PYRAMIDAL CELLS: ROLE IN PRIMATE PREFRONTAL CORTEX CIRCUITRY DURING POSTNATAL DEVELOPMENT AND SCHIZOPHRENIA

by

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Cognitive deficits constitute a core feature of schizophrenia, are persistent across the course of the illness and are the best predictor of long-term functional outcome. Dysfunction in certain cognitive processes, such as working memory, are common in subjects with schizophrenia and have been attributed to aberrant function of the dorsolateral prefrontal cortex **(DLPFC)**. This dysfunction appears to reflect, at least in part, alterations in excitatory neurotransmission. Cortical pyramidal neurons, the principal source of cortical glutamate neurotransmission, exhibit highly robust molecular and morphological alterations in schizophrenia. These alterations appear to be most pronounced in DLPFC deep layer 3, the same microcircuit necessary for the generation of neural oscillations in the γ-frequency range that sustain working memory function. Understanding how dysfunction in DLPFC cortical circuits in deep layer 3 might give rise to the pathophysiology of altered γ-frequency oscillations and working memory deficits in schizophrenia require an interrogation of the mechanisms by which these neuropathological alterations may arise, but also the normal developmental trajectories of these vulnerable microcircuits. In this dissertation, we provide evidence for pyramidal *cell type-specific* molecular disturbances and *synapse-specific* structural impairments in DLPFC deep layer 3, and *cell type-specific* and *layer-specific* nature of postnatal developmental refinements in pyramidal cells in the DLPFC, within the circuitry that subserves γ-frequency oscillations and working memory. Accordingly, we have identified alterations in the expression of numerous molecular regulators of the actin cytoskeleton in a *layer-specific* and *cell type-specific* manner in DLPFC deep layer 3 in individuals with schizophrenia that might be a critical “upstream” cause

in the pathogenesis of the illness. Additionally, using novel triple-label fluorescence immunohistochemistry and spinning-disk confocal microscopy, we characterize specific synaptic connections onto DLPFC deep layer 3 pyramidal cells in schizophrenia. Finally, we demonstrate that the developmental trajectories of primate DLPFC deep layer 3 pyramidal neurons are *protracted, and layer-specific* and posit that the molecular maturation of GABA synapses on pyramidal cells may account, at least in part, for the maturation of synchronized pyramidal cell firing which is crucial for γ-frequency oscillations.

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ABBREVIATIONS

2pFLIM: Two-photon fluorescence lifetime imaging microscopy

2PLSM: Two-photon laser scanning microscopy

ADF: Actin-depolymerizing factor/ cofilin

AIS: Axon initial segment

AMPARs: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

ARC: Activity-regulated cytoskeleton-associated scaffold protein

ARG: Tyrosine protein kinase Abl-related gene

ARP2/3: Actin-related protein-2/3

BDNF: Brain-derived neurotrophic factor

BMP: Bone morphogenetic protein

BOLD: Blood oxygenation level-dependent

CB: Calbindin

CB1R: Cannabinoid receptor 1

CCK: Cholecystokinin

CCS: Corticostriatal

CDC42: Cell division cycle 42

CDC42EP: CDC42 effector proteins

CNTRICS: Cognitive neuroscience approaches to the treatment of impaired cognition in schizophrenia

CNVs: Copy number variations

CPn: Corticopontine

CR: Calretinin

D1Rs: D1 receptors

DLPFC: Dorsolateral prefrontal cortex

DNMT1: DNA methyltransferase 1

DSI: Depolarization-induced suppression of inhibition

E/I: Excitatory-inhibitory

(F)-actin: Filamentous actin

FRET: Fluorescence resonance energy transfer

(G)-actin: Globular actin

GABA: γ-aminobutyric acid

$GABA_AR$s: γ-aminobutyric acid Type A receptors

GAD65: 65-KkDa isoform of glutamic acid decarboxylase

GAD67: 67-kDa isoform of glutamic acid decarboxylase

GAPs: GTP-ase activating proteins

GAT1: GABA membrane transporter 1

GDIs: Rho guanine nucleotide dissociation inhibitors

GEFs: Guanine nucleotide exchange factors

GWAS: Genome-wide association analyses

HDACs: Histone deacetylases

HSP: Homeostatic synaptic plasticity

IPSCs: Inhibitory postsynaptic currents

KCC2: K^+-Cl^-co-transporter 2

LIMK: LIM domain containing serine/threonine protein kinases

LTD: Long-term depression

LTP: Long-term potentiation

MARCKS: Myristoylated alanine-rich-C-kinase substrate

MATRICS: Measurement and treatment of research to improve cognition in schizophrenia

MD: Mediodorsal

mGIuR2/3: Group II metabotropic glutamate receptor

N-WASP: Neuronal Wiskott-Aldrich syndrome proteins

NARP: Neuronal activity-regulated pentraxin

NKCC1: N^{+}-K^{+}-Cl^{-}co-transporter 1

NMDARs: N-methyl-D-aspartic acid receptors

NPF: Nucleation-promotion factors

ODR: Oculomotor delayed response

OXSR1: Oxidative stress response kinase 1

PAK: p21-activated serine/threonine protein kinases

PANSS: Positive and negative scale score

PING: Pyramidal interneuron network gamma

PNNs: Perineuronal nets

PV: Parvalbumin

PVb: Parvalbumin basket

PVCh: Parvalbumin chandelier

RAC1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog family member A

ROCK: Rho-associated protein kinase

SNARE: Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

SNPs: Single nucleotide polymorphisms

SST: Somatostatin interneurons

synGAP: Synaptic GTPase activating protein

VGCCs: Voltage-gated Ca^{2+} channels

Vglut: Vesicular glutamate transporter

VIP: Vasoactive intestinal peptide

VTA/SNc: Ventral tegmental area and substantia nigra pars compacta

WNK3: With no K (lysine) protein kinase

PREFACE

My journey through graduate school is one that I will always treasure. It is a journey that has served to sharpen my intellectual faculties, made me challenge my presuppositions and inspired me to be a more compassionate scientist, all skills that are necessary in order to be successful in a highly competitive academic climate. Like the journey of many others, it is one that has its fair share of trials and tribulations. However, the Center for Neuroscience (CNUP) and Translational Neuroscience Program (TNP) at the University of Pittsburgh have provided me with an outstanding and exhilarating academic environment, from which I have benefited greatly. Richer from my experiences, I believe that I am poised to make a significant and meaningful contribution to biomedical research. It has been an absolute privilege to work with other like-minded graduate students, faculty members and staff, who are all committed to excellence.

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fascinated I was with the various topics in neuroscience when I took my first class at Colgate University. I hope that enthusiasm and awe-inspiring mentality can stand the test of time.

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“I am a part of all that I have met
Yet all experience is an arch wherethro'
Gleams that untravell'd world whose margin fades
For ever and forever when I move.....”

- from 'Ulysses' by Lord Alfred Tennyson

“The most beautiful experience we can have is the mysterious. It is the fundamental emotion that stands at the cradle of true art and true science”

- from 'The World As I See It' by Albert Einstein

“Man can will nothing unless he has first understood that he must count on no one but himself; that he is alone, abandoned on earth in the midst of his infinite responsibilities, without help, with no other aim than the one he sets himself, with no other destiny than the one he forges for himself on this earth”

- Jean Paul Sartre

“Learn as if you were going to live forever. Live as if you were going to die tomorrow”

– Mahatma Gandhi

1.0 GENERAL INTRODUCTION

1.1 OVERVIEW OF SCHIZOPHRENIA

1.1.1 Burden of schizophrenia

Schizophrenia is a debilitating, chronic neuropsychiatric disease that is highly prevalent and afflicts a large population (0.5-1%) globally (Lewis & Lieberman 2000). The illness is conceptualized as a neurodevelopmental disease whose symptoms are first manifested during late adolescence or early adulthood with most patients experiencing a life-long course of the disease (Lewis & Lieberman 2000). The disease is associated with personal suffering for the patients and is associated with problems related to employment, personal relationships, and self-care. Subsequently, patients with schizophrenia have a greater propensity to suffer from homelessness, poverty, unemployment, comorbid medical issues such as depression, excessive use of nicotine, alcohol and cannabis and increased susceptibility to commit suicide with approximately 30% of patients attempting suicide (Insel & Scolnick 2006, Radomsky et al 1999). As a result of these issues, the average life expectancy in patients with schizophrenia is reduced by about 1-3 decades (Saha et al 2007). Moreover, schizophrenia ranks #14 as the leading cause of disability worldwide and accounts for 3% of the total years of healthy life lost due to disability (WHO 2008).

In addition to the personal suffering endured by patients themselves, the illness is linked with significant emotional burden on caregivers, many of whom are family members, and society

at large (Gibbons et al 1984). Family members who are often the primary caretakers have to also bear immense financial costs associated with the illness. Based on the last estimate in 2002, the costs associated with schizophrenia in the United States amassed to $62.7 billion dollars (Wu et al 2005). The financial costs to society as a whole include both direct (inpatient and outpatient hospitalization and long-term medication costs) and indirect costs (years of lost productivity from patients with the disease and community at large) (Wu et al 2005). Indeed, comprehensive evaluation of the high societal financial burden of schizophrenia globally has revealed that the illness accounts for 1-3% of the total national healthcare expenditure in developed economies (Knapp et al 2004). The burden of schizophrenia is compounded by the general stigma associated with the disease and lack of empathy towards patients suffering from the illness (Penn et al 1994). The stigmatization of individuals with schizophrenia has negative consequences such as loss of independent functioning and reduced life opportunities (Penn et al 1994). In spite of the efforts to increase public awareness in order to reduce the stigmatization of patients with schizophrenia, this problem still persists and places a significant burden on patients (Thornicroft et al 2009).

1.1.2 Etiology of Schizophrenia

Schizophrenia is a highly heterogeneous syndrome and the disease pathogenesis is multifactorial, involving numerous etiological factors that aggravate the chance for developing the disease (Insel & Scolnick 2006, Lewis et al 2005). There is a general view that the illness involves interplay between several genetic liabilities, environmental risk factors and developmental processes to unleash pathogenetic mechanisms which produce a pathological entity, a constrained set of neuropathological alterations in brain structure (Lewis & Gonzalez-Burgos 2006). These cellular and molecular perturbations alter brain circuits and normal physiology and the resulting pathophysiology manifests in the various heterogeneous symptoms

of the disease (Lewis & Gonzalez-Burgos 2006). Genetic predisposition is certainly a major driving force in the pathogenesis of schizophrenia with variable estimate measures suggesting that it can account for 70-80% of cases (Cardno & Gottesman 2000). Monozygotic “identical” twins who share exactly the same genes have a 48% chance of developing schizophrenia, whereas dizygotic “fraternal” twins who share half the number of genes have a significantly lower 17% chance of developing schizophrenia (Gottesman 1991). Moreover, adoption studies have revealed that there is an elevated risk for developing schizophrenia in adopted-away biological offspring of schizophrenia patients (Gottesman & Shields 1976, Rosenthal 1971). Recent breakthroughs involving genome-wide association analyses **(GWAS)** from the *Schizophrenia Working Group of the Psychiatric Genomic Consortium*, analyzing more than 150,000 people, have revealed 108 risk loci identified by single nucleotide polymorphisms **(SNPs)** to be associated with schizophrenia which is thought to contribute to disease susceptibility (International Schizophrenia et al 2009, Schizophrenia Working Group of the Psychiatric Genomics 2014). Exome sequence variations and copy number variations **(CNVs)** in the form of microdeletions or microduplications have also been proposed to confer increased risk for schizophrenia (Malhotra & Sebat 2012, Rippey et al 2013, Walsh et al 2008). In addition, linkage disequilibrium studies have identified chromosomal translocations and duplications in various regions such as 3q29, 5q33, 6q21-22, 15q13.3, 16p11.2, 22q11 (Berry et al 2003, Purcell et al 2014). In spite of the advancements in genomic studies in schizophrenia, the individual contribution of individual genes is low due to limited penetrance and the polygenic burden involving thousands of common alleles increase risk for the illness (International Schizophrenia et al 2009).

A plethora of environmental risk factors and insults during prenatal and postnatal development can confer enhanced risk for schizophrenia (Hoftman & Lewis 2011, van Os et al 2008). Epidemiological studies have shown that maternal immune activation due to infection and obstetrical complications pose an increased risk for the illness (Cannon et al 2002). A wide

variety of postnatal factors that can increase risk for the development of schizophrenia include minority group position, urbanicity, childhood trauma and cannabis use (van Os et al 2010). These findings support the notion that gene-environment interactions could be pivotal in driving pathogenesis of schizophrenia and genetic predisposition, in conjunction with various environmental insults, can alter brain development. As a result, clinical manifestation of the disease may not represent the onset of the illness, but a downstream consequence of years of pathogenic processes in operation.

1.1.3 Epidemiology and clinical features of schizophrenia

Around a century ago, Dr. Emile Kraepelin originally described an assemblage of symptoms as *"dementia praecox"*, which was coined as *"schizophrenia"* later by Eugene Bleuler. Although our understanding of disease pathogenesis has greatly advanced over the course of time, the prevalence and incidence of schizophrenia has remained almost the same (Insel 2010). Meta-analysis studies using population-based data suggest that the prevalence and incidence of schizophrenia is a global phenomenon affecting people across cultures (Messias et al 2007). Interestingly, there is a greater incidence rate in men than women and also some indication that the prognosis is worse in men compared to women (Grossman et al 2008, McGrath et al 2008). Epidemiological studies suggest that numerous factors can predict the severity of schizophrenia. For example, family history is a positive factor that influences several negative and cognitive symptoms (Malaspina et al 2000, Tabares-Seisdedos et al 2003). Moreover, patients who have a lower age of onset typically have a more severe course of disease (Hollis 2000). In addition, patients diagnosed with schizophrenia, as opposed to schizoaffective disorder, which is characterized by symptoms of schizophrenia along with symptoms of mood disorders, have more severe positive, negative, and cognitive symptoms (Cheniaux et al 2008).

The constellation of clinical symptoms of subjects with schizophrenia can be grouped into three categories: *positive*, *negative* and *cognitive (Psychiatric Association 1994)*. The most striking feature of the illness and the clinical symptom that first brings individuals to clinical attention are the positive symptoms, and include disturbances such as delusions, hallucinations and thought organization. Positive symptoms typically reflect the *presence* of abnormal behaviors that create altered perceptions of reality and falsify normative behaviors. Delusions are erroneous beliefs that persist despite significant evidence to suggest otherwise, and include a wide range of themes such as persecution, reference and grandeur. For example, delusions of persecution might force an individual with schizophrenia to believe that they are being treated with malicious intent. Hallucinations are internal sensory perceptions experienced in the absence of external stimuli and can occur in any sensory modality, but are most typically auditory in nature (Andreasen & Flaum 1991, Thomas et al 2007). Auditory hallucinations are present in the majority of subjects with schizophrenia who may hear multiple voices that are distinguishable, yet may lack the ability to discern if they were internally generated or externally generated (Andreasen & Flaum 1991). Thought disorganization usually involves the inability to connect thoughts into logical sequences and is also associated with disorganized inappropriate behavior.

In contrast, negative symptoms reflect the *absence* of certain behaviors that are present in normal individuals and include flattened or inappropriate affect (affect dysregulation), lack of initiative (avolition), poverty of speech (alogia), inability to experience pleasure (anhedonia) and social withdrawal (asociality). These symptoms often result in various emotional and social impairments and are clinically difficult to recognize as part of the disorder since they can be mistaken for other neuropsychiatric conditions such as depression.

Lastly, cognitive deficits, although not currently included in the diagnostic criteria for schizophrenia, but highlighted importantly in the DSM-IV-TR (2000) description of the disease, are thought to represent a *core feature of dysfunction in schizophrenia* (Elvevag & Goldberg

2000). Cognitive deficits refer to abnormalities related to thinking and intellectual skills that allow one to perceive, acquire, understand and respond to information. Over the last decade, the National Institute of Mental Health has formalized various programs to combine cognitive remediation with pharmacological interventions in treating the cognitive deficits in schizophrenia. These include the Measurement and Treatment of Research to Improve Cognition in Schizophrenia **(MATRICS)** and more recently, Cognitive Neuroscience Approaches to the Treatment of Impaired Cognition in Schizophrenia **(CNTRICS)**. These initiatives have emphasized a handful of cognitive domains that deserve special attention in relation to schizophrenia such as working memory, attention, perception, episodic memory, executive function and social/emotional processing (Carter et al 2008). In light of recent developments to understand cognitive impairments in schizophrenia, I will primarily focus on this symptom domain and provide evidence for why they are considered to be core features of the disease in the following sections.

1.1.4 Cognitive impairments as a critical component of schizophrenia

Cognitive deficits emerge prior to the onset of psychosis, persist throughout the lifetime of individuals with schizophrenia and are thought to be present in a majority of patients (Elvevag & Goldberg 2000, Kahn & Keefe 2013, Lesh et al 2011, Reichenberg et al 2010). Approximately 70-80% of patients with schizophrenia show impairments in a battery of neuropsychological tests, although this data might have actually underestimated the prevalence (Kremen et al 2000, Palmer et al 1997). Almost all cognitive domains are affected in the illness with patients with schizophrenia performing ~1-2 standard deviations below the mean performance level of comparison subjects (Heaton et al 2001, Wilk et al 2004). Genetic assessments in identical twins discordant for schizophrenia suggest that the twin with schizophrenia fared poorly compared to the healthy twin in all metrics (Goldberg et al 1995). Cognitive dysfunction is

present in medication-naïve, first-episode patients and also present in a milder form in first-degree relatives of subjects with schizophrenia, indicating that it is not an artifact of neuroleptic treatment and is indeed intrinsic to genetic predisposition and the disease process (Sitskoorn et al 2004). Moreover, when cognitive dysfunction is defined as falling below predicted levels (based on premorbid intellectual functioning and parental education), nearly all schizophrenia subjects (98%) show significant disturbances (Goldberg et al 1990).

Cognitive deficits are the *best predictor of long-term functional outcome* (Green et al 2000). Recent meta-analysis results suggest that cognitive function, but not positive symptom severity, was the best predictor of employment outcome (Tsang et al 2010). Furthermore, cognitive deficits predict the ability of individuals with schizophrenia to integrate into society and future employment potential (Addington & Addington 1999). In sum, cognitive impairments are considered to be a core feature of schizophrenia.

1.1.5 Treatment and outcome

Historically, the treatment of schizophrenia has revolved around the use of antipsychotic drugs that were discovered serendipitously. Originally, drugs such as chlorpromazine, fluphenazine and haloperidol belonging to the class of “typical” antipsychotics were effective in treatment of positive symptoms by blocking dopamine D2 receptors. Unfortunately, blockade of D2 receptors also causes extrapyramidal symptoms which include parkinsonian symptoms (e.g., tremor), dystonia, dyskinesia, akathisia, and tardive dyskinesia. Development of pharmacotherapy resulted in the advent of “atypical” antipsychotics such as clozapine and olanzapine, which in addition to blockade of dopamine, also blocked serotonin receptors. These drugs caused a lower incidence of extrapyramidal symptoms and are associated with fewer side effects such as weight gain, diabetes and hypertension (Howes & Kapur 2009). Although these antipsychotic drugs ameliorate, to some degree, the positive symptoms of the illness, *they have*

little to no effect on negative and cognitive features (Buchanan et al 1998, Harvey & Keefe 2001).

In spite of reasonable success in treating positive symptoms of schizophrenia, the long-term functional outcome for patients is poor and subjects with schizophrenia have limited "recovery". Longitudinal studies support the notion that ~40% of schizophrenia subjects have *limited* social and functional recovery (Lambert et al 2008, Menezes et al 2006). Partial recovery enables some patients to integrate into society to a greater extent by living independently and involves improvements in social and vocational functioning. However, only 14% of patients undergo *prolonged* recovery within the first 5 years of the first psychotic outbreak whereas an additional 16% experience late-phase recovery (Harrison et al 2001, Robinson et al 2004). Therefore, the discovery of novel pharmacotherapy approaches to treat these cognitive deficits and improve long-term functional outcome in patients with schizophrenia is of utmost importance.

1.2 DORSOLATERAL PREFRONTAL CORTEX AND PYRAMIDAL CELLS: CRITICAL MEDIATORS OF WORKING MEMORY AND COGNITIVE FUNCTION

1.2.1 Cognitive dysfunction and the dorsolateral prefrontal cortex in schizophrenia

As described above, schizophrenia is characterized by an overarching deficit in cognitive function across a variety of domains and these impairments are the best predictor of long-term functional outcome. Impaired cognition in schizophrenia may reflect a general perturbation in cognitive control, the ability to adjust thoughts or behaviors in order to achieve goals (Lesh et al

2011). Cognitive control is contingent on the activity of numerous brain regions such as the dorsolateral prefrontal cortex **(DLPFC)**, anterior cingulate cortex and parietal cortex (Cohen et al 1997). Due to its extensive extrinsic and intrinsic anatomical connectivity across numerous sensory and motor brain regions, the prefrontal cortex occupies a critical hub in modulating top-down control of behavior (Miller & Cohen 2001).

Of the various domains of cognitive control dysregulation in schizophrenia, working memory, the ability to actively maintain and manipulate a limited amount of information in a transient fashion to guide future thought or behavior, is a prototypical example of a cognitive control process that has been extensively interrogated in rodent and primate models. Working memory can be divided into the following components: 1) Central executive, the dominant attention-controlling system; 2) Visuospatial sketch pad, to manipulate visual images; 3) Phonological loop, to store and rehearse speech-based information (Baddeley 1992, Baddeley 2003). In schizophrenia, an extensive body of literature has revealed robust alterations that are primarily pronounced in central executive function (Barch 2006, Kim et al 2004), and these alterations are associated with dysfunction of the DLPFC which is known to be crucial for executive function (Callicott et al 2003). A myriad of studies using regional cerebral blood flow and functional magnetic resonance imaging, reflective of neural activity, have documented *hypoactivity* in the DLPFC in schizophrenia (Cannon et al 2005, Glahn et al 2005, Ingvar & Franzen 1974, Minzenberg et al 2009, Perlstein et al 2001). Furthermore, in healthy volunteers, there appears to be an “inverted U” shaped relationship between DLPFC activation and working memory, exhibiting marginal activation at low and high loads, and significantly elevated activity at intermediate loads (Callicott et al 1999). In schizophrenia, the “inverted U” appears to be left-shifted such that they have elevated DLPFC activity and normal performance at low memory loads, but reduced activation and altered performance at higher memory loads (Callicott et al 2003). In aggregate, these findings support a clear connection between disturbances in working memory function and DLPFC activity in subjects with schizophrenia.

1.2.2 Deep layer 3 pyramidal cells: “cellular” basis of working memory

Within DLPFC microcircuits, a cellular mechanism that may subserve working memory function involves the spatially and temporally synchronized activity of ensembles of pyramidal cells during the delay period of working memory tasks. Pyramidal cells are the principal source of cortical glutamate neurotransmission, as well as the targets of the majority of cortical glutamate axon terminals and partake in a myriad of complex circuit operations. These cells constitute ~75% of total cortical neurons and can be readily discerned from other cell-types based on their triangular shaped cell bodies and well defined apical dendrites directed towards the pia mater and numerous short basilar dendrites. In particular, the persistent neural firing during the delay period of working memory is thought to arise from *recurrent excitation* between DLPFC pyramidal cells in deep layer 3 (Goldman-Rakic 1995). The seminal work from Fuster, Kubota and Goldman-Rakic using *in vivo* electrophysiology in the DLPFC in monkeys performing working memory tasks, such as the oculomotor delayed response **(ODR)** task, showed highly tuned, persistent activity during the delay period even in the absence of external stimulation (Funahashi et al 1989, Fuster 1973, Fuster & Alexander 1971, Kubota & Niki 1971). The ODR task is a spatial working memory task that has been used to scrutinize the physiological profile of DLPFC circuits in monkeys (Hikosaka & Wurtz 1983). The task requires subjects to remember the spatial position of the most recent cue over a delay period of multiple seconds, followed by a saccade to the memorized position. Deep layer 3 pyramidal cells in the DLPFC are often referred to as “Delay” cells because they show persistent, spatially tuned firing during the delay period in the ODR task (Arnsten 2009). Therefore, the activity of deep layer 3 pyramidal cells is believed to constitute the *“cellular”* basis of working memory (Goldman-Rakic 1995).

Anatomical tract tracing experiments have suggested that pyramidal neurons in deep layer 3 furnish extensive horizontal ramifications enabling recurrent excitatory connections in the

DLPFC for pyramidal cells with similar spatial tuning characteristics (Kritzer & Goldman-Rakic 1995). These studies corroborated previous experiments which found a similar columnar innervation pattern from the parietal association cortices suggesting that deep layer 3 pyramidal cells with similar spatial tuning properties were reciprocally connected across brain regions (Cavada & Goldman-Rakic 1989, Schwartz & Goldman-Rakic 1984). Moreover, direct physiological evidence support the opinion that spatially segregated clusters of pyramidal cells with similar spatial tuning and temporal profiles of activation across task epochs maintain the network of recurrent connections during working memory tasks (Constantinidis et al 2001). Recent data from iontophoresis studies in monkeys argues that the persistent firing properties of deep layer 3 pyramidal cells depends on N-methyl-D-aspartic acid **(NMDARs)** receptors that are enriched for the GluN2B subunit, found primarily on dendritic spines of long and thin spines (Paspalas et al 2013, Wang et al 2013). GluN2B-containing NMDARs are particularly adapted to maintain DLPFC network firing in the absence of sensory stimulation due to the slower kinetics whereas the faster kinetics of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors **(AMPARs)** can lead to dynamic instability and network collapse (Wang et al 2013, Wang 2001). Consistent with these findings, computational modeling studies propose that persistent neuronal firing requires slower kinetics of NMDAR as opposed to AMPARs for reverberating excitation (Compte et al 2000, Lisman et al 1998, Wang 1999).

1.2.3 Cortical pyramidal neurons in different laminar locations subserve unique functions

Pyramidal cells have often been conceptualized as one homogenous entity which differs starkly from inhibitory neurons, which can be demarcated into subgroups based on unique morphological, neurochemical and electrophysiological properties. However, pyramidal neurons can be divided into subgroups based on the brain region targeted by their principal axonal and

sources of excitatory inputs (**Figure 1)** (Shepherd 2011, Spruston 2008). Pyramidal cells occupy discrete laminar locations and the specification and differentiation of pyramidal neuron subtypes involves precise temporal and molecular regulation during embryonic and postnatal development to impart laminar positional information (Greig et al 2013, Molyneaux et al 2007, Rakic 1988, Rakic et al 1974). In general, superficial pyramidal cells (layers 2-3) primarily target other cortical areas via extrinsic and long-range intrinsic collaterals; pyramidal cells in deeper layers project to the striatum (layer 5) and thalamus (layer 6) (Jones 1984). Quantitative reconstruction of axon collaterals and principal axon projections furnished by pyramidal cells in the supragranular layers of the monkey PFC have elegantly defined the extent of the local axon collaterals (within 300μm of the cell body) and long-range axon collaterals that project through the gray matter before forming distinct stripe-like clusters in the same cortical region (Levitt et al 1993, Pucak et al 1996). Interestingly, consistent with the general "inside-out" pattern of cortical neurogenesis and migration, pyramidal neurons in the infragranular layers (layer 5 and 6) are generated first and achieve adult size of dendritic arbor earlier than pyramidal cells in the supragranular layers (layers 2 and 3) (Rakic 1988, Rakic 2008).

The molecular, morphological and functional properties of pyramidal cells vary substantially between different cortical layers, regions and across species and these differences are most pronounced for pyramidal cells in deep layer 3. For example, studies examining dendritic complexity in primates suggest that the number of spines in deep layer 3 pyramidal cells in the PFC of humans is significantly greater than macaque and marmoset monkeys (Elston 2000). PFC pyramidal cells are more spinous and branched than temporal or occipital lobes and the differences in magnitude are particularly striking in the basilar dendrites of deep layer 3 pyramidal cells, with the PFC having a ~10 fold increase in spine number compared to V1 (Elston 2003, Elston & Rosa 2000). The electrophysiological profiles of pyramidal cells in layer 3 also show great areal specialization with significant differences in input resistance, depolarized resting membrane potential, action potential firing rates and kinetics between V1

and the PFC in monkeys using whole-cell, patch clamp *in vitro* recordings (Amatrudo et al 2012, Luebke et al 2015). These findings reveal important differences in deep layer 3 pyramidal cells across species and between brain regions, substantiating the specialized role of deep layer 3 pyramidal cells in higher order cognitive processes such as working memory.

Pyramidal cells in deep layer 3 that are most critical for working memory receive presynaptic afferents from several different sources **(Figure 1)**: 1) local axon collaterals from surrounding pyramidal cells (Levitt et al 1993), 2) long-distance axon collaterals from pyramidal cells in the same cortical region (Melchitzky et al 1998, Pucak et al 1996), 3) association and calossal projections from pyramidal neurons from several different anatomical regions (Felleman & Van Essen 1991), 4) innervations from the mediodorsal **(MD)** nucleus of the thalamus, although other thalamic nuclei such as the pulvinar nucleus have minor contributions (Giguere & Goldman-Rakic 1988). Therefore, among the different laminar locations of pyramidal cells, those located in deep layer 3 appear to be of particular importance to neuronal network function since they are critically positioned to modulate the flow of excitatory neurotransmission through thalamocortical and corticocortical circuits.

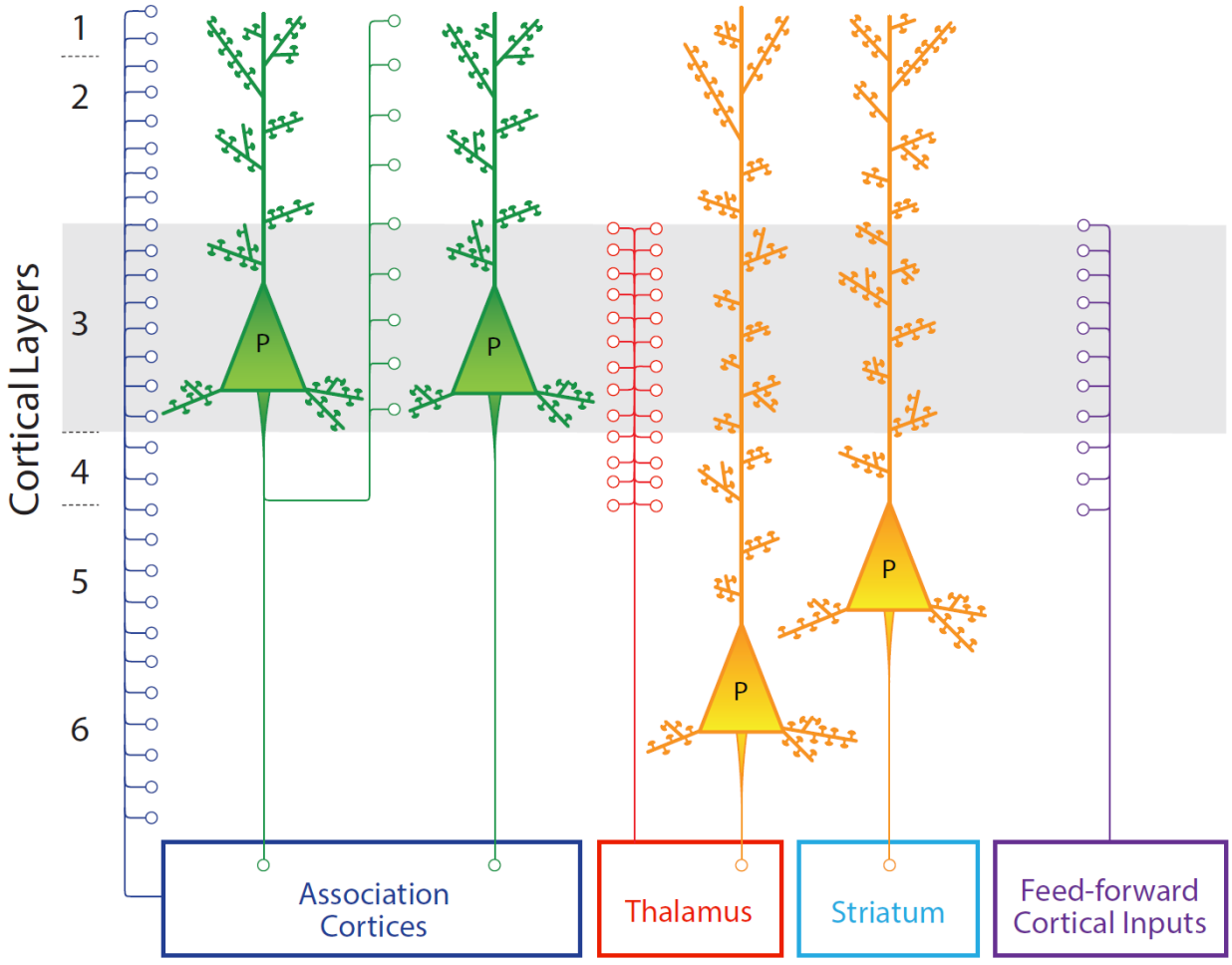

Figure 1. Cytoarchitecture of pyramidal cell organization across laminar locations in the primate DLPFC.

Based on size and packing density of neurons, the cerebral cortex can be stratified into six layers or lamina, from the pial surface of the cortex to the underlying white matter. Pyramidal cells, the primary source of excitatory neurotransmission in the brain, are distributed across these layers and comprise ~75% of total cortical neurons. Pyramidal cells occupying discrete laminar locations subserve unique functions and exhibit specific anatomical connectivity patterns. For example, pyramidal cells in superficial layers such as layer 3 primarily innervate other cortical regions via ipsilateral (same hemisphere) or contralateral (opposite hemisphere) projections and also furnish local, intrinsic axon collaterals that innervate local pyramidal cells. Pyramidal cells in deeper

layers such as layer 5 project to the striatum and brainstem, and layer 6 pyramidal cells primarily target the thalamus. These pyramidal cells also differ in the source of their excitatory inputs. For example, pyramidal cells in layer 3 receive feedforward cortical inputs from reciprocally connected cortical areas and also axonal inputs from the mediodorsal nucleus of the thalamus, whereas inputs from association cortices are distributed throughout all layers of the cortex. This figure was modified from Glausier and Lewis (2013).

1.2.4 Spatial tuning of deep layer 3 pyramidal cells is determined by GABA interneurons

The *synchronization* of pyramidal cell activity and specificity of *spatial tuning* of deep layer 3 pyramidal cells required for working memory, arises from local feedback and lateral inhibition provided by γ-aminobutyric acid **(GABA)** interneurons (Constantinidis et al 2002, Rao et al 2000, Wilson et al 1994). Ultrastructural analyses in monkey PFC have found that pyramidal cells in deep layer 3 furnish intrinsic axon collaterals that contact local GABA interneurons which in turn provide feedback inhibition to pyramidal cells to modulate postsynaptic response properties (Melchitzky et al 2001, Melchitzky & Lewis 2003). GABA interneurons in the monkey DLPFC demonstrate delay period activity that is isodirectionally tuned to nearby pyramidal cells in deep layer 3 (Rao et al 1999). According to the working model, the preferred direction of GABA interneurons is opposite to the preferred direction of pyramidal cells, such that the firing of pyramidal cells increases as the firing of GABA interneurons decreases (Constantinidis et al 2001). Furthermore, pharmacological application of GABA antagonists in the DLPFC suppresses the *spatial tuning* of DLPFC deep layer 3 pyramidal cells required for task related neuronal firing consistent with this working model (Rao et al 2000). Therefore, the sharpening of stimulus selectivity required for spatial tuning is

contingent on concomitant interactions between deep layer 3 pyramidal cells and local GABA interneurons (Constantinidis & Goldman-Rakic 2002). In addition to determining the spatial properties of pyramidal cells, GABA interneurons also subserve a *temporal* function and control when pyramidal cells are active during different phases of working memory (Goldman-Rakic 2002). Therefore, GABA interneurons perform the critical function of modulating the strength of persistent firing during working memory and enhance signal to noise.

1.2.5 Role of DLPFC GABA interneurons in sculpting pyramidal cell activity for cognitive control

GABA interneurons are highly heterogeneous and can be classified into non-overlapping populations based on several characteristics which include axonal and dendritic connectivity patterns, morphology, intrinsic physiological properties, molecular and neuropeptide expression patterns, developmental origins and temporal firing properties during various phases of network activity (DeFelipe et al 2013, Kepecs & Fishell 2014, Klausberger & Somogyi 2008). Distinct subclasses of GABA interneurons are particularly suited to modulate the postsynaptic firing properties of pyramidal cells (Buzsaki & Wang 2012). Based on electrophysiological properties, GABA interneurons can be subdivided into two groups: *fast-spiking* and *non-fast spiking* cells. The feedback and lateral inhibition required to spatially tune pyramidal cells in DLPFC deep layer 3 for sustained, synchronized neural activity is primarily mediated by fast-spiking GABA interneurons that express the calcium-binding protein parvalbumin **(PV)** (Bartos et al 2007, Gonzalez-Burgos & Lewis 2008). PV interneurons are preferentially localized in deep layer 3 and 4 of the cortex and are often referred to as fast-spiking cells because they have fast, non-accommodating firing patterns and fast membrane time constants (Galarreta & Hestrin 2002, Gonzalez-Burgos et al 2005, Hu et al 2014). PV interneurons comprise ~25% of GABA cells in

the primate PFC (Conde et al. 1994) and consist primarily in two separate classes: basket **(PVb)** and chandelier **(PVCh)** cells that innervate the perisomatic compartment and axon initial segment **(AIS)** of pyramidal cells, respectively. As a result of their specific regulation of the site of action potential generation in pyramidal cells, PV interneurons are thought to be critical determinants of the output of pyramidal cells (Hu et al 2014). Additionally, PV interneurons make multiple synaptic contacts with numerous pyramidal cells forming a widespread inhibitory network to modulate the postsynaptic excitatory input mediated depolarization of pyramidal cells (Hu et al 2014, Lee et al 2012). This extensive divergent arborization of PV interneurons allows it to synchronize the activity of multiple pyramidal cells (Bezaire & Soltesz 2013).

There appear to be a plethora of different non-fast spiking GABA interneurons that contribute to the heterogeneity of GABA interneurons. For example, the activity of pyramidal cells and PV interneurons in DLPFC deep layer 3 is influenced by inputs from another functionally and anatomically distinct subtype of GABA basket interneurons that are immunoreactive for cholecystokinin **(CCK)**. In contrast to the unique electrophysiological profiles of PV interneurons, CCK cells have regular-spiking properties due to accommodating firing patterns and slower time constants. In the PFC, the terminals of CCK basket cells are highly enriched with the cannabinoid receptor 1 **(CB1R)** and activation of these receptors with endogenous and exogenous cannabinoids can suppress GABA release following depolarization of postsynaptic pyramidal cells, a process termed depolarization-induced suppression of inhibition **(DSI)** (Eggan et al 2010, Pistis et al 2002, Wilson & Nicoll 2001).

Another class of GABA interneurons that exhibits exquisite spatial specificity in terms of innervations patterns includes those that express the calcium-binding protein calbindin **(CB)**. These cells can be subdivided into subgroups such as Martinotti, neuragliaform and double bouquet cells. Calbindin GABA interneurons are regular-spiking and have accommodating firing patterns and slower time constants. In contrast to PV interneurons, calbindin cells primarily innervate the distal dendrites of pyramidal cells (Kawaguchi & Kubota 1998, Zaitsev et al 2005).

Finally, the last group of GABA interneurons that comprise ~50% of GABAergic cells in the monkey DLPFC express the calcium-binding protein calretinin **(CR)** and neuropeptide vasoactive intestinal peptide **(VIP)** and have similar firing properties as calbindin cells (Kawaguchi & Kubota 1996, Melchitzky et al 2005, Pi et al 2013). In addition to innervating distal dendrites of pyramidal cells, calretinin cells also target other GABAergic interneurons (Melchitzky et al 2005).

1.2.6 Molecular heterogeneity in GABA receptors

The postsynaptic compartment of GABA inputs is also highly heterogeneous and GABAergic inhibition is primarily mediated by type A receptors (Farrant & Nusser 2005, Jacob et al 2008). Type A GABA receptors **($GABA_ARs$)** are ionotropic channels that permit the passage of chloride and bicarbonate ions. $GABA_ARs$ operate by producing a minor change in membrane potential but generate large conductances that shunt excitatory input-mediated depolarization following GABA activation. $GABA_ARs$ mediate fast synaptic GABA neurotransmission and are heteropentamers that are typically composed of 2α, 2β, and 1γ subunits from 7 different families (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3) (Farrant & Nusser 2005). $GABA_ARs$ with different subunit composition are spatially organized in different subcellular compartments along the pyramidal cell somatodendritic axis and have different physiological and pharmacological properties. The assembly, membrane trafficking and spatial organization of GABA receptors is determined by multifarious trafficking mechanisms to produce the immense heterogeneity for postsynaptic regulation (Jacob et al 2008). $GABA_ARs$ have been particularly relevant in clinical settings for pharmacological drug targets that have anesthetic, sedative and anticonvulsant function (Henschel et al 2008).

Different subtypes of GABA interneurons selectively target different domains on pyramidal cells and regulate their activity through different $GABA_ARs$ **(Figure 2)** (Farrant &

Nusser 2005, Huang 2014, Huang et al 2007, Klausberger & Somogyi 2008). For example, PVb cells preferentially innervate $GABA_A$Rs that contain the α1 and β2 subunits which are specifically localized to the perisomatic compartment and proximal dendrites (Doischer et al 2008, Nusser et al 1996). In contrast, PVCh cells form unique vertical terminal arrays called cartridges and preferentially innervate α2-containing $GABA_A$Rs at the AIS of pyramidal cells (Loup et al 1998, Nusser et al 1996). Distal dendrite targeting SST-containing Martinotti cells primarily innervates α5-containing $GABA_A$Rs localized on the dendritic shaft of pyramidal cells (Ali & Thomson 2008, Serwanski et al 2006). Finally, $GABA_A$Rs can also be delineated into different subtypes based on proximate localization to a GABAergic presynaptic input. For example, γ2-containing $GABA_A$Rs co-assemble with the α1, α2, α5 and β2 subunits to mediate *phasic* inhibition which involves the synchronized activation of receptors following GABA release into the synaptic cleft (Farrant & Nusser 2005, Jacob et al 2008). In contrast, δ-containing $GABA_A$Rs are spatially dispersed in a stochastic manner in extrasynaptic locations and are activated by ambient GABA to mediate *tonic* inhibition (Farrant & Nusser 2005, Jacob et al 2008). Importantly, the molecular heterogeneity of $GABA_A$Rs has significant functional repercussions, since α1-containing $GABA_A$Rs have much faster decay kinetics than currents generated by $GABA_A$Rs containing other α subunits (Lavoie et al 1997).

Given the sheer complexity of GABA interneuron subtypes and the myriad of physiological properties that have important ramifications for network operations, elucidating the specific subpopulations that are preferentially affected in schizophrenia has been an area of active investigation. Moreover, elucidating the developmental trajectories of GABA inputs to pyramidal cells across laminar location in a cell-type specific fashion is critical in understanding the maturation of pyramidal cells, which are essential for working memory function. The next few sections will focus on abnormalities related to glutamate and GABA signaling in schizophrenia.

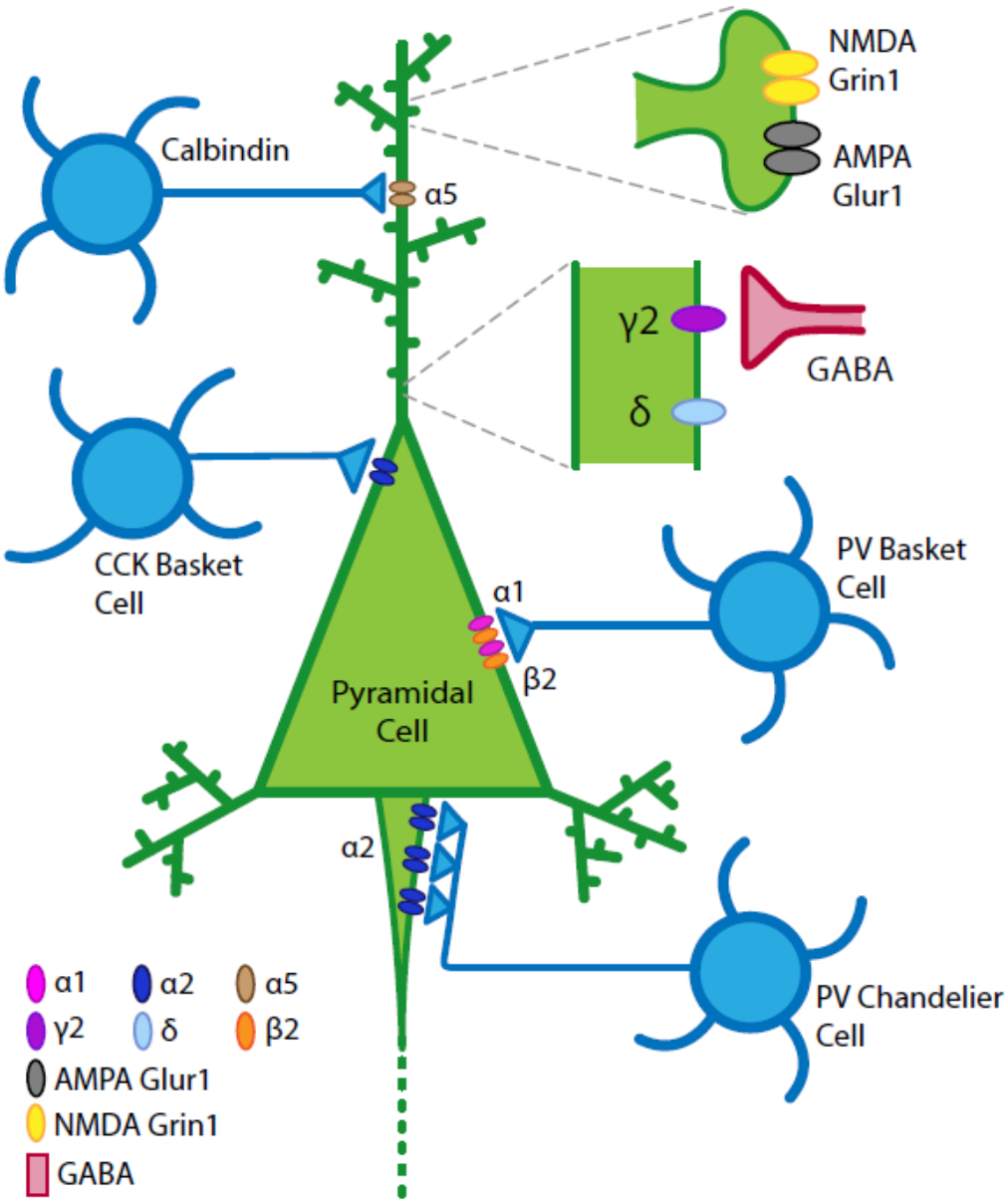

Figure 2. Canonical subcellular distribution of postsynaptic GABA and glutamate receptor subunits in cortical pyramidal cells.

The diagram illustrates the exquisite specificity by which different morphological classes of GABA interneurons innervate and regulate the output of pyramidal cells by targeting different subtypes of $GABA_A$Rs. Different subtypes of GABA interneurons target discrete subcellular compartments along the somatodendritic arbor of pyramidal cells to enable spatial control. Parvalbumin (PV) and the cholecystokinin (CCK) expressing basket cells primarily innervate the perisomatic compartment of pyramidal cells and signal through α1- and α2-containing $GABA_A$Rs. PV chandelier cells specifically target the AIS of pyramidal cells and signal through α2-containing $GABA_A$Rs. By contrast, calbindin-expressing cells (double bouquet, neurogliaform

and Martinotti) provide inhibitory inputs to the distal dendrites of pyramidal cells and signal through α5-containing $GABA_AR$s. $GABA_AR$-mediated synaptic neurotransmission is primarily mediated by γ2-containing $GABA_AR$s which when bound to GABA released presynaptically produces synchronous opening of ion channels. However, low GABA concentrations in the extracellular space can also produce persistent or tonic activation of $GABA_AR$s mediated by δ-containing $GABA_AR$s. As opposed to the spatial heterogeneity of $GABA_AR$s, glutamate neurotransmission is primarily mediated by AMPA Glur1 and NMDA Grin1 containing AMPA and NMDA receptors, respectively, localized on dendritic spines.

1.3 EVIDENCE FOR ALTERED EXCITATION AND INHIBITION IN SCHIZOPHRENIA

1.3.1 Aberration in excitatory neurotransmission in schizophrenia

A constellation of findings support the idea that DLPFC cortical alterations in schizophrenia may arise, at least in part, from aberrant excitatory neurotransmission. Previous postmortem studies have identified morphological disturbances in pyramidal cells in subjects with schizophrenia. These morphological changes include a lower density of dendritic spines, which reflect the number of excitatory innervations to pyramidal cells. For example, basilar dendritic spine density in Golgi-impregnated pyramidal cells was significantly lower by ~25% on deep layer 3 pyramidal cells in subjects with schizophrenia relative to normal comparison subjects and lower by 16% in psychiatrically ill comparison subjects (Glantz & Lewis 2000). Within the same subjects, these alterations are laminar-specific since the findings in the DLPFC are most pronounced in deep layer 3, present to a milder degree in superficial layer 3 and unaltered in layer 5 and 6 (Kolluri et al 2005).The decrement in spine density in subjects with schizophrenia has been observed in multiple brain areas in addition to the DLPFC, such as the temporal cortex and subiculum within the hippocampal formation, although the magnitude of difference between subjects groups has varied considerably (Broadbelt et al 2002, Garey et al 1998, Konopaske et al 2014, Rosoklija et al 2000, Shelton et al 2015, Sweet et al 2009). For

example, recent studies using multi-label confocal microscopy approaches have identified putative dendritic spines using a combination of postsynaptic markers, and have found a marked decrease in dendritic spine density in deep layer 3 in the auditory cortex in schizophrenia (Shelton et al 2015, Sweet et al 2009). Importantly, dendritic spine density alterations were not found in monkeys chronically exposed to antipsychotic medication, suggesting that spine density perturbations observed in subjects with schizophrenia is not related to antipsychotic use (Sweet et al 2009).

In addition to morphological alterations in dendritic spines, somal size and dendritic arbor of deep layer 3 pyramidal cells is reduced in subjects with schizophrenia in the DLPFC and auditory cortices and unchanged in pyramidal cells in the deeper layers (Glantz & Lewis 2000, Pierri et al 2001, Rajkowska et al 1998, Sweet et al 2004). For example, mean somal volume of Nissl-stained DLPFC deep layer 3 pyramidal cells was ~10% smaller in subjects with schizophrenia relative to matched normal comparison subjects, an alteration that was not explained by either antipsychotic medication history or duration of illness (Pierri et al 2001). These findings are compelling because alterations in somal size may indicate disturbances in neural connectivity since somal size is correlated with measures of dendritic and axonal arbor (Hayes & Lewis 1996, Lund et al 1975). Thus, morphological alterations are specific to or at least most prominent in deep layer 3 in the DLPFC of subjects with schizophrenia, are found in other cortical areas, and reflect the underlying disease process and not confounding variables.

Subjects with schizophrenia have also been reported to have smaller whole brain volumes during the prodromal stage, around the onset of overt psychosis and throughout all stages of the disease (Lawrie & Abukmeil 1998, Steen et al 2006), reductions in gray matter (Glahn et al 2008), and neuropil (Dorph-Petersen et al 2009). Together, the morphological alterations in pyramidal cells such as reduced spine density and dendritic arbor are conserved pathological deficits across brain regions and may contribute to reduced brain volumes and cortical neuropil.

1.3.2 Dendritic spines: structure and function

Dendritic spines are morphologically unique and biochemically discrete entities that emanate from the dendritic shafts of cortical and hippocampal pyramidal neurons, cortical spiny stellate cells, cerebellar Purkinje cells and medium spiny neurons of the striatum (Gray 1959, Harris & Kater 1994). Santiago Ramon y Cajal originally described dendritic spines using a silver impregnation method developed by Camillo Golgi and hypothesized that “such spines could be the points where electrical charge or current is received” (Ramon y Cajal, 1888). Decades later, it was established that the vast majority of excitatory synapses in the central nervous system (approximately 80-95%) are formed onto dendritic spines (DeFelipe & Farinas 1992, Gray 1959), avoiding dendritic shafts. These morphologically refined structures standout as the anatomical hallmark of distributed neural networks and has been the focus of several lines of investigation.

Subsequent studies using electron microscopy have exquisitely reconstructed dendrites and spines from serial sections to reveal the three-dimensional ultrastructure of spines on dendrites. The prototypical dendritic spines are membranous protrusions from dendritic shafts and consist of a bulbous head linked to the shaft through a thin spine neck (Harris & Kater 1994). Based on such findings, dendritic spines have been categorized into different subtypes using morphological parameters such as spine head size and neck length (Peters & Kaiserman-Abramof 1970). As per the most widely used nomenclature, dendritic spines can be demarcated into the following subtypes: thin, stubby, mushroom and filopodia (Peters & Kaiserman-Abramof 1970). Dendritic spine morphology correlates with strength of synapse with larger spines containing higher levels of AMPARs, enhanced response to glutamate stimulation and greater postsynaptic density protein networks (Matsuzaki 2007). High frequency activity patterns that induce long-term potentiation **(LTP)**, a well-characterized memory consolidation paradigm, are also associated with morphological changes such as increases in synaptic efficacy and

enlargement of spine heads (Kasai et al 2003). More recently, live imaging *in vivo* studies using modern digital microscopy methods such as 2-photon laser scanning microscopy **(2PLSM)** suggest that some dendritic spines are highly dynamic structures changing in size and shape over timescales of seconds to days in rodents (Alvarez & Sabatini 2007, Holtmaat et al 2006, Stettler et al 2006). Therefore, although dendritic spines exist in various morphological subtypes, these *in vivo* studies have proposed the idea that dendritic spine subtypes could be a part of a continuum of morphologically plastic structures (Yuste 2011).

Time-lapse studies of dendritic spine structural plasticity *in vitro* and *in vivo*, have put forward the view that dendritic spine structural plasticity is strongly correlated with synaptic plasticity (Hayashi & Majewska 2005, Holtmaat & Svoboda 2009). Moreover, dendritic spines are dynamically regulated by activity-dependent and experience-dependent modulatory mechanisms (Trachtenberg et al 2002). For example, elimination of presynaptic input following deafferentation produces a striking loss in dendritic spine density lending credence to the idea that neurotransmission acts as a signal to maintain the structural integrity of spines (Valverde 1967). Dendritic spines are particularly adapted to respond to neural activity because they contain the postsynaptic density, an electron-rich region juxtaposed to the presynaptic bouton active zone where neurotransmitters are released into the synaptic cleft (Scannevin & Huganir 2000). Together, although the exact function of dendritic spines remains enigmatic, these highly specialized structures help neural circuits achieve three important goals: (1) spines help to make the circuit connectivity matrix more distributed by augmenting synaptic connectivity between pyramidal cells (Yuste et al 2000); (2) to make excitatory input integration nonsaturating and linear by electrically isolating inputs (Araya et al 2006, Bloodgood & Sabatini 2005); (3) spines perform the critical function of ensuring that these connections are independently plastic by ensuring input-specific synaptic plasticity (Yuste 2011).

1.3.3 Actin cytoskeleton: Crucial regulators of Dendritic Spines

Dendritic spine structure, shape and stability are determined internally by the actin cytoskeleton which is thought to be crucial in regulating the postsynaptic signaling machinery and molecular composition of synapses within spines (Sheng & Hoogenraad 2007, Tada & Sheng 2006). The actin cytoskeleton exists in a dynamic equilibrium between monomeric globular **(G)-actin** and polymerized fibrous, or filamentous, **(F)-actin** and exhibits a high turnover rate via continuous treadmilling (Honkura et al 2008). The actin meshwork is apposed to the spine membrane and long-term changes in synaptic efficacy require optimal regulation of F-actin (Koleske 2013). For example, actin-GFP Fluorescence Resonance Energy Transfer **(FRET)** experiments have shown that actin polymerization is necessary for LTP induction in spines, whereas long-term depression **(LTD)** induction is associated with actin depolymerization (Honkura et al 2008, Okamoto et al 2004). Moreover, stabilization of dendritic spines and arbors is inextricably linked to polymerization of F-actin, particularly during neurodevelopment, when nascent synapses are preserved or eliminated depending on the source of presynaptic input (Cline & Haas 2008, Wu et al 1999). In addition, numerous genetic and pharmacological experiments indicate that activity-dependent actin cytoskeleton reorganization is necessary for morphological plasticity of spines and long-lasting changes in synaptic efficacy (Cingolani & Goda 2008, Matus 2000).

A plethora of extracellular molecules bind to cell surface adhesion receptors such as cadherins, integrins, immunoglobulin superfamily receptor complexes and ephrin receptors to activate numerous signal transduction pathways. These signaling pathways have been proposed to regulate the actin cytoskeleton in order to influence the establishment, development and plasticity of dendritic spines. In particular, the Rho family of GTPases (Ras Homolog family member A **(RhoA)**, Ras-related C3 botulinum toxin substrate 1 **(RAC1)** and cell division cycle 42 **(CDC42)**) is a crucial mediator of the actin cytoskeleton and has a significant impact on spine

morphogenesis (Saneyoshi et al 2010). These GTPases are highly concentrated in spines and have differential effects on spine regulation. For example, constitutive activation of RAC1 and CDC42 promote spine growth and stability while RhoA inhibits spinogenesis (Hall 1998, Soderling et al 2007). CDC42 and RAC1 are both crucial for the formations of filopodia and lamellopodia which may develop into mature spines, suggesting that these Rho GTPases are needed for the establishment of the full complement of dendritic spines (Ridley 2006). Recent studies using two-photon fluorescence lifetime imaging microscopy **(2pFLIM)** have demonstrated that activation of Rho GTPases is restricted to stimulated spines undergoing structural plasticity and exhibited a steep gradient at the spine necks (Murakoshi et al 2011).

Rho family proteins can bind to both GTP and GDP and have intrinsic GTPase activity (Chardin 2006). In the GTP-bound state, they can interact with and activate numerous downstream effector proteins, whereas, in the GDP-bound state, they are held in an inactive conformation (Bourne & Harris 2008). The activity of Rho GTPases is primarily modulated by guanine nucleotide exchange factors **(GEFs)** and GTP-ase activating proteins **(GAPs)**. GEFs stimulate the release of GDP, allowing GTP to bind and priming it for activation to modulate various downstream effectors. GAPs catalyze GTP hydrolysis, transforming Rho GTPases to a GDP-bound inactive state. Finally, the activities of Rho GTPases are also determined by Rho guanine nucleotide dissociation inhibitors **(GDIs)** that control the dissociation of GTP and GDP and subcellular localization of Rho GTPases (Bourne & Harris 2008).

Rho GTPases use a multitude of molecular mechanisms to control actin polymerization and regulate the structural stability and functional plasticity of dendritic spines **(Figure 3)**. For example, Rho GTPases can induce actin depolymerization by modulating the activity of the cofilin family of proteins, an actin-filament severing and actin depolymerizing factor (Hotulainen et al 2009). The cofilin family of actin-severing proteins is densely packed in the spine shell juxtaposed next to the presynaptic bouton, where F-actin filaments are less densely packed but are more dynamic (Honkura et al 2008). Mechanistically, this pathway is mediated by Rho

GTPase activation of p21-activated serine/threonine protein kinases **(PAK)**, which in turn phosphorylate and activate LIM domain-containing serine/threonine protein kinases **(LIMK)** to regulate actin disassembly through cofilin (Arber et al 1998, Yang et al 1998). Similarly, RAC1 and CDC42 stimulate actin polymerization via the actin-related protein-2/3 **(ARP2/3)** complex which stimulates the formation of new actin filament branching off existing filaments (Goley & Welch 2006). The ARP2/3 complex is highly concentrated in spines (Racz & Weinberg 2008) and knockdown in *in vitro* preparations have revealed its critical role in filopodia initiation and spine structural integrity (Hotulainen et al 2009). The activity state of ARP2/3 is determined by several nucleation-promotion factors **(NPF)** such as the Neuronal Wiskott-Aldrich syndrome proteins **(N-WASP)** which are directly activated by Rho GTPases such as CDC42 (Millard et al 2004).

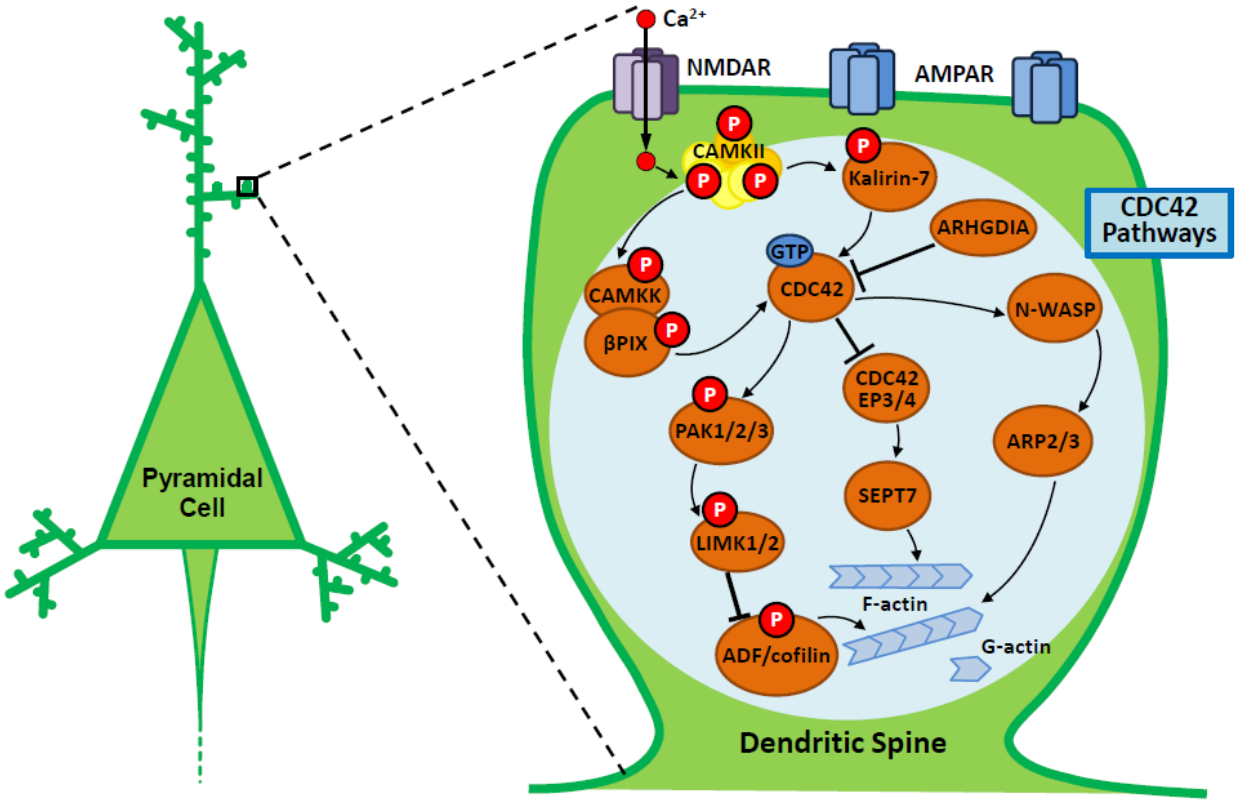

Figure 3. Regulatory actin cytoskeleton CDC42 signaling pathways for dendritic spine stabilization.

Calcium influx through NMDA receptors activates CAMKII which activates CDC42, a Rho GTPase that is a critical regulator of actin cytoskeleton dynamics in dendritic spines. The activity of CDC42 is regulated, at least in part, by ARHGDIA, a guanine nucleotide dissociation inhibitor that suppresses intrinsic GTPase activity. Once active, CDC42 can regulate the contribution of F-actin to dendritic spine structure through multiple pathways: **1) CDC42-CDC42EP pathway:** Activated CDC42 inhibits CDC42 effector proteins (CDC42EP), which dissociate the complex of septin filaments consolidated by SEPT7 in the spine neck. The transient disruption of the septin barrier allows the entry of various molecules from the dendritic shaft to the spine to facilitate F-actin mediated growth of spines in response to excitatory stimulation; **2) CDC42-PAK-LIMK pathway:** CDC42 activates the family of p21-activated serine/threonine protein kinases (PAK), which in turn activate LIM domain-containing serine/threonine protein kinases (LIMK) by phosphorylation. Activation of this signal transduction pathway inhibits the cofilin family of actin severing proteins that determine the recycling of F-actin necessary for spine stabilization; **3) CDC42-N-WASP-ARP2/3 pathway:** Activated CDC42 activates nucleation promotion factors such as N-WASP which promote actin nucleation of the actin-related protein-2/3 (ARP2/3) complex. The ARP2/3 complex is a critical determinant of activity-dependent structural plasticity of spines and stimulates *de novo* actin polymerization to generate F-actin branched filament networks to modulate spine morphogenesis.

1.3.4 Impairments in actin cytoskeleton in schizophrenia

Aberrant synaptic connectivity arising from destabilization of dendritic spines and arbors in DLPFC deep layer 3 pyramidal cells may be a consequence of alterations in the molecular mechanisms that establish the integrity of the actin cytoskeleton. Previous studies in total gray matter in the DLPFC have found decreased levels of CDC42 in subjects with schizophrenia, which were also found to be strongly correlated with spine density measurements from DLPFC deep layer 3 pyramidal cells (Hill et al 2006). Since CDC42 is instrumental in moderating actin polymerization required for the generation of mature spines, lower levels of CDC42 may impair the ability of pyramidal cells in the DLPFC in subjects with schizophrenia to maintain a full complement of excitatory inputs. Moreover, previous studies have also found lower mRNA levels of Kalirin, a RAC GEF that is highly concentrated in spines and regulates spine integrity through regulation of RAC1 and CDC42 (Cahill et al 2009, Hill et al 2006) and these changes were also correlated with spine density measures in deep layer 3 pyramidal cells (Hill et al 2006). These findings are compelling in light of genetic studies suggesting that the KALRN locus is associated with risk for schizophrenia (Ikeda et al 2011). Also, missense mutations in KALRN have been reported in subjects with schizophrenia, although future studies need to replicate these findings in larger cohorts (Kushima et al 2012). Recently, protein studies in the auditory cortex found no change in protein levels of Kalirin-5, -7, -12, but a significant upregulation in Kalirin-9 (Deo et al 2012), which is a negative regulator of dendritic length. In aggregate, alterations in CDC42 and Kalirin would be expected to result in an inability to form new spines and impaired maintenance of existing, mature spines.

The laminar specificity of the decrement in spine density in deep layer 3 may reflect the pathological disturbances in molecules that are expressed selectively in those layers. For

example, CDC42 effector proteins **(CDC42EP)** are preferentially expressed in the supragranular layers (layers 2-3) of the DLPFC (Arion et al 2007) and postmortem studies have found the expression levels of CDC42EP3 to be upregulated in schizophrenia (Ide & Lewis 2010). Activation of CDC42 following glutamate stimulation inhibits CDC42EP activity, which dissociates the complex of septin filaments in the spine neck, thereby facilitating the influx of postsynaptic molecules (cytoskeletal proteins and second messengers), from the dendritic shaft to the spine, required for F-actin mediated growth of spines and synaptic potentiation (Ide & Lewis 2010). In schizophrenia, lower levels of CDC42 and higher levels of CDC42EPs might lead to decreased capacity to dissociate the septin filament barrier, suppressing the influx of molecules required for spine plasticity, ultimately resulting in spine loss (Ide & Lewis 2010). In aggregate, these findings support the notion that reduced number of excitatory inputs to DLPFC deep layer 3 pyramidal cells may be a consequence of an *intrinsic abnormality* to these pyramidal cells which could preclude the maintenance of a normal complement of excitatory inputs. However, these prior studies have all been conducted in total gray matter tissue homogenates where transcript level measurements may be obscured by unaffected cell types. For example, in studies in total gray matter homogenates, changes in gene expression might reflect either a difference in relative number of cells expressing the transcript or altered level of expression in a *cell-type specific* fashion. Therefore, gene expression patterns in specific vulnerable neuronal populations in subjects with schizophrenia may be particularly illuminating in informing us about novel pharmacotherapy targets.

An increasing number of large-scale studies have proposed a genetic basis for dysregulation of the actin cytoskeleton in subjects with schizophrenia. Consistent with this idea, *de novo* mutations in proteins that regulate actin filaments dynamics and glutamate postsynaptic networks are preferentially found in individuals with schizophrenia (Fromer et al 2014). GWAS studies highlight several genes involved in glutamatergic neurotransmission in schizophrenia and several genetic risk factors for schizophrenia are related to the regulation of actin

polymerization and actin filament bundle assembly (Kirov et al 2012, Ripke et al 2013, Roussos et al 2012). These findings offer a plausible *proximal cause* for a primary alteration in dendritic spines and the excitatory inputs they receive in subjects with schizophrenia. Based on postmortem findings, this genetic predisposition could be moderated by *cell-type specific* and laminar-specific patters of gene expression to selectively induce morphological alterations in a laminar-specific manner in deep layer 3 pyramidal cells. In concert, the alterations in the actin cytoskeleton in schizophrenia support the notion that decreased excitatory drive to layer 3 pyramidal cells is “upstream” in the disease process (Lewis et al 2012).

1.3.5 Alterations in presynaptic afferents in the DLPFC: Potential source of dendritic spine alterations

The reduction in excitatory inputs to deep layer 3 pyramidal cells in the DLPFC in schizophrenia may also be a consequence of a laminar-specific reduction in the number of excitatory afferents. The thalamus is a key structure in gating sensory information and modulating the flow of information to the cortex (Steriade & Deschenes 1984). Excitatory inputs from the MD nucleus of the thalamus comprise the primary source of thalamic afferents to the DLPFC and these afferents primarily innervate deep layer 3 and layer 4 pyramidal cells and GABA interneurons (Barbas et al 1991, Giguere & Goldman-Rakic 1988). Intriguingly, the laminar-specific arborization of thalamic afferents from the MD nucleus to deep layer 3 and 4 parallels the laminar location of dendritic spine and somal volume alterations in schizophrenia. Therefore, reduction in inputs or activity from the thalamus could provide a plausible mechanism for the loss in dendritic spines in deep layer 3 pyramidal cells in schizophrenia. Original studies showed a decrement in the number of neurons in the MD nucleus (Byne et al 2002, Pakkenberg 1990, Young et al 2000), although follow up studies using larger sample sizes and appropriate stereological sampling techniques have failed to detect any differences (Cullen et al 2003,

Dorph-Petersen et al 2004). However, numerous diffusion-weighted neuroimaging studies have revealed alterations in the structural integrity between thalamocortical loops in schizophrenia (Wheeler & Voineskos 2014). The equivocal findings related to the number of neurons in the MD nucleus in schizophrenia do not rule out the possibility that the activity state of thalamocortical connectivity is reduced in schizophrenia especially since decreases in activity are associated with dendritic spine loss (Shi & Ethell 2006, Star et al 2002).

The vast majority of dendritic spines receive corticocortical afferents that are not as specific to laminar locations as thalamocortical afferents, but are thought to account for approximately 85-90% of total excitatory afferents in the DLPFC. Previous studies have shown reduced levels of synaptophysin, a marker of axon terminals, in the DLPFC in subjects with schizophrenia (Glantz & Lewis 1997), although this approach was not able to disentangle whether thalamocortical or corticocortical afferents were altered in the DLPFC. Moreover, it remains unknown whether these presynaptic boutons to DLPFC deep layer 3 pyramidal cells in subjects with schizophrenia never fully develop, are extensively eliminated during synaptic pruning periods, or are internalized later in life.

Furthermore, although dendritic spines primarily receive excitatory synaptic inputs from glutamate axon terminals, 15-30% of dendritic spines also receive synaptic input from GABA axon terminals (Beaulieu et al 1992, Jones & Powell 1969, Knott et al 2002, Kubota et al 2007, van Versendaal et al 2012). For example, EM studies have revealed that surprisingly large fractions of symmetrical synaptic appositions were formed onto dendritic spines that were co-innervated by an asymmetrical terminal (Beaulieu et al 1992, Jones & Powell 1969, Kubota et al 2007). In addition, a recent study using different isoforms of the vesicular glutamate transporter **(Vglut)**, Vglut1 and Vglut2, to discriminate between corticocortical and thalamocortical excitatory boutons, respectively, showed that the dual-innervated spines selectively received thalamocortical afferents but almost never corticocortical afferents (Kubota et al 2007). From a functional standpoint, GABA inputs onto spines, rather than merely suppressing depolarization

mediated by excitatory afferents, may actually sculpt neuronal activity at the subcellular level (Chiu et al 2013, Higley 2014). Pharmacological and computational studies suggest that GABA inhibition onto spines can suppress Ca^{2+} transients by inhibiting voltage-gated Ca^{2+} channels **(VGCCs)** and NMDA receptors (Chiu et al 2013). At a mechanistic level, GABA mediated inhibition onto spines can induce spine elimination by activating the actin-depolymerizing factor **(ADF)**/cofilin, a downstream target of the CDC42 pathway (Hayama et al 2013). However, previous studies in postmortem tissue in subjects with schizophrenia have not investigated if there are any deficits in the proportion of dual-innervated spines. Therefore, it remains to be determined if the spine deficits in DLPFC deep layer 3 pyramidal cells are associated with fewer axospinous afferents from particular sources.

1.3.6 Alterations in GABA interneurons are most pronounced in DLPFC deep layer 3 in schizophrenia

The *proximal deficit in excitatory activity in DLPFC deep layer 3* has been postulated to result in *homeostatic compensatory mechanisms to reduce feedback inhibition* of these pyramidal cells (**Figure 4**; Lewis et al. 2012). This idea is supported by various pre- and postsynaptic alterations related to GABAergic neurotransmission in schizophrenia in response to an intrinsic deficit in deep layer 3 pyramidal cells (Lewis et al 2012). For example, numerous studies using a plethora of different approaches such as quantitative PCR, *in situ* hybridization and DNA microarray, have consistently found lower levels of the gene encoding the 67-kDa isoform of GAD **(GAD67)**, the principal enzyme that synthesizes GABA (Akbarian et al 1995, Curley et al 2011, Mirnics et al 2000, Volk et al 2000). GAD67 expression is activity-dependent and is thought to account for ~90% of total GABA synthesized in the brain and deletion of the gene is embryonically lethal (Benson et al 1994, Lau & Murthy 2012). At the cellular and laminar level, the density of neurons with detectable levels of GAD67 mRNA was lower by 25-35%

across layers 1-5 in subjects with schizophrenia, with the remaining GABA neurons with detectable GAD67 mRNA levels showing no change (Volk et al 2000). Moreover, the deficits in GAD67 mRNA are accompanied by corresponding changes in the cognate protein in schizophrenia (Curley et al 2011, Guidotti et al 2000). In contrast to the decreased levels of GAD67, mRNA and protein levels of the 65-kDa isoform of GAD **(GAD65)**, the other enzyme responsible for GABA synthesis, are unaltered between comparison and schizophrenia subjects (Hashimoto et al 2008a). Furthermore, the density of GAD65-IR terminals is also unaltered in schizophrenia (Benes et al 2000).

The alterations in GAD67 seem to be pronounced in PV interneurons in DLPFC deep layer 3, the same layer where pyramidal cells show the most prominent molecular and morphological perturbations in schizophrenia **(Figure 4)**. For example, previous studies suggest that GAD67 mRNA decrement is strikingly altered ~30% of DLPFC interneurons, and dual *in situ* hybridization studies show that GAD67 mRNA is not detectable in ~50% of PV cells (Hashimoto et al 2003). Furthermore, levels of PV mRNA are also lower in subjects with schizophrenia in layer 3 and 4, although there is no change in the density of PV cells detectable by PV mRNA or the density of PV-IR neurons (Beasley et al 2002, Fung et al 2010, Hashimoto et al 2003, Mellios et al 2009). Consequently, these findings suggest that GAD67 mRNA levels are decreased in PV interneurons that have lower, but still detectable levels of PV mRNA. Moreover, using multi-label confocal light microscopy approaches, it appears as though the alterations in GAD67 and PV protein are more exacerbated in the presynaptic axon terminals of PVb cells, the primary site of GABA production and function, and these changes are specific to DLPFC layers 3 and 4 (Curley et al 2011, Glausier et al 2014). These alterations are accompanied by concomitant changes in the postsynaptic component of PVb inputs with a marked decrement in the $GABA_A$ α1 subunit within DLPFC layer 3-4 and the lower $GABA_A$ α1 subunit levels were found in deep layer 3 pyramidal cells but not interneurons (Beneyto et al 2011, Glausier & Lewis 2011). In addition, markers of PVCh inputs to pyramidal cell AIS are

also affected. For example, the density of GABA membrane transporter 1 **(GAT1)**-IR cartridges, the protein primarily responsible for reuptake of GABA into the axon terminals, is 40% lower in schizophrenia (Pierri et al 1999, Woo et al 1998). In the postsynaptic component of PVCh cells, there is a robust increase in $GABA_A$ α2 subunit protein levels in the pyramidal cell AIS (Volk et al 2002). These synergistic alterations have been hypothesized to decrease GABA reuptake and provide greater postsynaptic receptors to enable neurotransmitter binding to strengthen GABA signaling (Lewis et al 2012).

Various other subpopulations of GABA interneurons are also affected in the illness as the lower levels of GAD67 mRNA in the DLPFC are found in layers 1-2 and 5, where PV mRNA is unaltered (Hashimoto et al 2003). Among the various subpopulations of GABA interneurons, perisomatic targeting CCK interneurons have received considerable attention. Previous postmortem studies have found lower levels of CCK mRNA and CB1 mRNA and protein levels in subjects with schizophrenia, and the alterations in GAD67, CCK, and CB1 mRNA are strongly correlated in the same subjects (Eggan et al 2008, Hashimoto et al 2008a, Hashimoto et al 2008b). Additionally, dendrite targeting somatostatin **(SST)** interneurons, a subtype of CB cells, are also affected with previous studies reporting lower SST mRNA with corresponding changes in the $GABA_A$ α5 subunit-containing receptors that are postsynaptic to SST inputs (Beneyto et al 2011, Hashimoto et al 2008a, Hashimoto et al 2008b, Morris et al 2008) and these changes are also strongly associated with GAD67 mRNA levels in the same subject pairs. Contrary to this pattern of alteration, CR-containing GABA interneurons which comprise ~50% of GABA interneurons in the PFC, are relatively unaltered in CR or GAD67 mRNA levels (Hashimoto et al 2003).

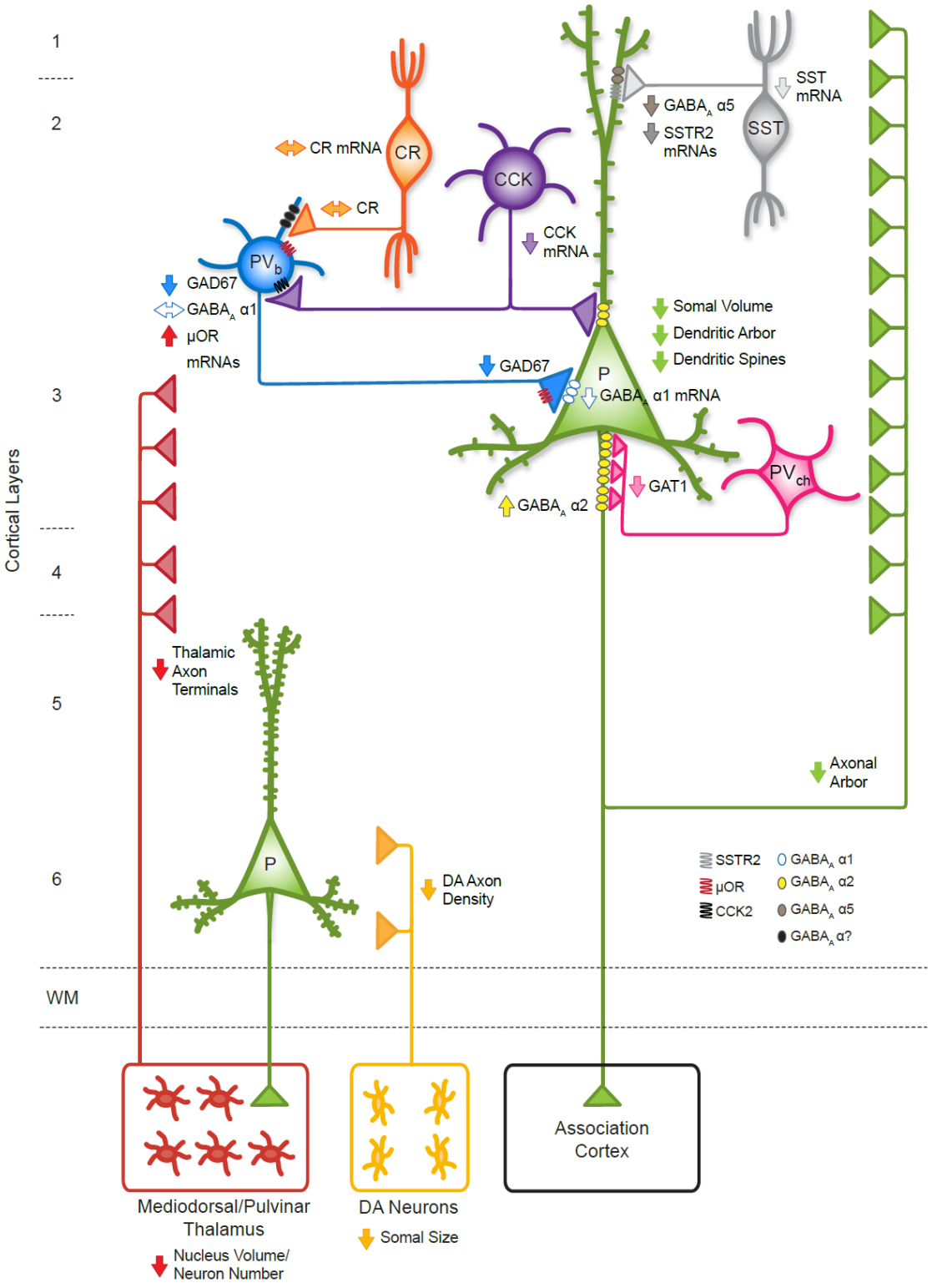

Figure 4. Schematic summarizing alterations in DLPFC circuitry in schizophrenia.

Pyramidal cells in deep layer 3 exhibit morphological abnormalities, such as smaller cell bodies, shorter dendrites and fewer dendritic spines. Reduced excitatory drive to deep layer 3 pyramidal

cells is balanced by compensatory, homeostatic mechanisms to reduce GABA inhibition onto pyramidal cells. Alterations in GABA neurotransmission by PV interneurons are shown by decreased expression of several gene products and are also associated with structural alterations in PV interneuron inputs. Structural alterations in PVCh cells include lower levels of GAT1 protein in the axon terminals and corresponding upregulation of α2-containing $GABA_A$Rs at the AIS. Although the density of PVb cell inputs are unchanged in schizophrenia, alterations in PVb cells include reduced levels of PV and GAD67 protein in PVb boutons and compensatory downregulation of α1-containing $GABA_A$Rs in the perisomatic compartment of pyramidal cells. Furthermore, lower cholecystokinin (CCK) and cannabinoid receptor 1 (CB1) transcript levels and lower CB1 protein in axon terminals reveal dysregulation of GABA neurotransmission in another subset of basket cells that also innervate the perisomatic region of pyramidal cells. Gene expression of neuropeptide somatostatin (SST) is also decreased in GABA interneurons along with postsynaptic α5-containing $GABA_A$Rs that are localized in distal dendrites of pyramidal cells. However, gene expression of calretinin-containing GABA interneurons, that primarily innervate other GABA interneurons, appears to be unaltered in schizophrenia. Presumed alterations in thalamic and DA cell bodies and their inputs to the DLPFC are also depicted. Adapted from Lewis and Sweet (2009).

1.4 DEVELOPMENTAL REFINEMENTS IN DLPFC DEEP LAYER 3 MICROCIRCUITS DURING POSTNATAL MATURATION

1.4.1 Protracted developmental maturation of pyramidal and GABA cells

Pyramidal cells and GABA interneurons in DLPFC deep layer 3 microcircuits, the same microcircuit responsible for working memory function where pathological alterations are most pronounced in schizophrenia, undergo robust changes during postnatal development which is conserved across species. For example, dendritic spine density on DLPFC pyramidal cells undergoes significant pruning during postnatal development in both monkeys and humans (Anderson et al 1995, Petanjek et al 2011). The density of dendritic spines peaks in expression early in development during the perinatal period when there is exuberant spinogenesis, reaches its zenith during childhood, and then declines by ~50% during adolescence. Consistent with these observations, the density of asymmetric excitatory synapses (defined morphologically by the presence of a thick postsynaptic density) declines in a similar manner during postnatal

maturation in both monkeys and humans (Bourgeois et al 1994, Huttenlocher 1979, Huttenlocher & Dabholkar 1997).

The elimination of supranumerary spines and synapses are determined by activity-dependent stabilization mechanisms and various hypotheses for the selective elimination of excessive morphological structures have been proposed (Le Be & Markram 2006). One such hypothesis has proposed that the refinement of excitatory inputs to spines in an age-related fashion primarily involves elimination of mature synapses rather than nascent, immature synapses (Gonzalez-Burgos et al 2008). Whole-cell patch clamp recordings in *in vitro* slice preparations of monkey PFC have revealed that excitatory inputs to deep layer 3 pyramidal cells in young monkeys (3-months of age) had immature functional properties such as lower AMPA/NMDA receptor ratio and much longer duration of NMDA receptor-mediated excitatory synaptic currents. However, excitatory inputs to older monkeys (15-month of age) had mature functional properties that persisted throughout postnatal development (Gonzalez-Burgos et al 2008). These findings led to the interpretation that the reorganization of excitatory connectivity involves the removal of mature synapses and that presynaptic factors or postsynaptic signals may tag mature synapses for pruning during postnatal development (Gonzalez-Burgos et al 2008). Developmental changes may also include concomitant changes involving synapse-specific stabilization following LTP and synapse-specific destabilization following LTD resulting in preservation of specific subpopulations of spines (Matsuzaki 2007, Zhou et al 2004). The rewiring of synaptic connections and activity-dependent stabilization of synapses may also be determined by molecular mechanisms involving actin cytoskeleton reorganization and related trafficking mechanisms (Calabrese et al 2006).

These structural age-related changes in synaptic pruning is thought to underlie the decline in cortical gray matter thickness that takes place primarily during adolescence in humans and presumably to an exaggerated degree, in subjects with schizophrenia (Feinberg 1982, Gogtay et al 2004). Structural comparisons across different populations of pyramidal cells

across laminar-location suggest that age-related developmental refinements are most striking in deep layer 3 pyramidal cells (Bourgeois et al 1994, Petanjek et al 2011) which might provide some insight into the laminar-specificity of spine alterations in schizophrenia. In schizophrenia, current theories postulate the idea that dysregulation of the molecular mechanisms associated with adolescence-related spine and synapse elimination may result in exaggerated pruning and lower spine density in the disease (Feinberg 1982, Hoffman & Dobscha 1989). Excessive synaptic pruning during postnatal development (particularly, during adolescence) has been linked to neurodevelopmental models of schizophrenia because it coincides with the emergence of the clinical symptoms of schizophrenia in early adulthood, suggesting that they may contribute to the pathophysiology of the disease (Lewis & Levitt 2002). Alternatively, the pathogenesis of schizophrenia may involve excessive production of immature axospinous synapses that are normally pruned.

Consistent with the structural alterations in pyramidal cells during postnatal development, several components of GABA inhibition undergo marked changes in primate DLPFC during postnatal maturation in deep layer 3. In particular, the terminals from PV interneurons undergo significant changes in layer 3 of the monkey DLPFC during postnatal development. For example, the density of PV-immunoreactive boutons presumably from PVb cells increases linearly from the perinatal period through adolescence (Erickson & Lewis 2002). The density of PVCh axon cartridges immunoreactive for either PV or GAT1 exhibits a complex pattern of changes during the same period, increasing from birth to childhood and then declining progressively during adolescence (Cruz et al 2003). Consistent with these findings, recent work using multi-labeling confocal microscopy suggests that PV protein levels within existing boutons from PVb cells increases during postnatal development while the mean number of PVCh cartridges per pyramidal cell AIS declines during postnatal development (Fish et al 2013). Interestingly, density of ankyrin-G and βIV spectrin, structural proteins localized to the AIS, also declines progressively until 1 year of age (Cruz et al 2009). Non-fast spiking interneurons show

complex patterns of maturation during postnatal development in the primate DLPFC. The density of CCK basket cells, particularly in the superficial layers, is high at birth and declines during the perinatal and childhood periods (Oeth & Lewis 1993). Similarly, SST mRNA declines from the perinatal period through childhood in monkey DLPFC during postnatal development (Hoftman et al 2015). These findings indicate *cell-type specific* mechanisms of GABA interneuron maturation during postnatal development in the monkey DLPFC.

At the postsynaptic level, $GABA_AR$ subunits also have distinctive developmental trajectories that vary across laminar location. Previous studies in total tissue homogenates have shown that the expression of α1, β2, δ, γ1, and γ3 $GABA_AR$ subunits increase whereas α2, α4, and β3 $GABA_AR$ subunits decrease in primate DLPFC during postnatal development (Duncan et al 2010, Fillman et al 2010, Le Magueresse & Monyer 2013, Maldonado-Aviles et al 2009). For example, expression of mRNAs encoding $GABA_A$ receptor α1 and α2 subunits in monkey DLPFC have revealed contrasting trajectories during postnatal maturation with striking changes between prepubertal and adult age groups (Hashimoto et al 2009). Importantly, given the differences in electrophysiological properties of different subunits, recent work examining $GABA_AR$-mediated inhibitory postsynaptic currents **(IPSCs)** from pyramidal cells in DLPFC deep layer 3 found decreases in the decay of IPSCs by the prepubertal period, while the amplitude increased until peripuberty (Gonzalez-Burgos et al 2014, Hashimoto et al 2009).

The myriad of molecular and structural changes in pyramidal cells and GABA interneurons during postnatal development, particularly in DLPFC deep layer 3, provides insight into understanding how circuit maturation might explain complex behaviors such as working memory. Previous work in humans, have revealed that working memory performance progressively improves through adolescence and this improvement is associated with increased participation of DLPFC circuity (Luna & Sweeney 2004). Similarly, studies in monkeys also suggest that the ability to perform working memory tasks first appears around 2-4 months of age and progressively increases to reach mature levels of performance around 3 years of age

(Goldman-Rakic 1987). The progressive improvement in working memory performance is predicated on greater activity of the PFC, since reversible cooling of the PFC has no effect on working memory performance during childhood (9-16 months of age), generates mild impairments during early adolescence (19-31 months of age), and significantly impedes performance in late adolescence (36 months of age) (Alexander 1982, Alexander & Goldman 1978). These findings are corroborated by *in vivo* electrophysiology studies in monkeys showing enhanced firing during the delay period of working memory tasks, primarily mediated by deep layer 3 pyramidal cells (Goldman-Rakic 1995). Moreover, the proportion of PFC neurons exhibiting delay-related firing dramatically increases from the perinatal period to early adolescence (Goldman-Rakic 1987).

1.4.2 Pyramidal cell and GABA interneuron circuitry in layer 3 is crucial for generating γ oscillations

Neural oscillations are a cardinal feature of neural circuits and occur across a range of frequencies (Buzsaki & Draguhn 2004, Buzsaki & Schomburg 2015). Particularly, oscillations in the γ-frequency (30-80 Hz), which involves the synchronized activity of ensembles of pyramidal cells, is crucial for higher order cognitive processes (Bartos et al 2007, Fries 2009). In the human DLPFC, γ-frequency activity is induced during the delay period of working memory tasks (Tallon-Baudry et al 1998) and power of γ synchrony increases in proportion to working memory load (Howard et al 2003). Subjects with schizophrenia exhibit decreased power of frontal lobe γ-frequency oscillations in EEG studies (Cho et al 2006, Minzenberg et al 2010) and also show alterations in phase-locking of γ-frequency oscillations at the onset of stimuli during cognitive tasks (Spencer et al 2003). These disrupted pathophysiological processes appear to be present throughout the duration of the disease and are even found in medication-naïve patients

(Minzenberg et al 2010) suggesting that it is not a manifestation of illness duration or medication use.

Interestingly, the genesis of γ-frequency oscillations appears to show laminar-specificity as well, with electrophysiological studies proposing that γ-frequency oscillations principally originate in layer 3 in monkey association cortices (Buffalo et al 2011, Quilichini et al 2010) suggesting a dependency on recurrent connectivity in layer 3 between pyramidal cells and GABA interneurons. Indeed, according to the Pyramidal Interneuron Network Gamma **(PING)** model, the generation of γ-frequency oscillations is inextricably linked to the crosstalk between pyramidal cells and GABA interneurons via recurrent synaptic connections (Whittington et al 2000). As per the PING model, GABA interneurons are recruited by phasic monosynaptic excitatory inputs from pyramidal cells, which synchronize the activity of pyramidal cells by feedback inhibition (Gonzalez-Burgos & Lewis 2012, Pouille & Scanziani 2001).

Among the various subpopulations of GABA interneurons, PV interneurons appear to be particularly well suited to mediate feedback inhibition to synchronize the activity of neural ensembles as discussed above. Experimental studies in rodents using optogenetic stimulation corroborate the importance of PV interneurons in generating γ-frequency oscillations, rendering the interpretation that pyramidal neuron connections to PV interneurons in deep layer 3 may be particularly important (Cardin et al 2009, Sohal et al 2009). PV interneurons are thought to partake in the PING model because they are rapidly recruited by excitatory synaptic inputs, generate synchronous high frequency train of action potentials, and discharge single action potentials phase locked to fast network oscillations (Armstrong & Soltesz 2012, Bartos et al 2007, Isaacson & Scanziani 2011). Therefore, local circuit abnormalities involving pyramidal cells and GABA interneurons (especially PV cells) in deep layer 3, essential for the generation of γ-frequency oscillations, support the logic that impairments in this cortical microcircuit may contribute to the neural substrate for working memory impairments in schizophrenia.

1.5 GOALS AND RELEVANCE OF THIS DISSERTATION

Understanding how dysfunction in DLPFC cortical circuits in deep layer 3 might give rise to the pathophysiology of altered γ-frequency oscillations and working memory deficits require an interrogation of the mechanisms by which these neuropathological alterations may arise, but also the normal developmental trajectories of these vulnerable microcircuits. First, although previous studies have elucidated plausible molecular mechanisms that can produce dendritic spine deficits in subjects with schizophrenia, little is known about the *cell-type specific alterations* in signal transduction cascades in deep layer 3 pyramidal cells and a few questions remain unanswered. Therefore, based on previous studies, are CDC42-related gene alterations specific to deep layer 3 or pyramidal cells in this location **(Chapter 2)**? Are there additional CDC42 signaling pathway components that are perturbed in subjects with schizophrenia? Second, the deficit in dendritic spines should be associated with fewer excitatory afferents. Thus, are the dendritic spine deficits in DLPFC deep layer 3 pyramidal cells associated with *fewer axospinous inputs from particular sources* **(Chapter 3)**? In other words, are particular subpopulations of dendritic spines vulnerable in schizophrenia? Third, the presence and timing of the activity of pyramidal neurons, is regulated by inhibitory inputs mediated by $GABA_A$Rs of varying subunit compositions that confer distinct functional properties to the receptor and influence the kinetics of GABA neurotransmission. Therefore, we sought to determine if the developmental trajectories of $GABA_A$R subunit expression in primate DLPFC deep layer 3 pyramidal neurons are *protracted, subunit- and layer-specific* **(Chapter 4)**? The developmental shifts in the expression of these subunits may modulate the characteristics of pyramidal cell firing and the maturation of behaviors such as working memory that depend on pyramidal cell activity. Understanding the normal maturation of cortical circuits in the monkey DLPFC is of paramount importance because aberrations in these circuits might underlie the core cognitive impairments in schizophrenia.

Therefore, the purpose of this dissertation is to ascertain the molecular **(Chapter 2)** and morphological **(Chapter 3)** alterations pertinent to dendritic spine pathology in deep layer 3 in subjects with schizophrenia and elucidate the developmental refinements **(Chapter 4)** in deep layer 3 pyramidal cells that are critical for the functional maturation of the DLPFC. **Chapter 5** concludes with a summary of the findings and proposed interpretation to our findings.

2.0 ALTERED EXPRESSION OF CDC42 SIGNALING PATHWAY COMPONENTS IN CORTICAL LAYER 3 PYRAMIDAL CELLS IN SCHIZOPHRENIA

Adapted from: Datta D, Arion D, Corradi JP and Lewis DA (2015). Altered expression of CDC42 signaling pathway components in cortical layer 3 pyramidal cells in schizophrenia. *Biological Psychiatry* **78** (11): 775-785.

2.1 INTRODUCTION

Cognitive deficits, such as impairments in working memory, represent a core feature of schizophrenia (Kahn & Keefe 2013) and these impairments appear to reflect altered circuitry in the dorsolateral prefrontal cortex (DLPFC;(Weinberger 1987)). In particular, convergent lines of evidence from postmortem (Kristiansen et al 2007), neuroimaging (Merritt et al 2013) and pharmacological studies (Javitt & Zukin 1991) implicate aberrant glutamate neurotransmission in cortical dysfunction in schizophrenia (Coyle 2004, Konradi & Heckers 2003, Moghaddam 2004). Pyramidal cells, the principal source of cortical glutamate neurotransmission, exhibit lower dendritic spine density (Garey et al 1998, Glantz & Lewis 2000, Konopaske et al 2014, Sweet et al 2009), shorter dendritic arbors (Glantz & Lewis 2000) and smaller somal volumes (Arnold et al 1995, Pierri et al 2001, Rajkowska et al 1998, Sweet et al 2003) in subjects with schizophrenia. These morphological aberrations appear to have laminar specificity as smaller pyramidal cell volumes and lower dendritic spine density were observed in deep layer 3 but not

in layers 5 or 6 (Kolluri et al 2005, Rajkowska et al 1998, Sweet et al 2004). None of these findings appeared to be attributable to antipsychotic medications or other co-morbid factors or potential confounds (Glausier & Lewis 2013) and therefore likely reflect the underlying disease process.

Previous postmortem studies suggest that these morphological alterations in layer 3 pyramidal cells may be the consequence of disturbed expression of genes that regulate the actin cytoskeleton (Hill et al 2006, Ide & Lewis 2010), which plays a critical role in dendritic spine formation and maintenance (Bonhoeffer & Yuste 2002, Koleske 2013, Negishi & Katoh 2005, Tada & Sheng 2006). For example, transcript levels of the Rho GTPase cell division cycle 42 (CDC42) are lower in DLPFC gray matter in subjects with schizophrenia and are positively correlated with layer 3 spine density measures (Hill et al 2006). The specificity of the spine density decrement on layer 3 pyramidal neurons may reflect the laminar-specific expression of certain molecules that interact with CDC42. Indeed, CDC42 effector proteins (CDC42EPs) are preferentially expressed in layers 2-3 of the human DLPFC (Arion et al 2007), and CDC42EP3 messenger RNA (mRNA) expression was reported to be upregulated in schizophrenia (Ide & Lewis 2010). The combination of lower CDC42 and elevated CDC42EP3 was hypothesized to alter the integrity of the barrier formed by the septin family of proteins in the spine neck (Joberty et al 2001), rendering it less permeable to the influx of postsynaptic molecules that are necessary for spine plasticity in response to glutamate stimulation (Ide & Lewis 2010).

These previous studies did not examine CDC42-related gene expression specifically in deep layer 3 or in pyramidal cells in this location. In addition, they did not address how altered CDC42 signaling could disrupt the regulation of the assembly and disassembly of actin filaments through cofilin, a family of actin-binding proteins **(Figure 5A)**. The activity of cofilin is downstream of a signaling pathway that involves the interaction between CDC42 and the p21-activated serine/threonine protein kinases (PAK) family of proteins (Edwards et al 1999,

Hotulainen & Hoogenraad 2010). These proteins are activated upon binding of the guanosine triphosphate (GTP)-bound forms of CDC42 and activate (among other targets) the LIM domain-containing serine/threonine protein kinases (LIMK1 and LIMK2) (Manser et al 1994, Parrini et al 2002) which in turn regulate the actin-depolymerizing activity of cofilin (Chen et al 2006, Hotulainen et al 2005, Sumi et al 2001). Additionally, the activity of CDC42 is regulated by guanine nucleotide dissociation inhibitors such as ARHGDIA **(Figure 5A)**, a class of molecules that inhibit the substitution of guanosine diphosphate (GDP) for GTP, suppressing GTPase activity (Newey et al 2005). Multiple regulators in the CDC42-PAK-LIMK pathway appear to be crucial for the actin cytoskeleton to maintain the stability of spine structure.

To determine if alterations in the CDC42-PAK-LIMK signaling pathway could contribute to the spine deficits on deep layer 3 pyramidal cells in schizophrenia, we conducted targeted gene expression analyses at laminar and cellular levels of specificity. We used laser microdissection to collect samples of DLPFC deep layer 3 from 56 matched pairs of schizophrenia and comparison subjects, and of individual deep layer 3 pyramidal cells in a subset of these subjects, and we measured the expression levels of CDC42-related mRNAs in these samples using reverse transcriptase quantitative polymerase chain reaction (qPCR) and microarray, respectively.

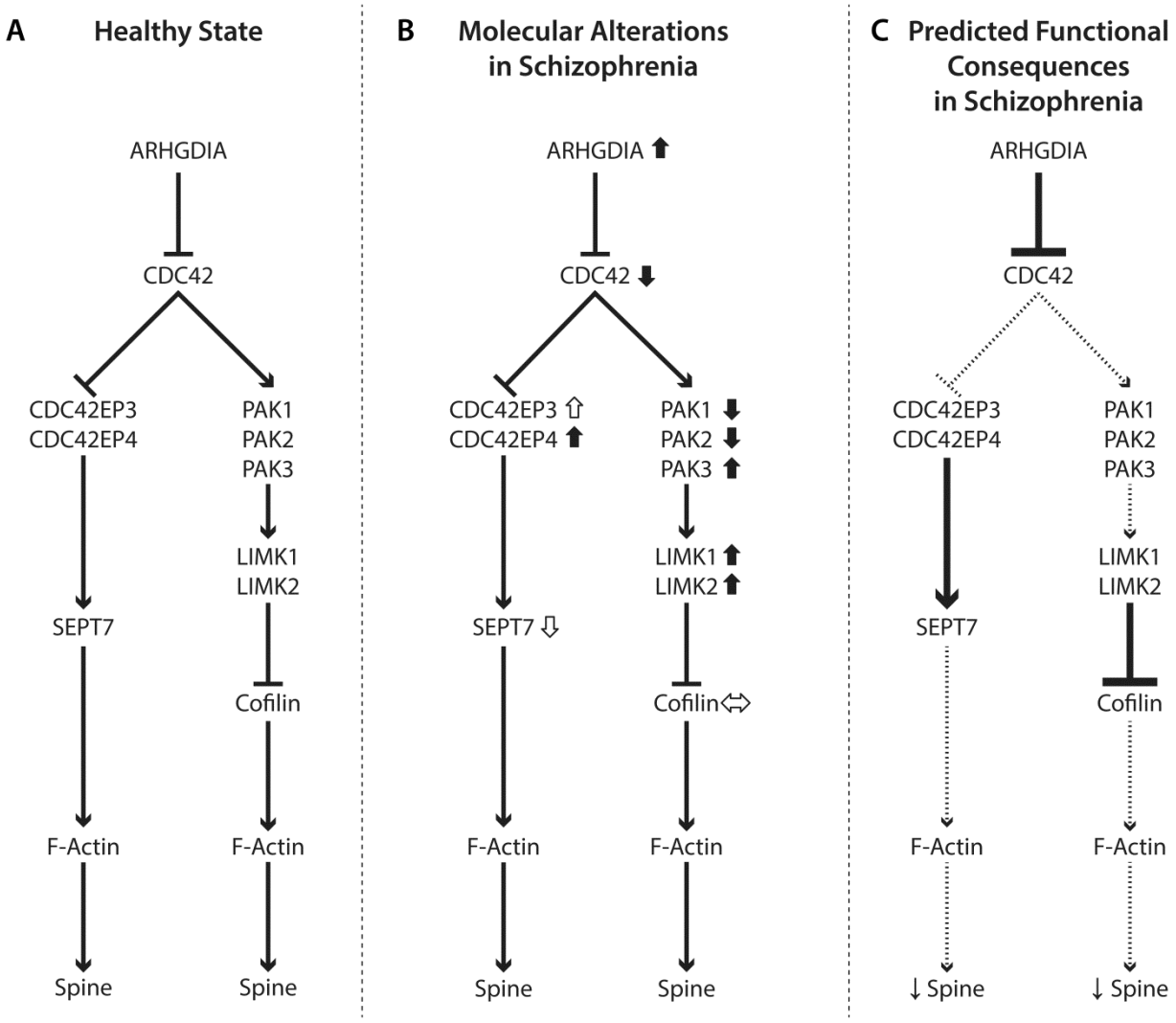

Figure 5. Schematic diagrams of cell division cycle 42 (CDC42)-CDC42 effector protein (CDC42EP) and CDC42-p21-activated serine/threonine protein kinases (PAK)-LIM domain-containing serine/threonine protein kinases (LIMK) signaling pathways and their proposed roles in spine deficits in schizophrenia.

(A) CDC42 signaling pathways that regulate the contribution of F-actin to dendritic spine structure. The activity of CDC42 is inhibited by ARHGDIA, a guanine nucleotide dissociation inhibitor that suppresses intrinsic GTPase activity. For the ***CDC42-CDC42EP pathway***, activated CDC42 inhibits CDC42EPs, which dissociate the complex of septin filaments

consolidated by SEPT7 in the spine neck. This opening of the septin barrier permits an influx of molecules from the parent dendrite that facilitate F-actin mediated growth of spines in response to excitatory inputs. For the ***CDC42-PAK-LIMK pathway***, CDC42 activates PAK, which in turn activate LIMK. Activation of this cascade inhibits the cofilin family of actin depolymerizing proteins that regulate the turnover of F-actin required for structural stability of spines. Arrows indicate activation and blunted lines indicate inhibition of each target. **(B)** Subjects with schizophrenia exhibit up- or down-regulation for multiple components of the *CDC42-CDC42EP* and *CDC42-PAK-LIMK* pathways in DLPFC deep layer 3 pyramidal cells. Solid short arrows next to transcript indicates reported evidence in this paper; open short arrows indicate previously reported evidence (Ide & Lewis 2010, Rubio et al 2012). **(C)** Predicted functional consequences in schizophrenia of altered CDC42 signaling pathways in DLPFC deep layer 3 pyramidal neurons. Higher levels of ARHGDIA would directly inhibit the activation of CDC42 holding it in an inactive GDP-bound state. In the *CDC42-CDC42EP pathway*, the effect of higher levels of ARHGDIA would be amplified by the combination of lower levels of CDC42 and higher levels of CDC42EPs, impairing the transient opening of the septin barrier in spine necks in response to excitatory inputs, and thereby suppressing the influx of molecules into the spine head required for spine growth and maintenance (Ide & Lewis 2010). In the *CDC42-PAK-LIMK pathway*, the combination of higher levels of ARHGDIA mRNA, lower levels of CDC42 mRNAs and lower levels of PAK mRNAs (given the much higher expression levels of PAK1 than PAK3, the down-regulation of PAK1 and PAK2 is predicted to have the dominant effect) would all converge to increase phosphorylation (inactivation) of cofilin family proteins and suppress actin depolymerization, resulting in F-actin destabilization and spine loss. The up-regulated levels of LIMK1/2 and PAK3 may represent compensatory, but inadequate, responses to mitigate the negative impact on F-actin dynamics of the upstream molecular pathology in this pathway.

2.2 MATERIALS AND METHODS

2.2.1 Human subjects

Human brain specimens (N=112) were obtained during routine autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA, USA) following consent obtained from the next-of-kin. An independent committee of experienced research clinicians made consensus DSM-IV (Psychiatric Association 1994) diagnoses or confirmed the absence of a psychiatric diagnosis for each using structured interviews with family members and review of prior medical records (Volk et al 2010). To control for experimental variance, each subject with schizophrenia (N=34) or schizoaffective disorder (N=22) was matched to one healthy comparison subject for sex and as closely as possible for age (**Appendix A**; see Supplemental Table S1 for details on individual subjects). As in prior studies (Curley et al 2011, Eggan et al 2012, Hashimoto et al 2008b, Hashimoto et al 2003, Kimoto et al 2014), we consider schizoaffective disorder to be a variant of schizophrenia based on the DSM-IV requirement for the class A criteria of schizophrenia to be present, in the absence of mood symptoms. Samples from both subjects in a pair were processed together throughout all stages of the study. Subject groups **(Table 1)** did not differ in mean age, postmortem interval (PMI), RNA integrity number (RIN), tissue storage time at -80°C or race. Brain pH significantly differed between groups ($t=2.51$; $df=55$, $p=0.015$), although the mean difference between groups was very small (0.1 pH unit) and the biological significance, if any, of this difference is unclear. Every subject had RIN ≥ 7.0 indicating an excellent quality of total RNA. All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

Table 1. Characteristics of human subjects

Characteristic	Quantitative PCR			Microarray		
	Comparison	Schizophrenia		Comparison	Schizophrenia	
Number	56	56		36	36	
Sex	43 M, 13 F	43 M, 13 F		27 M, 9 F	27 M, 9 F	
Race	47 W, 9 B	37 W, 19 B		30 W, 6 B	24 W, 12 B	
Age (years)	48.8 ± 13.7	47.6 ± 12.5		48.1 ± 13.0	46.9 ± 12.4	
Postmortem Interval (hours)	18.8 ± 5.6	19.3 ± 8.6		17.6 ± 6.1	18.0 ± 8.8	
RNA Integrity Number	8.1 ± 0.6	8.1 ± 0.6		8.3 ± 0.6	8.2 ± 0.6	
Brain pH	6.7 ± 0.2	6.6 ± 0.3		6.7 ± 0.2	6.6 ± 0.4	
Storage Time (months)	116.4 ± 55.4	114.2 ± 60.5		122.2 ± 49.8	125.7 ± 53.1	
Comorbid factor		Yes	No		Yes	No
Schizoaffective Disorder	-	22	34	-	13	23
History of Substance Abuse	-	38	18	-	23	13
Nicotine ATOD	-	38	12	-	23	7
Antidepressants ATOD	-	26	30	-	15	21
Benzo/VPA ATOD	-	25	31	-	14	22
Antipsychotics ATOD	-	49	7	-	31	5
Death by Suicide	-	15	39	-	10	26

*Age, PMI, RIN and Brain pH are represented as mean ± SD. M, Male; F, Female; W, White; B, Black.

For the schizophrenia subject groups, the table indicates the number of subjects for the potential confounding variables such as, diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death (ATOD); use of antipsychotics, antidepressants, or Benzo/VPA ATOD; or death by suicide. Benzo/VPA, benzodiazepines or valproic acid.

2.2.2 Laser microdissection procedure

We conducted two studies at different levels of resolution: 1) cortical layer-specific measures of gene expression in strips of tissue containing only DLPFC deep layer 3 (N=56 pairs; **Table 1**) and 2) cell-type specific measures of gene expression in DLPFC deep layer 3 pyramidal cells (N=36 pairs; **Table 1**).

For both studies, the right hemisphere of each brain was blocked coronally, frozen and stored at -80°C (Volk et al 2000). Cryostat sections (12 µm) were cut and thaw-mounted onto glass PEN membrane slides (Leica Microsystems, Bannockburn, IL) that had been UV-treated at 254nm for 30 minutes. The sections were dried and stored at -80 °C. On the day of the microdissection, sections were stained for Nissl substance with thionin **(Figure 6A)**. Using a Leica microdissection system (LMD 6500; 5x objective), DLPFC layer 3 was identified in portions of the section cut perpendicular to the pial surface. For the laminar microdissections, strips (~10 million μm^2) containing deep layer 3 (defined as the zone from the layer 3-4 border to 35% of the distance from the pial surface to the layer 6-white matter border) in DLPFC area 9 were collected from each subject using a 5x objective **(Figure 6B)**. In nearby tissue sections, individual deep layer 3 pyramidal cells (~200 pyramidal cells per subject), identified based on their characteristic somal morphology and the presence of a prominent apical dendrite directed radially toward the pia mater **(Figure 6C, D)**, were captured using a 40x objective as previously described (Arion et al 2015).

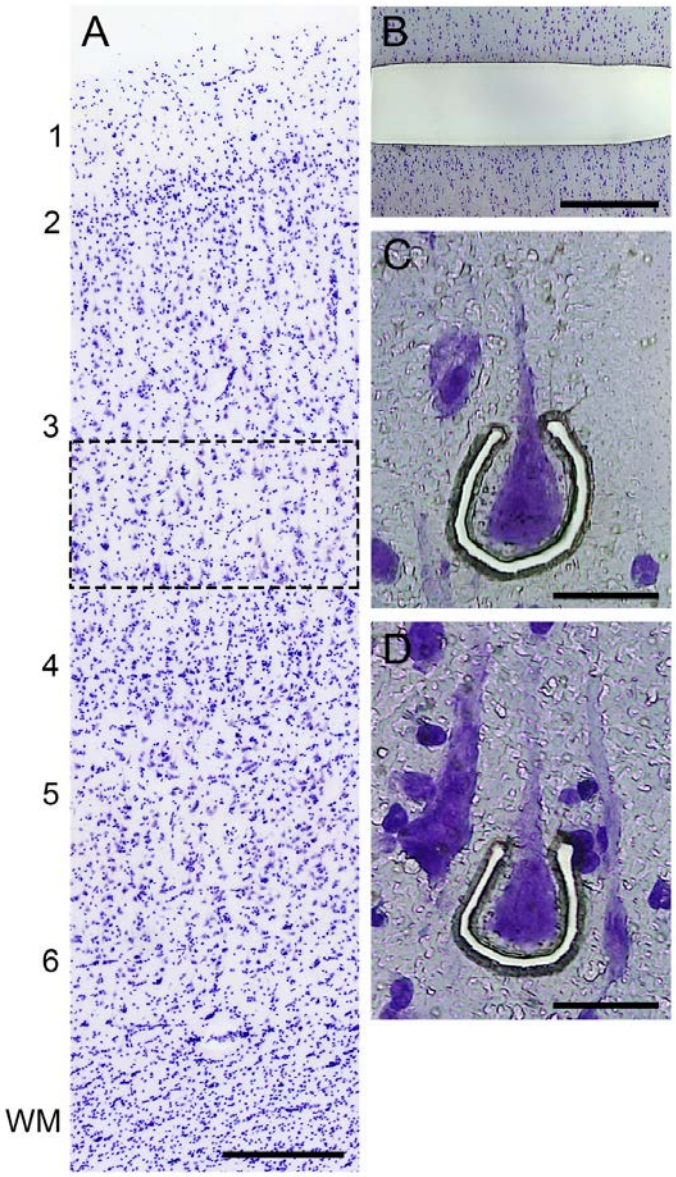

Figure 6. Laser microdissection approach.

(A) Nissl stain of human DLPFC showing the location (dashed rectangle) of deep layer 3 which was sampled. Numbers indicate cortical layers and WM indicates white matter. Calibration bar equals 300 μm. **(B)** Nissl stained section after removal of strip of deep layer 3 by laser microdissection. Calibration bar equals 700 μm. **(C and D)** Representative images of individual deep layer 3 pyramidal cells being captured by laser microdissection in DLPFC deep layer 3. Calibration bar equals 30 μm.

2.2.3 qPCR analyses

For each sample, RNA was extracted and purified using the QIAGEN RNeasy Plus Micro Kit (QIAGEN Inc, Valencia, California). Total RNA was converted to complementary DNA using the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Forward and reverse primers were designed for each target mRNA to generate PCR amplicons of 85-120 base pairs. The specificity and efficiency of qPCR amplification for each target mRNA was demonstrated by: 1) high amplification efficiency (>92%) across a wide range of cDNA dilutions (**Appendix A**; Supplemental Table S3) and 2) single products demonstrated in dissociation curve analysis.

Three internal reference transcripts (Beta-Actin [*ACTB*], Cyclophilin A [*PPIA*] and guanine nucleotide binding protein [*GNAS*]), selected based on their stable expression across the subjects in this cohort regardless of diagnosis (Volk et al 2014), were used to normalize data. Transcript expression levels of CDC42-related mRNAs (*ARHGDIA, CDC42*, *CDC42EP4, PAK1, PAK2, PAK3, LIMK1* and *LIMK2*) were quantified using qPCR using Power SYBR green dye (Applied Biosystems, Foster City, CA) and ViiA™ 7 Real-Time PCR system (Life Technologies, Carlsbad, CA). The cDNA samples from three matched pairs of schizophrenia and control subjects were processed together on the same 384-well qPCR plate with four replicates per primer set.

2.2.4 Microarray analyses

We used a microarray approach to quantify cellular expression of CDC42-related mRNAs in deep layer 3 pyramidal cells as previously described (Arion et al 2015). For transcriptome analysis, the RNA was extracted using the QIAGEN RNeasy Plus Micro Kit. The extracted RNA was transcribed into cDNA and subjected to a single round of amplification using

the Ovation Pico WTA System (Nugen Technologies, San Carlos, CA). After amplification, the cDNA was labeled using the Encore Biotin module and loaded on an Affymetric GeneChip® HT HG-U133+ PM Array Plate designed to assess expression levels of transcripts in the human genome (Affymetrix, Santa Clara, CA).

2.2.5 Antipsychotic-exposed monkeys

Experimentally naïve, male, young adult, long-tailed macaque monkeys (*Macaca fascicularis*) received twice daily oral doses of olanzapine, haloperidol or placebo (n=6 monkeys per group) for 17-27 months (Dorph-Petersen et al 2005). The doses of each antipsychotic medications produced trough serum levels known to be in the therapeutic range for the treatment of schizophrenia (Volk et al 2012). One monkey from each of the three groups was euthanized on the same day (Hashimoto et al 2008a), pyramidal cells were captured (Datta et al 2015), and subjected to microarray analysis (**Appendix A** in Supplement 1).

2.2.6 Data analysis and statistics

qPCR analyses. The comparative threshold cycle (CT) method was used in which transcript levels are normalized to the geometric mean of the three reference genes (*ACTB*, *PPIA* and *GNAS*). The difference in cycle threshold for each transcript was assessed by deducting the mean cycle threshold for the three reference genes from the cycle threshold of the target transcript. Because the difference in cycle threshold (dCT) represents the log2-transformed expression ratio of each target transcript to the geometric mean of the three reference genes, the relative expression ratio of each target transcript is determined as 2^{-dCT} (Volk et al 2010). We performed two analyses of covariance (ANCOVA) models on the expression ratio data for each transcript. Because subjects were selected and processed as

pairs, the first paired ANCOVA model included mRNA level as the dependent variable; diagnostic group as the main effect; subject pair as a blocking factor; and tissue storage time, brain pH and RIN as covariates. Subject pairing may be considered an attempt to balance diagnostic groups for sex and age, and to account for the parallel processing of tissue samples from a pair, and thus to not be a true statistical paired design. Consequently, a second unpaired ANCOVA model was performed that included all covariates (i.e., age, sex, postmortem interval, storage time, RIN and pH). All statistical tests were conducted with α-level= 0.05.

We also assessed the potential influence of other factors that are frequently co-morbid with the diagnosis of schizophrenia using ANCOVA models. For these analyses, we compared subjects with schizophrenia using each variable (sex; diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death; use of antipsychotics, antidepressants, or benzodiazepines and/or sodium valproate at the time of death; or death by suicide) as the main effect and age, tissue storage time, brain pH, PMI, and RIN as covariates.

Reported ANCOVA statistics include only those covariates that were statistically significant. As a result, the reported degrees of freedom vary across analyses.

Microarray analyses. The probe sets were filtered and paired t-tests were performed using the Random Intercept Model with Bayesian Information Criterion variable selection (Wang et al 2012). Differentially expressed gene discovery was conducted using meta-analysis and an adaptively weighted (AW) Fisher's method (Li & Tseng 2011) was applied. Meta-analyzed p-values from AW were then adjusted by the Benjamini-Hochberg procedure for multiple comparisons to control false discovery rate (Arion et al 2015, Benjamini & Hochberg 1995).

Antipsychotic-exposed monkey analyses. An ANCOVA model with the level of pre-specified mRNAs as the dependent variable, treatment group as the main effect, and triad as a blocking factor was employed. For each transcript of interest, the values of all probe sets targeting that transcript were averaged within each animal.

2.3 RESULTS

2.3.1 Expression of CDC42-related mRNAs in DLPFC deep layer 3

In subjects with schizophrenia, mean mRNA levels of ARHGDIA, a common upstream regulator of pathways related to CDC42 signaling **(Figure 5B)**, were higher in tissue homogenates of DLPFC deep layer 3 from the 56 subject pairs (+9.6%; paired: $F_{1,55}$=6.02, p=0.017; unpaired: $F_{1,108}$=1.99, p=0.161; **Figure 7A**) and in deep layer 3 pyramidal cells from the 36 subject pairs (+14.5%; p=0.041; **Figure 7B**). Although mean CDC42 mRNA levels were not significantly lower (-3.8%; paired: $F_{1,55}$=1.43, p=0.237; unpaired: $F_{1,110}$=0.29, p=0.585; **Figure 7C**) in the deep layer 3 tissue homogenates, they were significantly lower (-14.7%; p<0.001; **Figure 7D**) in DLPFC deep layer 3 pyramidal cells. Consistent with prior studies (Ide & Lewis 2010) of the CDC42-CDC42EP filament pathway **(Figure 5B)**, mean CDC42EP4 transcript levels were significantly higher in the subjects with schizophrenia in DLPFC deep layer 3 tissue homogenates (+30.2%; paired: $F_{1,54}$=7.85, p=0.007; unpaired: $F_{1,108}$=13.76, p<0.001; **Figure 7E**) and pyramidal cells (+39.4%; p=0.003; **Figure 7F**).

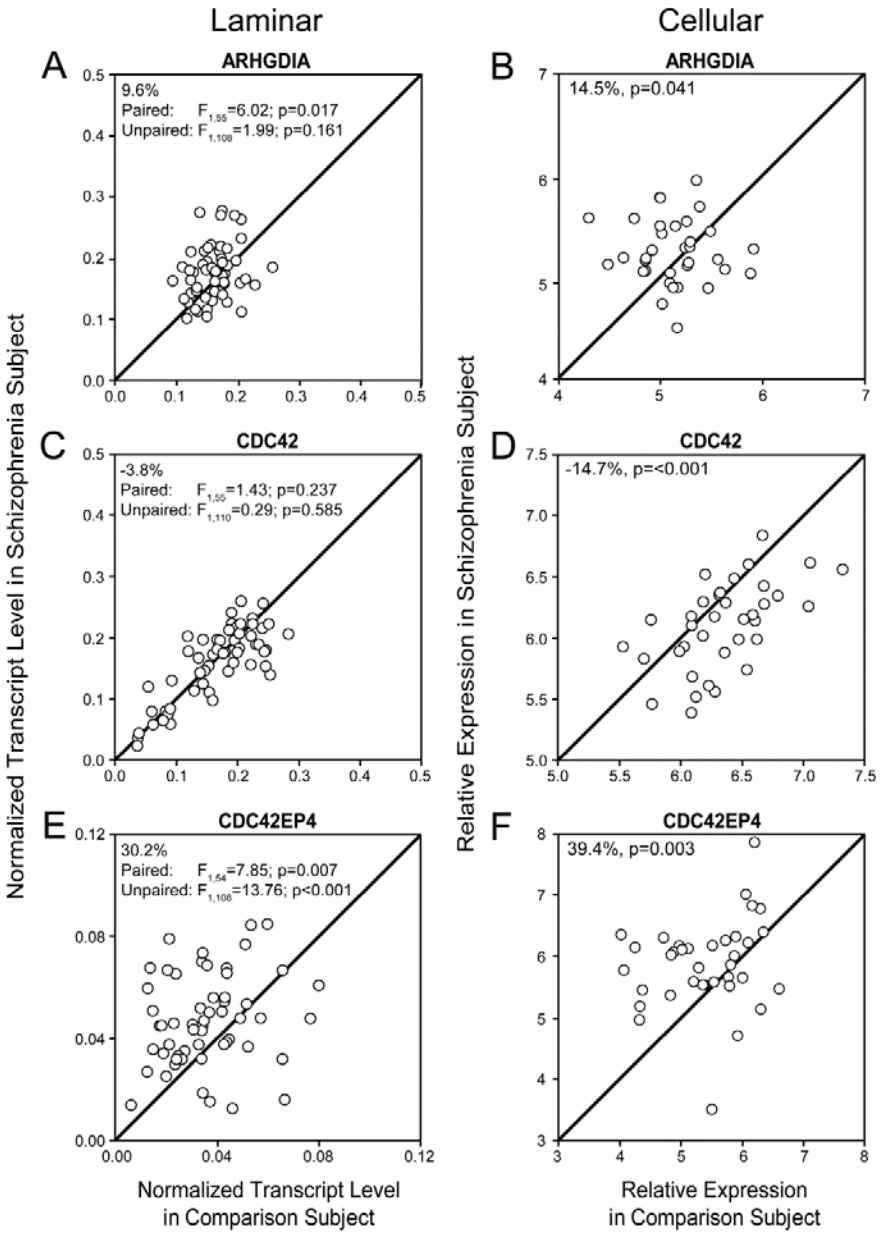

Figure 7. Expression levels of CDC42 signaling pathway components in DLPFC deep layer 3.

(A,B) Rho GDP dissociation inhibitor encoded by ARHGDIA **(C,D)** Cell division cycle 42 (CDC42), **(E,F)** CDC42 Effector Protein 4 (CDC42EP4) mRNA levels in DLPFC deep layer 3 tissue homogenates **(A,C,E)** and DLPFC deep layer 3 pyramidal cells **(B,D,F)** from schizophrenia and comparison subjects. Scatter plots show the transcript levels for each matched pair of a comparison and schizophrenia subject. Values above the unity line reflect pairs in which transcript levels are higher in the schizophrenia subject relative to the comparison subject. Values below the unity line reflect pairs in which transcript levels are lower in the schizophrenia subject relative to the comparison subject.

To interrogate the integrity of the CDC42-PAK-LIMK signaling pathway that regulates the assembly and disassembly of actin filaments through cofilin, we evaluated the expression level of several members of this pathway **(Figure 5B)**. Mean transcript levels for PAK1 did not differ between groups in tissue homogenates of DLPFC deep layer 3 (paired: $F_{1,55}$=0.08, p=0.774; unpaired: $F_{1,110}$=0.04, p=0.841; **Figure 8A**) but were significantly lower in pyramidal cell samples from schizophrenia subjects (-11.6%; p=0.002, **Figure 8B**). In contrast, in the subjects with schizophrenia mean transcript levels were higher for PAK2 in tissue homogenates of DLPFC deep layer 3 (+21.4%; paired: $F_{1,54}$=9.14, p=0.004; unpaired: $F_{1,108}$=12.80, p=0.001; **Figure 8C**) but were significantly lower in deep layer 3 pyramidal cells (-21.5%; p=0.013, **Figure 8D**). This discrepancy between layer-specific and cell type-specific measures for PAK2 could represent opposing patterns of change in pyramidal cells relative to other cell types (i.e., interneurons and glial cells) in the illness. Furthermore, the difference in sample size between qPCR (N=56 pairs) and microarray (N=36 pairs) analyses do not account for the marked difference in expression as the mean transcript levels were higher for PAK2 (+24.2%) in tissue homogenates from the same 36 pairs used for microarray analyses. Mean transcript levels for PAK3 were significantly higher in both tissue homogenates of DLPFC deep layer 3 (+18.6%; paired: $F_{1,54}$=13.03, p=0.001; unpaired: $F_{1,107}$=7.33, p=0.008; **Figure 8E**) and pyramidal cells (+15.8%; p=0.0027; **Figure 8F**). Finally, in the subjects with schizophrenia mean transcript levels for LIMK1 were modestly higher in deep layer 3 tissue homogenates (+9.9%; paired: $F_{1,55}$=3.70, p=0.060; unpaired: $F_{1,109}$=3.63, p=0.059; **Figure 8G**) and were significantly higher in pyramidal cells (+22.7%; p=0.031; **Figure 8H**). Consistent with these changes, mean transcript levels for LIMK2 were significantly higher in both deep layer 3 tissue homogenates (+63.4%; paired: $F_{1,55}$=29.53, p<0.001; unpaired: $F_{1,108}$=27.95, p<0.001; **Figure 8I**) and pyramidal cells (+22.2%; p=0.030; **Figure 8J**).

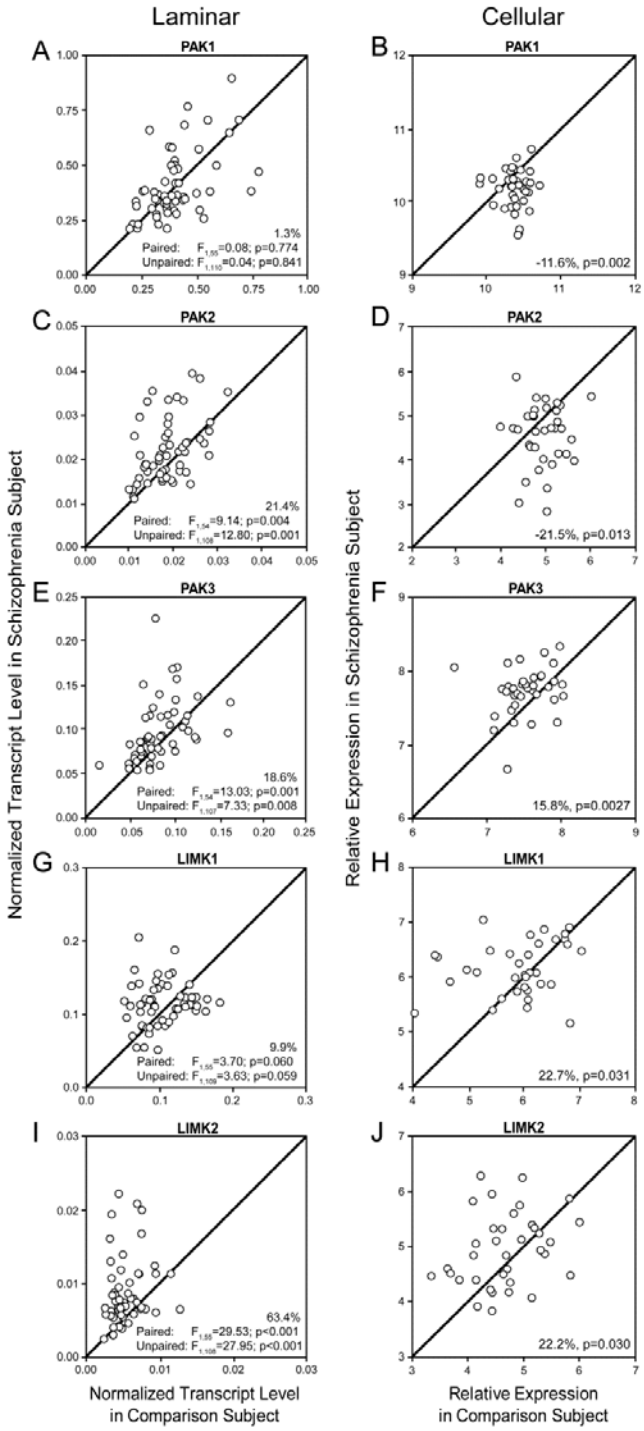

Figure 8. Expression levels of CDC42 signaling pathway components in DLPFC deep layer 3.

(A,B) p21 protein (CDC42/RAC)-activated kinase 1 (PAK1), **(C,D)** p21 protein (CDC42/RAC)-activated kinase 2 (PAK2), **(E,F)** p21 protein (CDC42/RAC)-activated kinase 3 (PAK3), **(G,H)** LIM domain kinase 1 (LIMK1), **(I,J)** LIM domain kinase 2 (LIMK2) mRNA levels in DLPFC deep

layer 3 tissue homogenates **(A,C,E,G,I)** and DLPFC deep layer 3 pyramidal cells **(B,D,F,H,J)** from schizophrenia and comparison subjects. Scatter plots show the transcript levels for each matched pair of a comparison and schizophrenia subject. Values above the unity line reflect pairs in which transcript levels are higher in the schizophrenia subject relative to the comparison subject. Values below the unity line reflect pairs in which transcript levels are lower in the schizophrenia subject relative to the comparison subject.

2.3.2 Effects of psychotropic medications and other confounding variables

For the transcripts that were significantly altered in the same direction in both the deep layer 3 tissue homogenates and pyramidal cell samples from the subjects with schizophrenia, we evaluated the effect of potential confounding variables in the qPCR data. In the 56 schizophrenia subjects, levels of ARHGDIA **(Figure 9A)**, CDC42EP4 **(Figure 9B)**, LIMK2 **(Figure 9C)** mRNAs did not differ as a function of sex, diagnosis of schizoaffective disorder; history of substance dependence or abuse; nicotine use at the time of death; use of antipsychotics, antidepressants, or benzodiazepines and/or sodium valproate at the time of death; or death by suicide (all $F_{1,48} \leq 2.39$; all $p \geq 0.13$). Similar results (all $F_{1,48} \leq 1.78$; all $p \geq 0.19$) were found for the other transcripts (i.e., PAK2, PAK3) that were significantly altered by RT-PCR.

To interrogate the potential effect of long-term exposure to typical or atypical antipsychotics, we also evaluated CDC42-related mRNAs in DLPFC deep layer 3 pyramidal cells from monkeys chronically exposed to olanzapine, haloperidol or placebo. Levels of CDC42-related mRNAs did not significantly differ among these three groups of monkeys **(Table 2)**. Serum levels of antipsychotic medications were not significantly correlated with any gene expression measures (all $lrl < 0.75$; all $p > 0.10$).

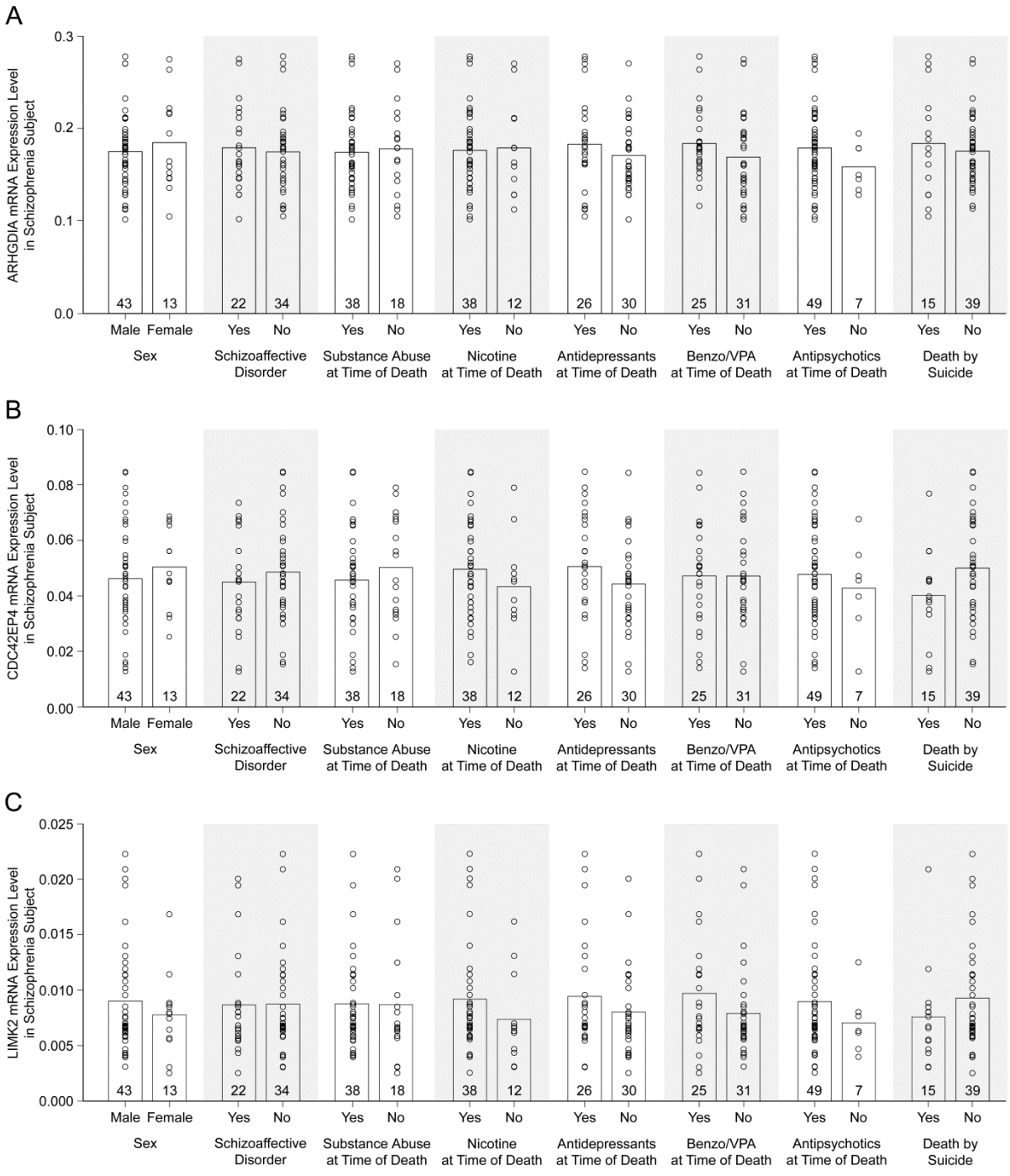

Figure 9. Effects of co-morbid factors on CDC42 signaling pathway components.

The effect of co-morbid factors on (A) Rho GDP dissociation inhibitor encoded by ARHGDIA (B) CDC42 Effector protein 4 and (C) LIM domain kinase 2 mRNA expression levels in subjects with schizophrenia in DLPFC deep layer 3 tissue homogenates. For each panel, the circles represent mRNA expression levels for individual schizophrenia subjects by qPCR and the bars

represent mean mRNA levels for the indicated group. Numbers at the bottom of bars indicate the number of subjects with schizophrenia per group. None of these confounding variables were significant for any of the transcripts (all $F_{1,48} \leq 2.39$, all $p \geq 0.13$). Two subjects had an undetermined manner of death and were not included in the death by suicide analysis. Six subjects had unknown nicotine use at the time of death and were not included in the nicotine analysis. Benzo/VPA, benzodiazepines or valproic acid.

Table 2. Summary of differences by transcript in antipsychotic-exposed monkeys

Transcript	Mean Expression ± Standard Deviation			ANCOVA
	Placebo	Olanzapine	Haloperidol	
ARHGDIA	8.2 ± 0.2	8.1 ± 0.2	8.1 ± 0.3	$F_{2,10}$=0.09, p=0.91
CDC42	9.9 ± 0.2	9.8 ± 0.2	10.0 ± 0.1	$F_{2,10}$=3.43, p=0.08
CDC42EP4	5.7 ± 0.5	5.2 ± 0.3	5.4 ± 0.4	$F_{2,10}$=3.02, p=0.09
PAK1	10.0 ± 0.1	10.2 ± 0.1	10.1 ± 0.1	$F_{2,10}$=2.51, p=0.13
PAK2	6.4 ± 0.4	6.4 ± 0.3	6.6 ± 0.3	$F_{2,10}$=0.59, p=0.57
PAK3	10.5 ± 0.2	10.7 ± 0.4	10.4 ± 0.2	$F_{2,10}$=3.21, p=0.09
LIMK1	8.9 ± 0.4	8.9 ± 0.3	8.8 ± 0.3	$F_{2,10}$=0.95, p=0.42
LIMK2	3.7 ± 0.2	3.7 ± 0.3	3.7 ± 0.2	$F_{2,10}$=0.03, p=0.97

ANCOVA, analysis of covariance

2.4 DISCUSSION

In this study, we found that subjects with schizophrenia exhibit altered gene expression, both up- and down-regulation, for components of the CDC42-PAK-LIMK pathway in DLPFC deep layer 3 tissue homogenates and pyramidal cells **(Figure 5B, right side)**. In addition, we confirmed and extended to the cell type-specific level, earlier findings of altered expression in the CDC42-CDC42EP pathway **(Figure 5B, left side)**. The levels of some transcripts (i.e., CDC42EP4, ARHGDIA, PAK3, LIMK1 and LIMK2) were significantly altered in schizophrenia, with the same direction and similar magnitude of difference from comparison subjects, in layer 3 and pyramidal cell samples, as measured with qPCR and microarray analyses, respectively. In contrast, the levels of other transcripts (i.e., CDC42, PAK1) were significantly altered only in deep layer 3 pyramidal cell samples, but showed the same direction of change in the layer 3 tissue homogenates, suggesting that these alterations are specific to or at least enriched in, pyramidal cells. These transcript alterations may reflect the disease process in schizophrenia because none of these changes were attributable to antipsychotic medications or other factors frequently co-morbid with schizophrenia. Our findings support the notion that altered signaling in the CDC42-PAK-LIMK pathway could perturb the regulation of the assembly and disassembly of actin filaments through cofilin, and in concert with alterations in the CDC42-CDC42EP pathway, could contribute to the lower density of dendritic spines that is most pronounced in deep layer 3 pyramidal cells in the DLPFC of subjects with schizophrenia.

2.4.1 Differences in layer-specific vs. cell type-specific pathology in schizophrenia

The assessment of molecular pathology at both laminar and cellular levels of resolution revealed differences in gene expression in schizophrenia that might be cell type-specific. For example, disease-related differences in the levels of CDC42 and PAK1 mRNAs detected in DLPFC deep layer 3 pyramidal neurons, but not in tissue homogenates from the same laminar location in the same subjects, support the idea that these findings are pyramidal cell-specific. Measures of gene expression in gray matter or even a specific cortical layer may obscure findings that are cell type-specific. Consistent with this idea, recent transcriptome analyses revealed substantial differential expression in schizophrenia of transcripts related to mitochondrial function in deep layer 3 pyramidal neurons and of transcripts related to the ubiquitin-proteasome system in layer 5 pyramidal cells (Arion et al 2014), alterations that were not detected in layer-specific transcriptome studies conducted in the DLPFC (Arion et al 2007). Similarly, previous studies in total gray matter tissue homogenates revealed a modest reduction (-6%) in levels of $GABA_A$ α1 subunit mRNA (Hoftman et al 2015), whereas dual-label *in situ* hybridization studies showed that mean $GABA_A$ α1 subunit mRNA expression was significantly 40% lower in deep layer 3 pyramidal cells, but was not altered in interneurons in the same layer (Glausier & Lewis 2011). Additionally, findings using laser microdissection to dissect neuronal populations in the thalamus from subjects with schizophrenia revealed lower expression of transcripts encoding glutamate receptor subunits and components of the postsynaptic scaffold in relay neurons, but not in a mixed population of glial cells and interneurons (Sodhi et al 2011).Thus, gene expression analyses at the level of individual cell types reveal distinctive alterations that are essential to understanding molecular pathology in the context of the neural circuits formed by different classes of neurons.

2.4.2 Contribution of CDC42-related signaling to dendritic spine abnormalities in DLPFC layer 3 pyramidal cells

Signaling through CDC42 pathways regulates the polymerization of the actin cytoskeleton and thus is essential for the maturation of filopodia into spines and for the maintenance of a normal complement of dendritic spines. The combined findings of the present and prior studies suggest at least three different patterns of molecular disturbances in CDC42 signaling that could contribute to spine deficits preferentially on deep layer 3 pyramidal cells in schizophrenia.

First, our findings highlight a schizophrenia-related alteration in a regulatory component of the Rho family of GTPases, including CDC42. Similar to other members of this family, CDC42 cycles between an active-GTP bound state and an inactive GDP-bound state (Luo 2000). The activity of these GTPases is regulated by guanine nucleotide dissociation inhibitors, such as ARHGDIA, which interacts with CDC42 (Gorvel et al 1998), suppresses the exchange of GDP for GTP and renders CDC42 inactive (Hoffman et al 2000). Our findings of higher expression levels of ARHGDIA in deep layer 3 pyramidal cells **(Figure 5B)** suggest that CDC42 is more likely to be held in an inactive-GDP bound state in subjects with schizophrenia. In combination with lower levels of CDC42 **(Figure 5B)**, the capacity of both the CDC42-CDC42EP and CDC42-PAK-LIMK signaling pathways to modulate the actin filaments that form the structural framework of dendritic spines would be predicted to be impaired in deep layer 3 pyramidal neurons in schizophrenia **(Figure 5C)**.

Second, the prominence of dendritic spine abnormalities in deep layer 3 pyramidal neurons of subjects with schizophrenia has been proposed to be the consequence of altered signaling through molecules that are expressed in a layer-specific fashion (Ide & Lewis 2010). For example, CDC42 effector protein (CDC42EP) mRNAs are preferentially expressed in layer

3 of the human DLPFC (Arion et al 2007). We previously demonstrated upregulation of CDC42EP3 in layer 3 of subjects with schizophrenia (Ide & Lewis 2010), and we showed in the present study that CDC42EP4 is upregulated in layer 3 pyramidal cells. According to the previously proposed model for the CDC42-CDC42EP pathway **(Figure 5A)**, the transient activation of CDC42 that normally occurs in individual spines after glutamate stimulation disrupts the CDC42EP-mediated assembly of the septin barrier consolidated by SEPT7 in the spine neck, enabling entrance into the spine head of the postsynaptic molecules, second messengers and cytoskeleton proteins necessary for F-actin mediated growth of spines and synaptic potentiation (Ide & Lewis 2010). In schizophrenia, the effect of higher levels of ARHGDIA would be amplified by the combination of lower levels of CDC42 and higher levels of CDC42EPs **(Figure 5B)** which impairs the opening of the septin barrier in response to glutamate stimulation, and thereby inhibits the influx of molecules into the spine head required for spine growth and maintenance, contributing ultimately to spine loss **(Figure 5C)**.

Third, in this study we identified altered expression of several genes in the CDC42-PAK-LIMK pathway. In this pathway, activated, GTP-bound CDC42 activates the PAK proteins which in turn activate LIMK proteins. This signaling cascade inhibits the cofilin family of actin depolymerizing proteins that regulate the assembly and disassembly of F-actin required for structural stability of spines (Chia et al 2013, Ouyang et al 2005, Scott & Olson 2007, Sumi et al 1999) **(Figure 5A)**. Our findings from deep layer 3 pyramidal cells indicate that schizophrenia is associated with lower expression of PAK1/2 proteins **(Figure 5B)** which would further reduce activity in the CDC42-PAK-LIMK pathway due to the combination of elevated expression of ARHGDIA and lower expression of CDC42 and contribute to spine deficits. Consistent with this interpretation, down-regulation of CDC42 and PAK1 proteins results in impaired long-term maintenance of spines (Murakoshi et al 2011), and over-expression *in vitro* of dominant negative forms of PAK1 reduces spine density (Hayashi et al 2007, Zhang et al 2005). A more recent study demonstrated that pharmacological manipulation of downstream signaling

components of the actin cytoskeleton such as the PAK proteins ameliorated synaptic deficits induced by DISC1 knockdown. Although this finding suggests that PAK proteins might serve therapeutic targets, future experiments need to delineate the pattern of alterations in multiple signaling cascades in subjects with schizophrenia that can converge on these downstream signaling components (Hayashi-Takagi et al 2014). In contrast, we found higher expression levels of LIMK1/2 and PAK3 in deep layer 3 pyramidal cells. We interpret this finding as a compensatory, but inadequate, response to the multiple upstream alterations that increase phosphorylation (inactivation) of cofilin family proteins and suppress actin depolymerization, resulting in F-actin destabilization and a reduction in spine number on deep layer 3 pyramidal cells **(Figure 5C)**.

In concert, these findings are consistent with other data suggesting that alterations in deep layer 3 pyramidal cells are “upstream” in the disease process of schizophrenia (Arion et al 2014, Lewis et al 2012) or perhaps even directly related to genetic risk for the illness. For example, recent findings highlight the CDC42-PAK-LIMK regulatory network as a pathogenetic factor in actin cytoskeleton dysregulation in schizophrenia (Zhao et al 2015). In addition, *de novo* mutations in proteins that regulate actin filament dynamics are preferentially found in individuals with schizophrenia (Fromer et al 2014), and copy number variations at the 15q11.2 locus implicate genes such as *CYFIP1* that regulate dendritic complexity and spine actin dynamics (Pathania et al 2014, Yoon et al 2014).

2.4.3 Conclusion

In concert with previous findings of alterations in other mediators (e.g., RhoA, Duo, Reelin, DISC1) of spine morphogenesis in schizophrenia (Arnsten et al 2012, Brandon & Sawa 2011, Erdely et al 2006, Guillozet-Bongaarts et al 2014, Hill et al 2006, Ide & Lewis 2010, Lipska et al 2006a, Lipska et al 2006b, Mirnics et al 2001, Rubio et al 2012), the findings of the

present study suggest that aberrant CDC42 signaling through two different pathways might represent a molecular pathology that converges on dendritic spine deficits specifically in deep layer 3 pyramidal cells (Glantz & Lewis 2000, Kolluri et al 2005) in subjects with schizophrenia. These observations support the notion that the actin cytoskeleton is dysregulated in schizophrenia. Because both spines and the axon terminals that innervate them are rich in actin, this dysregulation could result in lower excitatory drive to DLPFC deep layer 3 pyramidal cells (Lewis et al 2012) and a reduced need for energy production in these neurons (Arion et al 2014).

3.0 IDENTITY OF INPUTS TO DENDRITIC SPINES ON DEEP LAYER 3 PYRAMIDAL CELLS IN THE DORSOLATERAL PREFRONTAL CORTEX IN SCHIZOPHRENIA

3.1 INTRODUCTION

Cognitive impairments, including deficits in working memory, represent a core feature of schizophrenia (Kahn & Keefe 2013). Working memory is dependent on the sustained, synchronized firing of pyramidal cells in deep layer 3 of the dorsolateral prefrontal cortex (DLPFC), through recurrent excitatory interactions between spatially segregated clusters of cells (Goldman-Rakic 1995). Alterations in the circuitry of the DLPFC, such as morphological perturbations to pyramidal cells in deep layer 3, appear to contribute to the pathophysiology of working memory deficits in schizophrenia (Lewis et al 2012). For example, the density of dendritic spines, the principal site of excitatory inputs to pyramidal cells, is lower on the basilar dendrites of DLPFC deep layer 3 pyramidal cells in schizophrenia subjects (Garey et al 1998, Glantz & Lewis 2000, Konopaske et al 2014). These morphological alterations appear to be layer-specific as spine density in DLPFC layer 5 and 6 pyramidal cells did not differ between subject groups (Kolluri et al 2005). Consistent with these observations, the somal volume of deep layer 3 pyramidal cells in the DLPFC and other cortical areas, which correlates with the size of the dendritic tree and axonal arbor of a cell, is modestly reduced in subjects with schizophrenia, but showed no difference between subject groups in layer 5 (Pierri et al 2001, Rajkowska et al 1998, Sweet et al 2004). These layer-specific structural changes do not appear

to be attributable to antipsychotic medication history and are thought to reflect the disease process (Pierri et al 2001, Sweet et al 2009).

The layer-specificity of the morphological alterations in pyramidal cells in schizophrenia, such as lower dendritic spine density, raises questions about how this might arise. One possibility is that the spine deficits in DLPFC deep layer 3 pyramidal cells are the result of an intrinsic abnormality in pyramidal cells which prevents the maintenance of a normal repertoire of excitatory inputs (Glausier & Lewis 2013, Lewis & Gonzalez-Burgos 2006). Consistent with this idea, altered gene expression of certain proteins that are crucial in modulating dendritic spine structure and plasticity have been reported in layer-specific and cell type-specific studies in DLPFC deep layer 3 in schizophrenia and the mRNA expression levels of these genes correlate with spine density measures in deep layer 3 pyramidal cells (Datta et al 2015, Hill et al 2006, Ide & Lewis 2010). Furthermore, dysregulation of the molecular mechanisms that are necessary for the formation and maintenance of dendritic spines is also associated with cognitive, psychomotor and social impairments in transgenic mouse models, reminiscent of schizophrenia-like symptoms (Cahill et al 2009, Kim et al 2013, Kim et al 2015). However, an alternative possibility is that the reduction in excitatory inputs to DLPFC deep layer 3 pyramidal cells in schizophrenia is a consequence of a layer-specific reduction in the number of afferents.

Although dendritic spines receive excitatory synaptic inputs primarily from corticocortical glutamate axon terminals, 15-30% of dendritic spines also receive synaptic input from GABA axon terminals (Beaulieu et al 1992, Jones & Powell 1969, Kubota et al 2007, van Versendaal et al 2012). Interestingly, the magnitude of reduction in dendritic spine density in schizophrenia parallels the proportion of dually innervated dendritic spines that receive both Gray's Type I (asymmetrical; putatively excitatory) and type II (symmetrical; putatively inhibitory) synapses (Glantz & Lewis 2000, Gray 1959, Knott et al 2002, Kubota et al 2007, van Versendaal et al 2012). In addition, in layer 3 of the monkey PFC area 9, a large fraction (44%) of PV-immunoreactive (IR) axon terminals exclusively formed type II synapses onto dendritic spines

(Melchitzky et al 1999), the same layer where alterations in PV-basket interneurons are particularly pronounced in schizophrenia (Curley et al 2011, Glausier et al 2014, Hashimoto et al 2008, Hashimoto et al 2003). In a recent study, activation of $GABA_AR$s on individual dendritic spines by uncaging of caged GABA that mimics IPSCs or tonic application of $GABA_AR$ agonist muscimol, suppressed increases in cytosolic Ca^{2+} and was found to promote spine shrinkage and elimination by a mechanism that involved the actin cytoskeleton depolymerizing factor cofilin/ADF (Hayama et al 2013), a downstream target of the CDC42-PAK-LIMK pathway that is disrupted in schizophrenia (**Chapter 2**) (Datta et al 2015). Moreover, GABA inputs onto spines have significantly higher turnover rates than GABA inputs onto the dendritic shaft, suggesting that inhibitory inputs onto spines are dynamically regulated by activity in local circuits (Chen et al 2012, Knott et al 2002). Therefore, GABA interneurons appear to be primed to gate the postsynaptic activity in individual dendritic spines and directly modulate excitatory synaptic activity within dendrites (Chiu et al 2013). However, previous studies in postmortem tissue in subjects with schizophrenia in the DLPFC have not investigated the possibility of deficits in the proportion of dual-innervated spines.

In order to determine if a subpopulation of spines are preferentially affected in schizophrenia, we sought to examine if dendritic spine pathology in schizophrenia reflects fewer 1) dual-innervated dendritic spines receiving an inhibitory synapse and corticocortical input, and/or 2) dendritic spines receiving only a corticocortical input. To distinguish between these possibilities, we used recent advances in multi-label fluorescence immunohistochemistry and spinning disk confocal microscopy in postmortem human tissue (Fish et al 2008, Rocco et al 2015), to quantify the relative density of the various subpopulations of spines. These analyses were conducted in deep layer 3 and 4 of DLPFC area 9 in tissue sections from 20 matched pairs of schizophrenia and healthy comparison subjects and provide the first quantitative assessment of dendritic spine subpopulations in schizophrenia.

3.2 MATERIALS AND METHODS

3.2.1 Human Subjects

Brain specimens (N=40) were obtained during routine autopsies conducted at the Allegheny County Office of the Medical Examiner (Pittsburgh, Pennsylvania) after consent for donation was obtained from next of kin. An independent committee of experienced research clinicians made consensus DSM-IV diagnoses for each subject using structured interviews with family members and review of medical records. The absence of psychiatric diagnoses was confirmed in comparison subjects using the same approach. In order to control for experimental variance, subjects with schizophrenia or schizoaffective disorder (N=20) were matched individually to one healthy comparison subject for sex and as closely as possible for other covariates. All tissue samples from subjects in a pair were processed together throughout all stages of the study. The mean age, postmortem interval and tissue freezer storage time did not differ between subject groups **(Table 3)**. All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

Table 3. Characteristics of human subjects

Characteristic	Comparison	Schizophrenia
N	20	20
Sex	13 M/ 7 F	13 M/ 7 F
Race	17 W/ 3 B	12 W/ 8 B
Age (years)	44.7 ± 11.5	42.8 ± 9.1
Postmortem Interval (hours)	14.1 ± 5.5	12.0 ± 5.6
Storage Time (months)	122.4 ± 55.4	120.2 ± 60.5

*Age, PMI, and Storage Time are represented as mean ± SD. M, Male; F, Female; W, White; B, Black.

3.2.2 Immunohistochemistry

The left hemisphere of each brain from each subject was blocked coronally at ~1.0-2.0 cm intervals, fixed in cold 4% paraformaldehyde for 48 hours and immersed in a series of graded sucrose solutions. Blocks of tissue containing the superior frontal gyrus were sectioned coronally using a 40 µm block advance on a cryostat and stored in cryoprotectant solution at - 30°C until the day of the immunohistochemistry protocol. Two sections from DLPFC area 9 from each subject, spaced ~400 µm apart were used for the experiment, after identification of regions from Nissl-stained adjacent sections. Briefly, antigen retrieval methods were used to enhance immunoreactivity signal (Jiao et al 1999) by immersing sections in 0.01M sodium citrate solution (pH8.5) at 80°C in a water bath for 75 minutes, cooled to room temperature, rinsed in 0.1M phosphate buffer (pH7.4) and incubated in fresh 1% $NaBH_4$ (sodium borohydride) in 0.1M PBS for 30 minutes. After numerous rinses over 30 minutes, sections were permeabilized with 0.3% Triton X-100 in PBS for 30 minutes at room temperature, incubated in blocking solution containing 20% donkey serum, 1% bovine serum

albumin (BSA), 0.1% lysine and 0.1% glycine in PBS for 2 hours, and then incubated in scintillation vials for approximately 72 hours at 4°C in PBS containing 2% donkey serum and the following primary antibodies: vesicular glutamate transporter 1(**Vglut1**; guinea pig, 1:200, Cat#AB5905, Millipore, Billerica, MA, USA), spinophilin (rabbit, 1:1000, Cat#AB5669, Millipore, Billerica, MA, USA) and gephyrin (goat, 1:100, Cat#Sc6411, Santa Cruz Biotechnology, Dallas, TX, USA). Following brief rinses in PBS for 2 hours, the sections were then incubated in secondary antibodies (Donkey) conjugated to Alexa 488, and 647 (1:500, Invitrogen, Grand Island, NY, USA) or biotin (1:200, Fitzgerald, Acton, MA) for 24 hours. Sections were then incubated in streptavidin 405 (1:200, Invitrogen, Grand Island, NY, USA) for 24 hours. Finally, after rinses (4x30 min), sections were mounted using Prolong Gold Antifade reagent (Life technologies, Carlsbad, CA, USA) and #1.5 cover glass to minimize spherical aberration when using high-NA objectives. All slides were then coded to obscure diagnosis and subject number, and stored at 4°C until imaging.

3.2.3 Antibody Characterization

The polyclonal guinea pig anti-Vglut1 antibody directed against the C-terminus (amino acids 530-560), recognized a single band at the expected size of 60 kDa by western blot (Fremeau et al 2001) and preadsorption with immunogen peptide abolished immunolabeling (reported by manufacturer). The polyclonal rabbit anti-spinophilin antibody directed against a synthetic peptide (amino acids 286-390) recognized a single band of ~140 kDa by western blot and in primary neuronal cultures labeled only dendritic spines (Amateau & McCarthy 2002). The polyclonal goat anti-gephyrin antibody directed against the C-terminus of the protein (amino acids 710-760; clone R-20) labeled a single band of the appropriate molecular weight in western blots of mouse and rat brain tissue (reported by manufacturer). Secondary antibody specificity

was verified by omitting the primary antibody in pilot studies and has been shown to produce minimum cross-reactivity by the manufacturers.

3.2.4 Microscopy and sampling

Images were acquired on an Olympus (Center Valley, PA, USA) IX81 inverted microscope equipped with an Olympus spinning disk confocal unit, a Hammamatsu ORCA-Flash4.0 V2 digital CMOS camera (Bridgewater, NJ, USA), and a high-precision BioPrecision2 XYZ motorized stage with linear XYZ encoders (Ludl Electronic Products Ltd, Hawthorne, NJ, USA) using a 60x 1.40 NA SC oil immersion objective. All equipment was controlled by Slidebook 5.0 (Intelligent Imaging Innovations Inc., Denver, CO, USA) and Slidebook 6.0 (Intelligent Imaging Innovations Inc., Denver, CO, USA) was used for post-image processing. We generated three dimensional (3D) image stacks (2D images sequentially captured at defined 0.25 µm Z-step) that were 1024x1024 pixels over 25% of the total thickness of the tissue section starting at the coverslip. Data was collected using optimal exposure settings for every channel, yielding greatest dynamic range of the camera and negligible saturated pixels.

All images were acquired in deep layer 3 and 4 defined as extending between 35-60% of the distance from the pial surface to the layer 6-white matter border and was performed blinded to subject diagnosis. Using systematic random sampling, image stacks were sampled using a 200 x 200 μm^2 sampling grid. We imaged a total of 15 sites within deep layer 3 and 4. Moreover, a potential confound of quantitative fluorescence measures in human cortex is lipofuscin autofluorescence, and this was circumvented by taking advantage of recently developed methods (Rocco et al 2015). To eliminate this confound, lipofuscin was imaged using a customized fifth channel (excitation wavelength: 405nm; emission wavelength: 647nm) at constant exposure time across all sections and all object masks that overlapped with a lipofuscin mask were removed from analysis.

3.2.5 Image Processing

Each image stack is normalized for exposure time in each channel, and cropped to remove the top 10% of z-planes due to variability in the tissue surface as a result of the sectioning process on the cryostat. Preceding segmentation, the data set is deconvolved using AutoQuant Adaptive Blind Deconvolution algorithm (Media Cybernetics, Rockville, MD) as previously described (Rocco et al 2015). The deconvolution process combines statistical techniques of maximum likelihood estimation and a constrained iterative deconvolution algorithm to help increase signal-to-noise ratio by increasing the clarity of the data via improving resolving power. For data segmentation, a Gaussian channel was made for every deconvolved channel by calculating a difference of Gaussians using sigma values of 0.7 and 2 as described previously (Rocco et al 2015). Following deconvolution, a custom threshold/morphological segmentation algorithm is used to create object masks of labeled immunoreactive puncta, identified by morphological size ranging from 0.03 to 0.50 μm^3, which is appropriate for pre- and postsynaptic proteins as visualized by fluorescence microscopy (Fish et al 2008, Fish et al 2011). The segmentation protocol uses the Ridler-Calvard method to establish an initial binary threshold value for the iterative segmentation process, adjusted for every channel for each channel in each image stack as described previously (Fish et al 2008). Multiple iterations with subsequent threshold settings increasing by 50 gray levels are used for threshold segmentation in order to reach the maximum pixel intensity value in MATLAB (Natick, MA, USA). The combination of varying intensity thresholds in conjunction with morphological size gating is well suited for selection of objects without uniform and/or high fluorescence intensities, which can occur when proteins differ in their content within synaptic structures, because of disease states.

3.2.6 Definition of spine subtypes

We used mask operations in Slidebook to identify subtypes of dendritic spines based on the degree of overlap between voxels of different object masks in a multistep process. Spinophilin is a protein phosphatase 1-binding protein that is highly concentrated within dendritic spines in the postsynaptic density in the primate cortex (Allen et al 1997, Muly et al 2004), but the labeling is not exclusive to dendritic spines, with minor yet significant labeling of dendrites, shafts and glial cells (Muly et al 2004, Tang et al 2004). Presynaptic excitatory boutons were labeled using Vglut1, which specifically labels corticocortical boutons (Fremeau et al 2004, Fremeau et al 2001). Postsynaptic labeling for GABA synapses was labeled using gephyrin, which contributes to the multimeric scaffolds for the clustering of inhibitory receptors (Tyagarajan & Fritschy 2014). **Figure 10** shows confocal micrograph captured at 1024X1024 pixels showing immunoreactivity (IR) for Vglut1, spinophilin and gephyrin at 60X in the human DLPFC.

Dendritic spines receiving corticocortical input were defined as a spinophilin object mask that overlapped a Vglut1 object mask (Vglut1+/spinophilin+). Dual-innervated spines receiving corticocortical inputs were defined as spinophilin object masks that overlapped with gephyrin objects masks and Vglut1 objects masks (Vglut1+/spinophilin+/gephyrin+). **Figure 11** shows examples of different subpopulations of dendritic spines based on overlap between masked objects as described above. **Figure 12** shows an example of masks generated for each channel using the methods described above.

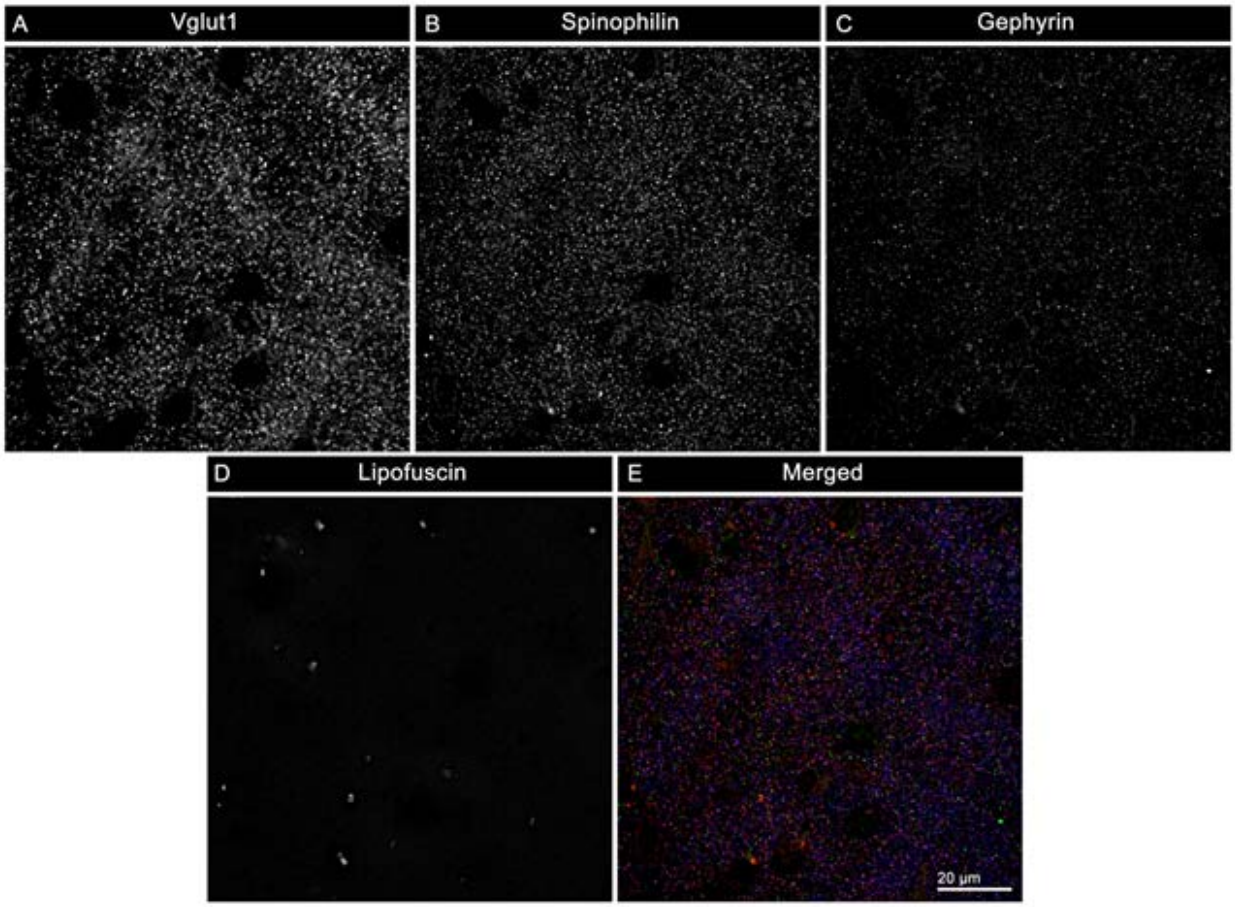

Figure 10. Triple-labeled human DLPFC tissue to assess dendritic spine subtypes.

Cryostat sections (40 µm) from schizophrenia and matched comparison subjects (2 sections/subject) were labeled for **(A)** Vglut1 (blue), **(B)** spinophilin (red) and **(C)** gephyrin (green) followed by post-image processing. **(D)** Custom filter settings were used to identify lipofuscin, a lipid-containing residue of lysosomal digestion, which is often associated as a potential confound in human postmortem tissue by presenting autofluorescent signal much greater than background. **(E)** Merged image showing Vglut1-IR, Spinophilin-IR and Gephyrin-IR puncta in human DLPFC deep layer 3. Bar = 20 µm.

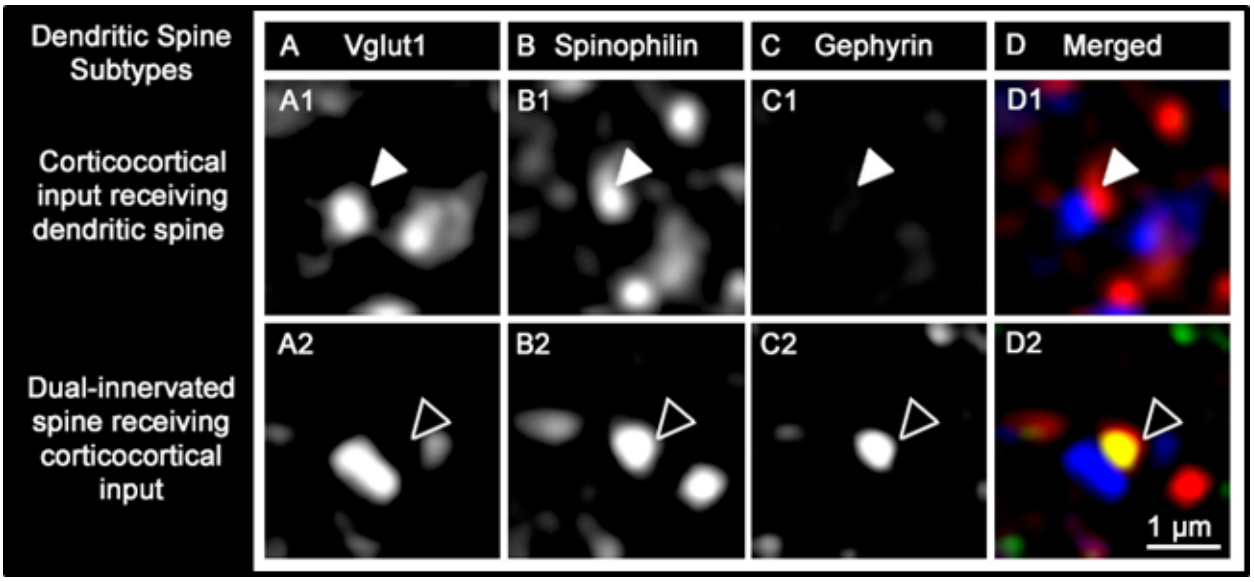

Figure 11. Identification of dendritic spine subtypes in human DLPFC.

(A1-D1) Dendritic spines (closed arrowheads) receiving a corticocortical input were defined as a spinophilin object mask that overlapped a Vglut1 object mask (Vglut1+/spinophilin+). **(A2-D2)** Dual-innervated spines receiving an inhibitory synapse and a corticocortical input (open arrowheads) were identified by spinophilin object masks that overlapped with gephyrin objects masks and Vglut1 objects masks (Vglut1+/spinophilin+/gephyrin+). Bar = 1 µm.

3.2.7 Statistical Analyses

We used two analyses of covariance (ANCOVA) models, paired and unpaired, to test the effects of diagnostic group on puncta density. The paired ANCOVA used puncta density as the dependent variable, diagnostic group as the main effect, subject pair as a blocking factor, and PMI, storage time and age as covariates. Subject pairing may be considered an attempt to account for the parallel processing of tissue samples from a pair and to balance diagnostic groups for sex, age and PMI and not a true statistical paired design. As a result, we also used a second model without subject pair as a blocking factor that included age, sex, PMI, storage time, and brain pH as covariates.

All statistical tests were conducted with α=0.05. Reported ANCOVA statistics include only those covariates that were statistically significant. Therefore, the reported degrees of freedom vary across analyses.

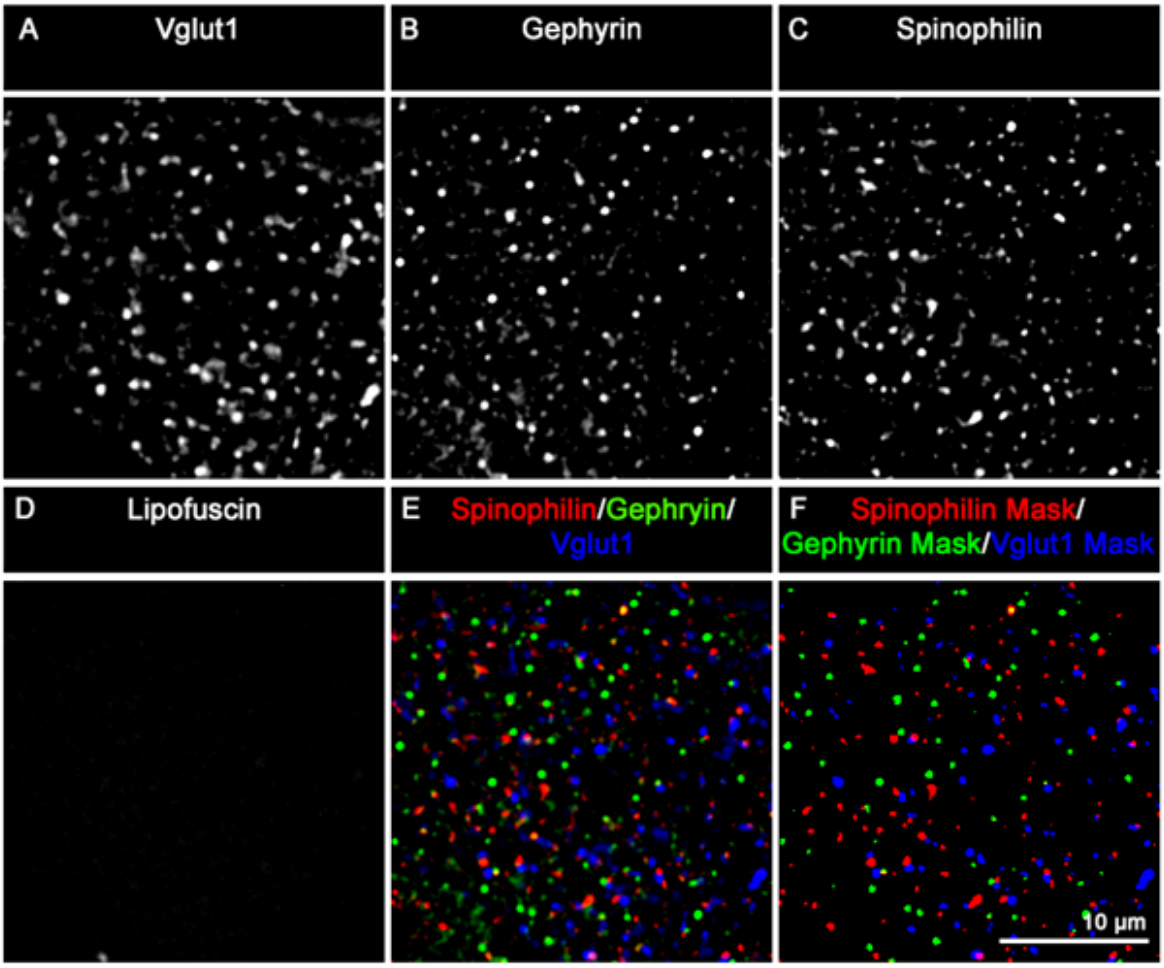

Figure 12. Generation of Vglut1-IR, gephyrin-IR and spinophilin-IR object masks.

Images of human DLPFC deep layer 3 and 4 labeled for Vglut1, gephyrin and spinophilin. Single channel images of **(A)** Vglut1, **(B)** gephyrin, **(C)** spinophilin, and **(D)** lipofuscin autofluorescence. **(E)** Merged image of A, B, and C. **(F)** Object masks corresponding to A, B, and C. Bar = 10 μm.

3.3 RESULTS

3.3.1 Density of Vglut1-IR puncta and gephyrin-IR puncta is unchanged in schizophrenia

We determined the density of Vglut1-IR and gephyrin-IR puncta in 20 pairs of comparison and schizophrenia subjects in layers deep 3 and 4. The density of Vglut1-IR puncta was not significantly different (-3.5%; paired: $F_{1,19}$=0.438, p=0.516; unpaired: $F_{1,38}$=0.536, p=0.468; **Figure 13A**) in DLPFC deep layer 3 and 4 of schizophrenia (0.033 ± 0.0048 objects/µm^3) relative to comparison (0.034 ± 0.0056 objects/µm^3) subjects. Likewise, the density of gephyrin-IR puncta was not significantly different (-2.9%; paired: $F_{1,19}$=0.224, p=0.641; unpaired: $F_{1,38}$=0.174, p=0.679; **Figure 13B**) in DLPFC deep layer 3 and 4 of schizophrenia (0.0061 ± 0.0011 objects/µm^3) relative to comparison (0.0063 ± 0.0016 objects/µm^3) subjects.

3.3.2 Density of dendritic spines receiving corticocortical input is unchanged in schizophrenia

In order to interrogate the subpopulation of spines that receive only a corticocortical input, the predominant form of excitatory afferents in the cortex, we determined the density of spinophilin object masks that overlapped with a Vglut1 object mask. The density of Vglut1+/spinophilin+ puncta was not significantly different (1.9%; paired: $F_{1,19}$=0.105, p=0.750; unpaired: $F_{1,38}$=0.133, p=0.718; **Figure 14A**) in layers deep 3 and 4 of schizophrenia (0.019 ± 0.0033 objects/µm^3) relative to comparison (0.019 ± 0.0031 objects/µm^3) subjects.

3.3.3 Density of dual-innervated dendritic spines receiving an inhibitory synapse and a corticocortical input is unchanged in schizophrenia

The density of Vglut1+/spinophilin+/gephyrin+ puncta was unchanged (-6.5%; paired: $F_{1,19}$=0.416, p=0.527; unpaired: $F_{1,38}$=0.398, p=0.532; **Figure 14B**) in schizophrenia (0.0002 ± 0.00007 objects/µm^3) relative to comparison (0.0002 ± 0.00008 objects/µm^3) subjects.

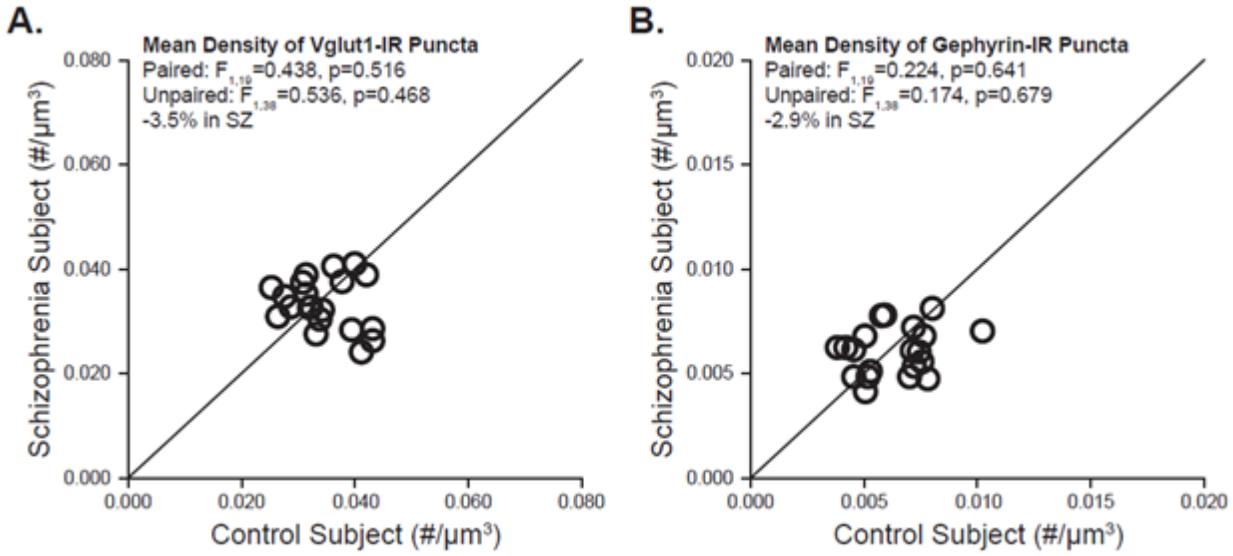

Figure 13. Vglut1-IR and gephyrin-IR puncta density are unaltered in DLPFC deep layer 3 and 4 of subjects with schizophrenia.

(A) Mean density of Vglut1-IR puncta. **(B)** Mean density of gephyrin-IR puncta. Each data point represents a schizophrenia and matched comparison subject pair. Data points below the unity line reflect pairs in which the measure is lower for the schizophrenia subject. F-statistics and p-values are provided for both paired and unpaired ANCOVA analyses.

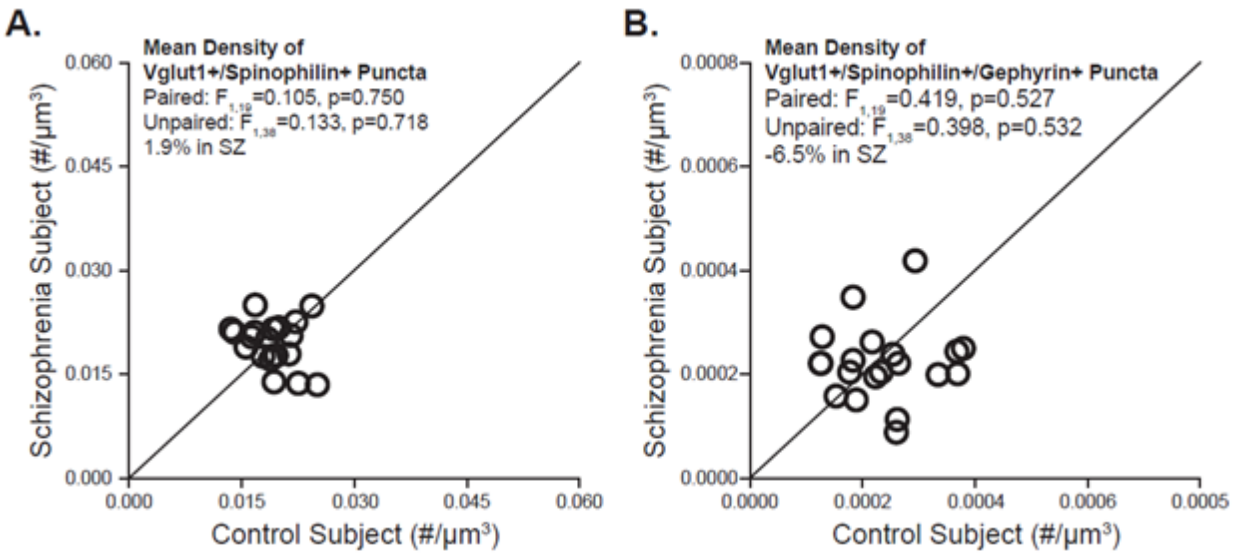

Figure 14. Vglut1+/spinophilin+ and Vglut1+/spinophilin+/gephyrin+ puncta density in DLPFC deep layer 3 and 4 between diagnostic groups.

(A) Mean density of Vglut1+/spinophilin+ puncta. **(B)** Mean density of Vglut1+/spinophilin+/gephyrin+ puncta. Each data point represents a schizophrenia and matched comparison subject pair. Data points below the unity line reflect pairs in which the measure is lower for the schizophrenia subject. F-statistics and p-values are provided for both paired and unpaired ANCOVA analyses.

3.4 DISCUSSION

In the present study, we sought to determine whether dendritic spine pathology in DLPFC reflects 1) a subpopulation of dual-innervated dendritic spines receiving an inhibitory synapse and a corticocortical input, and/or 2) dendritic spines that only receive a corticocortical input. In the DLPFC of subjects with schizophrenia, methodological limitations have precluded the assessment of neuropathological alterations in postmortem tissue with a high degree of

spatial resolution required to detect relatively small synaptic structures, and accurately quantify synapse-specific changes in these identified structures. To the best of our knowledge, the current study provides the first examination of dendritic spine subtypes in postmortem tissue in subjects with schizophrenia. We report that the density of dual-innervated dendritic spines receiving an inhibitory synapse and corticocortical input and density of putative dendritic spines only receiving a corticocortical input is unaltered in DLPFC layers deep 3 and 4 in the illness. Furthermore, we provide evidence that neither the density of Vglut1-IR nor gephyrin-IR puncta density is altered in DLPFC deep layer 3 and 4 in schizophrenia.

3.4.1 Subpopulation of dendritic spines receiving thalamocortical input may be decreased in schizophrenia

Excitatory synaptic input onto dendritic spines is mediated by corticocortical or thalamocortical afferents. Corticocortical inputs include local and long-range association projections, whereas thalamocortical inputs to the DLPFC originate in the mediodorsal (MD) nucleus of the thalamus and densely arborize in deep layer 3 and 4 (Giguere & Goldman-Rakic 1988). Although the subpopulation of dendritic spines receiving corticocortical inputs are unaltered in the disease, one possible interpretation of our findings is that reduced thalamocortical activity and/or inputs to DLPFC deep layer 3 and 4 lead to dendritic spine destabilization and loss of the spines targeted by these afferents. The thalamus is necessary for gating information between cortical areas, and in particular, the temporal synchronization of neuron ensembles in the PFC for cognitive processes (Saalmann & Kastner 2011). Interestingly, numerous studies have reported reduced activity of the MD thalamus, aberrant thalamocortical oscillations and thalamocortical dysconnectivity in subjects with schizophrenia (Minzenberg et al 2009, Woodward et al 2012). In fact, recent studies using diffusion tensor imaging and probabilistic tractography have shown reduced thalamic connectivity to the PFC in

schizophrenia, which was correlated with behavioral performance during working memory tasks (Marenco et al 2012). Importantly, selectively decreasing neural activity in the MD of mice performing cognitive tasks using the DREADD (designer receptor exclusively activated by a designer drug) pharmacogenetic approach, resulted in reduced beta-band synchronization between MD and PFC, and impairments in PFC-dependent learning and working memory tasks (Parnaudeau et al 2013). Thus, these studies suggest that diminished activity of the MD thalamus and disrupted MD-PFC functional connectivity might serve a critical role in the pathophysiology of the disease.

Several studies have interrogated the neuronal number and volume of the MD thalamus in postmortem tissue and the magnitude of reported differences vary considerably across studies. Initial studies by some groups reported decrease in volume and total neuron number of the MD thalamus (Byne et al 2002, Pakkenberg 1990, Young et al 2000), although follow-up studies overcoming methodological shortcomings showed no difference (Cullen et al 2003, Dorph-Petersen et al 2004). The discrepancy in the postmortem findings might be explained by size of subject cohorts and demographic, clinical and tissue characteristics of subjects examined, specific study of one or both hemispheres, and technical limitations in determining the anatomical boundaries of thalamic nuclei. However, in spite of the ambiguous anatomical findings in the MD thalamus in schizophrenia, axonal arborization from the MD thalamus to the PFC might still be reduced. Supporting this idea, a previous study from our group showed a marked decrement in the density of PV-IR varicosities in DLPFC area 9, specifically in the termination zone of axonal projections from the MD thalamus within deep layer 3 and 4 (Lewis et al 2001). Experiments conducted in macaque monkeys using immunocytochemical labeling for PV, have shown robust labeling in thalamic relay nuclei that project to the cortex (Jones 1998), and EM studies have shown that PV-IR terminals from MD thalamus constitute ~50% of total PV terminals in the middle cortical layers, and form Gray's Type I synapses (Melchitzky et al 1999).

The findings from the Lewis et al. 2001 study raise two interpretations: 1) Reduced PV-containing inputs from the MD thalamus and/or 2) reduced PV-basket cell boutons in DLPFC deep layer 3 and 4. A recent study demonstrated that the density of PV-basket cell inputs in the DLPFC is unchanged in schizophrenia (Glausier et al 2014). Although not definitive, these observations raise the idea that the decrement in the density of PV-IR varicosities in the DLPFC middle layers reflects fewer projections from the MD thalamus. As a result, decreased thalamocortical connectivity in the DLPFC in schizophrenia might contribute to the loss of spines within DLPFC deep layer 3 and 4 microcircuits. However, this needs to be tested using a multi-label fluorescence immunohistochemistry approach in DLPFC deep layer 3 and 4, using a combination of pre- and postsynaptic proteins to identify thalamocortical inputs onto dendritic spines.

3.4.2 Is there a redistribution of excitatory inputs from the dendritic spine to the dendritic shaft in schizophrenia?

An alternative explanation for a marked decrement in dendritic spine density observed previously, without a parallel change in corticocortical inputs (density of Vglut1-IR puncta) in the DLPFC as found in this study, is that excitatory inputs are relocalized onto the dendritic shaft as a consequence of a structural loss in spines. Various mechanistic studies exploring the functional consequences of dendritic spine loss, suggest that a decrement in dendritic spine density might cause a redistribution of excitatory synapses from dendritic spines to the dendritic shaft. For example, overexpression of Nr4a1, an activity-inducible gene encoding a nuclear receptor, produced a marked decrement in dendritic spine density without reducing excitatory synaptic transmission (AMPAR- and NMDAR-mediated EPSCs), which was produced by a redistribution of excitatory synapses onto the dendritic shaft in the absence of spines (Chen et al 2014). The downregulation of dendritic spines was mediated by the ability of Nr4a1 to alter gene

transcription of components of the actin cytoskeleton regulatory pathways, causing a reorganization of F-actin within dendritic spines (Chen et al 2014). Similarly, knockdown of hevin, an astrocyte-secreted synaptogenic protein that specifically induces thalamocortical synaptic connectivity without affecting corticocortical connectivity, produced a redistribution of excitatory synapses from dendritic spines to the shaft, resulting in no overall change in asymmetric synapse density compared to wild-type mice (Risher et al 2014). In the CA1 region of the hippocampus, chronic blockade of AMPARs resulted in a loss in dendritic spines but a concomitant 5-fold upregulation in the number of asymmetric shaft synapses, with a corresponding increase in mEPSC amplitude, but no change in mEPSC frequency or AMPA/NMDA ratio (Mateos et al 2007). The relocalization of excitatory synapses from spines to shafts is consistent with previous studies showing that PSDs can dynamically redistribute to the dendritic shaft, following spine retraction (Woods et al 2011). In fact, our findings showing no change in the density of Vglut1-IR puncta density is consistent with observations made in the auditory cortex in deep layer 3 in schizophrenia, where dendritic spine deficits have been reported (Moyer et al 2013). Taken together, these findings suggest a plausible explanation of how deficits in dendritic spines in schizophrenia might be associated with a spatial reorganization of excitatory inputs from the spines to the shaft. However, future studies using EM in postmortem tissue in schizophrenia, need to elucidate if such a phenomenon truly occurs and if the proportion of asymmetric excitatory synapses on dendritic shafts of DLPFC deep layer 3 pyramidal cells is higher in schizophrenia. Alternatively, attempts to explore intracellular filling of pyramidal cells in postmortem human tissue might be considered, although the scope of this approach is currently extremely limited (Benavides-Piccione et al 2013).

3.4.3 Limitations of this study

An important limitation of the current study is that by assessing dendritic spines using confocal microscopy, we cannot truly differentiate among the cellular source of the counted spines. Based on our previous results, the density of dendritic spines emanating from the basilar dendrites of deep layer 3 pyramidal cells were lower in the illness, however, dendritic spine density on the basilar dendrites of layer 5 and 6 pyramidal cells were unaltered (Glantz & Lewis 2000, Kolluri et al 2005). By imaging in a particular layer of the cortex (deep layer 3 and 4), we do not know if the counted spines are located on the basilar dendrites of pyramidal cells whose cell bodies are located in deep layer 3, or if these are the spines located on the apical dendrites of layer 5 or 6 pyramidal cells.

4.0 DEVELOPMENTAL EXPRESSION PATTERNS OF $GABA_A$ RECEPTOR SUBUNITS IN LAYER 3 AND 5 PYRAMIDAL CELLS OF MONKEY PREFRONTAL CORTEX

Adapted from: Datta D, Arion D, Lewis DA (2015). Developmental expression patterns of $GABA_A$ receptor subunits in layer 3 and 5 pyramidal cells of monkey prefrontal cortex. *Cerebral Cortex* **25** (8): 2295-2305.

4.1 INTRODUCTION

The primate dorsolateral prefrontal cortex (DLPFC) plays a critical role in cognition, particularly in tasks involving spatial working memory, the ability to transiently maintain and manipulate a limited amount of spatial information in order to guide thought or behavior. In both monkeys and humans, performance on spatial working memory tasks progressively improves from early childhood through adolescence, with mature levels of performance not achieved until late adolescence or early adulthood (Diamond 2002, Goldman 1971, Luna et al 2010). This age-related improvement in performance appears to reflect the maturation of the functional architecture of the DLPFC, and consequently its increased participation in the neural circuits that mediate spatial working memory (Alexander 1982, Alexander & Goldman 1978).

Within DLPFC circuitry, pyramidal cells in layer 3 appear to be preferentially involved in mediating working memory. For example, neural activity during the delay period of spatial

working memory tasks is most pronounced in the supragranular layers of the monkey DLPFC, with the greatest activity in layer 3 pyramidal cells (Friedman & Goldman-Rakic 1994, Sawaguchi et al 1990). In addition, reciprocal connections among populations of spatially-segregated clusters of layer 3 pyramidal cells are thought to provide the anatomical substrate for the recurrent excitation that sustains the firing of these neurons during the delay period of working memory tasks, serving as the cellular basis for keeping information “on-line” (Arnsten et al 2012, Goldman-Rakic 1995).

The time course of spatial working memory maturation is correlated with a developmental increase in the proportion of DLPFC neurons that exhibit delay period firing (Alexander 1982). This age-related change in neural activity patterns is associated with developmental refinements in structural markers of excitatory inputs to layer 3 pyramidal cells. For example, in layer 3 of both monkey and human DLPFC, the density of pyramidal neuron dendritic spines, the site of most excitatory synapses, and the density of axospinous synapses increase markedly during early postnatal life, remain at a plateau during childhood, and then decrease by 40-50% during adolescence (Anderson et al 1995, Bourgeois et al 1994, Huttenlocher 1979, Petanjek et al 2011).

Importantly, the activity of layer 3 pyramidal neurons during working memory tasks is also spatially tuned by inhibitory inputs. For example, interneurons in the monkey DLPFC demonstrate delay period activity that is isodirectionally tuned to nearby pyramidal neurons (Rao et al 1999). Furthermore, pharmacological blockade with GABA antagonists in the DLPFC disrupts both the spatial tuning of pyramidal neurons and behavioral performance during working memory tasks (Constantinidis et al 2002, Rao et al 2000). Consistent with this role of inhibition in spatial working memory, the axon terminals of the subset of GABA neurons (basket and chandelier cells) that express the calcium-binding protein parvalbumin also undergo substantial developmental refinements in layer 3 of monkey DLPFC. For example, parvalbumin protein levels in the axon terminals of basket neurons progressively increase during postnatal

development, whereas the density of chandelier neuron axon terminals decreases (Cruz et al 2003, Erickson & Lewis 2002, Fish et al 2013). Basket and chandelier neurons, and other classes of GABA neurons, selectively target different domains on pyramidal cells and regulate their activity through $GABA_A$ receptors with different subunit compositions (Farrant & Kaila 2007, Farrant & Nusser 2005, Jacob et al 2008). Moreover, the subunit composition of $GABA_A$ receptors contributes to the specificity of their electrophysiological properties. For example, α1-containing $GABA_A$ receptors generate currents with a much faster decay time relative to α2- or α5-containing $GABA_A$ receptors (Farrant & Nusser 2005). Thus, different $GABA_A$ receptor subunits appear to play specialized roles in regulating the activity of layer 3 pyramidal cells, and changes in the expression of these subunits with age may contribute to the maturation of spatial working memory performance.

Consequently, we sought to determine whether the expression of different $GABA_A$ receptor subunits in layer 3 pyramidal neurons have distinctive developmental trajectories, whether these trajectories differ from those of major markers (AMPA Glur1 and NMDA Grin1 subunits) of excitatory neurotransmission, and whether these developmental changes are specific to layer 3 pyramidal cells. In order to address these questions we used laser microdissection techniques to capture individual layer 3 pyramidal cells from the DLPFC of monkeys ranging in age from postnatal one week to 11.5 years and quantified the expression levels of the transcripts of interest by qPCR. Identical studies were conducted in layer 5 pyramidal cells which differ from layer 3 pyramidal cells based on their birth date, source of afferents, principal projection targets (Jones 1984) and role in working memory circuitry (Arnsten et al 2012). Moreover, available evidence indicates that recurrent excitatory connections between layer 3 and 5 pyramidal cells (Kritzer & Goldman-Rakic 1995) might be critical for the persistent firing of these cells that is required to sustain mental representation in the absence of ongoing sensory input (Arnsten et al 2012).

4.2 MATERIALS AND METHODS

4.2.1 Animals

We used 26 rhesus (*Macacca mulatta*) monkeys ranging in age from one week to 11.5 years (**Appendix B;** Supplemental Table 1). All monkeys were female except for one adult male. Monkeys younger than 6 months of age were housed with their mothers; juveniles 6-24 months were housed in groups; and those older than 24 months were housed either in pairs, or in single cages, in the same social setting as previously described (Erickson & Lewis 2002). All housing and experimental procedures were conducted in accordance with guidelines set by the U.S. Department of Agriculture and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the University of Pittsburgh's Institutional Animal Care and Use Committee.

Eleven monkeys were perfused transcardially with ice-cold artificial cerebrospinal fluid (ACSF) under deep anesthesia with ketamine and pentobarbital (Gonzalez-Burgos et al 2008); in two of these monkeys, a small tissue block was surgically excised from the rostral third of the principal sulcus in the left hemisphere for electrophysiology studies 2-4 weeks prior to perfusion. The remaining 15 monkeys, all experimentally naïve, were deeply anesthetized with ketamine and pentobarbital. After the monkey was deeply anesthetized, a scalpel and heavy duty scissors were used to quickly remove the head. Rongeurs were then used to remove the top of the skull and fine scissors used to resect the dura. The brain was then removed intact. Standard coronal blocks (~5 mm thick) were cut from the right hemisphere, placed on 2"x3" glass slides and immersed in

isopentane chilled on dry ice. Each frozen block was then placed in an appropriately labeled bag and stored in a -80°C freezer.

Monkeys were divided into the following four age groups based on the previously identified inflection points in the developmental trajectories of excitatory inputs to layer 3 pyramidal cells in monkey DLPFC (Anderson et al 1995, Bourgeois et al 1994): 1) perinatal (n = 6), monkeys from 0.1 to 1.5 months of age, within the period of a rapid increase in density of excitatory synapses and spines; 2) prepubertal (n = 7), monkeys from 3 to 9 months of age, within the period when the density of excitatory synapses and spines is at a plateau; 3) peripubertal (n = 7), monkeys from 16 to 32 months of age, within the period of excitatory synapse and spine pruning; 4) adult (n = 6), monkeys from 45 to 138 months of age, during the period when excitatory synapse and spine density are at stable adult levels.

4.2.2 Laser microdissection analyses

Cryostat sections (12 µm) were cut from coronal blocks containing DLPFC areas 9 and 46 **(Figure 15A-C)**, thaw-mounted onto glass polyethylene naphthalate membrane slides (Leica Microsystems, Bannockburn, IL) that had been previously UV-treated at 254 nm for 30 minutes, dried briefly, and stored at −80°C. On the day of the microdissection, slides were immersed in an ethanol–acetic acid fixation solution, stained with thionin, dehydrated through 100% ethanol, and air dried. Using a Leica microdissection system (LMD 6500; X40 objective; power, 15; aperture, 9; speed, 12; balance, 14; and offset, 180), individual pyramidal neurons in deep layer 3 or layer 5 were captured as previously described (Arion & Lewis 2011). Approximately 150 Nissl-

stained pyramidal cells from each of layer 3 and 5 were collected from each of three different slides per animal. Pyramidal cells were identified based on their characteristic somal morphology and the presence of a prominent apical dendrite directed radially towards the pia mater **(Figure 15D-E)**. The pyramidal cells from a given layer for each monkey were pooled together in 0.5-mL microtube caps (Ambion/Applied Biosystems, Foster City, CA), resulting in a sample of approximately 450 pyramidal cells per layer per subject, and lysed by vortexing for 30 seconds in 200 µL of RLT Buffer Plus (QIAGEN, Valencia, CA). The RNA was purified using the RNeasy Plus Micro kit (QIAGEN).

In order to validate the cell type specificity of the dissected neurons, and the absence of glial or GABA cell contamination, we obtained LMD samples of Nissl-stained 1) pyramidal neurons identified using the criteria described, 2) non-pyramidal neurons (presumably interneurons), and 3) glial cells from the same tissue sections. The cDNA from each sample was used to quantify levels of specific markers of interneurons (glutamic acid decarboxylase 67, GAD67) and glial cells (myelin basic protein, MBP) by qPCR. In pyramidal cells, expression levels of GAD67 were only 3.3% of those in interneurons and MBP only 0.6% of those in glial cells. In concert, these findings demonstrate that our dissection method results in the selective collection of pyramidal neurons without contamination by other cortical cell types.

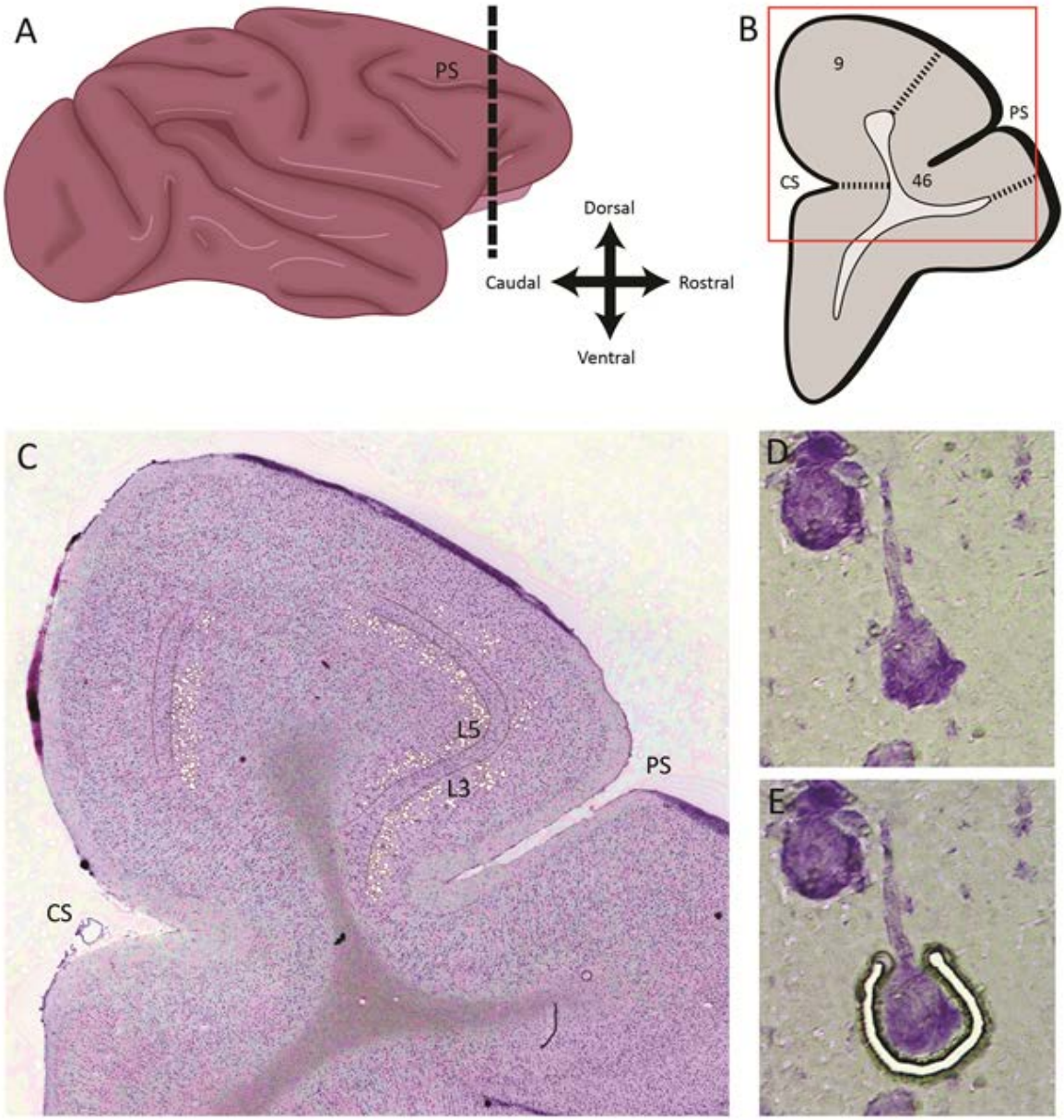

Figure 15. Dissection of individual pyramidal cells in different laminar locations from monkey DLPFC.

(A) Schematic drawing of the lateral view of the macaque monkey brain showing the approximate location (dotted black lines) of DLPFC sections used in the study. PS indicates principal sulcus and CS indicates cingulate sulcus. **(B)** Schematic drawing of a representative coronal section of monkey DLPFC, at the rostral-caudal level shown in panel A, showing the location of areas 9 and 46. Box indicates the portion of the section shown in panel C. **(C)** Thionin-stained coronal section showing the locations where pyramidal cells were captured in layer 3 and layer 5. **(D)** Digitally optimized representative image of a pyramidal cell in layer 3. **(E)** The same pyramidal cell in the process of being captured by laser microdissection.

4.2.3 Quantitative real-time polymerase chain reaction

Total RNA was converted to complementary DNA using the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Priming was performed with a mix of poly dT and random hexamers, according to the manufacturer's recommendations. The efficiency for each primer set was between 90% and 100%, and the amplified product resulted in a specific single amplicon in dissociation curve analysis (**Appendix B**; Supplemental Table 2). Given the limitations on the number of cells that could be captured, and the resulting limits on the amount of available cDNA from each sample, we were able to profile a total of 8 transcripts of interest (and two reference genes) from each sample. We determined transcript levels of the major postsynaptic $GABA_A$ receptor subunits (α1, α2, α5, β2, γ2 and δ) and of critical subunits for AMPA (Glur1) and NMDA (Grin1) glutamate receptors.

Samples from both layers of a given subject were always assayed on the same plate. For each sample, amplified product differences for each transcript were measured with 4 replicates using SYBR Green chemistry-based detection (Mimmack et al 2004). β-Actin and cyclophilin A were used as endogenous reference genes to normalize the expression levels of transcripts, as these two transcripts have been previously shown to have stable levels of expression across development in monkey DLPFC (Hoftman et al 2015, Volk et al 2012). The qPCR reactions were carried out in StepOnePlus thermal cycler (Applied Biosystems) using the StepOnePlus software with the automatic baseline and threshold detection options selected. These data were exported to Microsoft Excel (Microsoft, Redmond, Washington) and delta cycle thresholds (dCTs) were calculated for each sample by using the geometric mean of the two endogenous reference genes as the normalization factor (i.e., cycle threshold [CT] for each transcript in a sample minus the geometric mean of β-actin and cyclophilin A CTs for the same sample). Since the dCT represents the log2-transformed expression ratio of each transcript of interest to the geometric mean of the two reference genes we calculated the more intuitive

expression ratio (i.e., expression ratio=2^{-dCTs}) of each transcript and reported the results as expression ratios (Hashimoto et al 2008, Volk et al 2010).

4.2.4 Statistical Analyses

To assess the effect of age on transcript levels, we conducted both Pearson regression analyses for individual animal data and analyses of variance (ANOVA) for age group data for each transcript in layer 3 and layer 5 pyramidal cells. We also determined the mean and standard deviation of the expression ratios for each age group. Tukey's post hoc test was used for comparisons between age groups with $\alpha=0.05$. The reported p values for both the regression analyses and the ANOVAs were corrected for multiple comparisons (8 transcripts per layer times 2 layers equals 16 comparisons) using the Holm's simultaneous inference procedure (Volk et al 2000).

4.3 RESULTS

4.3.1 Postnatal expression of $GABA_A$ receptor subunit mRNAs in pyramidal cells

In monkey DLPFC, the levels of $GABA_A$ receptor α1 subunit mRNA significantly increased with age in both layer 3 ($r=0.93$, $p<0.001$) and layer 5 ($r=0.94$, $p<0.001$) pyramidal neurons **(Fig. 16A)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys **(Appendix B; Supplemental Table 1)**, mean levels of the α1 subunit mRNA increased by 137% in layer 3 and by 199% in layer 5 **(Table 4)**. Analysis by age group also revealed significant differences in mRNA levels in both layer 3 ($F_{3,22}=21.9$, $p<0.001$) and layer 5 ($F_{3,22}=26.5$, $p<0.001$) pyramidal neurons, with post hoc

analyses revealing significant ($p<0.05$) increases in expression of α1 subunit mRNA between the perinatal to peripubertal age groups in layer 3 and between the perinatal to prepubertal and the prepubertal to peripubertal age groups in layer 5 **(Fig. 16B)**. In addition, $GABA_A$ α1 subunit mRNA levels were higher in layer 3 than in layer 5 pyramidal cells in every animal, with expression levels approximately 2-fold higher in layer 3 in each age group.

In contrast, expression levels of the $GABA_A$ α2 subunit mRNA selectively decreased across postnatal development in layer 3 pyramidal cells ($r= -0.92$, $p<0.001$), and did not change with age in layer 5 pyramidal neurons **(Fig. 16C)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the α2 subunit mRNA decreased by 61% in layer 3 **(Table 4)**. Analysis by age group also revealed significant differences in mRNA levels in layer 3 ($F_{3,22}=61.5$, $p<0.001$), with post hoc analyses revealing significant ($p<0.05$) decreases in α2 subunit expression between each pair of adjacent age groups **(Fig. 16D)**. Although the level of $GABA_A$ α2 subunit mRNA was higher in layer 3 than layer 5 in every age group, this difference declined from approximately 4-fold higher in layer 3 in the perinatal age group to less than 2-fold in the adult age group.

In contrast to the developmental trajectories exhibited by α1 and α2 subunits, expression of α5 subunit mRNA significantly decreased in both layer 3 ($r= -0.83$, $p<0.001$) and layer 5 ($r= -0.70$, $p<0.001$) pyramidal neurons **(Fig. 16E)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the α5 subunit mRNA decreased by 74% in layer 3 and by 50% in layer 5 **(Table 4)**. Analysis by age group also revealed significant differences in mRNA levels in both layer 3 ($F_{3,22}=41.1$, $p<0.001$) and layer 5 ($F_{3,22}=13.5$, $p<0.001$) pyramidal neurons, with post hoc analyses showing significant ($p<0.05$) decreases in α5 subunit expression between the perinatal to prepubertal and prepubertal to adult age groups in layer 3 pyramidal cells and between the perinatal to prepubertal age group in layer 5 pyramidal cells **(Fig. 16F)**. The laminar pattern of $GABA_A$ α5

subunit mRNA expression also differed from α1 and α2 subunits as α5 levels were approximately 2-fold higher in layer 5 than layer 3 pyramidal cells across all age groups.

The $GABA_A$ β2 subunit, which preferentially assembles with the α1 subunit (Farrant & Nusser 2005), showed a developmental pattern of expression similar to that of the α1 subunit. Transcript levels of β2 significantly increased in both layer 3 ($r=0.64$, $p<0.001$) and layer 5 ($r=0.68$, $p<0.001$) pyramidal cells **(Fig. 16G)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the β2 subunit mRNA increased by 47% in layer 3 and by 89% in layer 5 **(Table 4)**. Analysis by age group also revealed significant differences in mRNA levels in both layer 3 ($F_{3,22}=6.4$, $p=0.003$) and layer 5 ($F_{3,22}=7.5$, $p=0.001$) **(Fig. 16H)** pyramidal neurons, with post hoc analyses revealing significant ($p<0.05$) increases in β2 subunit expression between the perinatal to peripubertal age groups in layer 3 and between the perinatal to prepubertal age groups in layer 5 pyramidal neurons **(Fig. 16H)**. Similar to the α1 subunit, laminar analyses showed that the expression of $GABA_A$ β2 subunit was approximately 2-fold higher in layer 3 than layer 5 pyramidal cells in every age group.

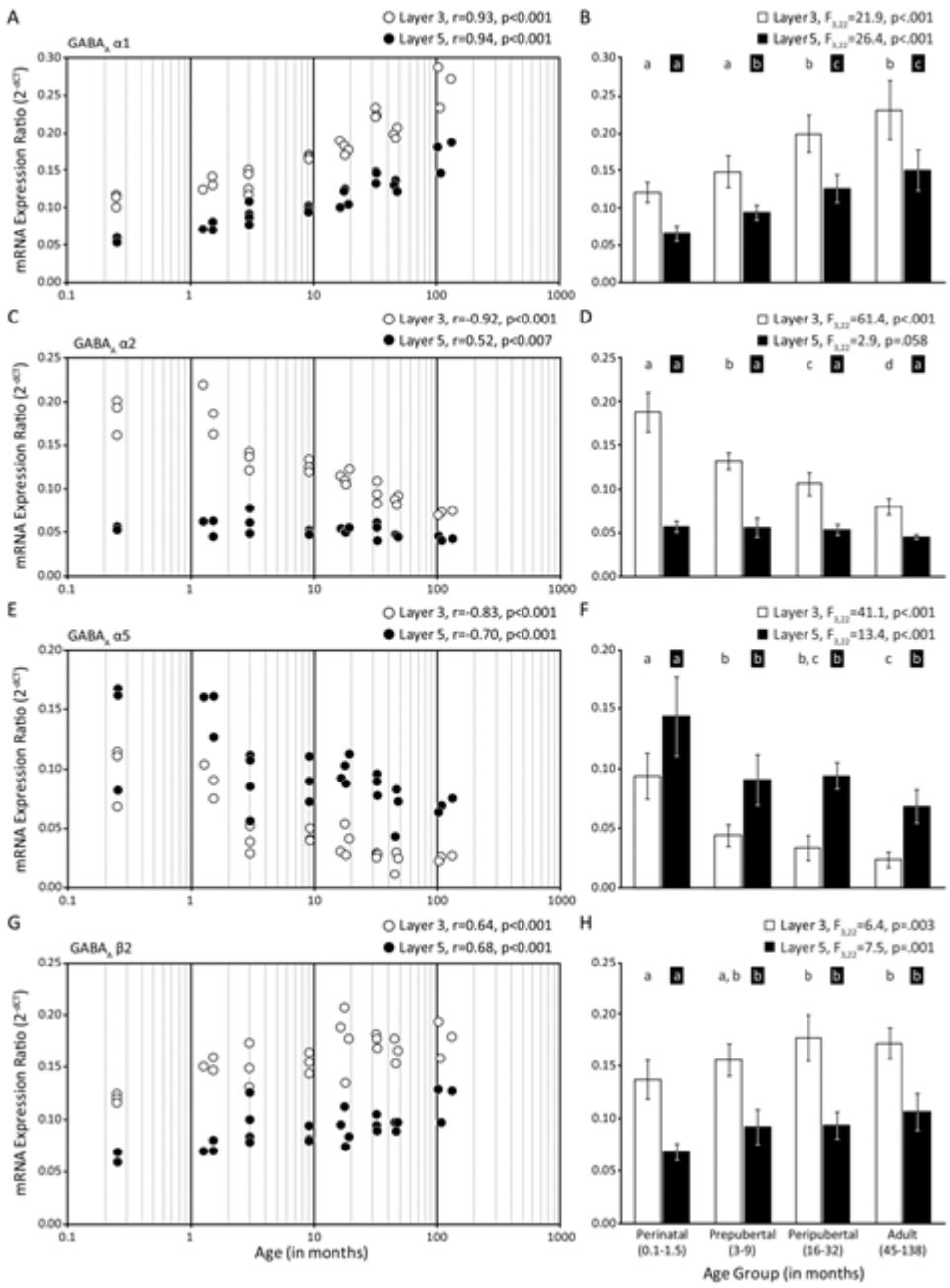

Figure 16. Developmental trajectories of GABA$_A$ receptor α1, α2, α5 and β2 subunit mRNAs in layer 3 and 5 pyramidal cells in monkey DLPFC.

The left panels show the expression ratios for each transcript in individual subjects for layer 3 (white circles) or layer 5 (black circles) pyramidal cells. The Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. The right panels show the mean (SD) expression ratios for each age group; age groups not sharing the same letter are significantly different ($p<0.05$). **(A, B)** GABA$_A$ α1 subunit mRNA expression increased throughout postnatal development. **(C, D)** GABA$_A$ α2 subunit mRNA expression decreased selectively in layer 3 pyramidal cells from perinatal to adult. **(E, F)** GABA$_A$ α5 subunit mRNA expression decreased in both layers throughout development. **(G,**

H) $GABA_A$ β2 subunit mRNA expression increased in both layers during development, paralleling the trajectory of $GABA_A$ α1 subunit mRNA.

In postsynaptic locations, most α1-, α2- and α5-containing $GABA_A$ receptors also contain a γ2 subunit (Farrant & Nusser 2005, Jacob et al 2008). Expression of $GABA_A$ receptor γ2 subunit mRNA significantly increased in both layer 3 ($r=0.89$, $p<0.001$) and layer 5 ($r=0.94$, $p<0.001$) pyramidal neurons **(Fig. 17A)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the γ2 subunit mRNA increased by 92% in layer 3 and by 163% in layer 5 **(Table 4)**. Analysis by age group also revealed significant differences in mRNA levels in both layer 3 ($F_{3,22}=18.5$, $p<0.001$) and layer 5 ($F_{3,22}=31.7$, $p<0.001$) pyramidal neurons, with post hoc analyses revealing significant ($p<0.05$) increases in γ2 subunit expression between the perinatal to peripubertal and peripubertal to adult age groups in both layer 3 and layer 5 pyramidal cells **(Fig. 17B)**. The magnitude of expression of the γ2 subunit was similar between layers with a slightly higher expression in layer 3 pyramidal cells at all developmental age groups.

Cortical $GABA_A$ receptors containing δ subunits are localized extrasynaptically, have a high affinity for GABA, and mediate tonic inhibition, defined as the constant activation of extrasynaptic receptors that, by increasing input conductance, reduces the probability of generating an action potential (Farrant & Nusser 2005, Nusser et al 1998, Wei et al 2003). Our results showed that the expression of the $GABA_A$ receptor δ subunit mRNA significantly increased in layer 5 ($r=0.91$, $p<0.001$) pyramidal cells, but did not change in layer 3 pyramidal cells across postnatal development **(Fig. 17C)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the δ subunit mRNA increased by 193% in layer 5 **(Table 4)**. Analysis by age group revealed significant differences in mRNA levels in layer 5 ($F_{3,22}=24.7$, $p<0.001$) pyramidal neurons, with post hoc analyses revealing significant ($p<0.05$) increases between the perinatal to prepubertal and prepubertal to adult age groups **(Fig. 17D)**. Expression level of the δ subunit was approximately

4-fold higher during the perinatal period in layer 3 compared to layer 5 pyramidal cells, with the levels of expression becoming similar in both layers in the adult age group.

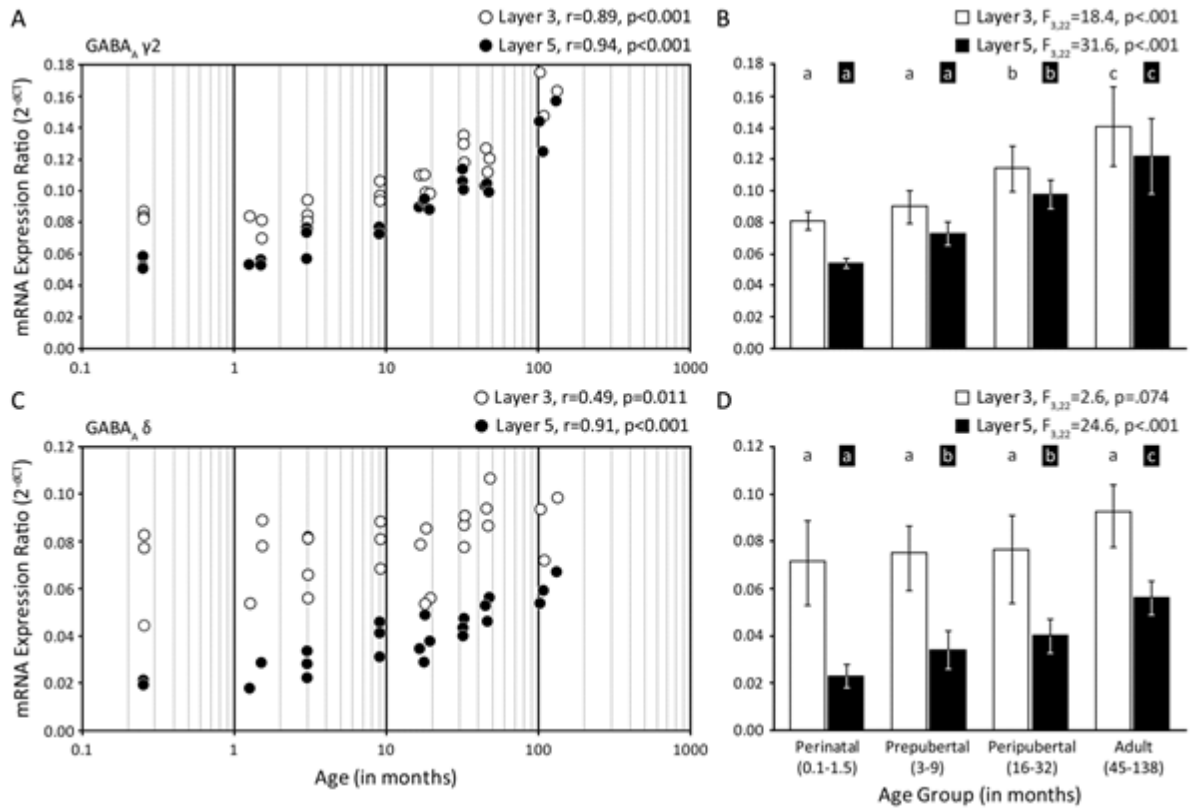

Figure 17. Developmental trajectories of GABA$_A$ receptor γ2 and δ subunit mRNAs in layer 3 and layer 5 pyramidal cells in monkey DLPFC.

The left panels show the expression ratios for each transcript in individual subjects for layer 3 (white circles) or layer 5 (black circles) pyramidal cells. The Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. The right panels show the mean (SD) expression ratios for each age group; age groups not sharing the same letter are significantly different ($p<0.05$). **(A, B)** GABA$_A$ γ2 subunit mRNA expression increased throughout postnatal development in both layers. **(C, D)** GABA$_A$ δ receptor subunit mRNA expression increased selectively in layer 5 pyramidal cells although the magnitude of expression was higher in layer 3 pyramidal cells.

4.3.2 Postnatal expression of glutamate AMPA and NMDA receptor subunit mRNAs in pyramidal cells

In order to determine the specificity of these developmental changes in expression of $GABA_A$ receptor subunits, we also evaluated the expression patterns for two glutamate receptor subunits that are critical mediators of excitatory neurotransmission (Cull-Candy et al 2001, Traynelis et al 2010). Expression of AMPA receptor Glur1 subunit mRNA significantly decreased in layer 3 ($r= -0.62$, $p<0.001$) pyramidal cells, and did not change in layer 5 pyramidal cells **(Fig. 18A)**. From the youngest (three animals one week of age) to oldest (three animals over eight years of age) monkeys, mean levels of the AMPA Glur1 subunit mRNA decreased by 37% in layer 3 **(Table 4)**. Analysis by age group revealed significant differences in mRNA levels in layer 3 ($F_{3,22}=7.2$, $p=0.002$) pyramidal cells, with post hoc analyses revealing significant ($p<0.05$), albeit modest, decreases in AMPA Glur1 subunit expression between the perinatal to peripubertal age group in layer 3 pyramidal cells **(Fig. 18B)**. Expression levels of the AMPA Glur1 subunit in layer 5 pyramidal cells were almost 2-fold higher in layer 5 compared to layer 3 pyramidal cells in every animal across all ages.

The developmental trajectory of the obligatory subunit for NMDA receptors, Grin1 (Monyer et al 1992, Schorge & Colquhoun 2003, Ulbrich & Isacoff 2008), did not show any significant changes in either layer 3 or layer 5 pyramidal cells during postnatal development **(Fig. 18 C, D)**. The expression of the NMDA Grin1 subunit was higher in layer 3 than layer 5 pyramidal cells across all age groups (perinatal=26.6%; prepubertal=46.7%; peripubertal=36.2%; adult=22.9%).

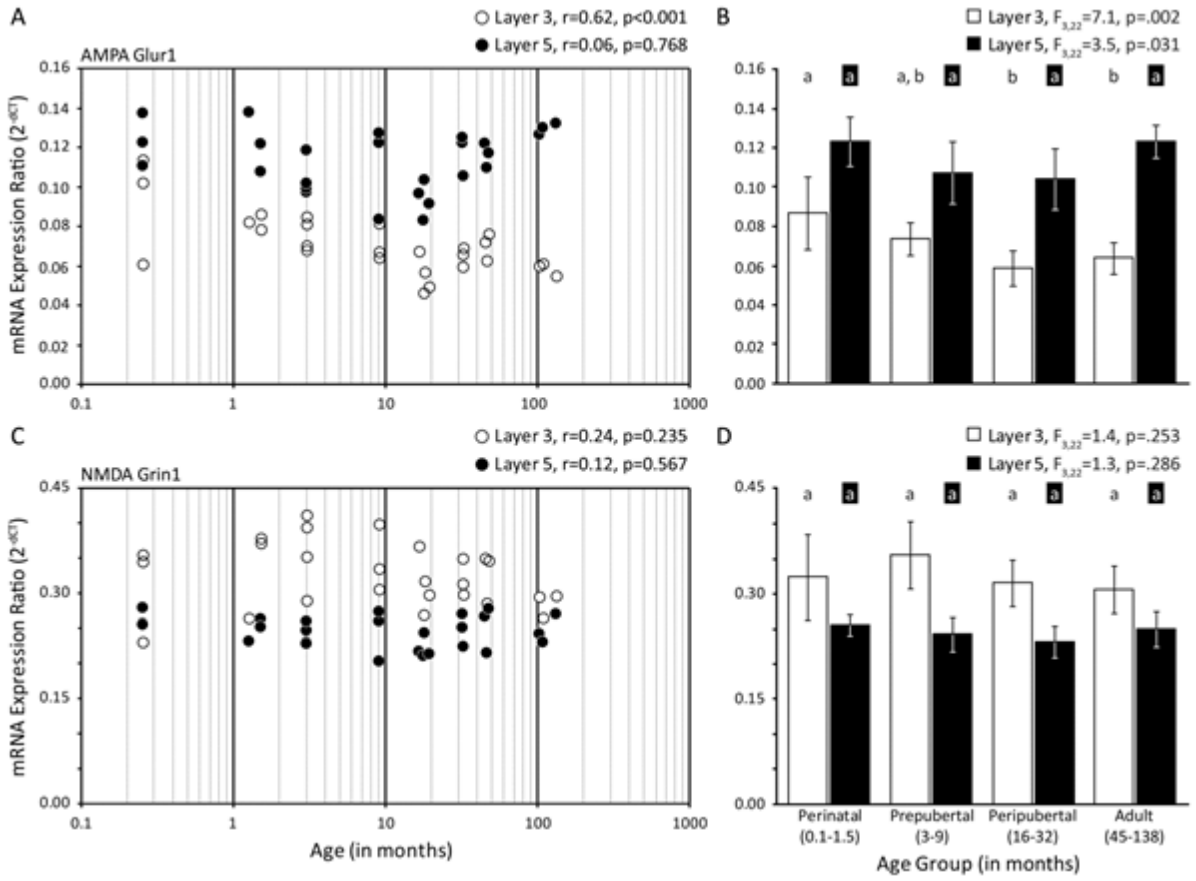

Figure 18. Developmental trajectories of glutamate receptor subunits AMPA Glur1 and NMDA Grin1 mRNAs in layers 3 and 5 pyramidal cells of monkey.

The left panels show the expression ratios for each transcript in individual subjects for layer 3 (white circles) or layer 5 (black circles) pyramidal cells. The Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. The right panels show the

mean (SD) expression ratios for each age group; age groups not sharing the same letter are significantly different ($p<0.05$). **(A, B)** AMPA Glur1 subunit mRNA expression decreased between the perinatal and peripubertal age groups during development in layer 3 pyramidal cells. **(C, D)** NMDA Grin1 subunit mRNA expression was largely unchanged throughout development.

Table 4. Summary of differences, by layer, magnitude, and time of maturation, of the developmental trajectories for GABA and glutamate receptor subunits in layers 3 and 5 pyramidal cells of the monkey DLPFC.

Transcript	Layer 3		Layer 5		Magnitude of expression
	Maximal % change from youngest to oldest	Period when adult level of expression achieved	Maximal % change from youngest to oldest	Period when adult level of expression achieved	
$GABA_A$ α1	137%	Adult	199%	Peripubertal	L3>L5
$GABA_A$ α2	-61%	Adult	-	Perinatal	L3>L5
$GABA_A$ α5	-74%	Peripubertal	-50%	Prepubertal	L5>L3
$GABA_A$ β2	47%	Peripubertal	89%	Prepubertal	L3>L5
$GABA_A$ γ2	92%	Adult	163%	Adult	L3>L5
$GABA_A$ δ	-	Perinatal	193%	Adult	L3>L5
AMPA Glur1	-37%	Peripubertal	-	Perinatal	L5>L3
NMDA Grin1	-	Perinatal	-	Perinatal	L3>L5

The youngest age group comprises of three animals of one week of age and the oldest animals comprises of three animals that were over eight years of age.

4.4 DISCUSSION

In this study, we used a cell type-specific approach to determine if the expression of $GABA_A$ receptor subunits specifically in layer 3 pyramidal neurons in monkey DLPFC exhibit distinctive developmental trajectories, whether these trajectories differ from those of excitatory receptor subunits in these neurons, and whether these developmental patterns of receptor transcript expression differ from those in layer 5 pyramidal cells.

Expression levels of all six $GABA_A$ receptor subunits studied changed over a protracted period of postnatal development. Some transcripts (e.g. $GABA_A$ receptor α1 subunit) in layer 3 pyramidal neurons progressively increased from birth until the adult age group, whereas others (e.g. $GABA_A$ receptor α2 subunit) declined across the same developmental period. In contrast, postnatal developmental changes in both glutamate receptor subunits were modest, with adult levels reached early in life. Some transcripts exhibited a similar direction of developmental change in both layer 3 and 5 pyramidal cells (e.g., $GABA_A$ α5 decreased in both layers and $GABA_A$ β2 increased in both layers), whereas others (e.g., $GABA_A$ receptor α2 and δ subunits and AMPA Glur1) changed with age only in one layer. Transcripts also differed in terms of whether expression levels were consistently higher in layer 3 (e.g. $GABA_A$ receptor γ2 subunit) or layer 5 (e.g. glutamate AMPA receptor Glur1 subunit) pyramidal cells.

Our focus on gene expression patterns in a specific population of neurons may be particularly informative since measures of transcript levels in total gray matter may be diluted by other cell types. For example, previous studies of total gray matter suggested a less robust increase in $GABA_A$ α1 subunit levels during development (Hoftman et al 2015) than those observed in layer 3 and 5 pyramidal cells in the present study. Moreover, the collection of the same number of neurons per subject also excludes the potential confound of gray matter

measures that age-related differences in transcript levels reflect changes in neuron number or density rather than changes in gene expression.

4.4.1 Relationships among developmental trajectories of GABA receptor subunits

The postsynaptic influence of GABA released into the synaptic cleft is determined, in part, by the subunit composition of the $GABA_A$ receptor to which it binds. The majority of $GABA_A$ receptors assemble according to a 2α:2β:γ stoichiometry (Farrant & Kaila 2007, Luscher et al 2011, Olsen & Sieghart 2009). The resulting receptor heterogeneity is associated with differences in two key parameters of synaptic transmission: the duration of inhibitory postsynaptic currents (IPSCs) and the nature of inhibition (i.e., phasic versus tonic). Because $GABA_A$ receptor α subunits contribute to differences in the decay rate of IPSCs, the developmental trajectories reported here may underlie important changes in the functional properties of DLPFC circuitry. For example, the decay time of GABA-activated currents is 6-fold faster when mediated by α1- than by α2 or α5-containing receptors (Farrant & Nusser 2005, Lavoie et al 1997, McClellan & Twyman 1999). Thus, the progressive increase in expression of $GABA_A$ α1 subunit and decrease in expression of α2 and α5 receptor subunits in layer 3 pyramidal cells across postnatal development is likely to substantially decrease the duration of IPSCs in these neurons with age. Consistent with this interpretation, the decay time constant of IPSCs in layer 3 pyramidal cells of monkey DLFPC was reported to decline significantly by approximately 25% between 16 and 44 months of age (Hashimoto et al 2009). A similar correlation between developmental changes in tissue levels of α $GABA_A$ receptor subunits and of IPSC decay time has been observed in rodent neocortex and hippocampus (Bosman et al 2002, Cohen et al 2000), although these changes are completed much earlier in rodents (Le Magueresse & Monyer 2013).

Whether these findings reflect a switch in the α subunit composition of $GABA_A$ receptors at particular synaptic sites or a change in the number of $GABA_A$ receptors containing a particular α subunit at different synaptic locations remains an important question for future studies. In either case, the apparent increase in the relative numbers of receptors containing fast versus slow decay kinetics may contribute to the substrate for developmental changes in cortical network oscillations. For example, gamma band (30-80 Hz) oscillations require a fast decay of GABA currents, such as that associated with the $GABA_A$ α1 subunit, in order for ensembles of pyramidal cells to be quickly released from synchronous inhibition in order to fire at a fast frequency (Bartos et al 2007). The developmental increase in the relative abundance of $GABA_A$ α1 subunits that confer fast IPSC decay properties shown here may increase the capacity of prefrontal networks to generate gamma oscillations. In addition, our finding that the increase in the relative ratio of α1 to α2 or α5 subunit expression is more prominent in layer 3 than in layer 5 pyramidal cells is particularly interesting given recent findings that gamma oscillations originate principally in layer 3 of adult monkey association cortices (Buffalo et al 2011). Because prefrontal gamma oscillation power increases in proportion to working memory load (Howard et al 2003, Roux et al 2012, Tallon-Baudry et al 1999), our findings may represent an important factor in the postnatal developmental increase in the power of gamma oscillations in humans (Uhlhaas et al 2009) and in working memory performance from 3 months of age to adulthood in monkeys (Alexander & Goldman 1978, Goldman 1971).

It is important to note that the subcellular location of the receptors affected by the opposed developmental trajectories of $GABA_A$ α1 and α2 subunits in layer 3 pyramidal cells and the expression levels of the cognate proteins cannot be determined from the present study. However, in adult animals, α1-containing receptors predominate postsynaptic to PV basket cell inputs, whereas α2-containing receptors predominate postsynaptic to PV chandelier cell inputs (Klausberger et al 2002, Nusser et al 1996). Previous studies using electron microscopy revealed that the overall density of symmetric GABAergic synapses does not change during

postnatal development in monkey DLPFC (Bourgeois et al 1994). However, recent work using quantitative, multi-label, confocal microscopy suggests that the pruning of axon terminals may be a cell type- and membrane domain-specific phenomenon which is not detectable when all GABAergic terminals are assessed without regard to cell type (Fish et al 2013). For example, the mean number of chandelier cell boutons per pyramidal neuron axon initial segment was significantly lower in adult compared with 3-month-old monkeys, whereas the density of basket cell boutons did not differ (Fish et al 2013). In contrast, the levels of parvalbumin protein in basket cell axon terminals were approximately two-fold higher in adult relative to 3-month-old animals, whereas no age-related differences were found for parvalbumin protein levels in chandelier cell axon terminals (Fish et al 2013). Although speculative, these findings raise the possibility that the $GABA_A$ receptors postsynaptic to these two types of GABA axon terminals may also exhibit distinctive types of developmental changes in response to the changes in their presynaptic inputs. For example, due to the developmental pruning of chandelier cell terminals, the number of $GABA_A$ α2-containing postsynaptic receptors may similarly decline, leading to a progressively lower demand for expression of α2 mRNA. Similarly, based on findings that parvalbumin levels are correlated with the probability of GABA release (Eggermann & Jonas 2012), the parallel increase in parvalbumin protein levels in basket cell terminals and the number of postsynaptic $GABA_A$ α1-containing receptors suggested by the current findings would provide a synergistic mechanism for increasing the strength of inhibitory inputs from parvalbumin basket cell inputs and thus a greater capacity to generate gamma oscillations with age.

4.4.2 Comparison of perisomatic and dendritic inhibition in pyramidal cells during postnatal development

In contrast to the perisomatic location of $GABA_A$ α1 and α2 subunits, $GABA_A$ receptors containing α5 subunits are localized on pyramidal cell apical dendrites (Ali & Thomson 2008) postsynaptic to GABA inputs from the Martinotti class of GABA neurons that express the neuropeptide somatostatin (SST) (Kawaguchi & Kubota 1996, Melchitzky & Lewis 2008). The SST-containing interneurons are low threshold-spiking cells that form electrically-coupled networks which oscillate at theta frequency (4-8 Hz) (Beierlein et al 2000, Gibson et al 2005). Moreover, these inputs are critical mediators of dendritic inhibition which controls the local integration of inputs and mediates strong disynaptic inhibition in cortical circuits (Kapfer et al 2007, Murayama et al 2009, Silberberg & Markram 2007). Our findings of a developmental decline in expression of the $GABA_A$ α5 subunit in both layer 3 and layer 5 pyramidal cells suggests that dendritic inhibition decreases progressively during postnatal maturation in the primate DLPFC (Caraiscos et al 2004). Expression of the mRNA for SST, which inhibits pyramidal neurons (Boehm & Betz 1997), also decreases significantly during postnatal development in the human PFC (Fung et al 2010). Although the developmental trajectories of molecules regulating GABA release from SST terminals and of SST receptors in pyramidal neurons remain unknown, the available data suggest that both presynaptic (Fung et al 2010) and postsynaptic components of dendritic inhibition onto layer 3 and 5 pyramidal cells decrease during maturation of the DLPFC. Thus, our results may provide the substrate for a progressive developmental shift from dendritic inhibition to perisomatic inhibition as the principal means to modulate pyramidal cell output. Alternatively, the apparent decrease in dendritic inhibition could parallel the decrease in density of dendritic spines and excitatory axospinous synapses during postnatal development (Anderson et al 1995, Bourgeois et al 1994, Petanjek et al 2011). This

notion is supported by recent findings suggesting that a significant proportion of dendrite-targeting GABA synapses are made onto individual spines to focally regulate Ca^{2+} signals and that GABA inputs onto spines have high rates of turnover *in vivo* (Chen et al 2012, Chiu et al 2013, Kubota et al 2007, van Versendaal et al 2012). Hence, the progressive decline in dendritic inhibition during postnatal development could be a response to the pruning of dendritic spines in apical dendrites.

4.4.3 Contrasting development of markers of phasic versus tonic inhibition of pyramidal cells

The γ2 subunit co-assembles with α1 and β2 subunits to form ~60% of $GABA_A$ receptors in the adult cortex (Mohler 2006). Our data indicate that these three subunits all increase in a similar fashion across postnatal development, suggesting a progressive increase in phasic inhibition with age in both layer 3 and 5 pyramidal neurons. In contrast, expression of the $GABA_A$ δ receptor subunit increased exclusively in layer 5 pyramidal cells during postnatal development, consistent with the findings of a prior *in situ* hybridization study showing a selective increase in $GABA_A$ δ receptor subunit expression in layer 5 relative to layer 3 (Maldonado-Aviles et al 2009). Interestingly, the γ2 to δ subunit ratio increased by 49% from the youngest (three animals one week of age) to the oldest animals (three animals over 8 years of age) in layer 3 pyramidal cells and decreased by 10% in layer 5 pyramidal cells. These comparisons suggest that the ratio of phasic to tonic inhibition becomes increasingly different between layer 3 and 5 pyramidal cells across postnatal development. The laminar-specific pattern of changes in the nature of GABA inhibition suggest that phasic inhibition predominates over tonic inhibition with age in layer 3 pyramidal cells during circuit maturation while the ratio of phasic to tonic inhibition remains unchanged in layer 5 pyramidal cells.

4.4.4 Development trajectories of molecular markers of inhibition versus excitation of DLPFC pyramidal neurons

Our experiments revealed marked differences in the magnitude of developmental changes in $GABA_A$ and glutamate receptor subunits in monkey DLPFC. In contrast to the robust developmental changes in $GABA_A$ receptor subunits, the developmental trajectories for the AMPA Glur1 and NMDA Grin1 subunits were relatively flat in both layers 3 and 5 pyramidal cells. Among other possibilities, the difference in trajectories may contribute to the molecular substrate for electrophysiological findings that excitatory postsynaptic currents (EPSCs) in layer 3 pyramidal cells of monkey DLPFC achieve mature properties much earlier in postnatal development than do IPSCs (Gonzalez-Burgos et al 2008, Hashimoto et al 2009).

4.4.5 Conclusion

In aggregate, the developmental trajectories of transcripts critical in mediating GABA neurotransmission in pyramidal cells in the DLPFC support a dynamic and complex process of circuit maturation. Our findings suggest that the molecular determinants of GABA neurotransmission have distinctive developmental trajectories that differ in magnitude and direction of change, whereas the molecular determinants of glutamate neurotransmission are relatively stable and achieve adult expression levels early in development. As a result, the components of GABA neurotransmission in DLPFC circuitry may have distinctive developmentally sensitive periods during which environmental events may have a particular strength in either enriching or impairing circuit development.

5.0 GENERAL DISCUSSION

In this dissertation, we provide evidence for pyramidal *cell type-specific* molecular disturbances and *synapse-specific* structural impairments in DLPFC deep layer 3, and *cell type-specific* and *layer-specific* nature of postnatal developmental refinements in pyramidal cells in the DLPFC, within the circuitry that subserves γ-frequency oscillations and working memory. In order to understand layer-specific and *cell type-specific* molecular mechanisms that are perturbed in schizophrenia, we investigated dysregulation of the actin cytoskeleton required for dendritic spine formation and maintenance and examined the integrity of the CDC42-PAK-LIMK signaling pathway using laser microdissection in DLPFC deep layer 3 **(Chapter 2)**. In order to reveal *synapse-specific* structural impairments in DLPFC deep layer 3, we conducted studies to determine if a subpopulation of dendritic spines is preferentially affected in schizophrenia using triple-label fluorescence immunohistochemistry and spinning-disk confocal microscopy **(Chapter 3)**. Finally, in order to understand *cell type-specific* and *layer-specific* postnatal developmental refinements in pyramidal cells in the DLPFC, we examined the developmental trajectories of $GABA_AR$ subunit expression in layer 3 and 5 pyramidal cells using laser microdissection in the monkey DLPFC **(Chapter 4)**.

We reported upregulation and downregulation of mRNA levels for various components of the CDC42-PAK-LIMK signaling pathway that were not attributable to psychotropic medications or other comorbid factors, suggesting that *cell type-specific* impairments in the actin cytoskeleton may substantially contribute to dendritic spine deficits in DLPFC deep layer 3 pyramidal cells **(Chapter 2)**. We report that the density of putative dual-innervated dendritic

spines receiving an inhibitory synapse and corticocortical input, and dendritic spines receiving a corticocortical input are unaltered in the illness, although other subpopulations of spines receiving thalamocortical input may be affected **(Chapter 3)**. We also found that the developmental trajectories of $GABA_AR$ subunit expression in primate DLPFC deep layer 3 pyramidal neurons are *protracted, subunit- and layer-specific* and posited that the molecular maturation of GABA synapses may account, at least in part, for the maturation of synchronized pyramidal cell firing which is crucial for γ-frequency oscillations (30-80 Hz) and emergent properties such as working memory **(Chapter 4)**.

In the following discussion, I will consider these topics and present a plausible cascade of events that might explain local-circuit abnormalities observed in the DLPFC and systems-level alterations in different neurotransmitter systems observed in the illness. I will also weigh in on alternative hypotheses that have been postulated for the disease process and mechanisms that can produce the reported data not addressed in the individual chapters. Lastly, I will offer some insight into future directions that are vital in establishing cause-consequence-compensation relationships in the pathogenesis of schizophrenia and speculate on the scope of innovative therapeutic strategies.

5.1 DECIPHERING THE DISEASE PROCESS IN SCHIZOPHRENIA

A fundamental challenge with making deductions from observed alterations in postmortem tissue in subjects with schizophrenia is knowing whether the observed changes represent a 1) Cause, an upstream primary problem related to the pathogenesis of the illness; 2) Consequence, a downstream detrimental manifestation of a cause; 3) Compensation, a response to the pathophysiology induced by pathological alterations; 4) Confound, an artifact of technical limitations; 5) Comorbidity, an outcome of factors frequently associated with the

disease process (Lewis & Gonzalez-Burgos 2008). Determining in which category a particular alteration lies is imperative for clarifying what cellular and molecular disturbances are most proximal to the pathophysiology of specific clinical symptoms (e.g., γ-frequency oscillations and working memory deficits).

5.1.1 Molecular evidence for a cell-autonomous intrinsic deficit in DLPFC deep layer 3 pyramidal cells: Dysregulation of the actin cytoskeleton

Given the critical importance of dendritic spine structural stability and functional integrity in mediating neural plasticity, a major unmet need in basic neuroscience is explicating the cellular and molecular mechanisms that establish dendritic stability and illuminating how these are perturbed in disease. The actin cytoskeleton provides essential structural support for maintaining F-actin configuration and dynamic remodeling of spine shape. The morphological properties of dendritic spines are determined by an intricate balance between actin polymerization and depolymerization (Honkura et al 2008, Okamoto et al 2004). In particular, the cofilin/ADF family is particularly important in establishing the equilibrium for G-actin and F-actin concentration by disassembling actin filaments to generate new barbed ends for F-actin assembly (Carlier et al 1997, dos Remedios et al 2003). In this dissertation, we have identified alterations in the expression of numerous molecular regulators of the actin cytoskeleton in a *layer-specific* and *cell type-specific* manner in DLPFC deep layer 3 in individuals with schizophrenia that might be a critical “upstream” cause in the pathogenesis of schizophrenia. Our results suggest that altered signaling through CDC42-PAK-LIMK pathway might be expected to reduce F-actin turnover in spines through cofilin/ADF and decrease dendritic spine density. Moreover, we confirmed and extended to the *cell type-specific* level earlier findings of disruptions in the CDC42-CDC42EP pathway. We have argued that morphological alterations such as lower dendritic spine density is most pronounced in DLPFC deep layer 3, because of a

cell-autonomous intrinsic abnormality in DLPFC deep layer 3 pyramidal cells, reflected by impairments in genes related to the actin cytoskeleton that are specifically dysregulated in DLPFC deep layer 3 pyramidal cells. Although alterations of CDC42 signaling pathway components are expected to significantly destabilize actin filament dynamics, in the following section, I will provide other plausible mechanisms that might impair the function of the actin cytoskeleton in pyramidal cells and merit further interrogation in schizophrenia.

5.1.2 Role of other actin cytoskeleton signaling proteins in F-actin regulation

Changes in the expression of several additional cytoskeleton regulatory molecules occur following changes in neuronal activity to modulate the dynamic equilibrium between F-actin and G-actin. For example, inhibition of RhoA target, Rho-associated protein kinase **(ROCK)** can suppress spine loss resulting from enhanced RhoA signaling (Xing et al 2012). Other molecules such as Drebrin, myristoylated alanine-rich C-kinase substrate **(MARCKS)** and β-adducin have important roles in dendritic spine morphogenesis and transition of immature spines to more stable structures (Koleske 2013). In addition, brain-derived neurotrophic factor **(BDNF)** and its postsynaptic partner TrkB receptors have also been documented to be crucial for spine enlargement and stabilization by inactivating cofilin/ADF (Baquet et al 2004, Gorski et al 2003, Rex et al 2007) and both BDNF and TrkB mRNA are lower in the DLPFC of subjects with schizophrenia (Hashimoto et al 2005, Weickert et al 2003). Another mechanism which can attenuate the activity of cofilin/ADF in dendritic spines involves synaptic GTPase activating protein **(synGAP)**, an inhibitor of the Rho GTPase RAC and important molecule for trafficking of glutamate receptors (Carlisle et al 2008). For example, heterozygous or homozygous deletion of synGAP in rodents was sufficient to cause altered activity-dependent phosphorylation of cofilin/ADF, resulting in an excess of mushroom-shaped spines (Carlisle et al 2008). Finally, F-actin binding proteins such as cortactin that are activated by tyrosine protein kinase Abl-related

gene **(ARG)**, can stabilize newly generated F-actin filaments by stimulating nucleation of the ARP2/3 complex (MacGrath & Koleske 2012). Selective inactivation of the ARP2/3 complex *in vivo* in forebrain excitatory cells leads to a decrement in spine and synapse density reinforcing its central role in orchestrating actin nucleation for long-term spine stability (Kim et al 2013, Kim et al 2015). The ARP2/3 complex has emerged as an important molecular hub, acting downstream of multiple genes implicated in the pathogenesis of schizophrenia and might be an important candidate for future examination **(Figure 19)** (Clement et al 2012, De Rubeis et al 2013, Hayashi-Takagi et al 2010, Russell et al 2014).

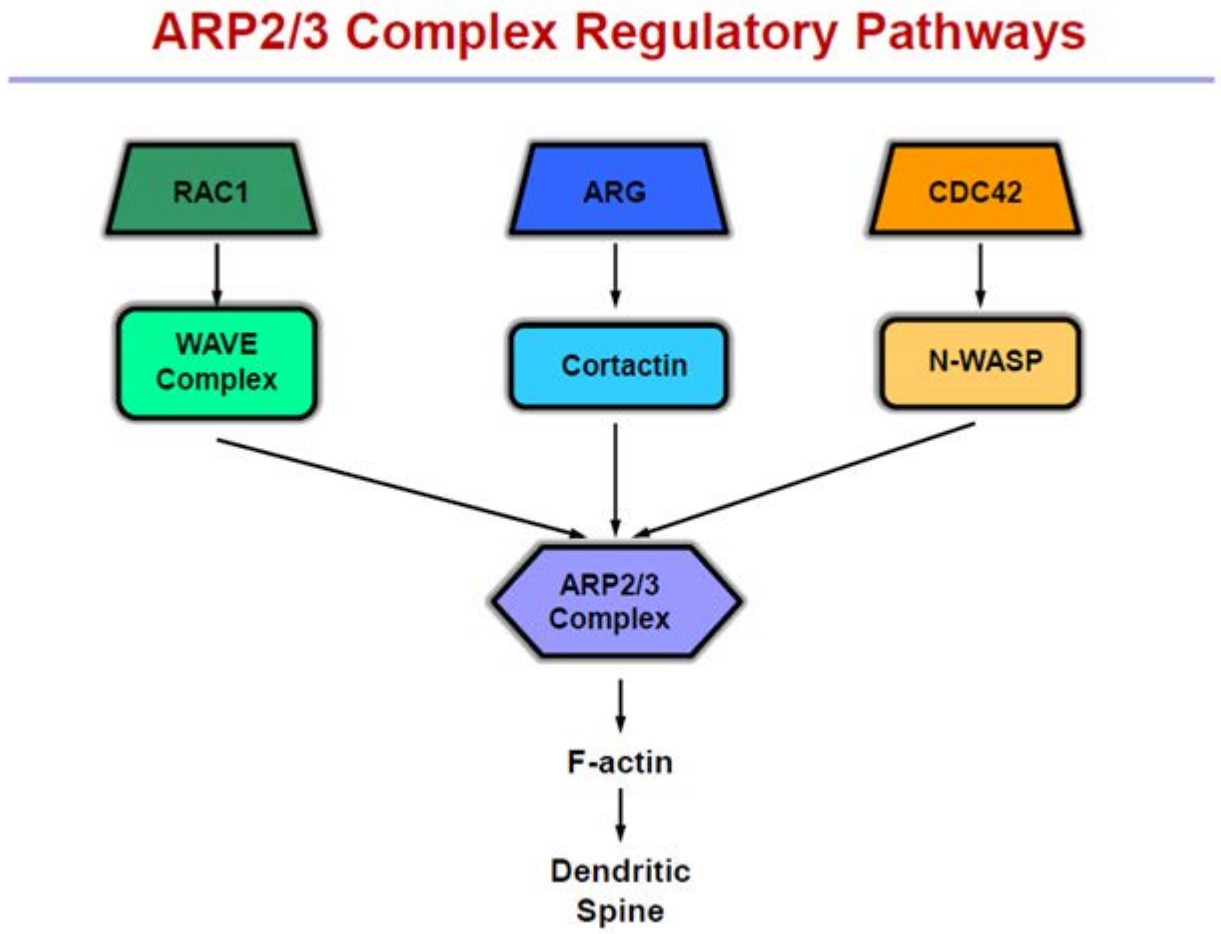

Figure 19. Model for molecular mechanisms that determine F-actin polymerization through ARP2/3 complex.

The activity of the ARP2/3 complex is regulated by several nucleation-promotion factors (NPFs) such as the Wiskott-Aldrich syndrome (WASP) proteins, WASP family Verprolin-homologous (WAVE) proteins and cortactin that act downstream of Rho GTPases such as CDC42 and RAC1, and tyrosine protein

kinase such as ARG, respectively. The ARP2/3 complex may represent a common final output for actin cytoskeleton regulatory pathways and binds to the sides of existing actin filaments to induce actin nucleation, resulting in the formation of branched actin filament networks. The ARP2/3 complex is comprised of seven subunits (actin binding proteins, ARP2 and ARP3; ATP-binding proteins, ARPC1, ARPC2, ARPC3, ARPC4, ARPC5) that are activated downstream of known risk genes for schizophrenia. Although beyond the purview of this dissertation, our preliminary findings suggest a marked decrement in 6 out of 7 subunits of the ARP2/3 complex (unpublished data) in DLPFC deep layer 3 pyramidal cells in schizophrenia. We are currently in the process of determining what upstream mechanism (CDC42-N-WASP, ARG-cortactin and RAC1-WAVE) might produce the downregulation of the ARP2/3 complex in schizophrenia.

5.1.3 Synaptic scaffolding proteins contribute to dendritic spine and synapse maintenance

The structural support required for the long-term maintenance of dendritic spines and the actin cytoskeleton is also provided by various scaffolding proteins and stabilizing mechanisms provided by cell-adhesion molecules. For example, Shank/Homer/PSD95 complexes interact with NMDARs to recruit IP_3 receptors and F-actin to excitatory synapses within dendritic spines, and modulate spine enlargement necessary for dendritic spine stabilization (Naisbitt et al 1999, Sala et al 2001). Knock-out Shank mice have diminished basal synaptic transmission, decreased dendritic spine density and social behavioral impairments (Hung et al 2008, Wohr et al 2011), and mutations in the promoter of the Shank1 gene have been associated with working memory deficits in subjects with schizophrenia (Lennertz et al 2012). Furthermore, cadherin-catenin, neurexin/neuroligin and ephrin receptor bidirectional adhesion signaling complexes are functionally coupled to the actin cytoskeleton to promote structural stabilization necessary for spine and synapse maintenance (Arikkath & Reichardt 2008, Craig & Kang 2007, Elia et al 2006, Ethell et al 2001, Penzes et al 2003). In addition, these scaffolding proteins and cell-adhesion complexes might serve as an important activity-dependent molecular control for concurrent spine maturation and pruning during neural circuit refinement, particularly during adolescence when the symptoms of schizophrenia emerge (Bian et al 2015).

5.1.4 Potential genetic basis for actin cytoskeleton impairments in schizophrenia

Several lines of evidence suggest that the neuropathological alterations observed in schizophrenia may be a product of genetic predispositions that impair the regulation of actin filament dynamics and the capacity to maintain a normal complement of excitatory inputs (Fromer et al 2014, Kirov et al 2012, Roussos et al 2012, Schizophrenia Working Group of the Psychiatric Genomics 2014). Copy number variation studies, GWAS, and exome sequencing studies have particularly identified enrichment in signaling complexes formed by the activity-regulated cytoskeleton-associated scaffold protein **(ARC)** of the postsynaptic density, PSD protein complex and NMDAR-signaling, which are central elements to regulating synaptic strength at glutamatergic synapses, and are thought to be associated with increased risk for developing schizophrenia (Crowley et al 2013, Kirov et al 2012, Purcell et al 2014, Stefansson et al 2008, Timms et al 2013). In addition, *de novo* mutations in schizophrenia are over-represented among loci encoding cytoskeleton-associated proteins that modulate *actin filament dynamics* and *actin bundle assembly,* and could contribute to the pathogenesis of the illness (Fromer et al 2014). The robust consistency of these genetic findings converge upon a constrained set of signaling pathways related to the regulation of synaptic plasticity, particularly on glutamatergic synapses. As the burgeoning body of literature establishing an etiological genetic basis for impairments in *actin cytoskeleton pathways* and *glutamate postsynaptic proteins* continues to grow, it is plausible that a genetic liability might provide a potential cause for a proximal deficit in dendritic spines in schizophrenia, which may be moderated by cell type-specific gene expression patterns.

5.1.5 F-actin interaction with presynaptic active zone proteins

The impairments in actin cytoskeleton signaling pathways in schizophrenia raise the possibility that destabilization of F-actin localized within presynaptic boutons *and* postsynaptic specializations could result in reduced excitatory drive to DLPFC deep layer 3 pyramidal cells. An emerging idea in the field proposes that the F-actin meshwork within presynaptic compartments of axonal boutons might serve as an important scaffold to organize the neurotransmitter release machinery, establish protein regulators at active zones, and enable presynaptic vesicle docking and endocytosis (Chia et al 2012, Dillon & Goda 2005, Zhang & Benson 2001). For example, in addition to their normative functions in controlling postsynaptic dendritic spine structures, the LIMK proteins are thought to also perform an important role in the presynaptic compartment. The LIMK proteins have been localized in the presynaptic compartment of the neuromuscular junction and loss of function of LIMK1 causes presynaptic motor neuron terminal degeneration, by acting downstream of the bone morphogenetic protein **(BMP)** receptor (Eaton & Davis 2005). Therefore, is a similar phenomenon for F-actin regulation of presynaptic compartments observed in the central nervous system? In fact, a recent report demonstrated that overexpression of neuregulin 1 during early postnatal development causes synaptic dysfunction and schizophrenia-like behavioral deficits (hyperactivity, impaired sensorimotor gating, social and cognitive deficits), by increasing levels of synaptic LIMK1 (Yin et al 2013a). Overexpression of neuregulin 1, similar to that observed in schizophrenia (Hashimoto et al 2004, Weickert et al 2012), produced glutamatergic hypofunction by seemingly suppressing presynaptic vesicle fusion at the active zone through a LIMK1-cofilin/ADF-mediated actin depolymerization mechanism (Yin et al 2013a). Moreover, the glutamatergic hypofunction phenotype produced in these mice was also paralleled by GABAergic hypofunction with a decrease in the amplitude of mIPSCs and expression of $GABA_A$ α1 receptors in pyramidal cells (Yin et al 2013a). Interestingly, dysfunction in the presynaptic neurotransmitter exocytic

machinery involving the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor **(SNARE)** complex has been reported in postmortem studies in schizophrenia (Castillo et al 2010, Ramos-Miguel et al 2015). Thus, it is plausible that impairments in the presynaptic release machinery might arise from molecular alterations in the actin cytoskeleton that disrupts F-actin organization within presynaptic compartments.

In concert, it appears as though there is a great deal of functional redundancy for actin cytoskeleton signaling pathways which might be necessary for optimal control of actin filament dynamics required for spine morphogenesis and synaptic activity. At any rate, understanding the extent of neuropathological alterations in actin cytoskeleton signaling pathways in schizophrenia and the spatial and temporal relationship between different regulatory aspects of signal transduction should remain an ongoing area of scrutiny.

5.2 ASSEMBLING A CASCADE OF ALTERATIONS IN THE PATHOPHYSIOLOGY OF SCHIZOPHRENIA

Elucidating the neuropathological alterations in DLPFC circuits in postmortem tissue in subjects with schizophrenia could shed light on pathophysiological impairments such as lower γ-frequency oscillations and working memory deficits. Numerous compelling hypotheses have been postulated, but understanding cause-consequence-compensation relationships for local circuit abnormalities and systems-level alterations in the illness, remains an ongoing challenge. As part of this section of the dissertation, I intend to summarize some of these hypotheses and provide some insight on how the findings presented in this dissertation may be integrated into the different models. Understanding the *proximal pathology* to the pathophysiology of working memory dysfunction can foster a better understanding of what animal models or model systems

might be beneficial in testing the efficacy of novel pharmacological compounds to ameliorate the symptoms of the illness.

5.2.1 Proximal deficit in schizophrenia is reduced excitatory drive to DLPFC deep layer 3 pyramidal cells

A major idea in the field proposes that a *cell-type autonomous* intrinsic deficit in deep layer 3 pyramidal cells, resulting from dysregulation of the actin cytoskeleton, induces morphological impairments (fewer dendritic spines, reduced somal volumes, shorter dendritic arbors) to these cells (Lewis et al 2012, Lewis & Gonzalez-Burgos 2006). These *cell type-specific* alterations may also be associated with *hypofunctional NMDARs* (and/or AMPAR signaling). These molecular and morphological alterations have been hypothesized to decrease excitatory drive to DLPFC deep layer 3 circuits and render DLPFC deep layer 3 pyramidal cells in a *hypoactive* state with a reduced need for mitochondria-mediated energy synthesis (Arion et al 2015, Gonzalez-Burgos et al 2015). Consistent with this idea, transcriptome analyses of deep layer 3 and 5 pyramidal cells have revealed marked dysfunction in mitochondrial and ubiquitin-proteasome system signatures, respectively, suggesting that these cells are hypometabolic, and hence less active (Arion et al 2015). Moreover, many of these alterations were not present, or found to a lesser degree, in samples of DLPFC gray matter from the same subjects, suggesting that the pattern of pathology is *pyramidal cell-specific* (Arion et al 2015). Therefore, these observations invoke the idea that dendritic destabilization due to actin cytoskeleton impairments and a decrement in the number of spines to receive glutamate inputs, lead to mitochondrial dysfunction in DLPFC deep layer 3 pyramidal cells **(Figure 20)**.

The decrement in excitatory activity of pyramidal cells in DLPFC deep layer 3 may involve a myriad of homeostatic, *compensatory* responses to reduce feedback inhibition of these pyramidal cells to restore *excitatory-inhibitory* **(E/I)** *balance* in DLPFC microcircuits. The

stability and integrity of circuit activity is dependent on E/I balance in order to prevent runaway excitation or dying out of cortical activity. This balance is maintained through the scaling of excitatory and inhibitory strength by virtue of a process termed *homeostatic synaptic plasticity* **(HSP)** (Turrigiano 2008, Turrigiano et al 1998). A plethora of scaling factors has been implicated in recent years and the diverse HSP mechanisms involve alterations in neurotransmitter content and postsynaptic receptor number (Pozo & Goda 2010, Turrigiano 2011).

In schizophrenia, an important HSP mechanism may involve the activity-dependent downregulation of phasic excitation of PV interneurons, in response to decreased network excitatory activity due to hypoactive DLPFC deep layer 3 pyramidal cells. Consistent with this idea, reduced excitatory drive to deep layer 3 pyramidal cells leads to decreased mRNA levels of neuronal activity-regulated pentraxin **(NARP)**, an immediate early gene important in the formation of excitatory inputs onto PV (and possibly SST) interneurons, in the DLPFC in subjects with schizophrenia (Kimoto et al 2015). NARP is secreted in an activity-dependent fashion at presynaptic glutamatergic axon boutons and facilitates the clustering of GluR4-containing AMPARs on PV interneurons (Chang et al 2010, Gu et al 2013). In fact, recent studies found that in mice with genetic deletion of the NARP gene and its associated receptor, there was a marked reduction in GluR4 AMPARs on PV interneurons, resulting in decreased feedback inhibition and disruptions in hippocampal rhythms and working memory (Pelkey et al 2015). Importantly, in the schizophrenia specimens, NARP mRNA was positively correlated with GAD67 mRNA (Kimoto et al 2015). Interestingly, the GAD67 promoter contains a conserved binding site for Zif268 (Szabo et al 1996, Yanagawa et al 1997), an immediate early gene, and Zif268 activation is accompanied by increased GAD67 expression (Luo et al 2008). Levels of Zif268 are also lower in the DLPFC of subjects with schizophrenia and are positively associated with GAD67 mRNA levels in the same subjects (Kimoto et al 2014). Thus, reduced Zif268 in response to lower excitatory activity of DLPFC deep layer 3 pyramidal cells, might be a crucial factor that contributes to lower GAD67 levels, altered GABA synthesis and compromised

cognition in schizophrenia. Given the fact that GAD67 expression in PV interneurons are activity-dependent (Benson et al 1994, Lau & Murthy 2012), decreased excitatory drive to deep layer 3 pyramidal cells might lead to an activity-dependent reduction in the secretion of NARP, thereby contributing to lower excitatory drive onto PV interneurons and reduction in Zif268, and a concomitant activity-dependent downregulation in GAD67 and PV expression in schizophrenia (Curley et al 2011, Glausier et al 2014, Volk et al 2000, Volk et al 2012a).

Supporting these findings, recent postmortem studies have also found elevated mRNA levels of the μ opioid receptor in schizophrenia (Volk et al 2012b). This receptor is intriguing for a couple of reasons: 1) Subcellular localization suggests that it is present in the perisomatic compartment and axon boutons of *PV interneurons* in the hippocampus (Drake & Milner 2002, McQuiston & Saggau 2003, Stumm et al 2004, Torres-Reveron et al 2009), 2) activation of the μ opioid receptor activates G protein-coupled inwardly rectifying K^+ channels that hyperpolarize the membrane potential and dampen responsiveness to excitatory drive, suppressing GABA release (Capogna et al 1993, Glickfeld et al 2008, Wimpey & Chavkin 1991). In fact, activation of the μ opioid receptor has been shown to impair γ-frequency oscillations (Gulyas et al 2010, Whittington et al 1998). The homeostatic upregulation of the μ opioid receptor in subjects with schizophrenia is predicted to further suppress *presynaptic* GABA release into the synaptic cleft (Volk et al 2012b) and GABA neurotransmission is further decreased by lower levels of $GABA_A$ α1 receptors in pyramidal cells in the *postsynaptic* compartment (Beneyto et al 2011, Glausier & Lewis 2011).

Consistent with these observations, recent studies suggest that the driving force for Cl^- influx into pyramidal cells in deep layer 3 may be altered in schizophrenia. Although expression levels of the two transporters (N^+-K^+-Cl—co-transporter 1 **(NKCC1)** and K^+-Cl—co-transporter 2 **(KCC2)**) that primarily establish the Cl^- equilibrium potential (Kahle et al 2008) are unaltered in schizophrenia (Arion & Lewis 2011), their activity may be altered, by virtue of existing in a hyper-phosphorylated state. Concordant with this interpretation, the expression levels of two

kinases, oxidative stress response kinase **(OXSR1)** and with no K (lysine) protein kinase **(WNK3)** is elevated in subjects with schizophrenia which is expected to decrease the activity of KCC2 and increase the activity of NKCC1, thereby increasing Cl^- levels intracellularly in layer 3 pyramidal cells (Arion & Lewis 2011). As a result, activation of $GABA_AR$s by GABA is expected to produce less influx of Cl^-, diminishing the hyperpolarizing nature of GABA signaling.

In concert, an array of pre- and postsynaptic alterations has been observed in GABA interneurons and these alterations are most pronounced in PV interneurons in DLPFC deep layer 3, the same laminar location where the molecular and morphological aberrations in pyramidal cells are most prominent. Taken together, the most parsimonious interpretation for the constellation of findings related to GABA neurotransmission alterations in schizophrenia, suggest *reduced feedback inhibition* of postsynaptic pyramidal cells, in response to *reduced excitatory drive* to DLPFC deep layer 3 pyramidal cells **(Figure 20)**, although other interpretations are plausible (discussed in section **5.3**). Although the compensatory changes in GABA interneurons restores E/I balance at a new level, the diminished strength of excitation and inhibition is at an insufficient level to synthesize γ-frequency oscillations (30-80 Hz) to properly mediate cognitive control required for working memory function (Lewis et al 2012).

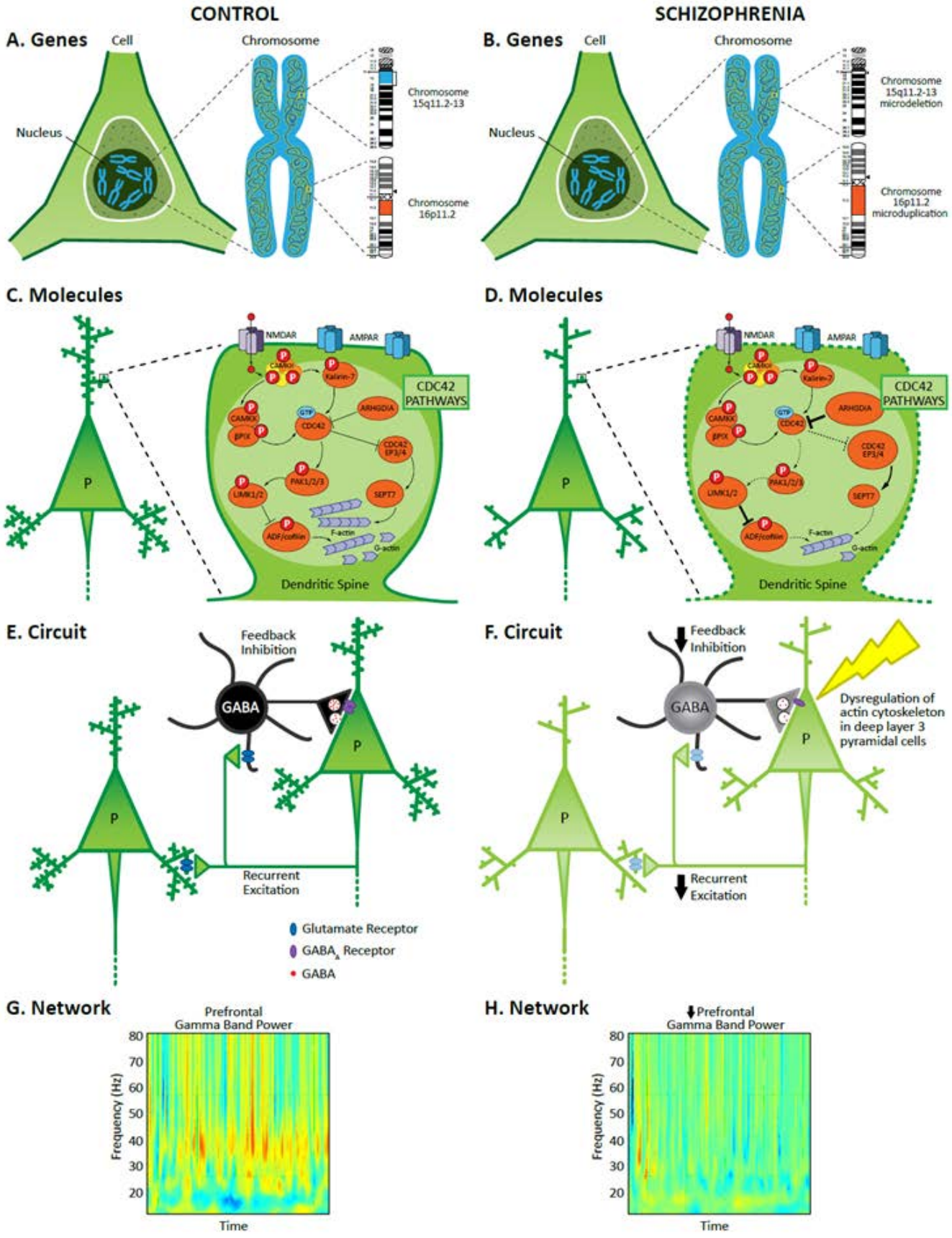
CONTROL
SCHIZOPHRENIA
A. Genes
Cell
Chromosome
Nucleus
Chromosome 15q11.2-13
Chromosome 16p11.2
B. Genes
Cell
Chromosome
Nucleus
Chromosome 15q11.2-13 microdeletion
Chromosome 16p11.2 microduplication
C. Molecules
D. Molecules
NMDAR
AMPAR
CDC42 PATHWAYS
Dendritic Spine
E. Circuit
F. Circuit
Feedback Inhibition
GABA
P
Recurrent Excitation
Glutamate Receptor
GABA$_A$ Receptor
GABA
Dysregulation of actin cytoskeleton in deep layer 3 pyramidal cells
G. Network
H. Network
Prefrontal Gamma Band Power
Frequency (Hz)
Time

Figure 20. Altered E/I balance in schizophrenia.

(A and B) Schematic showing copy number variations at risk alleles 15q11.2-13 (chromosome microdeletion) and 16p11.2 (chromosome microduplication), which implicate dysregulation of actin filament dynamics in the pathogenesis of schizophrenia. **(C and D)** Molecular alterations and predicted functional consequences of altered CDC42 signaling pathway components (CDC42-CDC42EP and CDC42-PAK-LIMK) in DLPFC deep layer 3 pyramidal cells in schizophrenia (see **Figure 5** in **Chapter 2**). The synergistic alterations in CDC42 signaling pathways is predicted to destabilize actin dynamics and produce spine deficits preferentially in DLPFC deep layer 3 pyramidal cells in schizophrenia. The size of molecules in schizophrenia relative to control subjects, reflect the pattern of changes observed in the illness. Arrows indicate activation, and blunted lines indicate inhibition of each target. **(E and F)** Schematic summarizing the organization of the DLPFC microcircuit between deep layer 3 pyramidal cells and GABA interneurons. Deep layer 3 pyramidal cells (green) are reciprocally connected to other pyramidal cells by local axon collaterals to mediate recurrent excitation and GABA (black) interneurons (primarily PV interneurons) to generate feedback inhibition. The integrity of these connections maintains normal γ-frequency oscillations (30-80 Hz) and E/I balance in healthy subjects. In schizophrenia, molecular alterations such as dysregulation of the actin cytoskeleton in deep layer 3 pyramidal cells is predicted to induce morphological perturbations such as lower dendritic spine density and somal volume. These alterations have been hypothesized to lower excitatory drive in the network and decrease the need for ATP production, resulting in a hypoactive circuit (light green). A primary upstream deficit in deep layer 3 pyramidal cells induces compensatory mechanisms [less presynaptic glutamatic acid decarboxylase (GAD67); lower number of postsynaptic $GABA_A$Rs] to reduce feedback inhibition from local GABA interneurons (light grey). Adapted from Lewis et al (2012) **(G and H)** In concert, the changes at the gene, molecular and circuit level, result in a reset E/I balance in schizophrenia with diminished excitation and inhibition, precluding normal circuit operations required for the generation of network oscillations such as γ-frequency oscillations, thereby producing cognitive impairments. Heat maps are reproduced from Cho et al (2006).

5.2.2 Reconciling DLPFC circuit changes with system-level alterations in schizophrenia

One of the functional consequences of reduced excitatory drive in DLPFC deep layer 3 might involve deficient excitatory output from the DLPFC. In addition to alterations in glutamate and GABA neurotransmission, alterations in dopamine neurotransmission have been well documented in schizophrenia. Traditionally, psychosis-related phenotypes were thought to be a consequence of *excessive subcortical dopamine* and the effectiveness of antipsychotic drugs is correlated to their inhibition of dopamine D2 receptors. However, a few studies have postulated that cognitive control impairments related to working memory might be associated with *reduced*

dopaminergic drive to the PFC (Davis et al 1991, Weinberger 1987). Elegant studies first conducted by Goldman-Rakic and later examined by other groups, demonstrated the importance of dopamine neurotransmission in regulating cognitive control (Sawaguchi 2001, Sawaguchi & Goldman-Rakic 1994), and in particular, in increasing the excitability of pyramidal cell ensembles in the DLPFC in layer 3, which might be important for sustaining the activity of DLPFC neuron activity during the delay-period of working memory tasks (Gonzalez-Burgos et al 2002, Henze et al 2000, Urban et al 2002). Indeed, regional depletion of dopamine in the prefrontal cortex of monkeys produces cognitive deficits, suggesting that dopamine neurotransmission in the DLPFC is critical for cognitive function (Brozoski et al 1979). The postsynaptic compartment of dopamine signaling is mediated primarily through D1 receptors **(D1Rs)** in the DLPFC during working memory tasks and pharmacological antagonism of D1Rs impairs delay-period activity (Sawaguchi & Goldman-Rakic 1991, Williams & Goldman-Rakic 1995). In essence, dopamine stimulation of D1Rs exhibits an inverted U-shaped influence on working memory, with blockade and excessive activity impairing spatial working memory, and optimal stimulation enhancing spatial tuning by suppressing delay-related firing in the cell's non-preferred orientation (Sawaguchi & Goldman-Rakic 1991, Vijayraghavan et al 2007, Zahrt et al 1997). Studies in schizophrenia suggest a *decrement in dopamine inputs* in the DLPFC with a concomitant compensatory *upregulation of D1 dopamine receptors* revealed by *in vivo* imaging studies (Abi-Dargham et al 2002, Akil et al 1999).

A current theory has proposed that hypoactive pyramidal cells and reduced excitatory drive to deep layer 3 pyramidal cells in schizophrenia, could explain a *hypodopaminergic* phenotype in the cortex and a *hyperdopaminergic* phenotype in subcortical areas **(Figure 21)** (Lewis & Gonzalez-Burgos 2006). This is because tracing studies using high-resolution electron microscopy in rats have shown that PFC pyramidal cells innervate dopamine cells in the ventral tegmental area in the ventral mesencephalon, which furnish reciprocal projections back to the PFC and inhibit, through GABA interneurons, dopamine cells that project to the nucleus

accumbens in the striatum (Carr & Sesack 2000a, Carr & Sesack 2000b). If this circuit is conserved across species, a plausible hypothesis in schizophrenia is that *reduced excitatory output* of pyramidal cells in the cortex can produce *decreased* dopamine activity in the cortex through mesocortical loops and *increased* dopamine activity in the striatum through mesostriatal loops **(Figure 21)**. Consistent with this hypothesis, recent *in vivo* studies in mice involving *functional loss of the actin cytoskeleton* in excitatory pyramidal cells of the forebrain recapitulated synaptic and behavioral phenotypes reminiscent of schizophrenia (Kim et al 2013, Kim et al 2015). Using a combination of optogenetics, viral anterograde and retrograde tract tracing, whole-cell patch clamp recording, the recent study involved conditional deletion of the ARP2/3 complex, and demonstrated spine loss and abnormal synaptic contacts in forebrain pyramidal cells (Kim et al 2015). This alteration resulted in increased stimulation of dopamine neurons in the ventral tegmental area and substantia nigra pars compacta **(VTA/SNc)**, thereby producing elevated striatal dopamine and antipsychotic-responsive psychomotor disturbances in conjunction with behavioral abnormalities such as cognitive deficiencies, sociability deficits, reduced sensorimotor gating and locomotor hyperactivity (Kim et al 2015). Moreover, the study suggests that the increased dopamine subcortically maybe a *consequence* of a proximal alteration in pyramidal neurons in the PFC, since viral re-expression of the ARP2/3 complex in frontal cortical neurons lowered striatal dopamine and reduced hyperlocomotor activity (Kim et al 2015). In this context, the mechanistic manipulation has face validity since administration of antipsychotic drugs such as haloperidol and clozapine rescued the behavioral deficits, lending credence to the idea that *actin cytoskeleton perturbations* maybe the *primary deficit* in the pathogenesis of schizophrenia **(Figure 21)**. This elegant proof-of-principle study raises several additional questions that need to be delineated: Can mouse models that produce aberrant actin cytoskeleton dynamics in PFC pyramidal cells recapitulate *compensatory* pre- and postsynaptic *alterations in GABA neurotransmission*, similar to those seen in schizophrenia? How do these

mechanistic alterations relate to other brain regions that are disrupted in schizophrenia such as the hippocampus?

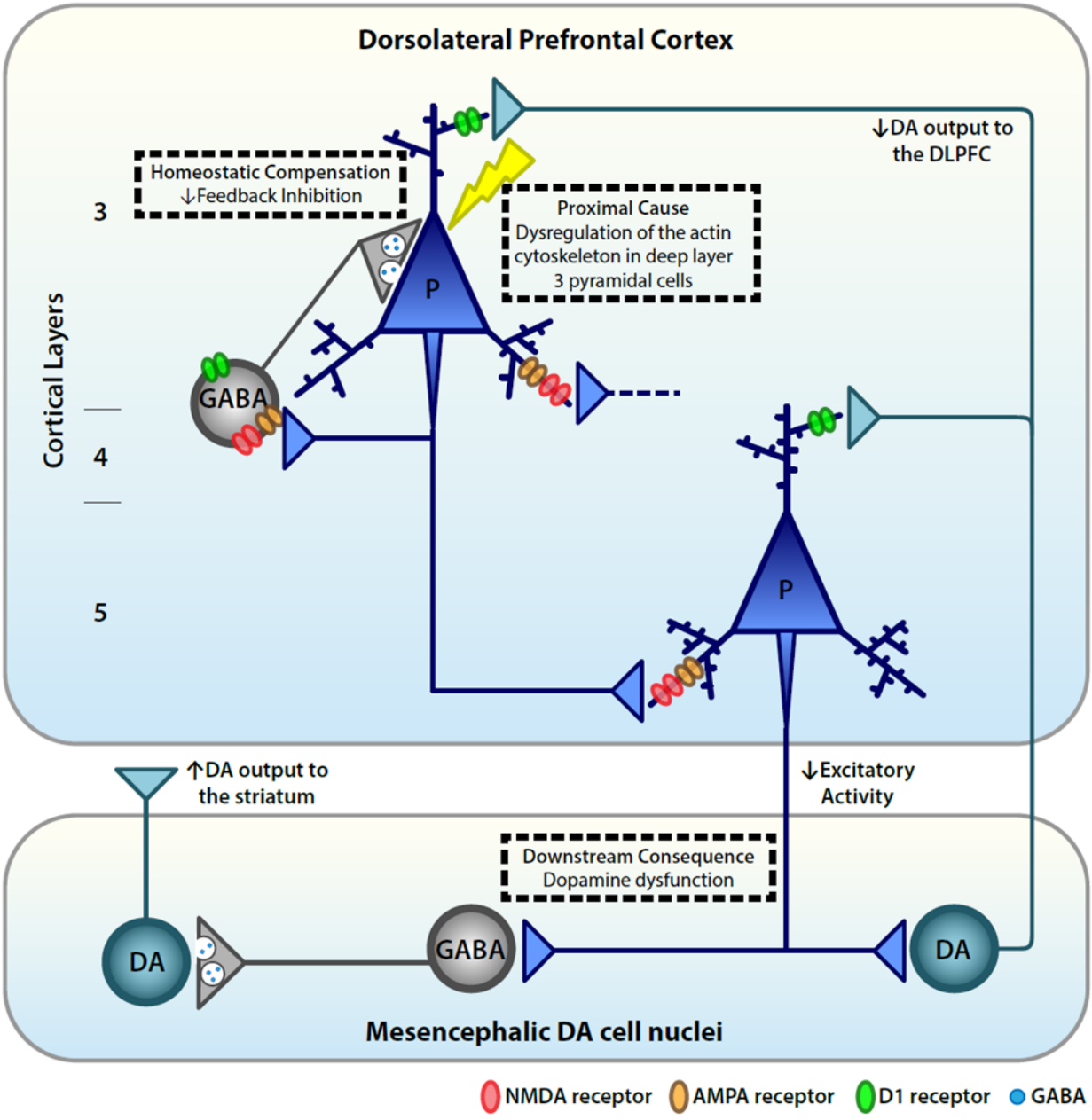

Figure 21. Schematic depicting local-circuit and systems-level abnormalities in schizophrenia.

Dysregulation of the actin cytoskeleton in DLPFC deep layer 3 pyramidal cells and morphological impairments in dendritic spines is predicted to *decrease* excitatory drive to DLPFC deep layer 3 cortical circuits. The decrease in excitation is balanced by homeostatic *compensatory* mechanisms to *decrease* feedback inhibition by GABA interneurons, in order to restore E/I balance in DLPFC microcircuits. Hypofunctional DLPFC pyramidal cells in deep layer 3 in schizophrenia lead to decreased output from the DLPFC layer 5 pyramidal cells, and

dysfunction of dopamine neurons located in the mesencephalon as a downstream *consequence*. Alterations in dopamine neurotransmission in schizophrenia include *decreased* activity of mesencephalic dopamine neurons projecting back to the cortex and *increased* activity, through disinhibition, of dopamine neurons projecting to the striatum. A hypodopaminergic phenotype in the cortex produces a compensatory, but functionally insufficient, upregulation of D1 receptors. Abbreviations: P, Pyramidal cells; GABA, γ-Aminobutyric acid interneurons; DA, Dopamine cells. Figure adapted from Lewis and Gonzalez-Burgos (2006).

5.3 IS THE PROXIMAL DEFICIT IN SCHIZOPHRENIA IN GABA INTERNEURONS?

An alternative hypothesis in the field posits that a *proximal deficit in GABA interneurons* might be an upstream event in the disease pathogenesis (Lisman et al 2008, Nakazawa et al 2012). A myriad of cellular, molecular and structural alterations have been extensively characterized in GABAergic circuits in subjects with schizophrenia (reviewed in section **1.3.6**, **Figure 4**). Particularly, extensive research has been focused on fast-spiking PV interneurons since these cells are critical in regulating the activity of pyramidal cells in layer 3 microcircuits and entrainment of DLPFC neural networks at γ-frequency oscillations (30-80 Hz) required for working memory (Cardin et al 2009, Sohal et al 2009). As a result, reduced GABA signaling from PV interneurons might contribute to the pathophysiology of lower prefrontal γ-frequency oscillation power during working memory tasks (Cho et al 2006, Howard et al 2003) and cognitive dysfunction in schizophrenia (Kahn & Keefe 2013). A number of sophisticated studies over the last decade have advocated different mechanisms by which PV interneurons might be dysfunctional in schizophrenia. The following sections will briefly discuss the various mechanisms by which this could occur.

5.3.1 Genetic liability for PV interneuron dysfunction

The decrement in GAD67 mRNA observed in schizophrenia might be due to causes that regulate the *GAD1* gene. Allelic variants in the GAD1 gene and epigenetic mechanisms involving chromatin structure perturbations have been associated with increased risk for schizophrenia and lower levels of GAD67 (Huang et al 2007, Straub et al 2007), suggesting that GAD67 changes may be developmentally controlled during the prenatal period. For example, using 3D genome architectures, it has been observed that the *GAD1* gene contains promoter-enhancer chromosomal loops that allow various distal regulatory elements to control GAD67 transcription in an activity-dependent manner. In the prefrontal cortex in subjects with schizophrenia, this loop was disturbed, providing a plausible explanation for activity-dependent downregulation of GAD67 in the illness (Bharadwaj et al 2013). Consistent with these findings, increased numbers of GABA interneurons expressing the DNA methyltransferase 1 **(DNMT1)** mRNA with a concomitant downregulation of GAD67 and reelin mRNAs have been observed in the PFC in schizophrenia subjects, suggesting that hyper-methylation of gene promoters might lead to gene expression changes observed in the illness (Ruzicka et al 2007). In fact, the upregulation of DNMTs that are highly expressed in GABA interneurons might lead to gene silencing of various other GABA-related markers by interacting with CpG binding repressor proteins and histone deacetylases **(HDACs)** (Grayson & Guidotti 2013). Interestingly, a recent large-scale genomic study found enrichment for CNV's related to GABA neurotransmission, although this was restricted to the $GABA_AR$ signaling complex, while replicating and extending the CNV enrichment for genes involved in glutamate neurotransmission in schizophrenia (Pocklington et al 2015).

Decreased signaling through putative risk genes related to signal transduction pathways such as neuregulin 1 and ErbB4, which affect NMDAR signaling, have also been implicated in the pathogenesis of schizophrenia (Lewis & Moghaddam 2006). Recent interrogation of this

pathway, by conditional deletion of ErbB4 in PV interneurons, a tyrosine kinase receptor, has demonstrated cellular, physiological and behavioral deficits suggestive of schizophrenia (Allen et al 2008, Fazzari et al 2010, Neddens et al 2011, Sullivan et al 2012). These deficits included pre- and postsynaptic alterations in PV interneurons, decreased spine density of pyramidal cells and altered γ-frequency oscillations (Del Pino et al 2013, Yin et al 2013b). Although these studies provide an interesting mechanistic link to PV interneuron dysfunction in schizophrenia, caution must be used in extrapolating too much from these findings because total ErbB4 mRNA levels appear to be unaltered in schizophrenia, but the expression levels of two minor splice variants are significantly altered (Chung et al 2015, Law et al 2007). However, dysregulated splicing of ErbB4 variants might function as dominant negatives in relation to ErbB4 signal transduction, therefore altered ErbB4 splice variant expression might be simulated by deletion of ErbB4 in rodent models.

5.3.2 NMDAR hypofunction in PV interneurons

Although the exact causes of PV interneuron dysfunction in schizophrenia is still under scrutiny, a widely popular hypothesis posits that hypofunction of NMDAR-mediated signaling specifically within PV interneurons, might be a potential primary pathological mechanism in the disease process (Lisman et al 2008, Nakazawa et al 2012). Consistent with this idea, initial results showed that administration of NMDAR antagonists such as phencyclidine and ketamine to healthy adult subjects produced behavioral changes reminiscent of positive, negative and cognitive symptoms of schizophrenia (Javitt & Zukin 1991, Krystal et al 1994, Lahti et al 1995). Follow up studies involving *in vivo* electrophysiology in the rat PFC revealed that systemic administration of NMDAR antagonist decreased the activity of putative GABA interneurons, which at a delayed rate, increased the firing rate of pyramidal cells (Homayoun & Moghaddam 2007). Importantly, these findings have been interpreted under the premise that PV cell

activation is more sensitive to NMDAR antagonists than pyramidal cells for their activation, by virtue of greater contribution of NMDARs at glutamate synapses onto PV interneurons, than pyramidal cells. As a result, these findings led to the speculation that disinhibition of pyramidal cells was mediated by preferential NMDAR hypofunction in PV GABA interneurons (Homayoun & Moghaddam 2007, Lisman et al 2008, Seamans 2008). Indeed, NMDAR antagonists in rodent studies produced a marked reduction of PV and GAD67 expression in PV interneurons similar to that seen in subjects with schizophrenia (Behrens et al 2007, Cochran et al 2003, Kinney et al 2006). Moreover, conditional knockout of the GluN1 (NR1) subunit during *early* postnatal development in a majority of cortical and hippocampal GABA interneurons, presumably mostly PV interneurons, produced similar downregulation of PV and GAD67 protein levels (Belforte et al 2010). In fact, specific knockout of GluN1 subunit of the NMDAR in PV interneurons generates disruptions in cortical rhythms such as γ-frequency oscillations (Carlen et al 2012, Korotkova et al 2010) that are also perturbed in subjects with schizophrenia (Cho et al 2006).

Despite these findings, there are certain fundamental challenges with the *NMDAR hypofunction on PV interneuron* hypothesis in schizophrenia: First, the relative contribution of NMDAR to EPSCs in fast-spiking interneurons compared with pyramidal cells is significantly *smaller* in several cortical regions including the PFC (Gittis et al 2010, Hull et al 2009, Lamsa et al 2007, Lu et al 2007, Rotaru et al 2011). In fact, the contribution of AMPAR to EPSCs in fast-spiking interneurons was significantly *larger* than in pyramidal cells due to the contribution of GluA2-lacking rapidly deactivating AMPARs (Hull et al 2009, Rotaru et al 2011, Wang & Gao 2010). The greater contribution of AMPARs to EPSCs in fast-spiking interneurons might prime these cells for fast synaptic activation in response to pyramidal cell activity for rapid depolarization and repolarization required for precise high-frequency firing, favoring the integration of coincident inputs necessary for the generation of γ-frequency oscillations (Galarreta & Hestrin 2001, Hu et al 2010). In addition, computational network models comparing fast AMPAR-mediated vs. slow NMDAR-mediated excitation of fast-spiking interneurons

suggest that AMPAR-mediated fast-spiking interneuron excitation is sufficient to support γ-frequency oscillations (Rotaru et al 2011). Supporting these findings, GluA1- or GluA4-KO in PV interneurons in mice had a marked decrement in spiking, reduced temporal precision and lower power of γ-frequency oscillations by preventing rapid coupling of excitation with inhibitory output (Fuchs et al 2007). In fact, NMDAR antagonists enhance rather than decrease the power of γ-frequency oscillations in rodent and human models (Hakami et al 2009, Hong et al 2010). Second, conditional deletion of GluN1 subunit expression in PV interneurons in *adult* animals produces marginal changes in glutamate neurotransmission and no change in PV and GAD67 protein levels, consistent with the evidence that in adult mice NMDARs provide a minor contribution to PV interneuron EPSCs (Belforte et al 2010). Third, serial electron microscopy studies using quantitative postembedding immunogold techniques analyzing synapses on interneuron dendrites and pyramidal cell spines have found a significantly lower density of GluN1-containing NMDARs onto PV interneurons compared to pyramidal cells (Nyiri et al 2003). Similarly, in the adult human cortex, ~70% of PV interneurons lack detectable levels of NMDAR subunit mRNAs (Bitanihirwe et al 2009). Fourth, systemic NMDAR antagonist application to awake behaving monkeys does not produce disinhibition (Wang et al 2013). Moreover, repeated exposure to NMDAR antagonist dizocilpine maleate (MK-801) in rats during adulthood does not produce disinhibition of cortical circuits assessed by analyzing local field potentials (Thomases et al 2013). Fifth, the disinhibition produced as a result of NMDAR hypofunction in PV interneurons is predicted to result in a hyper-excitable circuit, however substantial evidence points to a *decrease*, rather than an *increase* in the activity of the DLPFC. In fact, a recent meta-analysis of working memory in schizophrenia has “converged on *hypoactivation* of the dorsolateral prefrontal cortex as the most common finding” (Kern et al 2013), which is in striking contrast to the notion of disinhibited cortical circuits (Lisman et al 2008, Nakazawa et al 2012). In sum, these studies suggest that NMDAR hypofunction on PV interneurons as a proximal primary deficit and cortical disinhibition seem implausible and the effect of NMDAR antagonists

is unlikely to be mediated by NMDARs at glutamate synapses in mature cortical PV interneurons.

5.3.3 Role of other mechanisms underlying reduced GABA neurotransmission

Intrinsic abnormalities in interneurons may arise from alterations in gene products that are specifically expressed in these cells. Interneurons are known to depend on the expression of voltage-gated potassium channels for the accurate recognition of coincident excitatory synaptic inputs that may be critical for the synchronization of cortical networks. Intriguingly, PV interneurons exclusively express the Kv9.3 voltage-gated K^+ channel modulatory α subunit, encoded by the KCNS3 gene (Georgiev et al 2012). These Kv9.3 subunits form heteromeric channels with Kv2.1 α subunits, encoded by the KCNB1 gene (Kerschensteiner & Stocker 1999, Patel et al 1997). In comparison to homomeric Kv2.1 channels, heteromeric Kv2.1/Kv.9.3 channels have electrophysiological properties that are particularly suited for coincident input detection such as fast synaptic activation, slower deactivation, accelerated closed-state inactivation and a maximum of cumulative inactivation shifted towards higher frequencies (Kerschensteiner & Stocker 1999, Patel et al 1997). Recent studies using *in* situ hybridization have reported mRNA reductions in both KCNS3 and KCNB1, in the DLPFC of subjects with schizophrenia, signifying a decrement in heteromeric Kv2.1/Kv9.3 channels in PV interneurons (Georgiev et al 2014, Georgiev et al 2012). Furthermore, microarray analysis revealed 40% lower levels of KCNS3 mRNA specifically in PV interneurons captured by laser microdissection, further validating dysregulation of PV interneurons in schizophrenia (Georgiev et al 2014). Consistent with these results, another report suggests significant reductions in the mRNA and protein levels of Kv3.1b, the predominant splice variant of the Kv3.1 channel, which is involved in the repolarization of action potentials in neurons and contributes to the fast-spiking phenotype of PV interneurons (Yanagi et al 2014). In addition, murine models with disruptions in Kv3.1 and

Kv3.2 channels in fast-spiking PV interneurons exhibit alterations in firing patterns and synchrony for network oscillations (Joho et al 1999, Lau et al 2000). Genetic studies in schizophrenia have also revealed a SNP associated with KCNH2 gene, which encodes for another voltage-dependent K^+ channel Kv11.1, specifically expressed in the primate brain (Huffaker et al 2009). As a result, reduction in the complement of Kv2.1/Kv9.3 and Kv3.1 channels is expected to slow the time course of EPSPs in PV interneurons, impairing the ability of these cells to respond to temporally convergent excitatory inputs from DLPFC deep layer 3 pyramidal cells. This is turn, will preclude the summation of EPSPs within narrow time windows required for synchronizing neural ensembles of pyramidal cells in the range of γ-frequency oscillations.

Other current studies have established a connection between alterations in structural components of PV interneurons. PV cells are ensheathed by complex extracellular structures called perineuronal nets **(PNNs)** which consist of chondroitin sulfate proteoglycans and extracellular matrix components and these components are generated in an activity-dependent fashion (Dityatev et al 2007, Kwok et al 2011). PNNs act as cation sinks which promote the fast-spiking phenotype of PV interneurons and are also implicated in establishing the opening and closing of critical periods during development to regulate synaptic plasticity (Morishita et al 2015). Using *Wisteria floribunda* agglutinin which binds to the carbohydrate component of PNNs, the density of PNNs was reduced in the PFC in subjects with schizophrenia (Mauney et al 2013). In experimental rodent models, these PNN structures are also protective against oxidative stress (Cabungcal et al 2013), which might be crucial since redox dysregulation and oxidative stress has been implicated in the pathogenesis of schizophrenia (Do et al 2009). Consistent with reduced levels of cortical glutathione, an antioxidant, in subjects with schizophrenia (Gawryluk et al 2011), these studies provide a strong mechanistic basis for PV interneuron dysfunction involving PNN alterations.

Finally, deficits in GABA neurotransmission in schizophrenia might be a manifestation of impairments in GABA neuron ontogeny during gestation, which precludes normal phenotype specification and development. In contrast to pyramidal cells that undergo radial migration from the ventricular zone of the pallium during differentiation and maturation (Nadarajah et al 2001, Rakic 1995), GABA interneurons undergo tangential migration from the ventricular zone of the subpallium (Marin & Rubenstein 2001). The maturation of cortical neurons is adjusted by highly intricate extracellular signaling systems (e.g., netrin/DCC, Slit/Robo, Wnt3a) and transcription factor cascades (e.g., Pax6, Nkx2.1, Sox6, Lhx6, Dlx5/6, and Gsx1/2) that dictate fate specification (Hebert & Fishell 2008, Marin & Rubenstein 2001). Recent studies in postmortem tissue in the DLPFC in subjects with schizophrenia have found robust decreases in Lhx6 (Volk et al 2012a), which is necessary for the tangential migration of PV and SST interneurons, and mRNA levels of Lhx6 and GAD67 were correlated in the same subjects (Volk et al 2014). In fact, rodent studies using heterozygous Dlx5/6 mice, homeobox transcription factors that are critical for the maturation of PV interneurons, exhibit abnormal PV interneuron-specific intrinsic electrophysiological properties, decreased task-evoked γ-frequency oscillations, and compromised PFC-dependent cognitive flexibility following adolescence (Cho et al 2015a). These findings also demonstrate that stimulation at γ-frequency oscillations can restore cognitive flexibility in adult Dlx5/6$^{+/-}$ mice, proposing a potential causal link between disrupted ontogenetic factors and GABA interneuron dysfunction (Cho et al 2015a).

Ultimately, the determination of which hypothesis provides the most persuasive explanation of pathophysiological relationships (e.g., alterations in γ-frequency oscillations) in the disease process requires mechanistic tests in tractable paradigms to evaluate cause-consequence-compensation relationships. Given the heterogeneity of symptomatology in schizophrenia, it is also conceivable that a certain hypothesis (e.g., cell-autonomous intrinsic abnormality in DLPFC deep layer 3 pyramidal cells) may be more applicable to certain subpopulations of patients, whereas an alternative hypothesis (e.g., primary deficit in GABA

interneurons) may be more relevant to others. It is also imperative to understand how these hypotheses may shed light on developmental processes to see how they align with post-adolescence onset of schizophrenia endophenotypes.

5.4 DISSECTING THE MATURATION OF CORTICAL CIRCUITS THAT SUBSERVE WORKING MEMORY FUNCTION IN THE PRIMATE DLPFC

A thorough understanding of normal brain maturation is critical for advancing our knowledge of neurodevelopmental disorders such as schizophrenia. Due to technical limitations, direct probing of the normal postnatal refinements of neural cortical circuits in humans in a cell-specific manner is constrained. As a result, among the possible nonhuman primate models, the rhesus macaque (*Macaca mulatta*) has been extensively used as an experimental animal model to interrogate the fundamental neural mechanisms occurring throughout postnatal development. Moreover, due to their phylogenetic closeness to the human species, rhesus macaques provide an ideal species to investigate more complex cognitive functions and social behaviors. In comparison to rodents, rhesus macaques have an expanded PFC that continues to mature in a protracted fashion during postnatal development, similar to the human PFC (Nelson & Winslow 2009). In addition, primates and rodents have marked differences in the gene composition of neural progenitor cells, anatomical characteristics and physiological properties of circuit assembly (Molnar & Clowry 2012, Rakic 2009).

An important aspect of understanding the refinements in cortical circuits is to contextualize it in the framework for what the developmental trajectories of these circuits may tell us about vulnerabilities for the emergence of the various symptoms, cognition in particular, in schizophrenia. Over the last 3 decades, schizophrenia has been conceptualized as a disorder

of PFC cortical development and various hypotheses have been proposed to integrate aberrant developmental processes with the manifestation of clinical symptoms (Feinberg 1982, Keshavan et al 1994, Murray & Lewis 1987, Weinberger 1987). More recent theories have advocated that schizophrenia may be perceived as a “cognitive brain network disorder” with disruptions in integration across functionally similar brain regions, which might occur early in brain development (Dauvermann et al 2014). The sculpting of normative connectivity patterns during postnatal development is orchestrated by a myriad of intrinsic programming mechanisms that are temporally controlled by various guidance cues, signaling molecules, and activity-dependent pruning processes (Schubert et al 2015). In fact, recent genomic studies provide the first evidence that gene networks that undergo *de novo* mutations in schizophrenia are highly co-expressed in the fetal prefrontal cortex and functionally participate in migration, transcriptional regulation, *signaling* and *synaptic transmission* during development (Gulsuner et al 2013). Given the fact that PFC circuits involve sensitive periods of increased vulnerability for the pathogenesis of the disease, such that specific elements of the circuitry are selectively vulnerable to environmental insults during specific epochs in postnatal development (Hoftman & Lewis 2011), even a slight perturbation to the delicate balance of the system might result in deleterious consequences and contribute to neurodevelopmental disorders such as schizophrenia. In the following section, I will contrast the maturation of GABA and glutamate neurotransmission during postnatal development in the monkey DLPFC and based on the inflection points of GABA and glutamate postsynaptic markers, I will shed light on what components of DLPFC circuitry may be particularly prone to environmental insults during specific sensitive periods.

5.4.1 Molecular postsynaptic features of GABA neurotransmission undergo protracted developmental changes

In DLPFC area 9 and 46, using single-cell laser capture microdissection, in conjunction with qPCR gene expression profiling, we provide evidence showing that the expression of molecular postsynaptic determinants of GABA neurotransmission in pyramidal cells changes in a *subunit*- and *layer-specific* fashion during postnatal development. For example, in layer 3 pyramidal cells in the monkey DLPFC, the expression of the $GABA_A$ α1 subunit progressively increases from the perinatal period to adulthood, which is paralleled by a decline in expression of the $GABA_A$ α2 subunit during the same developmental period, in the same cells. In contrast, in DLPFC layer 5 pyramidal cells, we observed a similar, yet modest, increase in the expression of the $GABA_A$ α1 subunit, but no change in expression for the $GABA_A$ α2 subunit during postnatal development. The developmental trajectories of other GABA-related transcripts critical in mediating GABA neurotransmission (e.g., $GABA_A$ α5, $GABA_A$ β2, $GABA_A$ γ2, $GABA_A$ δ) in pyramidal cells in the DLPFC have similar dynamic and complex patterns of change. The developmental trajectories for these transcripts are protracted, extending from birth through adolescence, and show different patterns of change depending on the laminar-location of the pyramidal cell.

The developmental shifts in expression of $GABA_A$ receptor subunits may be particularly relevant for pyramidal cells in layer 3 that are part of the microcircuit that is crucial for the generation of γ-frequency oscillations. The progressive shift in the subunit composition to $GABA_A$ α1 subunit-containing receptors is predicted to result in faster inhibitory decay kinetics, which is particularly important since γ-frequency oscillations depend critically on the decay time course for inhibition (Bartos et al 2007, Buzsaki & Wang 2012). Consistent with these findings, recent studies investigating the functional development of GABA synapses using

electrophysiology in *in vitro* living slice preparations in monkey DLPFC layer 3 pyramidal cells during postnatal development, found that the decay of $GABA_AR$-mediated inhibitory postsynaptic currents **($GABA_AR$-IPSCs)** decreased while the amplitude of $GABA_AR$-IPSCs increased until the peripubertal period (Gonzalez-Burgos et al 2014). In particular, this progressive shift towards *faster* and *stronger* inhibitory currents may be necessary for fast-spiking PV interneurons to temporally synchronize pyramidal cell ensembles through feedback inhibition, thereby providing the functional capacity to support γ-frequency oscillations (Bartos et al 2007, Buzsaki & Wang 2012). In fact, in rodent cortical and hippocampal pyramidal cells, a similar decrease in $GABA_AR$-IPSCs has been attributed to a developmental upregulation of $GABA_A$ α1 subunit-containing receptors, although the maturation of circuits in primates occurs in a prolonged fashion compared to rodents (Bosman et al 2002, Cohen et al 2000, Pinto et al 2010). Moreover, simulations in computational network models predicted that mature levels of γ-band power are attained at late stages of development in the monkey DLPFC (Gonzalez-Burgos et al 2014). Therefore, both the *molecular determinants of GABA neurotransmission* and *functional inhibitory synaptic properties* of DLPFC layer 3 pyramidal cells, continue to progressively develop in a *protracted* manner during circuit maturation.

The molecular and functional maturation of GABA synapses during postnatal development may have significant ramifications for emergent behaviors that depend on the circuitry of the DLPFC in primates, such as working memory. Behavioral performance involving working memory tasks first appears around 2-3 years of age and continues to improve during postnatal development and involves increased recruitment of activity in the DLPFC (Alexander 1982, Alexander & Goldman 1978). Individuals who later go on to develop schizophrenia, fail to show this normal age-related improvement in working memory in late childhood and during adolescence, roughly between 7-15 years (Reichenberg et al 2010). Moreover, working memory load is proportional to PFC γ-frequency oscillations, in particular power of γ-frequency oscillations (Howard et al 2003). Subjects with schizophrenia fail to show bilateral increase in

the power of γ-frequency oscillations during working memory tasks (Cho et al 2006, Minzenberg et al 2010). Therefore, it is clear that the capacity for working memory is increasing during postnatal development, and working memory is dependent on γ-frequency oscillations. These findings highlight a critical question: How is the maturation of γ-frequency oscillations changing during postnatal development? Recent studies suggest that the power of 40 Hz auditory steady-state responses progressively increases with age, reaching a peak during adolescence (Cho et al 2015b). In addition, γ-frequency oscillation synchrony using a Gestalt perception task in parietal regions, increased in a monotonic fashion from childhood to adulthood (Uhlhaas et al 2009, Uhlhaas & Singer 2006, Uhlhaas & Singer 2010). Consistent with these findings, fMRI studies using blood oxygenation level-dependent **(BOLD)** signal which correlates positively with γ-frequency oscillation activity (Magri et al 2012, Niessing et al 2005), have observed that working memory performance and DLPFC activity continue to develop through late adolescence (Luna & Sweeney 2004, Scherf et al 2006). However, the development of functional oscillatory activity in the DLPFC has not been directly explored and remains an important question for future experiments.

In aggregate, the findings presented in this dissertation might provide an important molecular substrate for the protracted maturation of GABA synapses required for sustaining γ-frequency oscillations and the emergent property of working memory function during postnatal development. Since numerous environmental exposures from birth through adolescence have been associated with increased risk for schizophrenia (Hoftman & Lewis 2011, van Os et al 2010), the trajectories for different GABA transcripts may be susceptible to the influence of unique environmental exposures which operate during specific sensitive periods, to affect specific components of cortical circuits. This might explain why the cumulative effect of multiple environmental insults (e.g., cannabis exposure, stress) can exacerbate the risk for the development of schizophrenia, since different environmental insults affect different components of neuronal circuits that are rapidly changing during postnatal maturation.

5.4.2 Molecular postsynaptic features of glutamate neurotransmission remain static

In contrast to the dynamic and complex developmental trajectories of the molecular determinants of GABA neurotransmission, the postsynaptic molecular determinants of glutamate neurotransmission (AMPA GluA1 and NMDA GluN1, encoded by AMPA Glur1 and NMDA Grin1, respectively) revealed an intriguing pattern in pyramidal cells across laminar location. The expression level of the NMDA GluN1 subunit was consistently higher in layer 3 pyramidal cells compared to layer 5 pyramidal cells, although the developmental trajectory is relatively stable and achieve adult levels of expression early during the perinatal period **(Chapter 4)**. In contrast, the expression of the AMPA GluR1 subunit was consistently higher in layer 5 pyramidal cells compared to layer 3 pyramidal cells **(Chapter 4)**. Together, the NMDA GluN1/AMPA GluA1 subunit ratio was at least >2-fold higher in layer 3 pyramidal cells compared to layer 5 pyramidal cells in the DLPFC of monkeys throughout postnatal development, at every age group. The mean NMDA GluN1/AMPA GluA1 subunit mRNA ratio increased by 67% from the perinatal (3 animals 1 week of age) to peripubertal monkeys (3 animals ~32 months of age). Analysis by age group revealed significant differences in NMDA GluN1/AMPA GluA1 subunit mRNA ratio levels in layer 3 ($F_{3,22}$=11.6, P<0.001) pyramidal cells, with post hoc analyses revealing significant (P<0.05) increases between perinatal to peripubertal age groups **(Figure 22)**. However, NMDA GluN1/AMPA GluA1 subunit mRNA ratio levels in layer 5 ($F_{3,22}$=2.0, P=0.141) showed no change during postnatal development **(Figure 22)**.

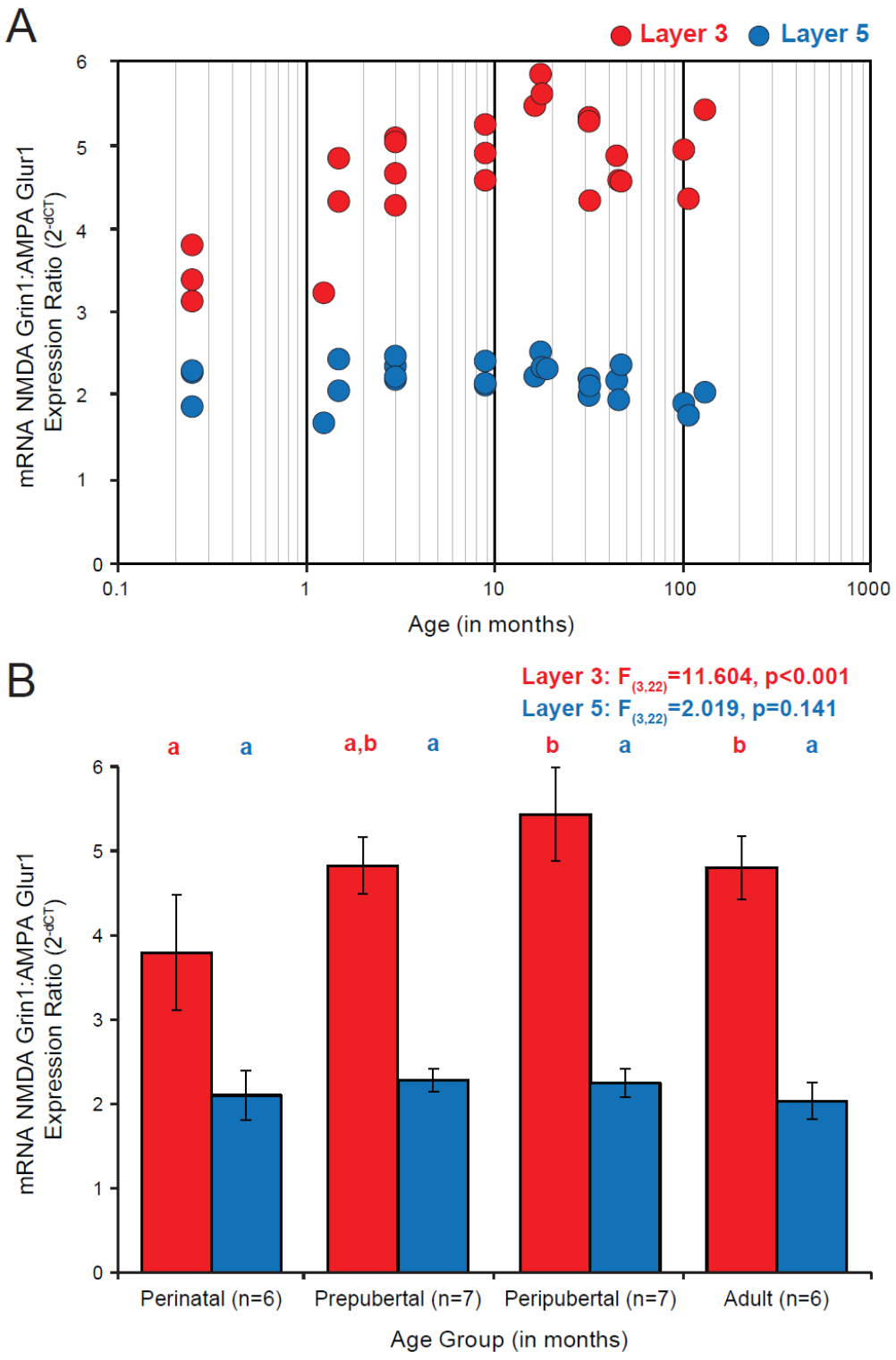

Figure 22. Developmental trajectories of glutamate receptor NMDA GluN1/AMPA GluA1 subunit mRNA ratio levels in layers 3 and 5 pyramidal cells of monkey DLPFC.

(A) Panel shows the expression for the NMDA GluN1/AMPA GluA1 subunit mRNA ratio in individual subjects for layer 3 (red circles) or layer 5 (blue circles) pyramidal cells. **(B)** Mean (SD) expression ratios for each age group; age groups not sharing the same letter are significantly different ($P<0.05$).

This dichotomous relationship between the expression of the principal subunits of NMDA and AMPA receptors in layer 3 and 5 pyramidal cells is congruent with recent findings suggesting that the persistent firing from recurrent excitation within the network of DLPFC layer 3 "Delay" cells is *dependent on NMDA receptor stimulation (Wang et al 2013)*. DLPFC layer 3 "Delay" cells are usually spatially tuned and maintain persistent firing during the delay period of working memory tasks, and convey information to "Response" cells primarily localized in layer 5 which fire in anticipation of, during and after the motor response during working memory tasks (Arnsten et al 2012, Sawaguchi et al 1989). Iontophoresis of specific antagonists for GluN2A and GluN2B receptor subunits in monkeys performing spatial working memory tasks suggests that NMDA GluN2B subunits are critical for DLPFC layer 3 "Delay" cells, since persistent firing was eliminated by local GluN2B NMDAR blockade (Wang et al 2013). Similar to our findings suggesting that the molecular composition of AMPARs is higher in layer 5 "Response" cells, the data from Arnsten and colleagues also revealed that layer 5 "Response" cells are selectively sensitive to AMPAR blockade (Wang et al 2013). These findings were validated by immunoelectron microscopy studies showing that NMDA GluN2B subunits are found exclusively within the postsynaptic density of DLPFC layer 3 spinous synapses in adult monkeys (Wang et al 2013).

The molecular and electrophysiological findings are consistent with an emerging notion from theoretical computation models with biophysically realistic simulations of activity in cortical neuronal networks, suggesting that spatial working memory is more stable, if network recurrent excitation between pyramidal cells is primarily mediated by slow voltage-gated NMDA receptors in DLPFC deep layer 3 microcircuits (Compte et al 2000, Lisman et al 1998, Wang 1999). NMDA receptor activation supports slow reverberating excitation and this pattern of excitation is crucial for the sustained firing of DLPFC deep layer 3 pyramidal neurons in the absence of

external stimulation (Compte et al 2000, Lisman et al 1998, Wang 1999, Wang 2001). In contrast, the decay of AMPA-receptor mediated EPSCs are three times faster than the decay of $GABA_A$ receptor mediated IPSCs (Aksay et al 2001, Hestrin 1993). As a result, a system with fast-positive and slow-negative feedback is dynamically unstable and can lead to network collapse. Thus, persistent activity mediated through NMDA-receptors might provide the necessary stability to sustain network oscillations in the γ-frequency range, which is generated in layer 3 association cortices (Buffalo et al 2011, Compte et al 2000, Quilichini et al 2010).

These molecular findings are relatively consistent with functional development of glutamate synapses in monkey DLPFC deep layer 3 pyramidal cells. Whole-cell patch clamp recordings in *in vitro* slice preparations of monkey DLPFC have revealed that excitatory inputs to deep layer 3 pyramidal cells had mature functional properties by 15-months of age which persisted throughout postnatal development (Gonzalez-Burgos et al 2008). The molecular and functional maturation of glutamate synapses in monkey DLPFC deep layer 3 pyramidal cells differ starkly from the structural maturation of excitatory inputs. For example, previous studies have shown that the density of dendritic spines located on the basilar dendrites on layer 3 pyramidal cells increase substantially during late gestation which reaches a plateau during late childhood, and then declines during adolescence (Anderson et al 1995). Similarly, in the human DLPFC, pyramidal neuron spine density undergoes a dramatic increase after birth to peak in childhood, and then declines during adolescence until the third decade of life (Petanjek et al 2011). In sum, it appears as though the molecular and functional maturation of glutamate synapses in deep layer 3 pyramidal cells occur *prior* to the pruning of synapses during late adolescence. Importantly, based on the inflection points of the developmental trajectories of GABA and glutamate synapses, it seems as though the molecular and functional maturation of glutamate synapses in DLPFC deep layer 3 occurs significantly *earlier* than the molecular and functional maturation of GABA synapses **(Figure 23)**. Although speculative, these contrasting patterns for GABA and glutamate neurotransmission suggest that glutamate synapses may not

be as susceptible as GABA synapses, to the influence of unique environmental exposures during specific sensitive periods.

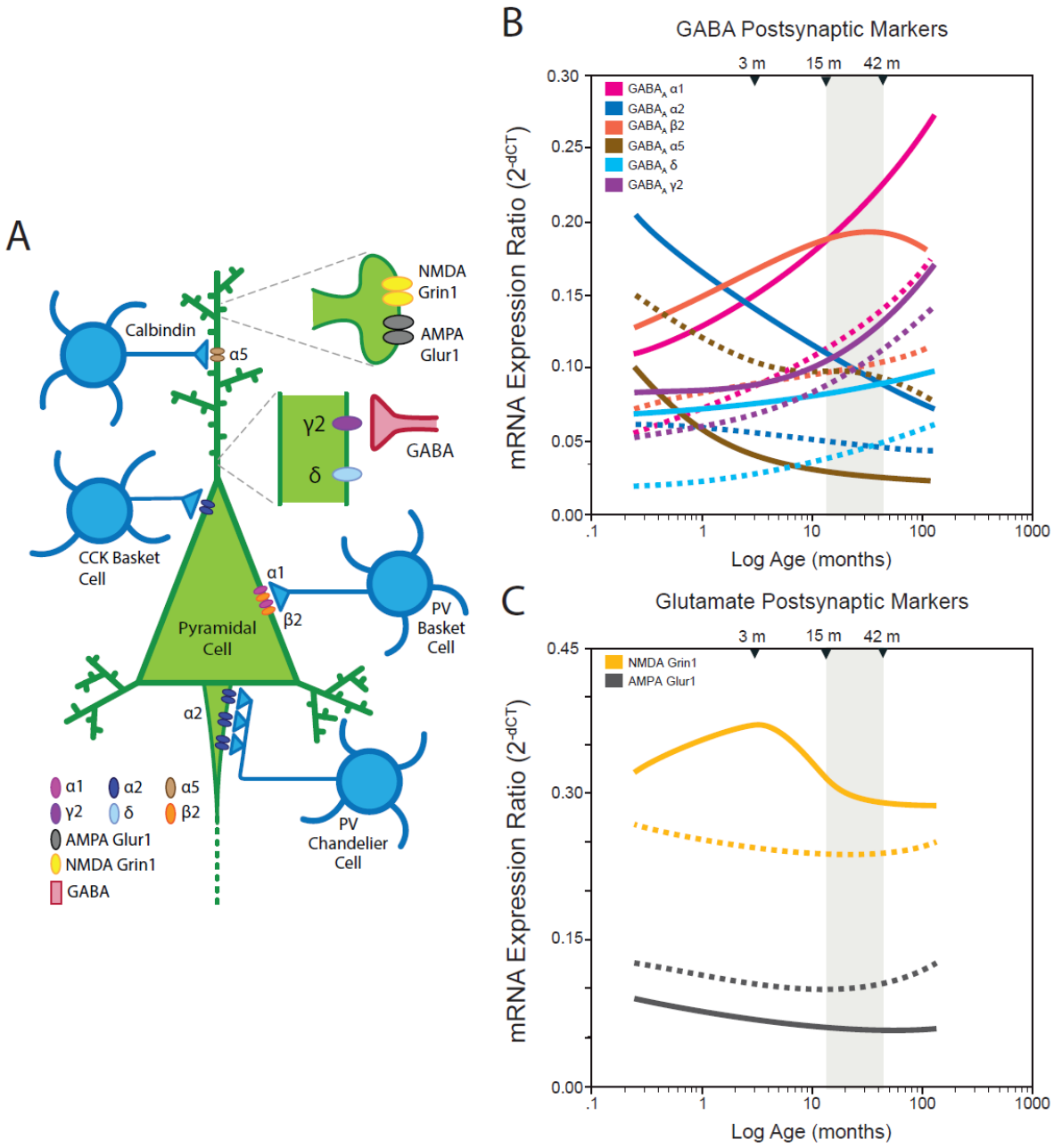

Figure 23. Developmental trajectories of GABA and glutamate postsynaptic markers in monkey DLPFC.

(A) Subcellular organization of GABA and glutamate postsynaptic markers in pyramidal cells. **(B)** Schematic summary of developmental trajectories of $GABA_A$ receptor subunits and **(C)** glutamate receptor subunit in DLPFC deep layer 3 (solid lines) and layer 5 (dashed lines) pyramidal cells. The protracted maturation profile for GABA postsynaptic markers during

postnatal development might explain why various environmental insults operating during sensitive time windows, can act on different components of DLPFC circuitry and increase the risk for schizophrenia. In contrast, the maturation profile for glutamate postsynaptic markers is relatively stable and less susceptible to environmental insults.

5.5 CONCLUDINGS REMARKS: THE NEXT FRONTIER IS UNDERSTANDING PATHOLOGY AT THE LEVEL OF SUBNETWORKS

The different lines of evidence presented thus far, provide compelling evidence of a *cell-autonomous intrinsic abnormality* in DLPFC deep layer 3 pyramidal cells that might be the primary deficit in the pathogenesis of schizophrenia. However, accumulating evidence supports the notion that even in an individual cortical layer, pyramidal cells are highly heterogeneous, have different local connection probabilities, distinct long-range projection targets, variability in intrinsic and functional properties, qualitative and quantitative differences in morphological properties and receive presynaptic inputs from different sources (Adesnik & Scanziani 2010, Beed et al 2010, Le Be et al 2007, Morishima & Kawaguchi 2006, Otsuka & Kawaguchi 2008). For example, using paired recordings and quantitative morphological analysis, recent evidence suggests that layer 5 forebrain corticopontine **(CPn)** projecting cells showed great reciprocal connectivity between each other, while avoiding corticostriatal **(CCS)** projecting cells (Morishima et al 2011). In contrast, in layer 5 of the visual cortex, the probability of feedforward connections from corticocortical cells to corticotectal cells is 4-fold higher than the probability of monosynaptic connections among corticocortical or corticotectal cells (Brown & Hestrin 2009). Therefore, it seems like different connectivity motif schemes may dictate the organization of subpopulations of excitatory cells depending on the brain region of interest. These connectivity motifs may be organized during neuronal differentiation as previous studies have shown that ontogenetic radially aligned sister excitatory cells have a propensity to be reciprocally connected

with each other rather than neighboring non-siblings, within a microcircuit of a functional cortical column (Yu et al 2009). Furthermore, a recent study puts forth the idea that genetically defined subpopulations of pyramidal cells share distinct neurogenesis and synaptogenesis time windows and are preferentially interconnected within a hippocampal microcircuit (dentate gyrus-CA3 and CA3-CA1) (Deguchi et al 2011).

Intriguingly, there is a growing consensus that pyramidal cells that can be subdivided into different subpopulations project to and are targeted by distinct subtypes of GABA interneurons, and may partake in separate information processing channels in neuronal networks (Bortone et al 2014, Krook-Magnuson et al 2012, Yoshimura & Callaway 2005). Supporting this idea, a recent study in the hippocampus using a combination of *in vitro* paired intracellular recordings, *in vivo* two-photon functional imaging in awake mice, and computational modeling showed that PVb cells evoked several times greater postsynaptic currents in CA1 pyramidal cells located in the deep layers compared to the superficial layers of the stratum pyramidale (Lee et al 2014b). In addition, the study showed that PVb cells preferentially innervated pyramidal cells projecting to the amygdala but received excitatory innervation from pyramidal cells that projected to the cortex (Lee et al 2014b). Similarly, CCK basket cells selectively innervate calbindin expressing pyramidal cells in layer 2 of the medial entorhinal cortex that project to the contralateral entorhinal cortex, but avoid reelin expressing pyramidal cells that project to the ipsilateral dentate gyrus (Varga et al 2010). In the striatum, fast-spiking GABA interneurons, selectively target direct-pathway medium spiny neurons (MSNs) over indirect-pathway MSNs, providing a mechanism for pathway-specific regulation of striatal output pathways (Gittis et al 2010). Finally, in the visual cortex, corticocortical pyramidal cells receive ~8-fold greater inputs onto their AIS from axoaxonic cells than corticothalamic cells (Farinas & DeFelipe 1991).

This raises the question, is a similar phenomenon of GABA interneuron specialization observed in the PFC, such that certain subpopulations of GABA interneurons selectively

innervate subpopulations of pyramidal cells? Indeed, using optogenetic stimulation paradigms, an elegant recent study found that fast-spiking PV interneurons preferentially innervate layer 5 thick-tufted, subcortically projecting type A cells, with prominent h-current, while avoiding thin-tufted callosally projecting type B cells, lacking a prominent h-current (Lee et al 2014a). The specificity of PV interneuron innervation to pyramidal cell subpopulations may have important implications for the computational properties of these cells during network activity. These findings are consistent with previous studies showing that disynaptic inhibition occurs with a higher probability between thick-tufted layer 5 pyramidal cells that are reciprocally connected, than callosally projecting layer 5 pyramidal cells (Le Be et al 2007). However, these findings are not in agreement with other studies which suggest that GABA interneuron connectivity, primarily in layer 2/3 in the somatosensory and frontal cortex resembles a “blanket of inhibition” such that GABA interneurons contact nearby pyramidal cells very densely without any apparent specificity (Fino & Yuste 2011, Inan et al 2013, Packer & Yuste 2011). These differences may be explained by several plausible reasons including laminar-specificity, brain region under investigation and technical methods applied to assess connectivity patterns between GABA interneurons and pyramidal cells. Interestingly, a recent report postulates that rather than exerting homogeneous inhibition in a uniform manner in postsynaptic pyramidal cells, the strength of inhibition is matched in a heterogeneous fashion depending on the amount of excitation received by the postsynaptic pyramidal cell (Xue et al 2014). As a consequence, E/I balance ratios are equalized across pyramidal cells such that when excitation increases, inhibition increases proportionally through increased recruitment of inhibitory drive by independent adjustment of synapses, primarily from PV interneurons (Xue et al 2014). Moreover, it is also pertinent to mention that, connectivity patterns between molecularly defined subpopulations of GABA interneurons themselves show cell type-specificity. At least in the supragranular and infragranular layers of the visual and somatosensory cortex, PV interneurons strongly inhibit each other but provide little inhibition to other interneurons, whereas SST

interneurons inhibit all other interneuron subtypes but avoid one another and VIP interneurons selectively target only SST interneurons (Pfeffer et al 2013, Pi et al 2013).

In sum, it appears as though pyramidal cells with distinct long-distance axonal projection patterns may have unique connectivity patterns, forming subnetworks within spatially intermingled cortical circuits. Intriguingly, GABA interneurons show domain specificity, selectivity in targeting the subcellular compartment along the somatodendritic arbor of pyramidal cells, and subnetwork specificity, by innervating specific subpopulations of pyramidal cells. Although, the functional implication of these selective innervation patterns to pyramidal cell subnetworks remains to be elucidated, this interneuron specialization represents an unrecognized form of GABA microcircuit specificity (Harris & Shepherd 2015, Krook-Magnuson et al 2012). As described above, in the context of the disease process of schizophrenia, GABA interneurons exhibit pre- and postsynaptic alterations to reduce feedback inhibition in response to reduce excitatory drive to DLPFC deep layer 3 pyramidal cells. However, based on emerging evidence, is it plausible that certain pyramidal cell *subpopulations* that receive preferential innervation from *specific subtypes* of GABA interneurons in the local microcircuit are preferentially affected in schizophrenia? Our nuanced understanding of cortical microcircuits from newer experimental approaches available primarily in rodents provides exciting opportunities for future investigation, with the underlying hope that advances in basic neuroscience may be applied to probing pathological disturbances in schizophrenia at even higher levels of spatial resolution. Ultimately, understanding such complex relationships within cortical microcircuits in schizophrenia might eventually facilitate in the discovery of novel therapeutic targets.

APPENDIX A

Altered Expression of CDC42 Signaling Pathway Components in Cortical Layer 3 Pyramidal Cells in Schizophrenia

Supplemental Information

Antipsychotic-exposed Monkeys

From each monkey in a triad, 150 individually-dissected pyramidal cells were collected from DLPFC deep layer 3. Total RNA was extracted (QIAGEN RNeasy microkit Plus), cDNA synthesized (QUANTA BioSciences qScript^{TM} cDNA SuperMix) and microarray analysis conducted using GeneChip® Rhesus Macaque Genome Array (Affymetrix, Santa Clara, CA) with all samples from a given triad processed together. All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Statistical Analysis

Microarray Sample Probe Set Filtering and Correction for Multiple Comparisons

A detailed description of the statistical analyses used for the microarray data is provided in Arion *et al.* 2014. Briefly, the Affymetrix CEL files were normalized and log2

transformed using RMA Express. Using a previously published method to filter the probe sets, the microarray dataset was filtered to 1) remove low expression probe sets by eliminating the lowest 40% mean intensities across all samples, and 2) remove non-informative probe sets by eliminating probe sets with the lowest 40% standard deviation across all samples. The microarray data set was then analyzed using the Random Intercept Model with Bayesian Information Criterion variable selection (RIM-BIC). In order to combine the differential expression information for each transcript, an adaptively weighted Fisher's method was used. The meta-analyzed p-values were then corrected for multiple comparisons using the Benjamini-Hochberg protocol to control false discovery rate. Finally, the potential influence of comorbid or confounding variables (sex, age, schizoaffective disorder diagnosis, suicide, RIN, PMI, pH, benzodiazepine or valproic acid use ATOD, antidepressant use ATOD and tobacco use ATOD) on differentially expressed probe sets was determined.

Table 5. Table S1. Demographic, postmortem, and clinical characteristics of human subjects used in this study

Subject Group[a]		Case No.	S/R/A[b]	PMI[c]	pH	RIN	Storage Time[d]	Cause of Death[e]	DSM IV Diagnoses[f] Primary Substance[g]		Anti-psychotics ATOD	Anti-depressants ATOD	BZ/VPA ATOD[h]
1•	C	592	M/B/41	22.1	6.7	9.0	203	ASCVD	N				
	S	533	M/W/40	29.1	6.8	8.4	213	Accidental asphyxiation	US		Y	N	N
2•	C	567	F/W/46	15.0	6.7	8.9	208	Mitral valve prolapse	N				
	S	537	F/W/37	14.5	6.7	8.6	213	Suicide by hanging	SA		N	N	N
3•	C	1322	M/W/62	16.5	6.8	8.6	73	ASCVD	N				
	S	566	M/W/63	18.3	6.8	8.0	193	ASCVD	US	AAR	Y	Y	Y
4•	C	604	M/W/39	19.3	7.1	8.6	201	Hypoplastic coronary artery	N				
	S	581	M/W/46	28.1	7.2	7.9	206	Accidental combined drug overdose	PS	ADC; OAC	Y	N	Y
5•	C	546	F/W/37	23.5	6.7	8.6	211	ASCVD	N				
	S	587	F/B/38	17.8	7.0	9.0	204	Myocardial hypertrophy	US	AAR	Y	N	Y
6•	C	551	M/W/61	16.4	6.6	8.3	210	Cardiac tamponade	N				
	S	625	M/B/49	23.5	7.3	7.6	198	ASCVD	DS	AAC	Y	Y	N
7•	C	681	M/W/51	11.6	7.2	8.9	191	Hypertrophic cardiomyopathy	N				
	S	640	M/W/49	5.2	6.9	8.4	196	Pulmonary embolism	PS		Y	Y	N
8•	C	806	M/W/57	24.0	6.9	7.8	170	Pulmonary embolism	N				
	S	665	M/B/59	28.1	6.9	9.2	194	Intestinal hemorrhage	PS	ADC	Y	Y	N
9•	C	822	M/B/28	25.3	7.0	8.5	167	ASCVD	N				
	S	787	M/B/27	19.2	6.7	8.4	173	Suicide by gun shot	SA	ODC	Y	N	N
10•	C	727	M/B/19	7.0	7.2	9.2	184	Trauma	N				
	S	829	M/W/25	5.0	6.8	9.3	165	Suicide by drug overdose	SA	ADC; OAR	N	N	Y
11•	C	871	M/W/28	16.5	7.1	8.5	156	Trauma	N				
	S	878	M/W/33	10.8	6.7	8.9	156	Myocardial fibrosis	DS	ADC	Y	Y	Y
12•	C	700	M/W/42	26.1	7.0	8.7	188	ASCVD	N				
	S	539	M/W/50	40.5	7.1	8.1	212	Suicide by combined drug overdose	SA	ADR	Y	Y	Y
13•	C	988	M/W/82	22.5	6.2	8.4	135	Trauma	N				
	S	621	M/W/83	16.0	7.3	8.7	199	Accidental asphyxiation	US		N	N	N
14•	C	686	F/W/52	22.6	7.0	8.5	190	ASCVD	N				
	S	656	F/B/47	20.1	7.3	9.2	195	Suicide by gun shot	SA	ADC	Y	N	N

Subject Group[a]		Case No.	S/R/A[b]	PMI[c]	pH	RIN	Storage Time[d]	Cause of Death[e]	DSM IV Diagnoses[f] Primary Substance[g]		Anti-psychotics ATOD	Anti-depressants ATOD	BZ/VPA ATOD[h]
15•	C	634	M/W/52	16.2	7.0	8.5	197	ASCVD	N				
	S	722	M/B/45	9.1	6.7	9.2	185	Upper GI bleeding	US	ODR; OAR	Y	N	N
16•	C	852	M/W/54	8.0	6.8	9.1	159	Cardiac tamponade	N				
	S	781	M/B/52	8.0	6.7	7.7	174	Peritonitis	SA	ADR	Y	Y	N
17•	C	987	F/W/65	21.5	6.8	9.1	135	ASCVD	N				
	S	802	F/W/63	29.0	6.4	9.2	170	Right ventricular dysplasia	SA	ADC; ODR	Y	N	Y
18•	C	857	M/W/48	16.6	6.7	8.9	158	ASCVD	N				
	S	930	M/W/47	15.3	6.2	8.2	145	ASCVD	DS	ADR; OAR	Y	N	Y
19•	C	739	M/W/40	15.8	6.9	8.4	183	ASCVD	N				
	S	933	M/W/44	8.3	5.9	8.1	144	Myocarditis	DS		Y	Y	Y
20•	C	1047	M/W/43	13.8	6.6	9.0	126	ASCVD	N				
	S	1209	M/W/35	9.1	6.5	8.7	107	Diphenhydramine overdose	SA		Y	N	N
21•	C	1086	MW/51	24.2	6.8	8.1	120	ASCVD	N				
	S	10025	MB/52	27.1	6.7	7.8	99	ASCVD	DS	OAR	N	N	N
22•	C	1092	F/B/40	16.6	6.8	8.0	120	Mitral valve prolapse	N				
	S	1178	F/B/37	18.9	6.1	8.4	111	Pulmonary embolism	SA		Y	N	Y
23•	C	1336	M/W/65	18.4	6.8	8.0	85	Cardiac tamponade	N				
	S	1173	M/W/62	22.9	6.4	7.7	111	ASCVD	DS	ADR	Y	N	N
24•	C	1122	M/W/55	15.4	6.7	7.9	116	Cardiac tamponade	N				
	S	1105	M/W/53	7.9	6.2	8.9	118	ASCVD	SA		Y	N	N
25•	C	1284	M/W/55	6.4	6.8	8.7	95	ASCVD	N				
	S	1188	M/W/58	7.7	6.2	8.4	109	ASCVD	US	AAR; OAR	Y	N	Y
26•	C	1191	M/B/59	19.4	6.2	8.4	109	ASCVD	N				
	S	1263	M/W/62	22.7	7.1	8.5	98	Asphyxiation	US	ADR	Y	Y	N
27•	C	970	M/W/42	25.9	6.4	7.2	137	ASCVD	N				
	S	1222	M/W/32	30.8	6.4	7.5	105	Combined drug overdose	US	AAC	Y	Y	N
28•	C	1247	F/W/58	22.7	6.4	8.4	101	ASCVD	N				
	S	1240	F/B/50	22.9	6.3	7.7	101	ASCVD	US	ADR	Y	N	N
29•	C	1324	M/W/43	22.3	7.0	7.3	87	Aortic dissection	N				

Subject Group[a]		Case No.	S/R/A[b]	PMI[c]	pH	RIN	Storage Time[d]	Cause of Death[e]	DSM IV Diagnoses[f] Primary Substance[g]		Anti-psychotics ATOD	Anti-depressants ATOD	BZ/VPA ATOD[h]
	S	10020	M/W/38	28.8	6.6	7.4	101	Salicylate overdose	PS	AAC; OAC	Y	Y	Y
30•	C	1099	F/W/24	9.1	6.5	8.6	119	Cardiomyopathy	N				
	S	10023	F/B/25	20.1	6.7	7.4	100	Suicide by drowning	DS		Y	Y	Y
31•	C	1307	M/B/32	4.8	6.7	7.6	90	ASCVD	N				
	S	10024	M/B/37	6.0	6.1	7.5	99	ASCVD	PS		N	N	N
32•	C	1391	F/W/51	7.8	6.6	7.1	76	ASCVD	N				
	S	1189	F/W/47	14.4	6.4	8.3	109	Combined drug overdose	SA	AAR	Y	Y	Y
33•	C	1282	F/W/39	24.5	6.8	7.5	95	ASCVD	N				
	S	1211	F/W/41	20.1	6.3	7.8	107	Sudden unexpected death	SA		Y	Y	N
34•	C	1159	M/W/51	16.7	6.5	7.6	113	ASCVD	N				
	S	1296	M/W/48	7.8	6.5	7.3	93	Pneumonia	US		Y	Y	N
35•	C	1326	M/W/58	16.4	6.7	8.0	87	ASCVD	N				
	S	1314	M/W/50	11.0	6.2	7.2	89	ASCVD	US		Y	Y	Y
36•	C	902	M/W/60	23.6	6.7	7.7	152	ASCVD	N				
	S	1361	M/W/63	23.2	6.4	7.7	82	Cardiomyopathy	SA	ODC	Y	N	Y
37	C	1374	M/W/43	21.7	6.6	7.2	79	ASCVD	N				
	S	904	M/W/33	28.0	6.2	7.1	150	Pneumonia	SA		Y	N	Y
38	C	1555	M/W/17	15.1	6.9	7.9	44	Trauma	N				
	S	1649	M/B/17	21.4	6.9	8.1	29	Hanging	US		Y	Y	N
39	C	1268	M/B/49	19.9	7.1	7.9	96	ASCVD	N				
	S	1230	M/W/50	16.9	6.6	8.2	102	Doxepin overdose	US		Y	Y	N
40	C	1466	F/B/64	20.0	6.7	8.8	61	Trauma	N				
	S	1341	F/W/44	24.5	6.6	8.8	83	Trauma	SA	ODC	Y	N	Y
41	C	1518	M/W/50	20.7	6.4	7.7	50	ASCVD	N				
	S	1367	M/W/47	28.9	6.6	7.2	80	Combined drug overdose	SA	ADC; ODR	N	N	N
42	C	1386	M/W/46	21.2	6.7	8.3	75	ASCVD	N				
	S	1420	M/W/47	23.4	6.8	8.2	69	Jump	SA	AAR; ODC; OAR	Y	Y	N
43	C	1472	M/W/61	23.8	6.5	8.0	60	Pulmonary embolism	N				

Subject Group[a]		Case No.	S/R/A[b]	PMI[c]	pH	RIN	Storage Time[d]	Cause of Death[e]	DSM IV Diagnoses[f] Primary Substance[g]		Anti-psychotics ATOD	Anti-depressants ATOD	BZ/VPA ATOD[h]
	S	1453	M/W/62	11.1	6.4	8.2	63	Trauma	PS	ADR	Y	N	Y
44	C	1026	M/W/59	19.8	6.3	7.4	128	ASCVD	N				
	S	1454	M/W/59	24.1	6.1	7.6	62	Trauma	PS	AAR; ODC	Y	Y	N
45	C	694	M/W/38	20.7	7.0	7.7	189	Subarachnoid hemorrhage	N				
	S	1455	M/W/42	8.2	6.4	7.7	62	Peritonitis	PS	AAR; OAC	Y	N	Y
46	C	1350	M/W/21	24.2	6.4	7.3	82	Trauma	N				
	S	1474	M/W/37	39.9	6.7	7.0	60	Hanging	SA	ADR	N	N	N
47	C	1792	F/W/36	28.1	6.5	7.5	5	Pulmonary embolism	N				
	S	1506	F/W/47	14.1	6.6	7.5	55	Combined drug overdose	SA	ADC	Y	Y	N
48	C	1524	M/W/66	9.4	6.4	8.1	48	Intestinal infarction	N				
	S	1542	M/W/65	17.4	6.7	7.8	45	Combined drug overdose	PS		Y	Y	Y
49	C	1270	F/W/73	19.7	6.7	7.7	96	Trauma	N				
	S	1579	F/W/69	16.1	6.7	7.7	39	ASCVD	SA	ADR; ODC	Y	N	Y
50	C	1372	M/W/37	20.5	6.6	9.0	79	Asphyxiation	N				
	S	1581	M/W/32	18.4	6.8	9.0	39	ASCVD	PS	ODC; OAC	Y	Y	N
51	C	1543	F/W/45	17.9	6.8	7.4	45	Subarachnoid hemorrhage	N				
	S	10026	F/W/46	23.8	6.6	7.6	98	Thermal injuries	US		Y	Y	N
52	C	1583	M/W/58	19.1	6.8	8.2	39	Trauma	N				
	S	1686	M/B/56	14.1	6.2	8.3	22	ASCVD	PS	AAR	Y	Y	Y
53	C	1554	M/W/50	23.2	6.5	7.6	44	ASCVD	N				
	S	1691	M/W/51	31.9	6.6	7.7	20	Combined drug overdose	PS	ADR; ODC	Y	N	Y
54	C	1635	M/W/66	25.3	6.8	8.2	31	Cardiac tamponade	N				
	S	1706	M/B/60	28.1	6.8	8.4	17	Sepsis	SA	AAR; ODC; OAR	Y	N	N
55	C	1384	M/W/67	21.9	6.6	7.0	77	ASCVD	N				
	S	1712	M/W/63	15.1	6.2	7.1	15	ASCVD	SA	ADR; ODC	Y	Y	Y
56	C	1558	M/W/54	24.4	6.9	7.7	43	ASCVD	N				
	S	1734	M/W/54	28.6	6.1	7.7	12	Pneumonia	US	AAR; ODC; OAR	Y	N	N

● Subject pairs used for microarray study.

[a] C, normal comparison; S, schizophrenia;

[b] A, age in years; B, black; F, female; M, male; R, race; S, sex; W, white;

[c] PMI, postmortem interval (hours);

[d] Storage time (months) at -80°C;

[e] ASCVD, arteriosclerotic cardiovascular disease; GI, gastrointestinal;

[f] DS, disorganized schizophrenia; N, none; PS, paranoid schizophrenia; SA, schizoaffective disorder; US, undifferentiated schizophrenia;

[g] ADC, alcohol dependence, current at time of death; ADR, alcohol dependence, in remission at time of death; AAC, alcohol abuse, current at time of death; AAR, alcohol abuse, in remission at time of death; ODC, other substance dependence, current at time of death; ODR, other substance dependence, in remission at time of death; OAC, other substance abuse, current at time of death; OAR, other substance abuse, in remission at time of death;

[h] BZ, benzodiazepines; VPA, sodium valproate; ATOD, at time of death;

Y, yes; N, no.

Table 6. Table S2. Sequences and priming efficiency for all human qPCR primer sets used in this study

Symbol	Forward Primer	Sequence	Reverse Primer	Sequence	Primer Efficiency %
ACTB	ACTB-Hu-F1	GATGTGGATCAGC AAGCA	ACTB-Hu-R1	AGAAAGGGTGTAA CGCAACTA	100
PPIA	Cyclo-F2	GCAGACAAGGTCC CAAAG	Cyclo_R2	GAAGTCACCACCC TGACAC	98
GNAS	GNAS-Hu-F1	AGAGGCGATTGAA ACCATTG	GNAS-Hu-R1	GTCAAAGTCAGGC ACGTTCA	99
ARHGDIA	ARHGDIA-Hu-F2	GCCAAATTGCCA AAACTCAA	ARHGDIA-Hu-R2	GCCCGCTGAGACA GAAAA G	95
CDC42	CDC42-F2	CAAGGACATTTGTT TGCCATT	CDC42-R1	TTTGGTGCATTTCA AAGGTG	100
CDC42EP4	CDC42EP4-F4	AGGAAGGCCAGTG CAGAATA	CDC42EP4-R4	GGGAGGGAGGGAA TAACTCA	100
PAK1	PAK1-Hu-F2	TTGGGGATGTTTG CTACCTC	PAK1-Hu-R2	GACATGACAAGCC ACAATGC	98
PAK2	PAK2-Hu-F3	CTCACTGACTATGT G CCAACG	PAK2-Hu-R3	TCGCAACTACAAAT CCCA AA	100
PAK3	PAK3-Hu-F2	TGGGGGTTCTTTAC CTTTCA	PAK3-Hu-R2	CTGCGATTCAGGC TTACAAA	95
LIMK1	LIMK1-Hu-F2	GAAGAGGCCATCC TTTGTGA	LIMK1-Hu-R2	CTCCCAGAAACCT CTGTCCA	95
LIMK2	LIMK2-Hu-F1	GCAGGGGGA ATTGATAAAGG	LIMK2-Hu-R1	ACATCTGGTCCCA CA ACTCA	103

APPENDIX B

Table 7. Supplemental Table 1: Rhesus Macaque Monkeys used in this study

Age group	Subject	Age (in months)	Sex	Weight (kg)	Perfusion Status	Prior Biopsy Status	Storage Time (in months)
Perinatal (0.25 to 1.5)	315	0.10	F	0.7	-	-	2
	311	0.25	F	0.7	-	-	9
	285	0.25	F	0.6	-	-	21
	299	1.3	F	0.6	-	-	33
	306	1.5	F	0.7	+	+	16
	307	1.5	F	0.9	+	+	14
Prepubertal (3 to 9)	241	3	F	1.0	+	-	61
	245	3	F	1.2	+	-	53
	277	3	F	1.0	+	-	29
	310	3	F	1.1	-	-	9
	261	9	F	1.6	-	-	40
	262	9	F	1.8	-	-	40
	273	9	F	2.0	+	-	34
Peripubertal (16 to 32)	255	16	F	2.6	+	-	42
	317	16	F	2.7	-	-	1
	287	18	F	2.4	-	-	13
	286	19	F	2.4	-	-	13
	280	32	F	3.8	+	-	25
	281	32	F	3.7	+	-	25
	291	32	F	3.9	-	-	11
Adult (45 to 138)	289	45	F	5.7	-	-	13
	258	46	F	6.3	+	-	41
	288	47	F	5.0	-	-	13
	259	104	F	6.4	+	-	41
	282	108	M	11.7	-	-	24
	260	138	F	9.5	-	-	41

Table 8. Supplemental Table 2: Primer sequences for transcripts assessed during postnatal development in monkey DLPFC

Transcript	Forward Primer Sequence	Reverse Primer Sequence	Primer Efficiency
Cyclophilin-A	GCAGACAAGGTTCCAAAG	GAAGTCACCACCCTGACAC	100%
β-Actin	GATGTGGATCAGCAAGCA	AGAAAGGGTGTAACGCAACTA	96%
$GABA_A$ α1	TAAGCATCAGTGCCAGAAAC	ACACAAAGGCATAGCACACT	95%
$GABA_A$ α2	TCTCCCCAAAGTGGCTTATG	CCCATCCTCTTTTGGTGAAG	91%
$GABA_A$ α5	TGTTTCCAAGACGTCCCATT	TACTGGGCATCTGAGTGTCG	95%
$GABA_A$ β2	CCTCTCCTGGGTCTCCTTCT	AGGTGGGTGTTGATTGTGGT	96%
$GABA_A$ δ	ACCACGGAGCTGATGAACTT	AGGGCATGTAGGATTGGATG	95%
$GABA_A$ γ2	CCCCAAGGTCTCCTATGTCA	AATGCAAGGTGCCATACTCC	94%
NMDA Grin1	TCCCTGTCCATCCTCAAGTC	CGCGAGTCACATTCCTGATA	90%
AMPA Glur1	CTGTGAATCAGAACGCCTCA	GCCCAGATTTGCAAGAATGT	98%

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