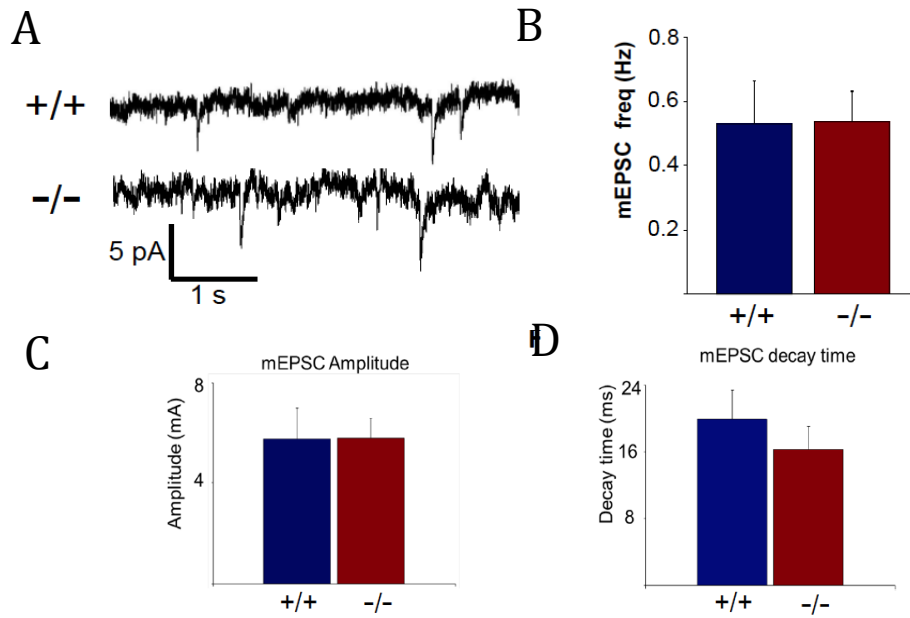
Figure 3-6: Excitatory synaptic inputs to the CA1 Pyramidal cells were not changed in the cD2 null.
A) Sample trace showing miniature excitatory post synaptic current (mEPSC) recorded in CA1 pyramidal cells. **B)** Glutamatergic mEPSC frequency in CA1 pyramidal cells. **C, D)** mEPSC amplitude or decay time were not different between the cD2 null and WT mice.

CA1 pyramidal cells in cD2 null show slight but significant reduction in excitability in response to depolarizing currents: Although there was a trend towards a more depolarized resting membrane potential in CA1 neurons of cD2 nulls, these neurons paradoxically showed a significantly increased current threshold (rheobase) and reduced spike frequency in response to current injection. No significant differences were found in the input resistance or Ih current.
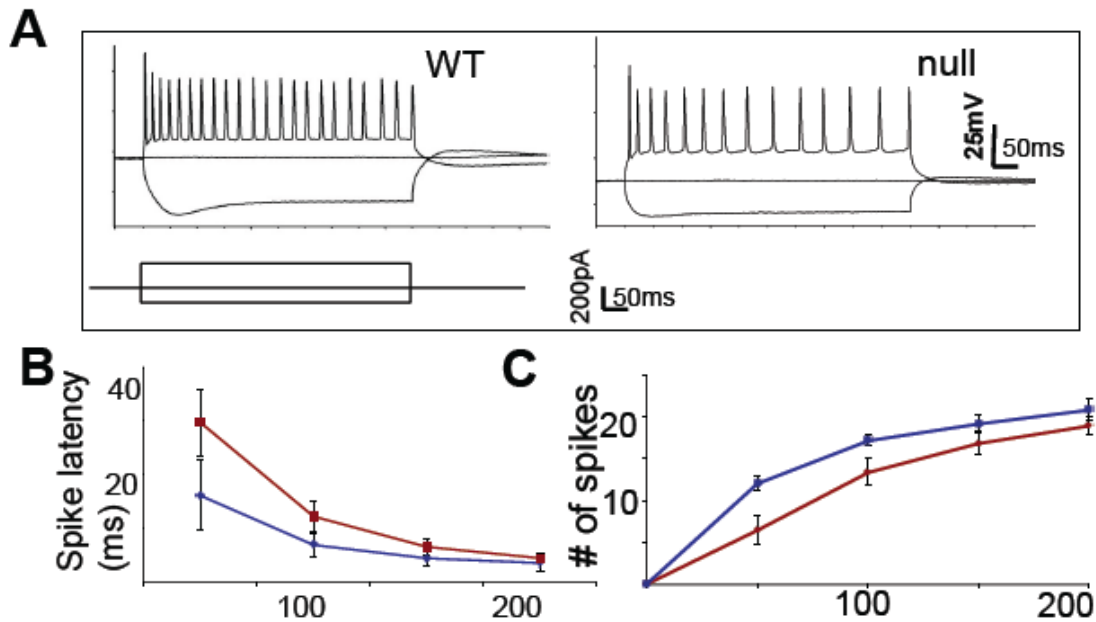
Figure 3-7 Reduced pyramidal excitability in the hippocampus in the cD2 null.
A) Representative traces from the cD2 null (red) and WT (blue) hippocampal pyramidal cells on injection of hyperpolarizing and depolarizing currents under current clamp conditions. **B)** Latency to 1st action potential spike and **C)** number of spikes in response to depolarizing current pulses. CD2 null show increased latency to spike and reduced spikes at a particular current step.

Discussion

Taken together, these findings show that cD2 null mice show loss of GABAergic inhibition in the hippocampus as evidenced by: 1) Reduced PV+ interneuron density in the CA1 region, 2) deficit in GABAergic synaptic transmission at CA1 pyramidal cells. This deficit is regionally heterogeneous and does not affect all regions of the cortex equally; we failed to find evidence for either reduction in PV+ interneuron or reduced mIPSCs in the PFC. Moreover, there was little or no evidence for compensation for the PV+ interneuron deficit, as we observed an equal-sized deficit in GABA-mediated inhibitory synaptic events, with no changes in post-synaptic aspects of GABA-mediated synaptic transmission or in excitatory synaptic transmission at hippocampal projection neurons.

PV+ interneuron deficit is regionally heterogeneous: varies across cortical

regions: Taken together with the previous reports (Glickstein et al., 2007), these data show that there are regional differences in PV+ interneuron loss in the cD2 null mice. A loss of around 40% was found in the hippocampus compared to about 30% in the somatosensory and motor cortical regions. There are laminar differences in PV+ interneuron deficit in the cD2, such that the superficial layers are more severely affected (42% for layer I – III vs 31% for IV – VI) layers of cortex (Glickstein et al., 2007).

Although a vast majority of PV+ interneurons in different neo-cortical and hippocampal regions share their origins from the MGE, different sets of progenitors within the MGE, may give rise to interneurons that migrate to and populate different regions of the brain. CD2 null mutation may differentially affect these progenitors within the MGE, thus explaining the heterogeneous distribution of PV+ interneurons within the cortex. Alternatively, temporal differences in origin of interneurons may explain these differences; cD2 null mutation is known to affect later born neurons (Glickstein et al., 2007; 2009), the areas of the brain, that receive most of their interneurons relatively late during development may be most severely affected by the mutation.

Tricoire et al showed that there is a spatio temporal division in the development of interneurons that inhabit different layers of the hippocampus, so that the CGE-derived interneurons primarily localize to strata lacunosum moleculare and deep radiatum, while MGE-derived interneurons show preference for strata pyramidale and oriens (Tricoire et al., 2011).

We hypothesize that the PV+ cells of the PFC, the somatosensory cortex and the hippocampus originate from different sets of progenitors, which are differentially affected by the loss of cD2, hence leading to heterogeneity of PV+ cell density in different parts of the cortex. These findings

are also consistent with reports of other interneuron deficit models that showed regional

selectivity in interneuron loss in the neocortex (Powell et al., 2003).

Little evidence for compensatory changes in the GABAergic system: Although the

cD2 null mutation affects the GABAergic system during development, there is a persistent and

lifelong deficit in GABAergic inhibition. Glickstein at al found that PV+ interneuron deficit in

the cD2 null is static and does not increase with age (Glickstein et al., 2007). In many instances,

developmental deficits are accompanied by homeostatic compensatory processes; surprisingly,

we didn't find evidence for compensation in the inhibitory system in these mice. Qualitatively, I

observed a decrease PV+ basket like structures forming around the pyramidal cells

corresponding to the loss of PV+ cell bodies in the cD2 null. Moreover, the decrease in mIPSC

frequency, a measure thought to reflect the presynaptic component of GABA transmission, was

not compensated by any changes in post-synaptic GABA transmission as reflected by no

increase in the amplitude or change in kinetics of mIPSC. This suggests that the cD2 null, in the

presense of reduced PV density, do not show a compensatory increase in connectivity by

sprouting more dendrites and axonal endings.

I have also examined the possibility of compensations in the excitability and glutamate inputs to

pyramidal cells. While I found that glutamate-mediated excitatory currents onto pyramidal cells

in CA1 were not altered in the cD2 null, I did observe a reduction in intrinsic membrane

excitability in response to current injection. These findings are similar to the findings from the

DLX1 null mouse, another developmental model of interneuron loss. DLX1 null mice loss of a

subset of hippocampal and neocortical somatostatin-, NPY-, VIP-, and calretinin-positive

interneurons at around one month of age, is followed by a the onset of onset of epilepsy (Cobos et al., 2005). These mice show also decreased excitability of CA1 pyramidal neurons (assessed in current-clamp recordings of input resistance and rheobase)(Howard et al., 2012)

Are different subtypes of PV+ cells differentially affected in cD2 nulls? In this study, we have not addressed the question: whether different subtypes of PV expressing interneurons are differentially affected in the cD2 null mice. There is no histological marker or antibody that could distinguish between these two classes. These cells are also identical in their electrical properties, and are derived from the same progenitors during development. Factors that may facilitate the speciation of chandelier cell phenotype as opposed to the basket are not understood well, however some evidence points to temporal differences in this speciation (Inan et al., 2013)

Conclusion

These data show that the cD2 null show a regionally selective deficit in the density of PV+ interneurons, the magnitude of which is greater in the hippocampus, as compared to other regions we studied in the cortex. This loss of PV+ interneurons corresponds with reduced GABAergic inhibition in these regions. The cD2 null, hence gives us a suitable model to test the hypothesis about the relationship between loss of PV+ interneuron function and hippocampal disinhibition and this in turn leading to dysregulation of the ventral tegmental dopamine system.

Activity in the cortex is often conceptualized as a balance between excitation and inhibition. A possible consequence of reduced inhibition in the cD2 null can be increased excitation exhibited as epileptiform/ seizure activity. We examine this possibility in Chapter 4. In Chapter 5, I examine whether cD2 null show increased cerebral blood flow or volume – which is thought to correlate with neural activity- and has frequently been shown to be elevated in patients with schizophrenia.

Chapter 4: MRI of Cerebral Metabolic Activity in the cD2 Null: An *In Vivo*, Clinically Relevant Manifestation of Hippocampal Disinhibition?

Introduction

A large number of studies over the years have shown that cerebral blood flow (CBF) and cerebral blood volume (CBV) are correlates of brain function and metabolism (Small et al., 2011; Schobel et al., 2013). CBV and CBF can be estimated using functional magnetic resonance imaging (fMRI) .CBV provides sub-millimeter resolution for mapping relatively stable metabolic states within the hippocampal formation. As reviewed in the General Introduction, the hippocampus is one of the most affected regions in schizophrenia. Structural changes such as reduction in hippocampal volume have been noted in the hippocampus for a long time (Wright et al., 2000; Heckers and Konradi, 2010) for review see (Heckers and Konradi, 2010). More recently, changes in resting cerebral blood volume have also been found in both schizophrenia as well as its risk states (Schobel et al., 2009). This increased blood flow can be used as a proxy for the 'metabolic state' of the hippocampus.

Hypothesis:

cD2 null mice will show increased cerebral blood volume in the hippocampus but will show normal CBV in the mPFC.

Brief Methods:

We used a CBV based fMRI technique, identical to the one that have been used in human schizophrenia (Moreno et al., 2006; Schobel et al., 2009; 2013). Relative CBV (rCBV) values were calculated for the hippocampus (HIPP), the medial prefrontal cortex (mPFC), and the cerebellum (CBL). Cerebral blood volume measurements were conducted in anesthetized mice using isoflurane anesthesia. (For detailed methods see Materials and Methods)

Results:

The cD2 nulls showed hippocampal hyperactivity:

cD2 null mice showed increased rCBV relative to the metabolic activity in the hippocampus (t_{14} = 3.3, p < 0.01) **(Figure 4-1a)**. Consistent with the smaller (non-significant) decreases in PV+ interneuron number and synaptic inhibition in the PFC, cD2 null mice did not show an increase in basal prefrontal cortical rCBV **(Figure 4-1 b)**. Moreover, there was no difference between genotypes in basal rCBV in the cerebellum (t_{14} = 0.3, p > 0.25) despite the reported alteration in cerebellar stellate interneuron number and function in cD2 null mice (Huard et al., 1999). The increase in hippocampal rCBV in cD2 null mice remained significant when normalized for cerebellar **(Figure 4-1 d)** or overall brain (data not shown) resting rCBV.
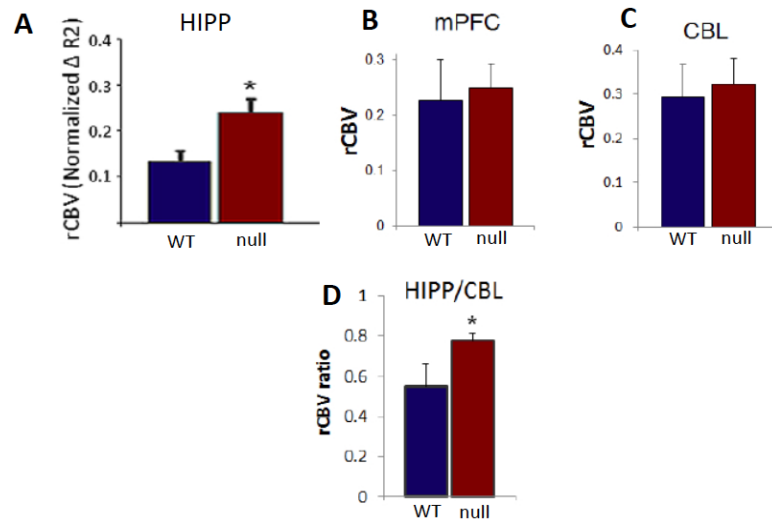
Figure 4-1: Increased hippocampal rCBV (Normalized R2) in the cD2 null.
A) cD2 null mice (n= 8) show hyper-metabolism as shown by relative cerebral blood volume measurements (rCBV) in the hippocampus (HIPP) but not in the medial Prefrontal cortex (mPFC) **(B),** or the cerebellum (CBL) **(C)**. **D)** Hippocampal rCBV normalized for cerebellar CBV.

Data expressed as means ± SEM. *$p < 0.05$, independent t-tests

Discussion

We found that loss of GABAergic inhibition in the cD2 null is accompanied by HIPP disinhibition *in vivo,* as evidenced by an increase in rCBV in the hippocampus. The hyperperfusion and presumed hypermetabolism was relatively selective for the hippocampus. The cD2 null did not differ in rCBV measurement in the mPFC, a region in which the PV+ interneuron deficit was found to be small and to lack impact on inhibitory synaptic inhibition. Notably, resting metabolism was also similar between genotypes in the cerebellum despite the fact that loss of cD2 leads to a deficits in cerebellar stellate neurons and deficits in evoked blood flow in this region (Yang et al., 2000). This suggests a pattern of hyper-metabolism specifically related to cerebral cortical PV+ interneuron deficits in which the hippocampus is disproportionately affected. Taken together with the finding that increases in glutamate can

induce and increase in rCBV in the hippocampus (Schobel et al., 2013), these data suggest that the hippocampus is abnormally activated in the cD2 null mice.

CBV measurements have been used as a measure of neural activity, however it is presently not clear whether spiking activity or afferent synaptic activity accounts for changes in this signal. Some studies have suggested that out of these factors, incoming input and local neural signal processing (indicated by local field potential) and not spiking activity are better predictors of the hemodynamic response (blood flow or volume) (Logothetis, 2003). Other studies have shown that glutamate efflux induced by the psychomimetic drug ketamine can drive an increase in rCBV in the hippocampus (Schobel et al., 2013).

In the context of our study, increased CBV corresponded to the relative reduction in PV+ interneuron density, being prominent in the hippocampus but not in the mPFC. The PV+ interneurons are the major source of GABAergic synaptic input to the cell body and axon hillock region of the pyramidal cells, and have a large influence on the spike firing activity of these cells. Hence in the cD2 null, a differential increase in CBV in regions that show PV+ loss may reflect enhanced spike activity of pyramidal neurons. On the other hand (or in addition), the possibility remains that increased CBV in the CA1 region of the hippocampus stems from an increase in afferent inputs. CA1 receives two major afferent inputs: the Schaffer Collateral input from the CA3, and the Perforant Path input from the entorhinal cortex. In the cD2 null, PV+ loss and in fact increased basal CBV was also found in the CA3 region. Hence it is possible that the increased CBV in the CA1 region reflects an increase in CA3 input to the CA1. *In vivo* electrophysiological methods can be utilized, in future studies to answer this question.

Chapter 5: Dysregulation of Mesolimbic Dopamine System Function in the cD2 Null

Introduction

As reviewed in the General Introduction, disinhibition or over-activation of the ventral hippocampus would be predicted to increase the activity of mesolimbic DA neurons and enhance responsiveness to amphetamine (AMPH). Specifically, ventral hippocampus activation leads to an increase in the number of spontaneously active DA neurons in the VTA (Floresco et al., 2001; 2003; Lodge and Grace, 2007) and increased DA efflux in the nucleus accumbens (Blaha et al., 1997; Legault and Wise, 1999; Howland et al., 2004). The locomotor response to AMPH, which depends on DA transmission within the ventral striatum (Kelly et al., 1975; Pijnenburg et al., 1975; Kelly and Iversen, 1976), is also exaggerated when the ventral hippocampus is disinhibited or activated (White et al., 2006; Lodge and Grace, 2007). Use of AMPH as a tool for these studies is particularly important, not only because it is a behavioral assay that is sensitive to ventral hippocampal disinhibition, but also because it is a psychostimulant which induces the positive symptoms of schizophrenia in humans (reviewed in (Lieberman et al., 1987).

In this section, I describe the experiments undertaken to examine mesolimbic dopamine function in the cD2 null. First, I examined locomotor response to amphetamine, followed by the experiments to determine the role of ventral hippocampal activity in this behavior. Lastly, I

describe the experiments to study ventral tegmental DA cell activity - using in vivo recording techniques.

Experiment 5.1: Amphetamine-induced locomotor activity in cD2 null mice

Hypothesis:

Impaired inhibition of hippocampal circuits (specifically projections from the ventral hippocampus) in cD2 –/– mice will lead to a hyper-responsiveness to amphetamine.

Brief Methods:

Mice are placed in a 17" x 17" open field box with a white floor. Movement of the mice is measured by the help of an automated setup and translated into distance. After 30 min of baseline activity, 2mg/kg amphetamine (dissolved in saline) is administered in an intraperitoneal (i.p.) injection. Activity is recorded for another 60 min, after which the mice are returned to their home cages. A separate cohort of mice, were pretreated with either saline or raclopride (0.5 mg/kg) 30 minutes before injection of AMPH (2.0 mg/kg, i.p.) and locomotor activity was measured as described above. (See also Materials and Methods)

Results:
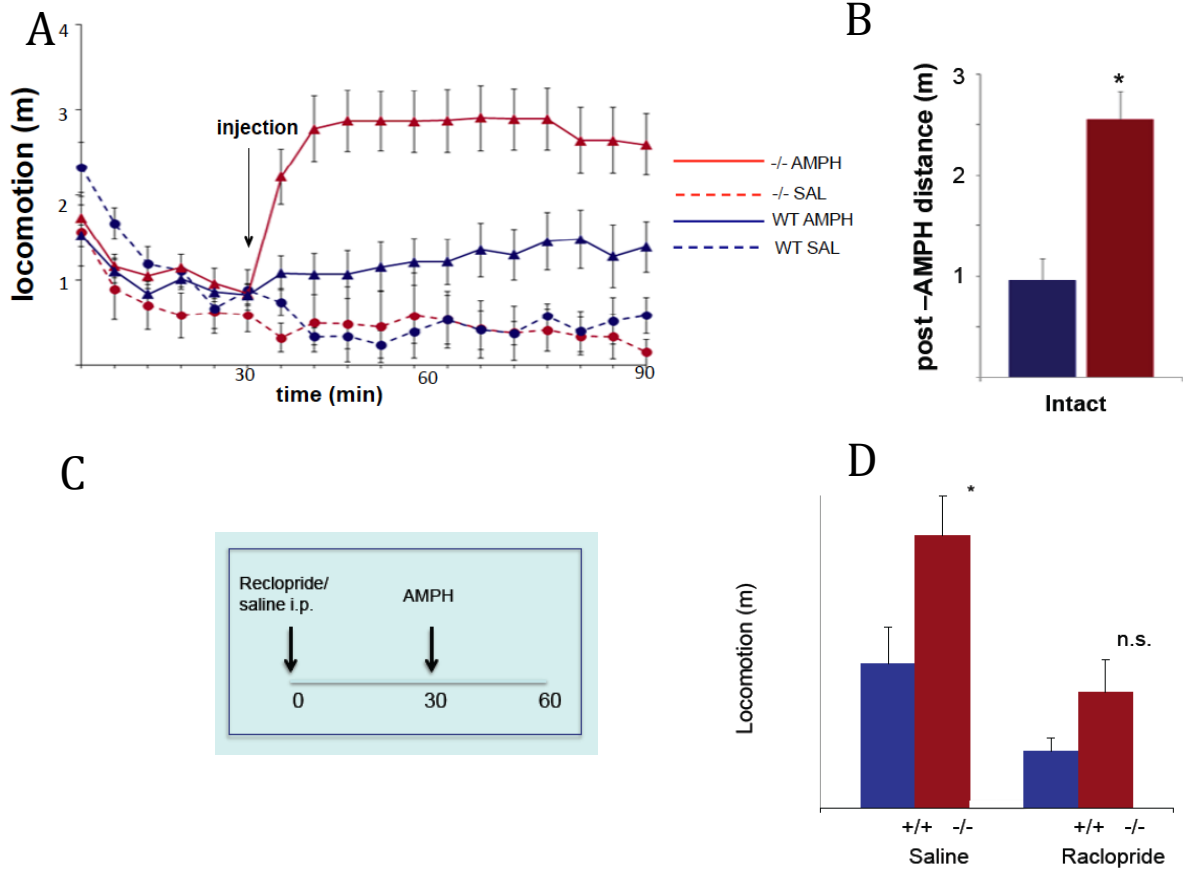
Figure 5-1: Increased psychomotor response to amphetamine in the cD2 null.
A) Locomotor activity pre- and post-injection of saline or amphetamine (AMPH; 2 mg/kg, i.p.)(n's = 13 and 4 per genotype for AMPH and saline, respectively); arrow indicates time of injection. **B)** Average locomotion after injection of amphetamine was significantly increased in the cD2 null mice. **C)** Basic design of the raclopride pretreatment experiment. **D)** Locomotor response to amphetamine with saline or raclopride pretreatment in cD2 null mice. (Student's t-test, * $P < 0.05$, n.s. $P > 0.05$)

CD2 nulls show increased locomotor activity to amphetamine: A marked increase in

locomotion was observed in both groups with administration of amphetamine. However the cD2

nulls showed a much greater response **(figure 5-1)**. Prior to amphetamine injection, the mice

showed gradual reduction in locomotor activity, perhaps showing habituation to the open field

environment. I did not observe any difference in baseline locomotor activity. Similarly, saline

injection did not elicit any change in locomotor activity in either group. A mixed ANOVA design with genotype and drug as factors, and time (before or after injection) as the repeated measure, was used. This was followed with planned Student's t-test comparisons of genotypes within drug condition separately for baseline and post-injection locomotion. An interaction between genotype and drug ($F_{1,29} = 6.0$, $p < 0.05$); genotype and time ($F_{1,29} = 11.2$, $p < 0.01$); and a trend for a 3-way interaction ($p = 0.078$). Planned comparisons show no differences between genotypes prior to injection ($t_{31} = 0.2$, $p > 0.25$) or in the response to saline ($t_6 = 0.13$, $p > 0.25$, drug groups combined). However there was a robust difference in response to amphetamine between the genotypes, with the cD2 null showing a much greater locomotion ($t_{23} = 4.5$, $p < 0.001$).

Amphetamine response in the cD2 null is attenuated by blockade of DA D2 receptors:

Locomotor response to amphetamine has been associated with increased dopamine release in the nucleus accumbens. We sought to find if locomotor response to amphetamine is dopamine dependent using pretreatment with raclopride, a selective D2 R antagonist (Ogren et al., 1986) . Mice were treated with intra-peritoneal injection of raclopride (0.5 mg/kg) at the beginning of the experiment, and immediately placed in an open field **(figure 5-1 c)**. We observed that pretreatment with raclopride reduced amphetamine response in the cD2 nulls down to the level of the WT. While with saline pretreatment, the cD2 null continued to show hyper-locomotion **(figure 5-1d)**. Raclopride also attenuated the baseline locomotor activity.

These data are in agreement with previous studies that showed reversal of increased locomotor activity induced by amphetamine, along with suppression of spontaneous activity (O'Neill and Shaw, 1999). Raclopride, a selective D2 antagonist was only partially able to reduce amphetamine induced locomotion, which is consistent with the findings of other studies showing

that both D1 as well as D2 receptors are involved in locomotor response to this psycho-stimulant (O'Neill and Shaw, 1999). Attenuation of baseline locomotor activity indicates dopamine dependence of this behavior.

Summary:

These experiments show that the hippocampal disinhibition in the cD2 null is accompanied by increased locomotor response to amphetamine.

Experiment 5.2: Ventral hippocampal modulation of AMPH-induced hyperlocomotion in the cD2 null

Next we studied the involvement of the hippocampus in amphetamine response.

Hypothesis:

If disinhibition of the hippocampus contributes to increased sensitivity to amphetamine in the cD2 null through an excitatory drive, then a lesion of the ventral hippocampus should attenuate amphetamine response.

Brief Methods:

Preliminary experiments showed that we could reliably induce selective lesions of the

ventrocaudal hippocampus with placements of guide cannulae (with occluded lumina, made from

0.6 mm outer diameter syringe needles) **(figure 5-2 a)**. Using this method, bilateral guide

cannulae were placed in the ventrocaudal hippocampus under general anesthesia. To control for

any non-specific effect of the lesioning procedure, a separate group of mice received guide

cannulae placements in the parietal cortex overlying the hippocampus. After allowing for

recovery (typically a week), mice were tested for locomotor response to amphetamine using

methods described above. Cannula placements were confirmed at the end of the experiment and

any subject with non-specific damage or extensive extra-hippocampal lesions (for the

hippocampal cannulation group) were not included in the analysis.

Results:
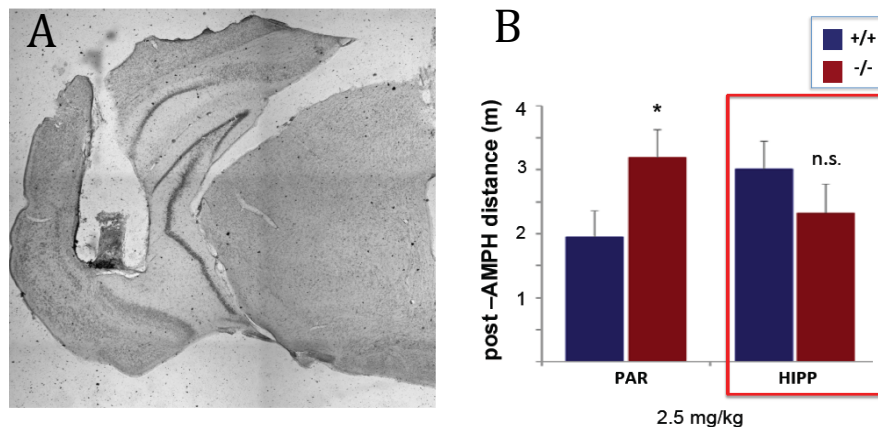
Figure 5-2: Effect of hippocampal lesion on amphetamine response in the cD2 null.
A) Coronal section of the hippocampus showing a representative example of hippocampus lesion. **B)**
Augmented response to amphetamine in cD2–/– mice is eliminated by partial lesions of the ventral
hippocampus (HIPP) but not that of the overlying parietal cortex (PAR).

Locomotor response to amphetamine is attenuated by hippocampal lesions: I observed that
while hippocampal lesions greatly attenuated amphetamine response in the cD2 null and
eliminated the genotypic difference between the cD2 null and WT mice (effect of genotype, t_{20} =
1.1, p > 0.25; n= 11) **(figure 5-2)**. This was not a non-specific effect to a brain lesion, as cD2
null mice with lesions of the parietal cortex, continued to show hyperlocomotion as compared to
WT mice (effect of genotype, t_{14} = 2.2, p < 0.05; n = 7-9) **(figure 5-2)**.

*Summary***:**

The enhanced amphetamine-induced locomotor activity in cD2 null mutant mice, is eliminated
by lesions of the caudoventral hippocampus, but not the overlying parietal cortex. This supports
the hypothesis that the abnormally exaggerated response to amphetamine in cD2 null mutant
mice is driven by disinhibition of the caudoventral hippocampal output.

Experiment 5.3: VTA DA neuron activity

Hippocampal activation can increase the population activity of dopamine (DA) neurons in the
ventral midbrain via a disinhibitory GABA-GABA striato-pallidal circuit. To determine if the
impairment in hippocampal inhibition in the cD2 –/– mice might increase DA neuron activity,
we performed extracellular single-unit recordings in the ventral tegmental area (VTA).

*Hypothesis***:**

Reduction in GABAergic transmission and disinhibition of vHIPP circuits will lead to an increase in the number of spontaneously active DA neurons in the VTA.

Brief Methods:

Spontaneously active dopamine cells were recorded in chloral hydrate-anesthetized mice as described in Chapter 2, General Materials and Methods.

Results:

VTA dopamine cell population activity is increased in the cD2 null: We found that the cD2 null mice exhibited a significantly greater number of spontaneously active cells per recording track ($t_{8.8} = 6.3$, $p < 0.01$; n = 6-7) **(figure 5-3)**. CD2 nulls showed an average of 3.4 (+/- SEM 0.1) spontaneously active DA neurons per track, while the WT litter mate controls showed an average of 1.7 (+/- SEM 0.25) Cells / track. No difference was found in the firing rate, frequency of burst firing, or number of spikes per burst.
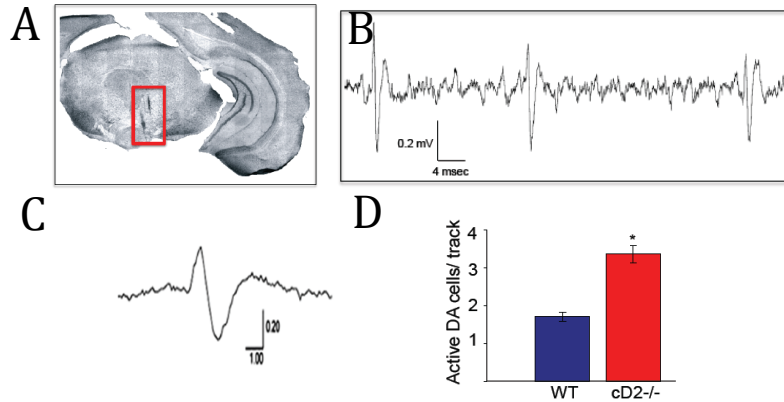
Figure 5-3: Ventral tegmental dopamine cell population activity is increased in the cD2 null.
A) Representative coronal section showing recording tracks through the central and lateral VTA..Scale bar 1mm, corrected for shrinkage. **B)** Representative trace from an extracellular recording of a single unit. **C)** The unit exhibits waveform and burst-firing characteristics of a DA neuron. **D)** Average number of spontaneously active dopamine neurons is increased in the cD2 null mice.

*Summary***:**

The cD2 null mice showed increased DA cell activity in the Ventral tegmental area. This is consistent with a disinhibited hippocampus.

Discussion**:**

We have found that cD2 nulls show an increase in population activity of VTA DA neurons, locomotor response to AMPH- a response mediated by DA projections from VTA to limbic striatum and largely normalized in cD2 nulls with a DA D2 blocker. The increased locomotor response is blocked by a lesion to the caudoventral HIPP indicating that HIPP output is necessary for this phenotype in the cD2 null.

These phenotypes are consistent with and further support the evidence for disinhibition of the ventral HIPP in the cD2 null mouse. These data are also consistent with dysregulation of the hippocampus-Nac pathway (Floresco et al., 2003) in the cD2 null. The cD2 null showed an elevation of spontaneously active Dopamine cells in the VTA. No elevation of firing rate or burst firing was observed, which has been seen to depend on pedunculopontine reticular system activity (Floresco et al., 2003). Future studies will test this hypothesized circuit by measuring electrical activity at the various nodes in the circuit, such as the hippocampus, nucleus accumbens and the ventral pallidum.

Analysis of electrode tracks showed that the electrical recordings in our studies targeted the medial and central midbrain DA neurons.

Although imaging studies in patients with schizophrenia have shown increased striatal dopamine, as shown by increased labeled L-DOPA, increase amphetamine induced displacement of D2 ligands and increased baseline occupancy at dopamine receptors, it is not known whether the activity of DA cells, such as the firing rate or the number of spontaneously active DA cells is higher in these patients.

Preliminary evidence from our study showed that systemic administration of amphetamine led to a decrease in firing in the VTA (data not shown). Since amphetamine leads to dopamine release by synaptic as well as other mechanisms, DA release is expected to lead to D1 receptor mediated auto-inhibition thus leading to depression of DA cell firing rate. This interpretation is also supported by data that showed that acute haloperidol injection activated non-firing DA neurons and conversely, DA microinjection inhibited DA cell firing. These data suggests that amphetamine leads to an increase in DA release and not an increase in dopamine cell firing. To test this idea further, DA can be directly measured by microdialysis.

Hippocampal modulation affects locomotor activity (Jarrard, 1968; Jarrard and Bunnell, 1968). Several studies have reported a baseline hyperactivity in mice supposed to have disinhibited hippocampi (Lodge and Grace, 2007; Bast et al., 2001a; Belforte et al., 2009). It is believed that novelty induced dopamine release contributes to this phenotype. In light of these previous findings, lack of baseline hyperactivity is intriguing in the cD2 null. We hypothesize that a possible reason may be some degree of compensation in response to a developmentally determined persistent disinhibition in the cD2 null. In a previous study, Jaholkowski et al. reported no difference in locomotor activity in the cD2 null (Jaholkowski et al., 2009). It is worth mentioning here that the same group reported baseline hyperactivity in the cD2 nulls in a recent study (Jedynak et al., 2012). Moreover, a colleague has found evidence for baseline hyperactivity in the cD2 null in a larger group of mice (Samuel Pasketwitz, personal communication). The conditions during which the cD2 null show enhanced baseline locomotion, and whether recruitment or dynamic differences in disinhibition under some conditions determine whether there is increased baseline locomotion need further study.

Chapter 6: Hippocampus and Prefrontal Cortical Dependent Cognition in the cD2 null mice

Introduction:

As reviewed in the General Introduction, patients with schizophrenia show a range of cognitive deficits (Sadock et al., 2007; Barch and Ceaser, 2012). Modeling these deficits in rodent models is a high priority given their relationship to functional outcome (Sadock et al., 2007), and given that we currently have no effective pharmacological treatments for impaired cognition in schizophrenia. Modeling these cognitive deficits in rodents is also tractable, given a rich cognitive neuroscience literature establishing conserved neurocognitive processes across rodents and humans (Moore and Robbins, 2008). Domains of cognition mediated by the medial temporal lobe (including the hippocampus), as well as those operations dependent upon the prefrontal cortex, are affected in schizophrenia, and these domains of cognition can be measured in the rodent.

Assessing hippocampus-dependent associative learning in rodent models.:

Tasks recruiting and dependent upon the hippocampus often encompass the encoding and association of "contexts" a spatiotemporal association of a set of cues (that form a gestalt context) with an event (Kim and Fanselow, 1992; Fanselow, 2000). Pavlovian fear conditioning experiment can be used to investigate both hippocampus dependent and hippocampus-

independent forms of memory. In this experiment, an innocuous stimulus such as a tone or light (called conditional stimulus, CS) is presented concurrently with an aversive stimulus such as a foot shock (unconditional stimulus, US). The mouse forms an association between the CS and US. A subsequent presentation of either the US or CS, elicits a response which consists of cessation of all movement (except breathing) referred to as freezing.

Lesion studies in rodents have shown that lesions of the hippocampus result in disruption of conditioning to compound stimuli such as the context (Kim and Fanselow, 1992). On the other hand, lesions of the amygdala leads to disruption of conditioning to discrete cues such as visual or auditory discrete cue conditioning (Campeau et al., 1995). In addition, the amygdala and the ventral peri-aqueductal grey (PAG) are important for the expression of fear response (as a behavioral response distal to cued or context conditioning) (Kim et al., 1993) **(figure 6-1).**

The formation of the representation of the context requires NMDA receptor function as well as protein synthesis in the hippocampus. Also, the involvement of the hippocampus in contextual conditioning corresponds well with the finding that place fields form in the hippocampus during exploration of an environment (O'Keefe and Dostrovsky, 1971).
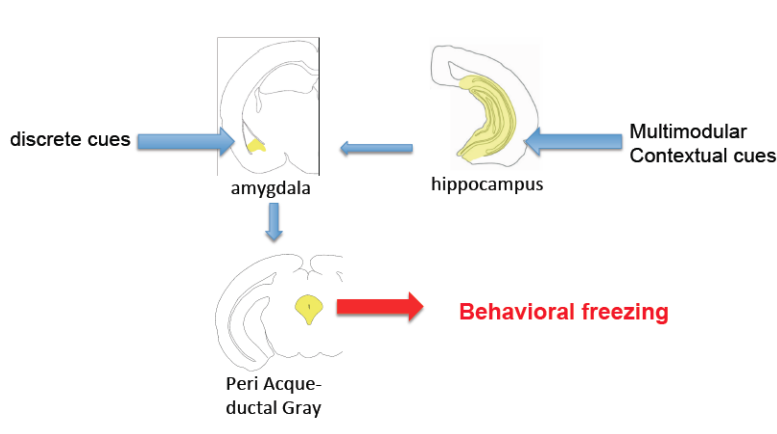
Figure 6-1: Fear conditioning circuit.
Schematic representation of the circuit thought to underlie hippocampus dependent fear conditioning.

Although the role of the dorsal hippocampus is best established in contextual conditioning and in trace conditioning, some reports have reported that the hippocampus contributes to freezing performance to a delay conditioned auditory stimulus when the strength of the association between the US and CS is relatively weak (fewer trials or low foot-shock intensity), but not when stronger conditioning parameters are used (Quinn et al., 2008)

These results suggest the presence of two parallel memory systems: one that is hippocampus independent and contributes to a direct tone-foot shock association, and a hippocampus-dependent memory system that maps a context or weak, discrete cue on to the unconditioned stimulus and response (Quinn et al., 2008).

Although lesion studies have focused on the role of dorsal hippocampus in contextual fear conditioning, some studies have reported that the ventral hippocampus/subiculum could also be involved in acquisition, retrieval and/or expression of conditioned fear (Maren, 1999; Richmond et al., 1999; Bast et al., 2001b). The connectivity of the ventral hippocampus corroborates with these findings, since the ventral hippocampus has intimate connections with the amygdala and the nucleus accumbens, which are involved in classical fear conditioning to explicit and contextual cues (Anagnostaras et al., 2001; Bast et al., 2001a).

Assessing prefrontal cortex-dependent cognition in rodent models:

One form of PFC-dependent cognition, captured in human neuropsychological testing by the Wisconsin Card Sorting Test (WCST) is *cognitive flexibility or 'set-shifting' ability.* In set-shifting tasks, the subject is required to make decisions based on one stimulus discrimination rule, then the rule (stimulus-outcome contingencies) spontaneously changes, and the subject

must adjust choices accordingly. Set-shifting can be testing in rodents by changing the relevance of stimulus modalities (e.g. olfactory versus tactile) cues. Lesion studies have shown that different aspects of this task have been shown to be dependent on different brain regions. Reversal learning (reversing the contingency for a given stimulus pair) recruits and depends on the orbitofrontal cortex while shifting responding to a new perceptual domain (extra-dimensional (ED) shift) depends on the dorsomedial frontal cortex in rodents (Birrell and Brown, 2000).

We used multiple variations of contextual and cued fear conditioning to test hippocampus-dependent cognition, and the rodent set-shifting paradigm to test PFC function, in the cyclin D2 null mice.

Experiment 6.1: Assessment of hippocampus-dependent learning and memory using contextual versus cued aversive conditioning

Hypotheses:

I imagined two competing scenarios with respect to hippocampus-dependent learning :

1) PV+ interneuron deficit leads to increased activity but hippocampal function is not disrupted. In this possibility, fear conditioning will be normal, possibly enhanced in the cD2 null mice.

2) PV+IN loss will leads to disruption of hippocampal function and consequently, fear conditioning will be disrupted.

*Brief Methods***:**

Mice were fear conditioned in a distinct context (context A) using five pairings of auditory tones (conditioned stimulus, CS+) and foot shock (unconditioned stimulus, US). On the following day, amygdala- and hippocampus-dependent conditioned fear responses were tested. First, in a novel context (Context B), we measured freezing to the first presentation of the tone CS+ (presented without shock), an amygdala-dependent response that does not require the hippocampus (20). Second, during repeated presentations of the CS+ in Context B, we monitored post-tone freezing. Six hours later, we measured the context-conditioned fear response (freezing in Context A without presentation of the tone CS+).

For statistical analysis, a mixed ANOVA design was used with retrieval phase as the repeated measure and genotype as the between-subjects factor. Independent (Student's) t-tests were used for planned comparisons to test the effect of genotype on each conditioned response type.

*Results***:**

<u>No difference in baseline freezing between the cD2 null and WT mice</u>: There was no genotypic difference in freezing response at baseline. After administration of the foot shock, both cD2 null and WT mice responded by profound locomotor activity (the unconditional reaction, UR). Following the foot shock, there was an increase in freezing response. The overall freezing increased with each shock administration. The final level of freezing reached at the end of the training session did not differ significantly between the genotypes.

These data suggest that the cD2 null were able to learn the association between the tone and the shock, showing normal acquisition of fear memory. In addition, it indicates that the cD2 null are not deficient in their freezing response.
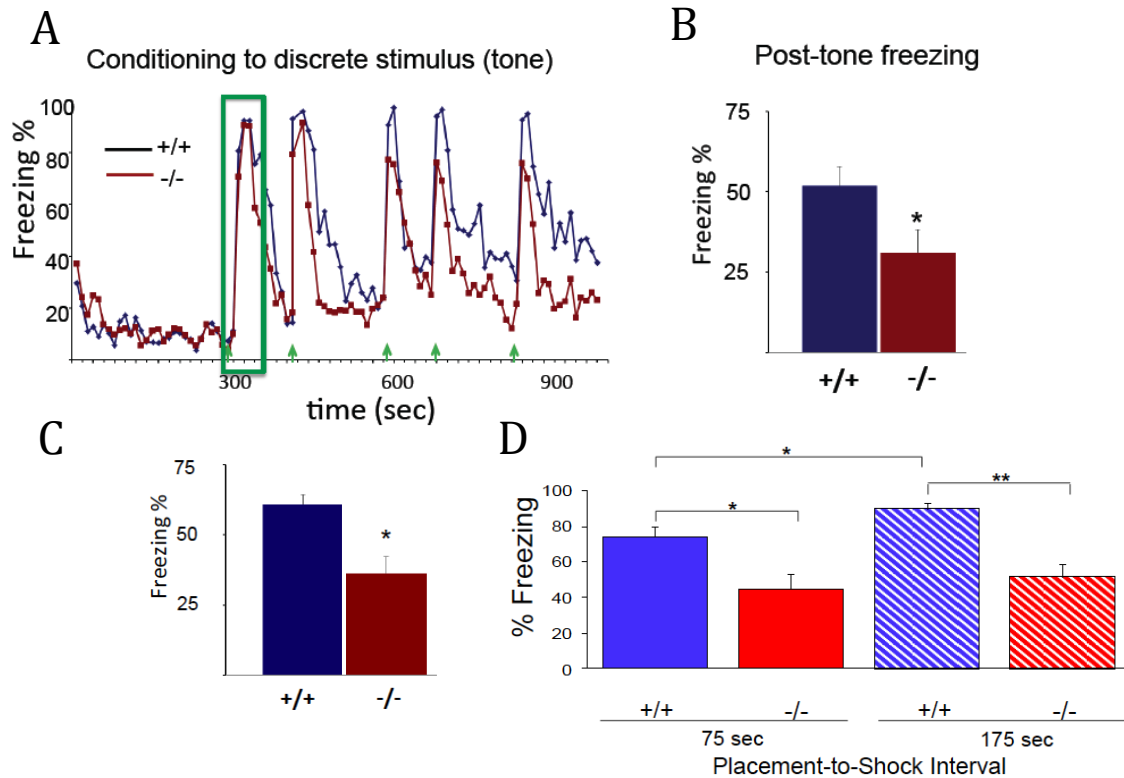
Figure 6-2: cD2 null show deficits in hippocampus dependent fear conditioning.
A) Behavioral freezing to tone CS+ in a novel context (Context B) (arrows indicate times of CS+ presentation). B) Average post tone freezing in the novel context is decreased in the cD2 null. C) Freezing during re-exposure to the conditioned context (Context A). D) Effect of increased exposure to training context on behavioral freezing. CD2 null mice did not increase conditioning with increasing time in the context. Data expressed as means +- SEM. * $p < 0.05$, mixed repeated measures ANOVA and planned comparisons with independent t-tests. The overall statistical model (12) showed a significant effect of genotype ($F_{1,16} = 14.4$, $p < 0.01$; $n = 9$ per genotype)

Differences in conditioning to discrete auditory stimuli in the cD2 null: The next day, the mice were placed in a novel context that differed from the training context in terms of olfactory, tactile and visual stimuli. The mice showed very little baseline freezing **(figure 6-2a)** indicating that

they were able to distinguish the novel context from the training context. Both groups show similarly robust conditioned responses to the first CS+ (arrowhead; average freezing during tone; $t_{16} = 1.3$, $p > 0.25$) **(figure 6-2a,b)**. This suggests intact amygdala dependent memory and cognition.

Subsequent administration of tones in the same context, showed diminished freezing response in the cD2 null during post-tone intervals in Context B ($t_{15.8} = 2.3$, $p < 0.05$) **(figure 6-2 a,b)**. This is consistent with the findings of Quinn et al, who showed that hippocampus dependence of freezing response in this phase of the experiment (Quinn et al., 2008).

__CD2 null shows deficits in contextual conditioning__: Six hours after placement in the novel context, the mice were placed in the original training context (context A). In contrast to the novel context, the mice showed a robust freezing response immediately after placement in the context, indicating conditioning to the context. The cD2 null showed greatly reduced freezing to the context ($t_{16} = 2.3$, $p < 0.05$) **(figure 6-2 c)**.

These data indicate a robust deficit in contextual conditioning in the cD2 null. As described earlier, we did not find a deficit in learning or freezing performance in these mice. Additionally, a robust freezing response to the auditory tone and a clear ability to distinguish between the novel and the training context, rules out any strong sensory deficit, which could have interfered in recognition of the discrete stimuli. In addition, freezing at the end of the training period indicates normal acquisition of the task.

__Increased exposure to the conditioning context fails to improve learning in the cD2 null__: Time spent in the conditioning context before the administration of the US is a critical determinant of conditioning. Increasing exposure to the conditioning context increases conditioning to the

training context (Fanselow, 1986,1990; Wiltgen et al., 2006). Previous work has shown that in hippocampus lesioned rodents, context conditioning can be increased by increased exposure to the conditioning context (Wiltgen et al., 2006).

Hence we asked whether the cD2 will be able to improve context learning, if given more time in the conditioning context. We systematically varied the placement to shock interval, and then determined the effects on contextual conditioning.

We observed that in WT mice contextual conditioning was enhanced by increasing the time the mice spent in the conditioning context before administration of the unconditional stimulus (shock) **(figure 6-2 d)**. However, the cD2 nulls failed to show any benefit from increased exposure to the context **(figure 6-2 d)**. A two way ANOVA revealed a significant main effect of genotype $F(1,30) = 53$; $p<0.001$, and placement to shock interval $F(1,30) = 4.8$; $p<0.05$, but no interaction. Post hoc comparisons showed increased conditioning within WT mice with longer placement to shock interval ($t_{17}=2.7$; $p< 0.05$), but not in the cD2 nulls ($t_{13}=0.74$; $p>0.05$).

Summary:

These experiments show that the cD2 null show deficits in hippocampal dependent fear learning and memory, however had relatively intact learning of discrete cues, which is hippocampus independent in most conditions.

Experiment 6.2: Assessment of PFC-dependent cognition using a cognitive set-shifting task

Hypothesis:

Given our finding of reduced PV+ interneuron density and $GABA_A$-mediated inhibition and altered metabolic activity in the hippocampus but not in the prefrontal cortex, we hypothesized that in contrast to hippocampus-dependent cognition, tasks that have been shown to be sensitive to intact PFC function would not be disrupted in the cD2 null mice. We used attentional set shifting experiment to determine if a prefrontal cortex dependent task is intact in the cD2 null mice.

Brief methods:

Animals dug for food reward in bowls containing various digging media (such as cage bedding or gravel) scented with spices. The task consisted of several phases: Day 1) simple discriminations between odors and media, Day 2) compound discrimination (CD), intra-dimensional shift 1 (ID1), Day 3) recall of ID1, reversal of ID1's reward-contingencies, Day 4) ID2, ID3 and extra-dimensional shift (ED). In the CD, two spices were pseudo-randomly paired from trial to trial with two digging-media; the spices predicted reward. For each ID, the spice-pair was changed; for the ED, all four stimuli were changed, and the digging-media predicted reward. Reversal learning in this paradigm has been shown to depend on the orbitofrontal cortex (Bissonette and Powell, 2012), and Extra-dimensional shift on the medial PFC.

Results:

We determined if cD2 nulls differed in 1) ability to form a cognitive set (improvement across ID phases), 2) cognitive-affective flexibility (reversal of a specific stimulus-response contingency) or 3) ability to shift cognitive set (mapping contingencies onto a new modality in the ED). ANOVA showed significant effects of task-phase and genotype on errors to criterion, but no interaction. CD2 nulls made more errors across the whole task, but without specific deficits in reversal learning or the ED (**figure 6-3**).
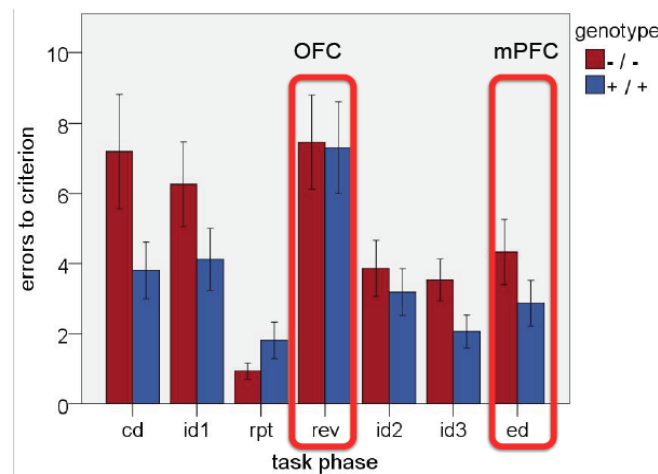
Figure 6-3: cD2 nulls show relative sparing of prefrontal cortical dependent cognitive set shifting task.
Bar graph showing errors to criterion (nine consecutive correct trials) for each discrimination. CD2 null mice showed an overall learning deficit but no selective deficit in orbitofrontal or medial PFC dependent phase of the task.

Each pair of bar is a phase in the task. There is a generalized learning deficit but no differential effect on the PFC dependent phases of the task the deficit doesn't seem to be mediated by oFC (reversal) or mPFC (ED shift) .

Discussion:

Given our findings that PV+ interneuron density is reduced in the hippocampus but not in the prefrontal cortex, we hypothesized that hippocampus dependent tasks will be disrupted in the cD2 null mice, with relative sparing of the prefrontal cortical dependent function. Alternatively, hippocampal dysregulation may disrupt prefrontal function because of heavy hippocampal afferents to this region (Rosene and Van Hoesen, 1977). In conclusion, cD2 nulls show deficits in hippocampus dependent fear memory and learning, a generalized learning deficit, but no specific deficit in PFC dependent cognition. The cD2 null show normal acquisition of fear and showed relatively intact freezing behavior. This rules out a severe generalized learning deficit in these mice. Our data support the hypothesis that even a small-to-moderate reduction of PV+ interneuron function such as that indicated by histopathological studies of schizophrenia, disrupts hippocampal function and cognitive operations that depend on hippocampal circuits.

Contextual learning can be hippocampus-independent under some conditions: Recent studies parse out the conditions during which contextual and cued conditioning depends upon the hippocampus. The hippocampus is thought to increase the efficiency of conditioning so that it plays a role in contextual conditioning when the strength of associating between the US and CS is weak (Wiltgen et al., 2006). Various studies have shown that learning deficits in the presence of a long-standing hippocampal lesion can be overcome by more rigorous training (more frequent US-CS pairing or increasing the time spend in the conditioning context) (Wiltgen et al., 2006). In short, learning is inefficient in the absence of the hippocampus but still possible. This suggests that the deficits in the cD2 null mice are different from the mice with hippocampal lesions (see below).

Hippocampal disinhibition in cD2 null does not mimic a hippocampal lesion: Consistent with previous studies, which show deficits in contextual conditioning in the presence of a hippocampal lesion immediately anterograde or retrograde (with respect to training) (for review see (Fanselow, 2000)), our study shows a selective deficit in hippocampus dependent contextual conditioning in the cD2 null.

However, in contrast to the lesion studies, which showed that in the presence of a hippocampal lesion, contextual learning requires more rigorous training (Wiltgen et al., 2006), the deficits in the cD2 null could not be improved by multiple tone-shock pairing or by increasing the time spent in the training context. We hypothesize that a disinhibited hippocampus leads to persistent aberrant signaling - which may prevent the delegation of the task of contextual conditioning to a extra-hippocampal area. This hypothesis can be tested by lesioning of the hippocampus in the cD2 null, which is expected to improve the contextual learning deficit in these mice.

Possible effect of hippocampal neurogenesis in fear conditioning in the cD2 null mice: Although a deficit in neurogenesis in the dentate has been related to deficits in contextual fear conditioning in some (Saxe et al., 2006; Drew et al., 2010) but not all studies (Clark et al., 2008), the magnitude of the deficit is small and the deficit is remediable by extensive training (Drew et al., 2010).

Our findings differ from the findings of Jaholkowski and colleagues (Jaholkowski et al., 2009), who reported no difference in contextual fear conditioning between cD2 null and WT mice. Although the general methods in our study were similar to those used by Jaholkowshi et al, some differences are worth noting.

1) Jaholkowski et al used 3 tone-shock pairs for training (20 sec tone, co-terminating with a 1 sec 0.7mA foot-shock), in comparison to 5 used in our study.

2) In addition, we have used a longer placement to shock interval (300 s, compared to 120 in Jaholkowski et al).

Consequently, we achieved a much higher freezing in WT mice in test of context (average freezing of over 60% compared to around 40% in Jaholkowshi et al.)

It is possible, that the cD2 null are able to sub-optimally learn about the context (average hovered around 40% in our as well as Jaholkowshi et al), a stronger level of training in the WT increases learning, while the cD2 null fail to improve learning any further. Post-tone freezing in novel context may be more sensitive to disruption of hippocampal function (Quinn et al., 2008), hence would have been worth exploring in the study by Jaholkowski and colleagues.

Possible sparing of PFC-mediated functions

The current results suggest that PFC-dependent cognitive flexibility may be spared cD2 nulls, relative to processes mediated by the temporal cortex. However, there are several caveats to this interpretation

The mice showed a discrimination learning deficit, indicating general deficits in striatal-based learning. It is difficult to interpret "sparing" of PFC function in the context of a possible deficit in striatal based learning

Future studies may investigate whether these animals show deficits in response-inhibition.

In summary, these data support the hypothesis that deficits in GABAergic inhibition in a certain cortical region disrupts the cognitive functions that depend on this region. For example, in schizophrenia, loss of PV+ interneuron function in the hippocampus is likely to contribute to cognitive deficits. Hence, efforts to normalize the GABAegic deficit may lead to improvement in the cognitive deficits in this disease.

Section III: A Study of Partial Restoration of Hippocampal Interneurons Using Progenitor Cell Transplantation

Chapter 7: Effects of Transplantation of Neuronal Precursors Derived from the Medial Ganglionic Eminence into the Hippocampus of the Adult cD2 Null

Introduction:

Survival and differentiation of MGE-derived interneuron precursors in recipient brain:

GABAergic precursor cells can be isolated from the developing MGE during embryonic life. These cells, when transplanted into the brains of young or adult recipient mice, migrate through the brain tissue (Wichterle et al., 1999; Alvarez-Dolado et al., 2006; Baraban et al., 2009) and differentiate into morphologically and functionally normal interneurons (Alvarez-Dolado et al., 2006; Baraban et al., 2009). The transplanted cells are able to survive for a long time in the host brains; survival for as much as a year has been reported (Zipancic et al., 2010).

Difference between MGE progenitors and stem cells:

Stem cells are characterized by two important properties 1) the ability to generate differentiated cells and 2) the ability for self renewal. Division of stem cells result in a certain proportion of daughter cells failing to differentiate and maintaining a stem cell fate. Progenitor cells are cells

that like the stem cells have an ability for self renewal and giving rise to differentiated cells, however unlike stem cells - which show seemingly endless regeneration - progenitor cells are depleted after only a few cycles of cell division (Seaberg and van der Kooy, 2003). In addition, progenitor cells are more fate restricted in their developmental potential and usually give rise to only a handful of cell types or sometimes even a single cell type (Brady and George J Siegel, 2011). Both stem cells and the more fate restricted progenitor cells are attractive candidates for cell based regenerative therapies to treat damage and disease.

Functional effects of transplanted GABAergic cells:

Several groups have reported successful MGE derived GABAergic cell transplant into neonate (Alvarez-Dolado et al., 2006; Baraban et al., 2009) (Southwell et al., 2010) as well as adult brains (Wichterle et al., 1999; Zipancic et al., 2010; la Cruz et al., 2011). Transplanted MGE cells form synaptic connections with pyramidal cells (Southwell et al., 2010), and enhance synaptic inhibition of the surrounding projection neurons (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010). Moreover, these transplants can block seizures (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010) and modulate the behavioral effects of NMDA receptor blockade (Tanaka et al., 2011).

MGE derived cells are functionally integrated into the cortical circuit, where they make and receive synaptic connections (Southwell et al., 2010) and show signs of activation in response to behavioral tasks (Tanaka et al., 2011). Transplantation of MGE derived cells in the visual cortex has been shown to open a developmental critical window, thereby allowing ocular dominance plasticity (Southwell et al., 2010).

MGE derived cells several features that indicate normal development of these cells. For example, these cells express markers of GABAergic fate, such as GAD 67, PV, SST, CR, etc. These cells have been reported to show normal interneuron firing characteristics including the fast firing of the PV+ interneurons (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Southwell et al., 2010). Gross morphological features of transplanted GABAergic interneurons are similar to native interneurons (Alvarez-Dolado et al., 2006).

Lastly, and perhaps most importantly, MGE derived GABAergic cells have been shown to increase GABA-mediated synaptic and extra-synaptic inhibition onto host brain pyramidal neurons (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010).

Behavioral changes following MGE transplants:

The potential therapeutic use of MGE derived GABAergic cells was first explored in seizure behavior in mice. In these studies, genetic (Baraban et al., 2009) or pharmacological manipulations (Zipancic et al., 2010) were used to induce seizure susceptibility. Transplantation of GABAergic cells was then performed to examine whether this procedure could ameliorate enhanced excitability (Baraban et al., 2009). Transplantation of MGE derived cells has also been shown to reduce anxiety like behavior in WT mice (Valente et al., 2012).

Time period required for maturation of transplanted cells:

The transplanted MGE derived cells may require time to reach functional maturation. De la Cruz et al found that the protective effect of MGE transplant on epileptogenesis was evident at 2.5

week post transplant but not at 1 week (la Cruz et al., 2011). Southwell et al reported that the transplants were not effective in increasing plasticity until over a month after the transplant (Southwell et al., 2010).

Transplant of MGE derived GABAergic progenitors into vHIPP in cD2 null mice:

In this series of experiments I sought to determine if live MGE-derived cells transplanted into the adult hippocampus could develop into new inhibitory interneurons and reverse the physiological and behavioral abnormalities hypothesized to be driven by hippocampal disinhibition in the cD2 null mice. To test whether the physiological, and behavioral phenotypes in the cD2 null arise from deficits in GABAergic particularly PV+ interneuron-mediated inhibition, we replaced some to the lost GABAergic cells in the cD2 null hippocampus (described below in detail).

Aims:

1. Whether interneuron deficits in the HIPP is *sufficient* to produce the psychosis-relevant imaging, cognitive and behavioral phenotypes: Our experiment show that, deficit of PV interneurons is associated with disinhibiting and behavioral, cognitive abnormalities. However these do not show that GABAergic deficit is necessary for producing these alterations. CD2 null mice feature various deficits apart from reduction in PV+ interneuron density, which could possibly account for the observed phenotypes.

2. To disambiguate the effects of PV-cell loss in HPP from that in other cortical areas: we increased PV+ cell density selectively in the hippocampus using GABAergic progenitor

transplant: Deficits in PV+ interneurons in the cD2 null is not restricted to the hippocampus, to isolate the contribution of hippocampal disinhibition in producing these deficits, we used transplant of GABAergic progenitors selectively in the hippocampus.

Hypotheses:

If reduction in GABAergic transmission contributes to AMPH hypersensitivity in the cD2 mice, then augmenting GABA transmission in the hippocampus should reduce the locomotor response to amphetamine **(Figure 7-1)**.
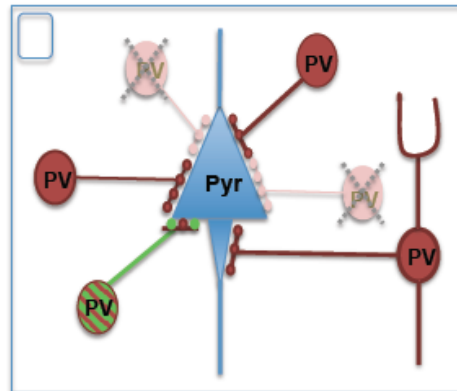
Figure 7-1: MGE derived progenitor cell transplant to increase PV+ interneuron density in the cD2 null.
Schematic of the basic hippocampal circuit, showing a pyramidal cell and its PV+ perisomatic GABAergic inhibitory interneurons. A portion of these neurons (indicated by cross signs) is lost in the cD2 null mice. We attempted to reverse the deficit with MGE derived progenitor cell transplants, which can develop into new PV+ interneurons (shown by the green cell).

Methods:

MGE was identified in acute brain sections made from pan GFP expressing E15.5 embryos. MGE tissue was triturated and washed with Hanks buffer and suspended in neurobasal medium. Around 20,000 cells were injected into each caudo-ventral hippocampus, using a nano-injector. Behavioral and electrophysiological experiments were conducted 6-8 weeks after transplantation. Histological analyses were conducted as much as six months after transplant.

Results:

Localization of the MGE transplant to the ventral hippocampus: Localization of the injections was confirmed in post-mortem histological analysis (**figure 7-2**). The injection procedure caused minimal damage to the hippocampus. We did not find evidence for any inflammatory response in the hippocampus around the injection site. Gliosis was not observed around the site of injections either in nissel stained sections (**figure 7-2c**) or with GFAP immunostaining. Consistent with previous reports, no neoplastic structures were found within the transplanted brains. The control mice which received killed MGE cells showed no GFP+ cells.

Survival and migration of MGE transplant cells in host brain We noticed that the transplanted GFP+ve cells survived in the host brain, An average of about 3706 GFP+ cells survived (± 573 [S.E.M.]; n=4) in the host brain. This represents about 10% of the total cells transplanted into the brains of host mice. No GFP+ cells were observed in the killed MGE transplanted brains.

MGE Progenitor transplant into the ventral hippocampus migrates throughout the hippocampus: Although the injection of the MGE derived progenitors was confirmed to be in

the caudo-ventral hippocampus, we observed that the transplanted GFP+ cells migrated throughout dorso ventral and rostro-caudal extent of the hippocampus (**figure 7-3**). The density of the transplanted cells was highest around the injection site. The GFP+ cells however remained largely restricted to the hippocampus; extra-hippocampal GFP+ cells were only occasionally found.
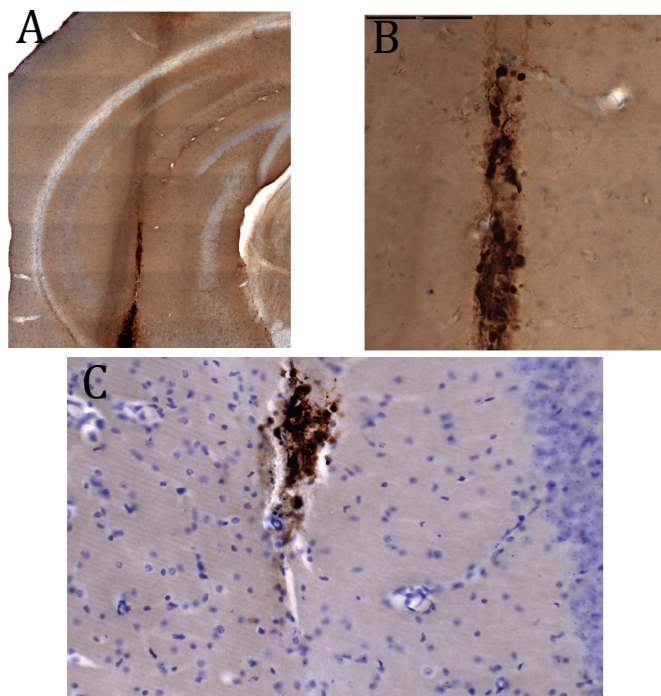
Figure 7-2: Injection site of MGE cells in the hippocampus.
A,B,C) Coronal section of the caudo-ventral hippocampus showing injection track. The mouse was sacrificed 1 day post injection, peroxidase staining was done for GFP. C) The section was counter stained with cresyl violet.

Transplanted Progenitor cells develop into GABAergic Interneurons: MGE derived progenitor cells differentiated into neurons that morphologically resembled normal interneurons (**figure 7-3a,c**) and expressed markers of mature IN fate such as GABA, PV and SST (**figure 7-4**).

Quantitative analysis of GFP cells in sections co-immunostained for GFP and interneuronal markers showed that more than 95% of the cells were positive for GABA. A majority of cells also expressed either PV (56% of all GFP+ cells) or SST (35% of GFP+ cells)(**figure 7-4 d**).
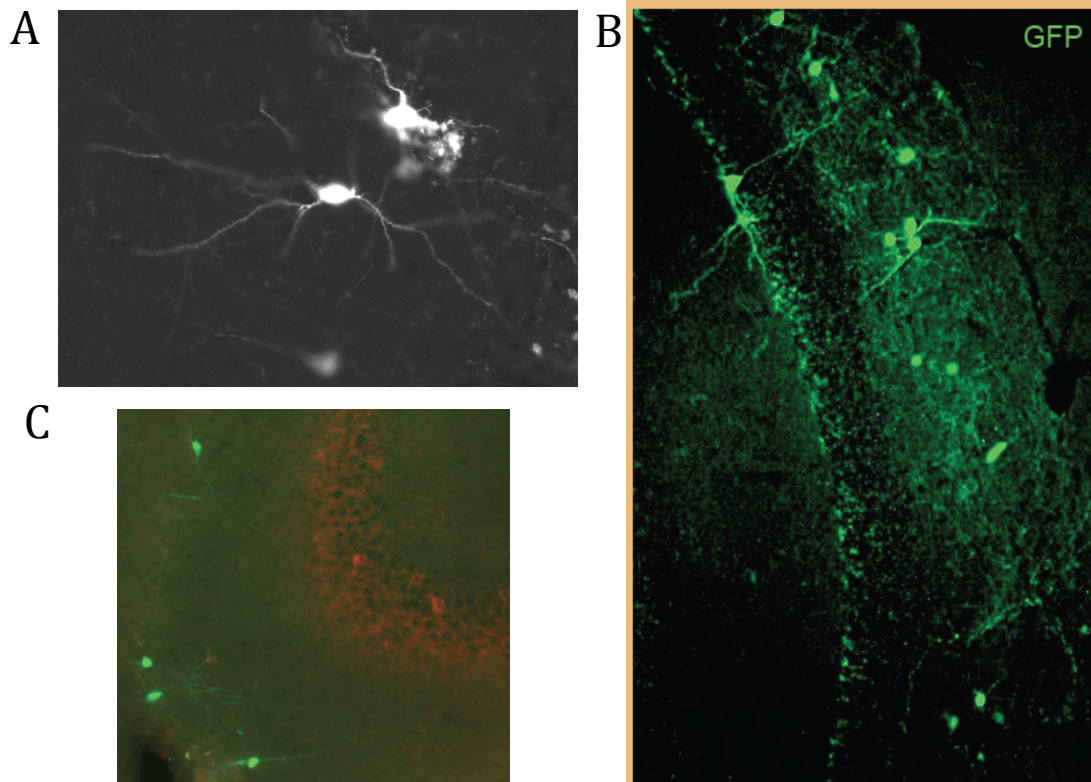
Figure 7-3: MGE-derived progenitors develop into interneurons in the host brain.
A) GFP+ cells show interneuron like morphology. **B)** Hippocampal section from the adult host brain several months after transplantation showing GFP+ grafted cells. **C)** GFP+ cells in the dorsal hippocampus.
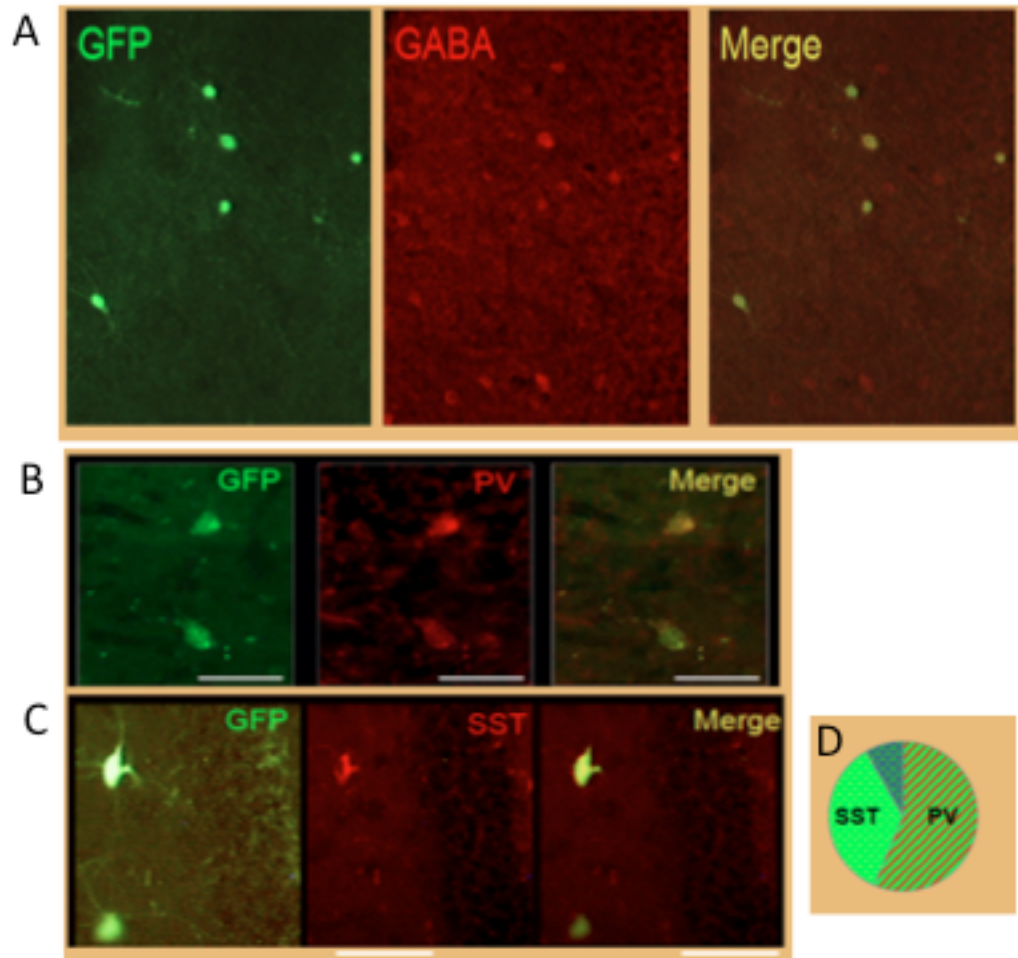
Figure 7-4: MGE derived progenitors express markers of interneurons.
(A, B, C) Transplanted GFP+ cells in the mature hippocampus showing co-expression of GABA **(A)**, Parvalbumin **(B)**, and somatostatin **(C). D)** Percentage of total GFP+ cells co-labeled with PV or SST. Co-labeling assessed separately for PV and SST, thus remaining portion likely includes a mixture of PV+, SST+ and other cell types

MGE transplant normalizes hippocampal metabolism in the cD2 null: First we looked at the

effect of MGE progenitor transplant on the cerebral imaging phenotype in the cD2 null.

Cerebellar (CBL)-corrected hippocampal CBV was calculated as described in Chapters 2 and 4.

As shown in Chapter 4, hippocampal rCBV is higher in non-manipulated cD2–/– relative to cD2

+/+ mice ($t14= 2.2$, $p < 0.05$) (**figure 7-5 a)**. We found that cD2 null mice that received the live

MGE cell transplant showed reduced hippocampal metabolic activity relative to the control

group (con) transplanted with killed MGE cells (live-cell < killed-cell: t2 = 21, p < 0.01) (**figure 7-5 b)**.
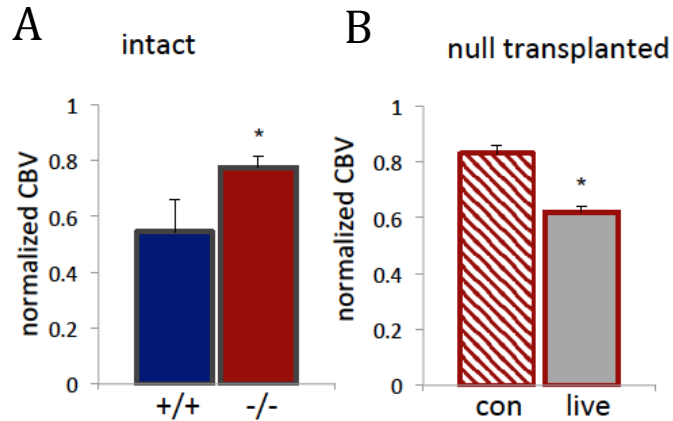
Figure 7-5: MGE transplant normalize hippocampal hypermetabolism
A) Cerebellar (CBL)-corrected CBV in the hippocampus in non-manipulated cD2 null. **B)** Hippocampal metabolic activity in cD2–/– mice with live-MGE cells (live) and the control group (con)

**MGE transplants reduce VTA dopamine cell activity**: Dysregulation of VTA dopamine cell activity is a sensitive measure of disinhibition in the hippocampus. We hypothesized that MGE-derived progenitor transplants would normalize enhanced population activity of dopamine neurons in the VTA. Consistent with this hypothesis, cD2 null mice that received live MGE cells showed a reduction in spontaneously active DA neurons (**figure 7-6)**. On the other hand those that received killed MGE cells (controls) continued to show a greater number of spontaneously active cells in the VTA. No significant change was seen in the WT with either live or killed cell transplants (**figure 7-6)**.
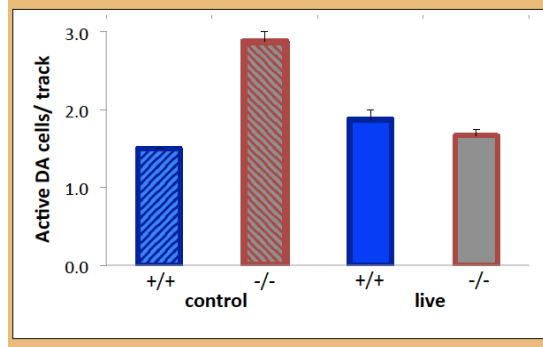
Figure 7-6: Normalization of enhanced dopamine cell activity by MGE transplants in the cD2 null.
Spontaneously active Dopamine cells in the VTA in cD2 null and WT mice with MGE progenitor cell transplant (live) and killed MGE cells (control).

__MGE transplants normalize amphetamine hypersensitivity in the cD2 null:__ We hypothesized that if reduction in GABAergic transmission contributes to AMPH hypersensitivity in the cD2 mice, then augmenting GABA transmission by MGE transplants in the hippocampus would reduce the locomotor response to amphetamine. Consistent with this hypothesis, we found that cD2 null mice that received the live MGE transplant showed reduction of amphetamine response ($t_{12} = 2.4$, $p < 0.05$), while the cD2 null that received killed MGE cells continued to show a high locomotor response (**figure 7-7).**
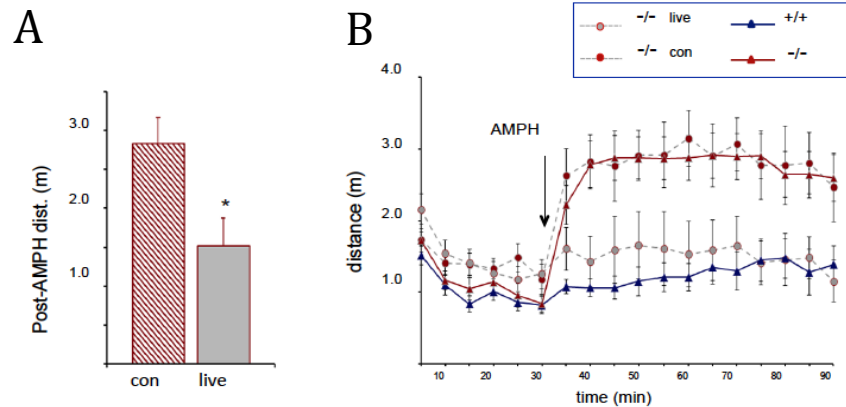
Figure 7-7: Normalization of amphetamine hypersensitivity by MGE transplants in cD2 null mice.
A) Average locomotor response to amphetamine in cD2 nulls receiving live MGE cells (live) was significantly decreased as compared to the controls (con), cD2 null mice that received killed MGE cells.
B) Time course of locomotor activity in live-cell transplanted (dashed gray) and transplant controls (dashed red) with overlay of expected values for intact WT (solid blue) and cD2 null (solid red) mice; arrow indicates time of injection

MGE transplant improves hippocampus dependent fear conditioning in the cD2 null: Both the dorsal as well as the ventral HIPP have been implicated in contextual fear conditioning. The cD2 null mice show evidence for deficits in contextual fear and has deficits in both ventral and dorsal HIPP. Although we targeted the transplants to the vHIPP, the transplanted cells migrated extensively throughout the hippocampus.

CD2 null mice that received MGE transplants showed improved hippocampus dependent memory as evidenced by increased post tone freezing response (Average, $t_{10} = 3.3$, $p < 0.01$), as well as increased conditioned freezing in the training context ($t_{10} = 2.6$, $p < 0.05$) (**figure 7-8**).

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The overall statistical model revealed a significant effect of transplant condition (F1, 11 = 5.3, p < 0.05).
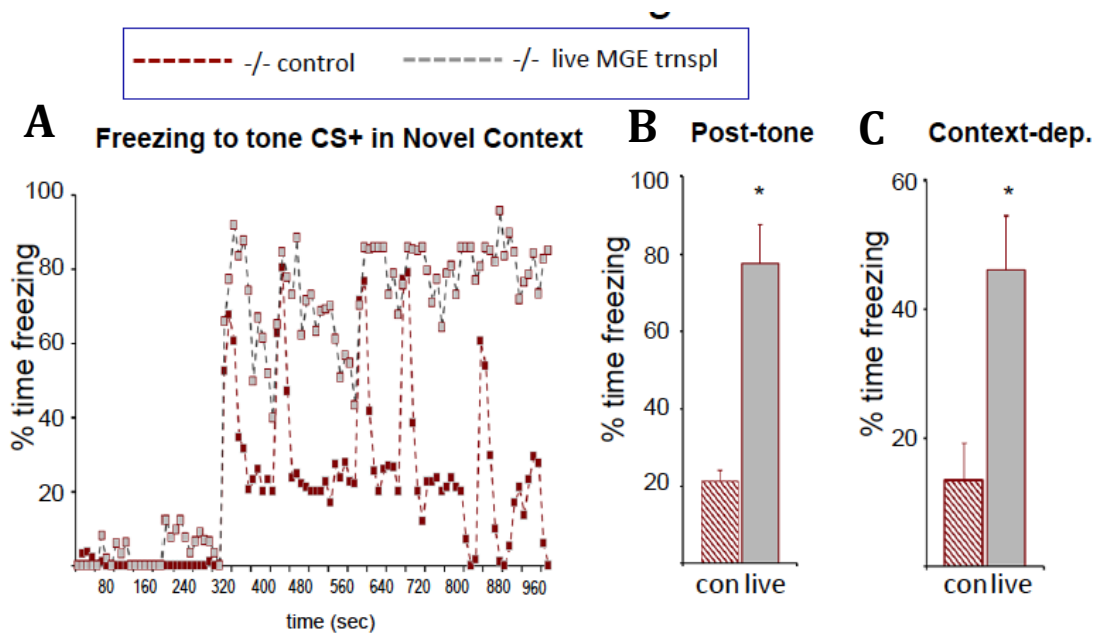
Figure 7-8 MGE transplants improve fear conditioning
A) Cue-conditioned freezing (arrow) in live-cell and control-transplanted cD2 null mice is similarly robust, but post-tone freezing is stronger in live-cell transplanted mice. **B)** Average post tone freezing in novel context in MGE transplanted (live) and control (con) mice. **C)** Average contextual freezing in novel context in MGE transplanted (live) and control (con) mice.

Discussion:

These data show that partial restoration of GABAergic interneurons to the adult hippocampus in a PV+ interneuron deficient mouse model leads to normalization of hypermetabolic imaging phenotype, normalization of VTA dopamine cell activity, normalization of amphetamine sensitivity and improvement in hippocampus dependent learning and memory.

These results raise the possibility that GABAergic transplants to the hippocampus could normalize hippocampal hyperactivity, improve cognitive deficits and positive symptoms of schizophrenia.

A small number of transplanted cells have a large impact: Some data suggests that the transplanted GABAergic cells make and receive three times as many connections as host inhibitory neurons (Southwell et al., 2010), which may partially explain how a small number of surviving transplanted cells may rescue behavioral deficits in the cD2 null mice. Surprisingly this study found that although the transplanted cells showed more connectivity, both excitatory synapses made on the transplanted cells, as well as inhibitory synapses made by the transplanted cells were weaker than those of host GABAergic cells (Southwell et al., 2010). In another study, De la Cruz et al found no relationship between the density of grafted cells and the degree of seizure attenuation (la Cruz et al., 2011). In light of these studies, the disproportionate impact of a small number of transplanted GABAergic cells on behavior remains unclear.

A preliminary analysis showed that, there was no increase in the number of PV+ GFP -ve cells following MGE transplant, ruling out the possibility that MGE transplant leads to a direct proliferative effect on GABAergic cell population or GABAergic precursors. Moreover, adult neurogenesis in the dentate gyrus was not increased in cD2 nulls that received MGE transplants. We noticed that while the cD2 null mice showed deficits in only PV+ cell population, MGE transplant led to the development of both PV+ as well as Somatostain+ve GABAergic cells. To observe whether rescue of behavioral deficits in the cD2 is specific to increase in PV+ cells and not due to another non-specific effect of the MGE transplant, future studies may use transplant of the caudal GE. I hypothesize that such transplant will not reverse the behavioral deficits in the cD2 null.

Taken together, these experiments show that the grafted MGE-derived progenitor cells successfully developed into GABAergic interneurons with normal morphology in the cD2 null hippocampus. CD2 null mice that received MGE transplants showed reversal of metabolic hyperactivity in the hippocampus. Functionally, MGE transplants reversed hyper-responsivity to amphetamine and improved hippocampus dependent learning in the cD2 null. Finally, transplant of MGE derived GABAergic cells normalized increased Dopamine population activity in the cD2 null.

Unanswered questions about GABAergic precursor transplant: Several questions remain unanswered about the MGE derived GABAergic transplants. For example, the mechanisms through which the transplanted GABAergic cells impact the circuit is far from clear. For example, it remains to be seen:

1) Whether the pattern of connectivity of the transplanted cells that express a certain marker such as PV, corresponds with the characteristic connectivity of these cells in native conditions.

2) Whether increase in GABAergic neurotransmission alone is responsible for the rescue of the behavioral phenotype in the MGE transplanted mice (in seizure models and in the cD2 null mouse). Genetic strategies that allow for termination of GABA release (for example, see (Murray et al., 2011)) - in MGE transplant derived GABAergic cells - may be used to answer this question.

3) Whether the survival, connectivity, speciation or function of the transplanted progenitor cells change with activity in the transplanted area, and if this process is affected by the disease state of the recipient mouse.

The effects of the MGE transplants in the adult cD2 null mouse support the rationale for targeting GABA neurotransmission within the hippocampus to treat psychosis. Understanding of the mechanisms through which these cells mediate their effect can open new vistas in the search for better therapeutic approaches in schizophrenia.

Section IV General Discussion and References

Chapter 8: General Discussion

In this thesis, I used an animal model of a disruption of cortical PV+ interneuron development to test whether the resulting PV+ interneuron deficits can give rise to persistent hippocampal disinhibition and ultimately lead to physiological and behavioral phenotypes analogous to brain imaging, cognitive, and behavioral abnormalities linked to hippocampal and DA system dysfunction in psychosis. This study also provides a rationale for designing new therapeutic interventions for psychosis that are aimed at the GABAergic neurotransmission in general and hippocampal interneurons in particular. In this section, I discuss implications of the findings of this thesis for the pathophysiology of psychosis and the potential of cortical interneuron-targeted, stem cell-based therapeutic strategies for this disorder.

A circuit-based model for a role for hippocampal PV+ interneurons in psychosis

To summarize the major rationale for the study, various lines of research implicate altered hippocampal function in the pathophysiology of psychosis in schizophrenia. Imaging studies have shown that the hippocampus in schizophrenia is marked by *hyperactivity*; yet cognitive studies have documented an apparent *hypofunction*. Patients with schizophrenia exhibit a hyperperfusion of the hippocampus (interpreted as increased metabolism) under resting conditions (Heckers et al., 1998; Heckers, 2001b; 2001a; Schobel et al., 2009; 2013) **(figure 8-1)**. This basal hippocampal hypermetabolism is predictive of psychosis in individuals at risk for

psychosis and correlates with the severity of psychotic symptoms (Friston et al., 1992; Liddle et al., 1992; Schobel et al., 2009; 2013). This baseline hyperactivity also corresponds with a less robust recruitment of the hippocampus (i.e. a decrease in the relative increase in metabolic activity) during performance of cognitive tasks that depend on the hippocampus (Heckers et al., 1998; Heckers, 2001b). Postmortem studies have offered a possible neural mechanism underlying this hypermetabolic phenotype by showing alterations in the GABAergic system, particularly in the PV+ interneurons, which implies deficits in GABAergic neurotransmission. However, at the onset of the current studies, it was unknown if PV+ interneuron dysfunction can lead to the hippocampal hypermetabolic phenotype observed in imaging studies in schizophrenia. Moreover, it was unknown whether such a form of hippocampal disinhibition would disruption cognitive function. However, a number of studies have established the importance of PV+ interneurons in regulating patterning and synchronization of neuronal activity within cerebral cortical network, including the hippocampus (Klausberger and Somogyi, 2008; Buzsáki, 2009),and have linked these PV+ interneuron-modulated 'rhythms' to cognitive function (Wang, 2010).Thus, I predicted that loss of PV+ interneurons in the hippocampus would result in disruption of hippocampus-mediated cognitive operations (**figure 8-2**).

Similarly, hippocampal hypermetabolism and excess striatal dopamine have been found to correlate with positive symptoms of psychosis, but in separate lines of studies. Work in rodent models has offered evidence of a neural circuit mechanism that might connect these two neural correlates of psychosis: abnormal hippocampal regulation of striatal dopamine neurotransmission. These studies show that the hippocampus 1) modulates the activity of subcortical dopamine systems particularly of the DA cells in the ventral tegmental area **(figure 8-2)** and 2) increases dopamine efflux in response to amphetamine, thus providing a possible link

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between hippocampal hyperactivity and increased striatal dopamine in patients with schizophrenia **(figure 8-1).** Specifically, reduction in hippocampal PV+ interneuron is proposed to lead to a loss inhibitory input to hippocampal projection neurons. This, in turn, leads to excessive, abnormally-patterned activity and abnormal excitatory signals from the hippocampus to its target regions within the basal ganglia, including nucleus accumbens and ventral pallidum. Through a circuit that has not been entirely characterized, excess hippocampal output to these limbic basal ganglia nodes disinhibits ventral tegmental DA neurons (**figure 8-2**). This model, along with existing empirical data, also predicts that disinhibition of the caudoventral hippocampus increases amphetamine-mediated striatal dopamine efflux and locomotion.

The overarching goal of this thesis was to determine if the above hypotheses might be inter-related and testable in an animal model. I aimed to examine first whether a loss of number or/and function of PV+ interneurons in the hippocampus could lead to disinhibition of the hippocampus and a hypermetabolic phenotype, observed as increased cerebral blood flow in with magnetic resonance imaging method. I then aimed to determine whether this condition could ultimately be related to disruptions of hippocampal-dependent cognition and mesostriatal dopamine system function similar to those characteristic of psychosis. The neural circuitry I hypothesized to mediate these effects of hippocampal disihnhibition is shown in **figure 8-2**.
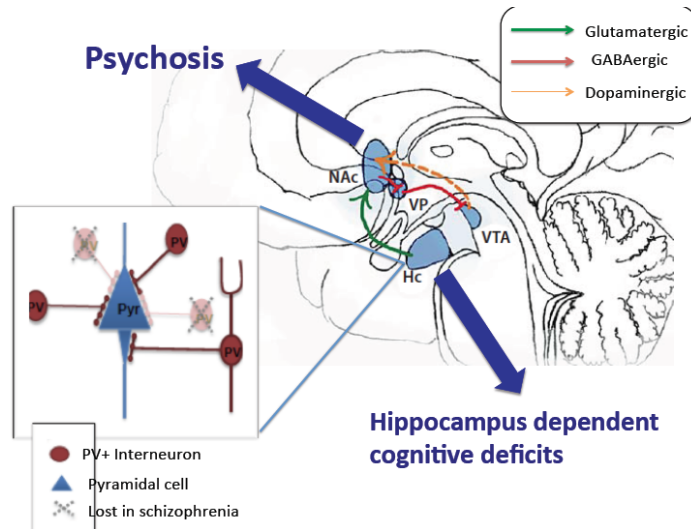
Figure 8-1: Schematic of the probable circuit through which hippocampal PV+ interneuron deficit can drive cognitive and behavioral abnormalities characteristic of psychosis.
NAc, nucleus accumbens, VTA, ventral tegmental area, VP, ventral pallidum, Hc, hippocampus. Figure adapted from (Mikell et al., 2009).

Direct test of this hypothesis is however not possible in humans due to various practical and ethical concerns, however, animal models can establish the plausibility of a hypothesis/ theory about pathogenesis or pathophysiology, originally devised from human findings. Animal models can be used to create a specific alteration in the cortical circuit, and then study the effect of this manipulation on the physiology and behavior. Hence, testing of the aforementioned hypothesis required the availability of an animal model with a selective deficit in the PV+ IN in the hippocampus.
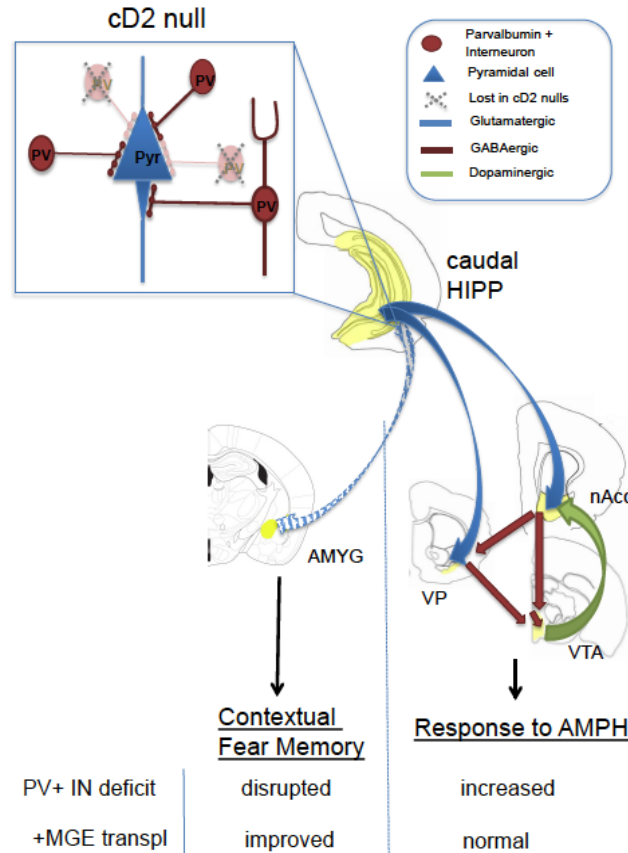
Figure 8-2: Conceptual model of the overall hypothesis of the study.

Loss of hippocampal PV+ interneurons produces cognitive deficits and physiological and behavioral phenotype characteristic of psychosis in the Interneuron deficit models. Summary of the behavioral findings in the cD2 null and the effect of MGE transplants

Generation of animal model of PV+ interneuron deficits

Deficits in PV+ interneurons can be produced in an animal model by various possible strategies.

Modeling of schizophrenia risk alleles in mice:

Several of the prominent risk alleles of schizophrenia, such as the Neuregulin/ ErbB4 and DISC 1, have been shown to regulate GABAergic function (see below). Neuregulin and its receptor ErbB4 have been shown to regulate interneuron migration in the cortex (Flames et al., 2004). Similarly, reductions in PV+ IN density was reported in the mPFC in a dominant-negative form of DISC1 (DN-DISC1) (Hikida et al., 2007). A recent study however reported an increase in

PV+ interneurons in the hippocampus in a DISC1 mutant mouse (Lee et al., 2013), most likely

due to abnormal migration (Steinecke et al., 2012). More studies need to be conducted to

understand the possible relation between schizophrenia susceptibility genes and GABAergic

interneuron development and function. However, in general, the effect of these manipulations is

not limited to the GABAergic system. In fact, the alterations in the GABAergic system were

subtle as compared to that in other systems (Cobos et al., 2005; Hikida et al., 2007; Murray et al.,

2011). In addition, mice models of schizophrenia risk alleles show a multitude of effects on

various cell types, only some of which are currently understood (Thomson et al., 2013). Thus

complicating the interpretations drawn from these models.

Approaches that disrupt interneuronal synaptic input or output: This approach involves

manipulation of synaptic inputs or outputs of interneurons; hence affecting the functional role of

these cells in the cortical circuit. This approach has been used to disrupt glutamatergic synaptic

neurotransmission (Fuchs et al., 2007), and disruption of synaptic release machinery of

interneurons (Murray et al., 2011) which can effectively disrupt the synaptic output of these

cells. However, these studies have not adequately answered the question whether deficits in PV+

interneuron function leads to hippocampal hyperactivity in a way that's analogous to that seen in

patients with schizophrenia. Furthermore the effect of these manipulations on hippocampal

control of mesostriatal dopamine system has not been explored. For example, Fuchs and

colleagues (Fuchs et al., 2007) generated mice with conditional ablation of the AMPA receptor

subunit GluR-A subunit in PV+ interneurons. These mice exhibited disruption of gamma band

oscillations and impairment of spatial working memory. Overall hippocampal metabolism and

regulation of the mesostriatal dopamine was not explored in these studies. Similarly, this

question was not studied by Murray and colleagues (Murray et al., 2011) who ablated GABA

release machinery in PV+ cells. In conclusion, the mice models with selected targeting of inputs or outputs of PV+ interneurons have increased understanding of the role of these neurons in the cortical circuit, however they have been insufficiently explored in testing hypotheses about schizophrenia pathophysiology.

Other approaches that disrupt interneuron development: These models used genetic or toxic interventions to disrupt the development of the interneurons. A targeted ablation of specific interneuronal populations is possible at least in theory because of the spatial and temporal separation of their progenitors and by the virtue of the fact that specific sets of developmental genes operate within different types of progenitors. Manipulation of these genes can help create mouse models with deficits in specific interneuron subclasses (Powell et al., 2003; Cobos et al., 2005; Moore et al., 2006; Penschuck et al., 2006; Fuchs et al., 2007; Glickstein et al., 2007; Xu et al., 2010). In practice however, this approach often gives rise to deficits in multiple GABAergic populations, a spontaneous seizure phenotype, and frequent embryonic or early life lethality, which prevents the use of these models in studies of psychosis-relevant behaviors.

It is notable that a number of developmental perturbations originally discovered to model other aspects of schizophrenia also affect interneuron development. One prominent model in this category is administration of the DNA alkylator methylazoxymethanol acetate at gestation day 17, the " MAM E17 model" (Moore et al., 2006). This model recapitulates the excess striatal dopamine transmission and related behavioral abnormalities, and also shows some hippocampus-dependent cognitive deficits (Lodge et al., 2009). Similar to the mouse models used in this thesis, the MAM E17 rat shows a significant decrease in the density of PV+ interneurons in the hippocampus, with apparent sparing of the somatostatin-expressing subpopulation (Penschuck et al., 2006).

In addition to developmental approaches, repeated exposure to ketamine or phencyclidine, NMDA antagonists known to induce psychosis in humans, also down-regulates PV+ interneurons in the hippocampus and other cerebral cortical regions (Behrens et al., 2007) (Schobel et al., 2013).

Importantly, in these developmental or pharmacological "animal models of psychosis" a direct link between the PV+ interneuron deficits and the cognitive and DA-system phenotypes has not been fully established.

Mutant mice models with developmental deficits of cortical PV+ interneurons

The above rodent models can be categorized as models of schizophrenia etiology or pathophysiology that also show interneuron deficits, or models of interneuron deficits that may have some phenotypes relevant to schizophrenia. However, the circuit model shown in **figure 8-2** had not been explicitly tested in these models. To address this gap,

I used mice with genetic mutations that directly affect interneuron development via well-characterized mechanisms operating in the MGE (Glickstein et al., 2007; 2009; Xu et al., 2010). I chose to study models featuring a prominent and relatively selective PV+ interneuron deficit in the since this is perhaps the most replicated interneuron deficit in post mortem studies of schizophrenia (Lisman et al., 2008; Konradi et al., 2011; Lewis et al., 2012). Moreover, although direct comparisons of studies using different patient cohorts can not be compared, the effect sizes of the PV+ interneuron deficit in post-mortem studies of the hippocampus are, in general, larger than those of prefrontal cortical regions. Consistent with this, I had begun to observe anecdotally that in the cyclin D2 and Six3Cre;Smo$^{f/f}$ models, the PV+ interneuron deficit seemed especially reliable in the hippocampus.

Using the methodology outlined above, I conducted a multi-level analysis of the histological, metabolic, physiological, and behavioral changes arising from disruption of PV+ interneuron development in the cD2 null mutant (Chapters 3-7) and the Six3Cre;Smo$^{f/f}$ mice (Appendix 2). Initial anatomical and physiological results showed a persistent deficit in GABAergic inhibition in the hippocampus, ruling out any significant compensation in these developmental models. While this result does not speak to the question of whether the deficit in PV+ interneurons in schizophrenia is developmental or adult onset, it does demonstrate that a developmental etiology of such a deficit is certainly possible, as it would persist into adulthood and produce persistent disinhibition.

PV+ interneuron deficits and a "hypermetabolic" MRI phenotype

Following this, we found that loss of PV+ interneurons led to hippocampal hypermetabolism as shown by functional imaging (fMRI). We used an imaging technique very similar to what has been used in patients with schizophrenia. Cerebral blood volume (CBV) closely correlates with brain function and metabolism (Small et al., 2011), hence increased CBV suggests increased metabolism in the hippocampus.

Hippocampal disinhibiton disrupts cognition

As mentioned previously, *hyperactivity* of the hippocampus in schizophrenia corresponds with an apparent *hypofunctionality* in performing cognitive operations .I found that the cD2 null mice also showed robust deficits in hippocampus dependent fear conditioning. Interestingly, the deficit in these mice was unlike the effect of a hippocampal lesion (Wiltgen et al., 2006). While learning of contextual fear – which usually requires the hippocampus - continues albeit less efficiently in the presence of an (anterograde) lesion of the hippocampus, the cD2 null showed

no such effect. This again suggested the presence of a hyperactive hippocampus with overdrive of its output structures (such as the PFC and the amygdala).

Hippocampal disinhibition leads to excessive activity in the mesolimbic DA system

Next I studied dopamine cell activity in the ventral tegmental area. Patients with schizophrenia are highly sensitive to the psychotomimetic effects of amphetamine (reviewed in (Lieberman et al., 1987)) - which is linked to striatal dopamine efflux. In rodents, the behavioral readout of this phenomenon is hyperlocomotion. Moreover, various studies have shown that pharmacological activation of the hippocampus leads to increase in the number of spontaneously active dopamine neurons. I predicted that hippocampal hyperactivity would lead to enhanced mesolimbic DA neuron population activity and locomotor response to amphetamine in the hippocampal PV+ interneuron deficit models.

As predicted, an increase in activity of the VTA DA cells was found **(figure 8-2)**, strongly suggesting dysregulation of the mesostriatal dopamine system. Previous literature has generally found a correspondence between increased DA activity, and enhanced amphetamine sensitivity. Hence, I predicted that the PV+ interneuron deficit models would show enhanced locomotor response to amphetamine. This effect was dopamine neurotransmission dependent, and required intact hippocampal activity, as hippocampal lesion eliminated the effect.

Further testing of the model of disrupted hippocampal circuit function using transplantation of neural stem cells from the MGE

The above results showed an overwhelming agreement with the hypothesis that loss of PV+ interneurons in the hippocampus in these mutant mice models led to hippocampal hyperactivity, cognitive deficits and behavioral and physiological phenotypes analogous to psychosis.

However, the question remained whether it was the PV+ interneuron deficit that led to these phenotypes. Both the cyclin D2 null mutation and MGE-selective knock out of Smoothened affect PV+ interneuron populations in other cortical regions. To address whether hippocampal interneuron function was critical to the phenotypes we observed, we adopted a novel approach that involved replacing PV+ interneurons to the hippocampus using transplantation of MGE derived progenitor cells. We showed that restoring GABAergic interneurons to the hippocampus normalized hippocampal hyperactivity, and, in turn, attenuated the excess activity in the mesolimbic DA system and improved hippocampus-dependent cognition (**figure 8-2**).

These results tested the plausibility of the circuit model (**figures 8-1 and 8-2**) that attempts to explain the relation between a disinhibited hippocampus and dysregulation of mesostiatal dopamine system function on one hand, and disruption of hippocampal circuits mediating contextual and episodic learning and memory on the other. Demonstration of this link, previously only hypothesized, not only tests and affirms the model of hippocampal control of the mesolimbic dopamine pathway, but also provides clear potential targets for therapeutic interventions in schizophrenia, as discussed below.

Transplantation of MGE-derived interneuron precursors: implications for novel treatment strategies for schizophrenia

Antipsychotic medications have minimal effect on negative symptoms and cognitive deficits of schizophrenia and inadequately control positive symptoms in as many as a third of all patients (Sadock et al., 2007). Consequently, improved therapeutic interventions are desperately needed for schizophrenia. Identification of novel therapeutic targets may facilitate the development of drugs that are more efficacious and are beneficial for negative symptoms and cognitive deficits. While various neurotransmitter systems have been implicated, all presently known drugs target the dopamine system.

GABAergic cell transplants as a potential treatment for schizophrenia

These results of this study show that partial restoration of GABAergic interneurons to the adult hippocampus in a PV+ interneuron deficient mouse model led to normalization of hypermetabolic imaging phenotype, normalization of VTA dopamine cell activity, normalization of amphetamine sensitivity and improvement in hippocampus dependent learning and memory. These results raise the possibility that GABAergic transplants to the hippocampus could normalize hippocampal hyperactivity, improve cognitive deficits and positive symptoms of schizophrenia.

Possible mechanisms of action of MGE derived cell transplants:

Previous studies had shown the efficacy of GABArgic progenitor cell transplants in controlling cortical disinhibition (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010; la Cruz et al., 2011). Recent findings from several groups suggest that MGE derived progenitors cells that are destined to become GABAergic interneurons- when transplanted into the cortex of

another mouse, survive, migrate in the host tissue and develop into morphologically and functionally mature interneurons. Moreover, the transplanted cells may form functional synapses with the host neurons (Southwell et al., 2010), and enhance GABAergic neurotransmission (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010). These characteristics suggest that the transplant of GABAergic cells may restore excitation/ inhibition balance in mice models of disinhibition (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Zipancic et al., 2010). Whether this is the only (or primary) mechanism by which MGE derived progenitor cells may mediate their action remains to be seen. For example, some studies have shown that embryonic stem cells may promote functional recovery after brain injury by secreting growth factors.

Embryonic stem cells as a potential source of GABAergic cells

The use of human embryonic tissue is fraught with ethical and legal issues, and can be obtained only in limited amounts, which makes it unsuitable for large-scale clinical application. A potential solution to this problem may lie in the use of alternative sources of GABAergic interneurons. One suitable candidate is an embryonic stem (ES) cell. ES cells are derived from the early and have the broadest developmental potential. That is, theoretically, they can develop into any cell type. In reality however, directing ES cells to a certain fate remains a challenging problem. Recently, some groups have shown success in generating GABAergic interneurons from ES cells (Liu et al., 2013). The success of such efforts may be instrumental for the clinical application of GABAergic cell transplants.

Other potential therapeutic approaches targeting the GABAergic system

Apart from GABAergic progenitor cell transplants, other therapeutic modalities can potentially be used to control hippocampal hyperactivity in schizophrenia. Pharmacological agents, which are active at the GABAergic receptor, can be used to enhance or inhibit GABAergic transmission and henceforth study its role on hippocampal function and the effect on hippocampal outputs to the mesolimbic DA system. One of these, the benzodiazepine class of drugs, has a long history of use, as a combination therapy in schizophrenia (Wolkowitz and Pickar, 1991). The use of benzodiazepines is however limited by their non-selectivity for the various types of $GABA_A$ receptors - with different subunit composition. A GABAergic modulator that selectively targets the hippocampus could augment GABAergic activity and control hippocampal hyperactivity. A promising development has been the use of a benzodiazepine positive allosteric modulator (PAM) that is relatively selective for the α5 containing receptors, which have a high level of expression in the hippocampus (Gill et al., 2011). Hence agonist at this receptor can be used systemically with limited non-selective effects. Gill at al studied the effect of this α5 selective PAM on hippocampal activity and mesolimbic dopamine function in the MAM E17 model. Treatment with this drug reduced the number of spontaneously active DA neurons in the VTA of MAM animals and lowered locomotor response to amphetamine, consistent with the idea that positive modulation of GABA receptors in the hippocampus leads to increase in inhibition and a reduction of hippocampal hyperactivity and attenuation of hippocampal hyperdrive of the mesostriatal dopamine system in this model. These interesting findings await further studies to understand the therapeutic potential of this drug.

Another interesting candidate is MK0777, a benzodiazepine-like agent with selective activity at GABAA receptors containing α2 or α3 subunits. Lewis and colleagues (Lewis et al., 2008)

reported improved in a cognitive task in a group of patients with schizophrenia with this drug. A subsequent larger randomized clinical trial however failed to find an improvement in cognitive deficits in patients with schizophrenia with the use of this drug (Buchanan et al., 2011).

General Conclusion

The studies described in this thesis provide strong evidence for the hypothesis that deficits in PV+ GABAergic synaptic neurotransmission in the hippocampus leads to hippocampal hyperactivity, and dysregulation of its output; the mesostriatal dopamine system. These effects may underlie some of the cognitive and positive symptoms of schizophrenia. These results also suggest that therapeutic interventions that target the GABAergic system – particularly the transplantation of GABAergic progenitor/ stem cells may be effective in ameliorating cognitive and psychotic symptoms of schizophrenia.

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Appendix 1: Seizure Susceptibility in the CD2 Null Mouse

Introduction

Seizure and Epilepsy

An epileptic seizure is a paroxysmal uncontrolled neural activity in the brain. The simplified model of epileptic seizures states that there is a balance of inhibition and excitation in the cortex, somewhat like brakes and accelerator in an engine, which when disturbed, leads to seizure activity. The relation between GABA and seizures came out of the knowledge that in many animal and human preparations, antagonists at the GABA receptors can lead to seizures, while its agonists can control seizures. Several studies have shown that the loss of GABAergic interneurons is accompanied by seizures or epilepsy (Cobos et al., 2005), (Powell et al., 2003)

Immediate early gene expression can be used to estimate neuronal activation:

Cellular fos, is a proto-onco gene, which is the cellular counter part of the viral oncogene, v-fos. cfos is rapidly and transiently expressed in many tissues following growth activation. In the neurons cfos is expressed in response to synaptic or electrical activation (Sagar et al., 1988). c-fos codes for a transcriptional factor, which couples extracellular signals to long term changes in cellular phenotype by affecting gene expression (Kovács, 1998). C-Fos expression has been used as a functional marker of activated neurons (Sagar et al., 1988; Kovács, 1998), and used as a tool to delineate individual neurons as well as extended circuits responsible to a wide variety of

external stimuli. Some data suggests that, cFos activity is sensitive to temporal features of action potential patterns, and may show self-regulation of its threshold of expression so that it is activated by 'supra-normal' stimuli (for review see (Kovács, 1998)). A loss of GABAergic inhibition would be expected to lead to an increased excitation of pyramidal cells in the hippocampus.

To reveal hyperexcitability of hippocampal neurons and seizure susceptibility secondary to loss of GABA neurotransmission, we administered the $GABA_A$-benzodiazepeine receptor (BZR) inverse agonist, DMCM (methyl 6,7-dimethoxy-4-ethyl-beta-carboline- 3-carboxylate), a drug that reduces the efficacy of $GABA_A$ receptors without blocking the GABA binding site (Chebib and Johnston, 2000). DMCM (and other benzodiazepine inverse agonists) reduces the affinity of the GABA binding site for GABA and reduce the frequency of opening of the chloride channel while GABA is bound; thus, their effect interacts with the level of endogenous GABA available to bind to the receptor. In these experiments we examined induced c-fos expression and seizure behavior under conditions in which GABA neurotransmission has been reduced with $GABA_A$-benzodiazepeine receptor (BZR) negative modulator.

Hypotheses:

Loss of GABAergic inhibition in the cD2 null will be reflected as increased c-fos expression in the hippocampus and increased seizure susceptibility following administration of a $GABA_A$-BZR negative modulator.

Methods:

Cyclin D2 null, hemizygous for cD2 (het) or WT mice were tested in age, sex and dose-matched sets. DMCM was dissolved at 0.25-0.5 mg/ml, in a solvent of 2-hydroxypropyl-b-cyclodextrin (HBC; 50 mg/ml in saline).

To assess DMCM-induced c-fos expression, DMCM was given at a dose sub threshold for seizures, to reveal patterns of excitation in excitatory frontal and limbic corticofugal pathways hypothesized to be affected in genetic models of Pv+ interneuron deficits. Mice were given Intra-peritoneal injections of DMCM (1.5-2.5 mg/kg in yolked pairs). Previous research has shown that c-fos mRNA is induced within couple of minutes after acute challenge and peaks between 30 and 60 min. Hence we perfused the mice at 60 min after drug administration. Perfusion, fixation and tissue processing used standard methods.

To assess seizures, DMCM was injected at 2 to 10 ml/kg to achieve 3 doses: 0.4 mg/kg, 4 mg/kg, 8 mg/kg. Behavior was videotaped and scored for 20 min post-injection. Onset latency, frequency and duration of seizures, were scored.

Results

CD2 nulls show greater cfos expression in the hippocampus in response to negative GABA receptor modulator: Consistent with the evidence for lack of spontaneous seizures in the cD2 null, no differences in cfos expression were found between the genotypes under baseline conditions. However, when given DMCM, the cD2 nulls showed robust cfos expression in several areas of the brain including the hippocampal formation most notably in the DG.
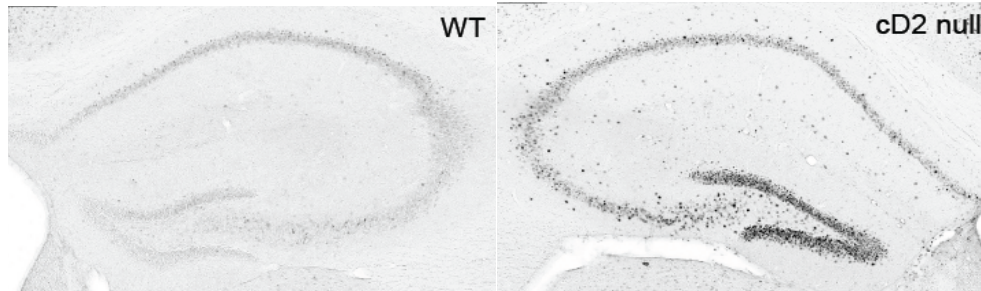
Appendix A1-1: Greater hippocampal activation in cD2 null as shown by cfos expression.
Coronal sections of the dorsal hippocampus, showing immuno-labeling for cfos. The mice were injected with sub-convulsant dose of DMCM and sacrificed 1 hour later.

Lack of spontaneous seizures in the cD2 null: The mice were observed in their home cages for spontaneous seizures/ convulsions at various ages. We did not observe any spontaneous seizures.

CD2 nulls show increased susceptibility to negative modulators of GABA receptor:

Consistent with our hypothesis, we saw that both cD2 nulls as well as WT mice showed seizes in administration of DMCM. The threshold DMCM dose to induce seizure was significantly lower for cD2 nulls, relative to WT, while the heterozygotes (+/-) exhibited an intermediate distribution. At each dose, cD2 nulls had a significantly shorter latency than wild types ($p < 0.05$). Heterozygotes differed from wild types at the 8 mg/kg dose (KS test p's < 0.05).

In order to see if seizure susceptibility in the cD2 null was specific to the GABAergic system, we observed the effects of strychnine- a glycine receptor antagonist. Both cD2 nulls as well as WT showed no difference in seizure latency or threshold with strychnine (data not shown).
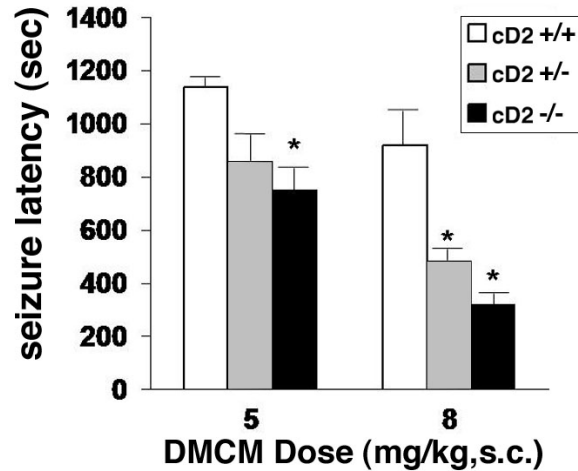
Figure A1-2: Increased seizure susceptibility in the cD2 null.
CD2 null showed reduced latency to seizures, as compared to WT on administration of DMCM, a negative modulator at the $GABA_A$-BZR.

Discussion

The cD2 null mutant mouse shows greater hippocampal cell activation as indicated by cFos expression, and greater susceptibility to seizures, in response to administration of a negative modulator of GABA activity.

The increased cell activation was demonstrated at doses of a $GABA_A$-BZR negative modulator that are sub-threshold for inducing seizures. The induced cFos activity was greatest in the dentate gyrus, a region thought to be involved in generation of spontaneous and evoked seizures (Houser et al., 2012). These data are consistent with a deficit in PV+ interneuron density (Glickstein et al., 2007) and a decrease in GABAergic tone in this region. Consistent with the cell activation pattern, the cD2 null mutant also shows a seizure susceptibility that can be revealed when GABA neurotransmission is partially compromised with the negative allosteric modulation of the $GABA_A$-BZR.

These data are consistent with a previous report (Glickstein et al., 2007) in which the cD2 nulls showed intermittent bursts of faster frequency discharges in cortical EEG recordings, and a shift in the power spectral density towards a higher frequency. The presence of this abnormal activity in the context of absence of spontaneous seizures may indicate that the cD2 null have a cortical irritability phenotype with abnormal electric activity that falls short of spreading widely to produce overt seizures. Absence of spontaneous seizures has also been observed in several other models of interneuron deficit (for example, see (Murray et al., 2011) (Belforte et al., 2009), It is possible that the GABA interneuron deficit is not sufficient to bring GABA tone below the threshold necessary to induce seizure activity.

Alternatively, a developmental loss of GABAergic interneurons during development may lead to compensatory changes in the cortical circuit that reduce the probability of seizure activity. It is important to remember that in most cases compensation is often insufficient to fully counteract the effects of the primary perturbation. Some of the possible compensatory changes have been explored in the cD2 null. The structural and functional studies of Chapter 3 indicated little compensation in the GABAergic innervation of the pyramidal neurons. The deficit in PV+ cell bodies was paralleled by an apparent reduction in PV+ axonal baskets around pyramidal neurons. Functionally, there was little evidence for compensations in GABA input, as evidenced by the deficit in mIPSC frequency with no post-synaptic changes (i.e. in mIPSC amplitude or kinetics). Finally, a previous study showed no change in the density of somatostatin, VIP, NPY expressing cells (Glickstein et al., 2007).

 I have also examined the possibility of compensations in the excitability and glutamate inputs to pyramidal cells. While I found that glutamate-mediated excitatory currents onto pyramidal cells in CA1 were not altered in the cD2 null mutant, I did observe a reduction in intrinsic membrane

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excitability in response to current injection. These findings are similar to the findings from the DLX1 null mouse, another developmental model of interneuron loss. DLX1 null mice loss of a subset of hippocampal and neocortical somatostatin-, NPY-, VIP-, and calretinin-positive interneurons at around one month of age, is followed by the onset of onset of epilepsy (Cobos et al., 2005). These mice show decreased excitability of CA1 pyramidal neurons (assessed in current-clamp recordings of input resistance and rheobase).

Overall, these data show that a developmental loss of PV+ interneurons and the associated reduction in GABAergic neurotransmission is associated with increased cell excitation and seizure activity under conditions when GABA neurotransmission is further compromised.

Appendix 2:   Studies In an MGE-Specific Knockout of the Sonic Hedgehog Effector Smoothened: An Alternate Mouse Model of PV+ Interneuron Deficits

Introduction

In addition to our studies with the cD2 null mice, we used an alternate model of a PV+ interneuron deficit for convergent validity. In this chapter, I introduce the Six3Cre; smo f/f mouse, a conditional mutant of sonic hedge hodge receptor - smoothened and describe the results of experiments performed on this model.

Development of the Six3Cre; smo f/f mouse model

Sonic hedgehog signaling is an important regulator of cell fate. The genesis of PV+ and SST+ interneurons requires the expression of NKX2.1, which is maintained by SHH signaling during neurogenesis. In the Six 3 Cre smof/f model, the elimination in the medial ganglionic eminence (MGE) of Smo, a key effector of SHH signaling, leads to conversion of some MGE progenitors to a caudal ganglionic eminence-like, bipolar calretinin- expressing cell fate. In addition, a higher level of SHH signaling promotes the generation of the somatostatin-expressing inter- neuron at the expense of a PV+ interneuron subgroup. These results indicate that cortical interneuron

diversity, a major determinant of cortical function, is critically influenced by differential levels of SHH signaling within the ventral telencephalon.

Selective reduction in PV+ interneurons in the Six3Cre;Smof/f

In the Six3Cre;Smof/f features a selective reduction in PV+ interneuron density and a subtle increase in a sub class of Calretinin expressing cells. Xu et al (Xu et al., 2010) proposed that a combination of cell autonomous and cell non autonomous effects results in selective reduction in PV+ cells in the cortex, while the number of SST+ cells remains unchanged.

In this model, Cre recombinase is expressed in a striped pattern with in the MGE, leading to excision of SHH effector Smoothened in a subset of MGE cells. As a result, Shh signaling is severely affected in these cells. Disruption of SHH signaling leads to loss of Nkx2.1 expression, resulting in severe disruption of further development along the PV or SST pathways. In turn, these cells show ectopic expression of GSX2, a homeodomain transcription factor, normally enriched in the dorsal LGE and in the CGE. This results in reduction of PV+ and SST+ populations that are normally produced y these progenitors, and increase in a subset of Calretinin+ cells.

In contrast to these cells, the adjacent cells that do not recombine Smo respond by up-regulating SHH signaling (as shown by increased Gli1, Ptch1, and Hhip1 expression). These transcripts are normally enriched in the dMGE.

As a result of these cell autonomous and cell non-autonomous processes, the overall density of SST+ cells is not altered in the Six3Cre; Smo f/f mutants. Accordingly, there is a cell-non autonomous expansion of SST production by MGE progenitors in this mutant. These results

suggested that SHH signaling is important for ventral patterning of the ganglionic eminence, while the development of the dorsal pallidal fates (LGE and CGE) is SHH independent.

Six3Cre; smo f/f show normal brain size and no reduction in cell density in the cortex

Six3Cre;Smof/f show no reduction in brain size and normal cortical thickness (Xu et al., 2010). Cortical lamination is intact and there are no gross changes in cell density (as determined by Tbr or NeuN immune-staining). These data show that the Six3Cre; smo f/f shows a much selective deficit than the cD2 null mouse.

Differences between Six3Cre:Smof/f and cD2 null models

Six3Cre:Smof/f mutants differ from cyclin D2 mutants in a few important ways. Firstly, this model features a much selective manipulation of sonic hedgehog (Shh) signaling. Shh signaling deficit is limited to the ganglionic eminence, instead of the global knockout of cD2 gene in the cD2 nulls. This suggests limited off target effects in this model.

Secondly, microcephaly present in the cD2 null mice is absent in the Six3Cre;smof/f. Thirdly, Six3Cre;smof/f mutants show an up-regulation in the calretinin-expressing interneurons, a subpopulation that synapses on other inhibitory interneurons that, in turn, synapse on dendrites of cortical pyramidal neurons (Freund and Buzsaki, 1996). This implies that whereas both the Six3Cre:Smof/f and cyclin D2 mutants may model a loss of perisomatic GABAergic inhibition to cortical pyramidal neurons, the Six3Cre:Smof/f mouse may additionally feature some disinhibition affecting the inputs to distal dendrites.

These data show that Six3Cre; smo f/f model features a developmental deficit in cortical PV+ interneurons. However, mechanism of the interneuron deficit is different in the conditional smoothened model as compared to the cD2 null. We reasoned that a correspondence between the results of the two models would argue that the phenotype stems from the PV+ interneuron deficit - which is shared by these two models- and not from a non-specific effect of the two mutations, which will be different in the two models.

Experiment A2-1: Stereological quantification of PV+ interneuron deficit in the hippocampus

Hypothesis:

Six3Cre smo f/f will show reduction in PV+ interneurons in the hippocampus.

Brief Methods:

Coronal sections were cut from formalin fixed brains from Six3Cre;Smof/f and control mice Six3Cre;Smof/+. Forty micron thick sections were cut on a vibratome, and 1 in every 5 sections were stained for PV using anti-parvalbumin antibody **(figure A2-1)**. Contours were drawn around the CA1 region using standard atlas (Paxinos and Franklin, 2001). Both dorsal as well as ventral hippocampus was included in the analysis. PV+ interneurons were counted in the entire ROI. The density measure was obtained by factoring in the area of ROI - found using the Cavelieri method. (See also Materials and Methods)

Results:

Six3Cre;Smof/f shows reduced cortical PV-cell density*:* Stereological counting of PV-IR cell

bodies in the CA1 region of the hippocampus showed that the Six3Cre;Smof/f mice showed a

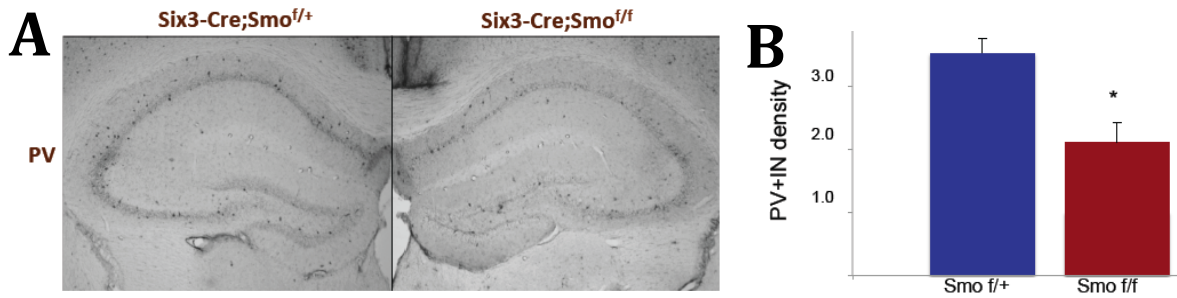reduction in PV+ interneuron density in this region. (t_3=3.6, p=0.037; n=3).

Figure A2-1: Reduced PV+ interneuron density in the hippocampus in Six3Cre;Smof/f mice.
A) Coronal sections of the dorsal hippocampus immunostained for PV. **B)** Average PV+ interneuron density in CA1 region of the hippocampus.

Experiment A2-2: GABAergic inhibitory neurotransmission

Hypothesis:

Six3Cre;Smof/f will show reduction in GABAergic synaptic neurotransmission at pyramidal

cells in the hippocampus.

*Brief Methods***:**

I measured miniature inhibitory post synaptic currents in pyramidal cells in the hippocampus and

the PFC. A mIPSC represents a post synaptic response due to action potential independent,

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stochastic release of a single GABA vesicle. mIPSC frequency is a close approximation of the density of GABAergic synapse on pyramidal cells in organotypic cultures (Hartman et al., 2006; Swanwick et al., 2006). Whole cell voltage clamp recordings were used to measure miniature inhibitory post-synaptic currents from CA1 pyramidal cells. A holding potential of -70mV was used. All recordings were conducted in the presence of TTX (to block fast Na channels), APV (to block NMDA) and CNQX (to block AMPA channels). The recording electrode contained Potassium gluconate or Cesium Chloride to block leak potassium channels. (See also Materials and Methods)

Results:

Six3Cre;Smof/f shows reduced GABAergic inhibition To study if a reduction in PV+ interneuron density in the hippocampus is associated with reduced GABAergic neurotransmission, we measured mIPSCs in voltage clamped pyramidal cells of CA1 region. We found that mIPSC frequency is reduced in CA1 pyramidal cells in Six3Cre;Smof/f. (ttest df 18 p < 0.05 n=12 ko (4 mice), 8 wt (3 mice). No difference was seen in the amplitude, rise time or decay time characteristics of these currents.
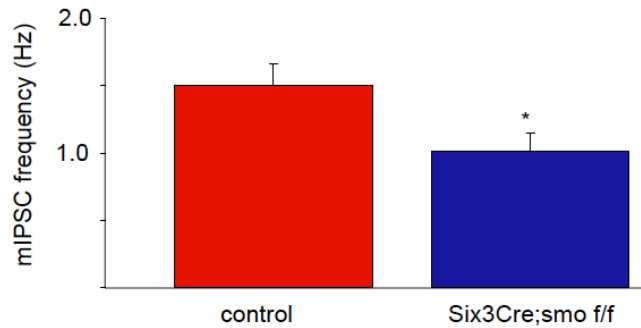
Figure A2-2: Reduced GABAergic synaptic neurotransmission in the hippocampus in Six3Cre;Smof/f mice.
Average GABAergic mIPSC frequency in CA1 pyramidal cells is reduced in the Six3Cre;Smof/f

These data show that a reduction in PV+ interneuron density in the Six3Cre;Smof/f is

accompanied by reduction in GABAergic inhibition in the hippocampus.

Experiment A2-3: Amphetamine sensitivity in the Six3 Cre; smo^{f/f} mice

Hypothesis:

Six3 Cre; smo f/f mice will show enhanced locomotor response to amphetamine.

Brief Methods:

Mice are placed in a 17" x 17" open field box with a white floor. Movement of the mice is

measured by the help of an automated setup and translated into distance. After 30 min of

baseline activity, 2mg/kg amphetamine (dissolved in saline) is administered in an intra peritoneal

injection. Activity is recorded for another 60 min, after which the mice are returned to their home

cages. In a separate cohort, mice were pretreated with either saline or raclopride (0.5 mg/kg) 30

minutes before injection of AMPH (2.0 mg/kg, i.p.) and locomotor activity was measured as

described above. (See also Materials and Methods)

Results:

Six3Cre;Smof/f mice show greater locomotor response to amphetamine.

Six3Cre;Smof/f mice showed an increase in baseline locomotor response and increased response

to amphetamine. A mixed ANOVA model showed a main effect of time $F_{(1,29)} = 4.616$ p=0.04

and a genotype x locomotion interaction $F_{(2,29)} = 7.7$ (p=0.002) (N=15 control, 16 smo
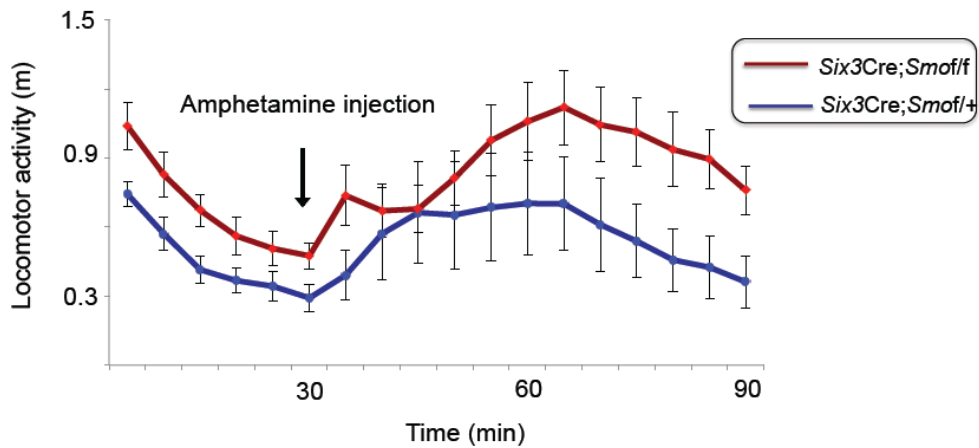
ko)(**figure A2-3**)

Figure A2-3: Enhanced amphetamine sensitivity in the Six3cre;Smo[f/f].
Conditional smoothened knockout mice showed greater baseline locomotion and an increased response to amphetamine. Arrow indicates time point at which 2mg/kg amphetamine was administered.

Discussion

These data show that Six3Cre:Smo$^{f/f}$ mice show a reduction in PV+ interneuron density in the hippocampus. This deficit in associated with loss of GABAergic inhibition on hippocampal pyramidal cells. Loss of GABAergic inhibition predicts disinhibition in the hippocampus. As described earlier, a disinhibited hippocampus and hyper excitation of its outputs will lead to dysregulation of dopamine system - leading to enhanced psychomotor response to amphetamine.

Taken together with the results from the cD2 null mouse, this data support the hypothesis that loss of PV+ interneurons can lead to disinhibition and dysregulation of the dopamine system.