DEVELOPMENT OF CORTICAL GABA CIRCUITRY: IDENTIFYING PERIODS OF VULNERABILITY TO SCHIZOPHRENIA

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University of Pittsburgh, 2013

The development of cognitive functioning is disrupted in many individuals who will later be diagnosed with schizophrenia, lagging behind healthy comparison subjects by 1-2 standard deviations at clinical onset. Cognitive dysfunction often appears years before clinical onset, is the best predictor of functional outcome, and is increasingly recognized as a central feature of schizophrenia. The domains of cognitive functioning affected in schizophrenia are mediated, at least in part, by prefrontal cortex (PFC) GABA neurons, which show molecular alterations in postmortem studies in schizophrenia. One common environmental risk factor for schizophrenia is chronic cannabis use, which disrupts cognitive function most prominently during adolescence, a time of flux in PFC circuitry that may be a sensitive period for the effects of cannabis use on neural circuit maturation. Parvalbumin (PV)-containing GABA neurons may be particularly vulnerable to risk factors for schizophrenia since they are altered in the disease, important for neural activity associated with cognitive functioning, and have a lengthy period of postnatal maturation. However, the nature of PV neuron subtype-specific developmental changes is not clear. Therefore, this dissertation focuses on understanding the *timing* of altered expression profiles of GABA-related mRNA levels in schizophrenia, the *impact* of chronic cannabis exposure during adolescence on GABA circuits of the monkey PFC, and the *cell type-specific nature* of postnatal maturation of influential GABAergic connections. Indeed, we find that the profile of GABA transmission markers in postmortem PFC tissue in schizophrenia can be explained by disrupted development of their mRNA levels; that chronic exposure to the psychoactive compound in cannabis during adolescence alters the GABAergic mRNA levels in monkey PFC; and that two populations of PV neurons have distinctive modes of maturation in monkey PFC.

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ABBREVIATIONS

AIS: Axon initial segment

PV: Parvalbumin

CB1R: Cannabinoid receptor 1

CCK: Cholecystokinin

CR: Calretinin

SST: Somatostatin

PVch: Parvalbumin-containing chandelier neuron

PVb: Parvalbumin-containing basket neuron

CCKb: Cannabinoid receptor 1-/cholecystokinin-containing basket neuron

vGAT: Vesicular GABA transporter

GABA: γ-aminobutyric acid

GAT1: GABA membrane transporter 1

GAD67: Glutamic acid decarboxylase, 67 kilodalton isoform

GAD65: Glutamic acid decarboxylase, 65 kilodalton isoform

$GABA_A$ α1: $GABA_A$ receptor subunit α1

$GABA_A$ α2: $GABA_A$ receptor subunit α2

$GABA_A$ α4: $GABA_A$ receptor subunit α4

$GABA_A$ γ or γ: $GABA_A$ receptor subunit γ

$GABA_A$ δ: $GABA_A$ receptor subunit δ

IR: Immunoreactive

THC: Δ^9-tetrahydrocannabinol

DSI: Depolarization-induced suppression of inhibition

DSE: Depolarization-induced suppression of excitation

MOR: μ-opioid receptor

mGluR1α: Metabotropic glutamate receptor subunit 1α

RGS4: Regulator of G-protein signaling

DEFINITIONS

Adolescence – the period between childhood and adulthood, which usually coincides with the onset of puberty and is behaviorally identified as a period of increased risk seeking and profound changes in behavior; The molecular "adolescence" of the prefrontal cortex is defined in this dissertation as the period of asymmetric synapse and dendritic spine density reductions that occurs between childhood and adulthood, and is independent of puberty status. In macaque monkeys, this period of dynamic pruning coincides on average between 15 and 45 months of age.

Critical period – a strict time window during which experience provides information that is essential for normal development and permanently alters behavior

Development – the extended period from conception to young adulthood when neural circuits and behaviors are maturing into their adult form

GABA-related markers – mRNA or proteins of molecules that are either directly involved in GABA neurotransmission or are identifiers of GABA neuron subpopulations; In this dissertation, GABA-related markers include GAD67, GAD65, GAT1, vGAT, $GABA_A$ receptor subunits, PV, CR, SST, CB1R, CCK

Neurodegenerative – classic gross pathologically identifiable loss of neurons accompanied by gliosis and most frequently associated with Alzheimer's disease, Huntington's disease, and Parkinson's disease

PV bouton – overlapping PV-IR and vGAT-IR puncta adjacent to a $GABA_A$ receptor γ2-IR cluster

Sensitive period – a limited period of development when brain function is highly influenced by the effect of experience

Working memory – the ability to maintain and manipulate on-line a limited amount of information for a short period of time in order to guide thoughts and behaviors toward a goal

PREFACE

I am extremely grateful for the guidance and support that I have received during graduate school, which has fueled my continued pursuit of becoming an incisive yet compassionate physician scientist. The Translational Neuroscience Program (TNP) has provided a thriving, collegial environment, both nurturing my scientific development and provoking me to seek excellence. I consider myself especially fortunate to work with an abundance of insightful and caring faculty members, students and staff, who are all driven to excel.

First, I would like to especially thank my academic advisor, mentor, and role model, David Lewis, for giving me sage advice throughout graduate school, and for always bringing humor to each encounter. He is a true visionary and an incredibly gifted manager of personalities. His guidance in scientific thinking and in both written and oral communication has outstandingly impacted my professional development. Dave is a model physician scientist and department chair, who embodies the leadership, drive, compassion and humility that I continuously seek to develop in my life.

I would also like to express my gratitude for Ken Fish, who has been a co-mentor and co-advisor to me. Ken's support, innovative thinking and his approach to problem solving—with the outlook that basically any problem has a solution—is quite remarkable and has instilled in me the confidence to approach any scientific or personal challenge with the belief that it can be resolved. I highly value these lessons.

Thanks to the many wonderful colleagues and friends—past and present—at the TNP who have shared in my successes and supported me during challenging times. Thank you, Holly Bazmi, for teaching me my first molecular techniques and for being incredibly patient. You are a true colleague, mentor and friend, and your unconditional support means so much to me. Thank you, David Volk, Guille Gonzalez-Burgos, Rob Sweet, Etienne Sibille, Dominique Arion, John Enwright and Colleen McClung, for bringing enthusiasm, expertise, and a highly motivating positive attitude every day. Thank you, my talented current and past co-trainees: Jill Glausier, Allison Curley, Brad Rocco, Sohei Kimoto, Diego Pafundo, Was Sheikh, and Mike Kitchens. Thank you to the many Lewis lab staff members who facilitated my projects. I would like to acknowledge Mary Brady for her excellent technical assistance. To the Lewis lab

chipmunks/musketeers, Dibs Datta and Wonjae Chung: Our many scientific, intellectual and philosophical conversations, lunches at Oishii, conference travels, and your support throughout this process have enriched my life. I am grateful to Sue Johnston, Mary Ann Kelly, and the entire Brain Bank Team, and of course, to the many families who made the important and generous decision to donate the brain tissue of their loved ones during an incredibly difficult time, and who patiently participated in our diagnostic interviews.

The Center for Neuroscience (CNUP) has been instrumental in my success as a graduate student. I am blessed to have encouraging and supportive committees of faculty experts who have given me fresh ideas and constructive feedback throughout my graduate training. From the reprint exam to my dissertation committee, I would like to extend my heartfelt gratitude to my dissertation committee chair, Pat Card, to Judy Cameron, Steve Meriney, Erika Fanselow, Don DeFranco, Paula Monaghan, and of course to Ken Fish and David Lewis, for the interest, time and thoughtful effort they have committed to my development. Pat Levitt, thanks for traveling across the country to be my outside examiner, and as a Bruin, I forgive you for being a Trojan and hope you will not hold that against me at the defense! Other members of the CNUP who have provided me with a "home away from home" include Karl Kandler, Elias Aizenman, Floh Thiels, and Jon Johnson. I appreciate the support and leadership from Alan Sved, Susan Amara, Linda Rinaman and Brian Davis. Thanks to my friends from the CNUP and MSTP, especially Dibs Datta, Meghan "Meggles" McCord, Nicole "Shifty" Scheff, Alison Kriesler, Anoopum Gupta, and Jeff Walch for making this journey incredibly fun and fulfilling.

The University of Pittsburgh has given me many opportunities to meet influential clinical and research mentors. Thanks to my career advisors: Tim Greenamyre, Richard Steinman, Clayton Wiley, and Tim Oury from the MSTP; Johnathan Engh and Mark Richardson from Neurosurgery; Ed Burton from Neurology; and Ray Cho, Jason Rosenstock, and Gretchen Haas from Psychiatry.

I have learned how important a dedicated staff is for helping me get through the variety of requirements and for keeping these large centers, departments and programs running smoothly. Special thanks to Joan Blaney, and thanks to Patti Argenzio, Joni Mainier, Marlene Nieri and Debbie Glumac from the CNUP and Department of Neuroscience; Jeanie Knox-Housinger, Robin Klapheke, Lisa Murphree, Laura English and Sharon Slovenec from the TNP and Department of Psychiatry; and Manjit Singh and Justin Markuss from the MSTP.

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“Out of your vulnerabilities will come your strength.”

—Sigmund Freud

“Portray [people with mental illness] sympathetically, and portray them in all the richness and depth of their experience as people, and not as diagnoses.”

—Elyn Saks

1.0 GENERAL INTRODUCTION

1.1 OVERVIEW OF SCHIZOPHRENIA

1.1.1 Human cost

Schizophrenia is a neuropsychiatric syndrome that affects roughly 26.3 million people worldwide (*Global Burden of Disease: 2004 Update,* 2008) and is recognized as a leading cause of all years lived with severe disability, accounting for 3% of the total years of healthy life lost due to disability (*Global Burden of Disease: 2004 Update,* 2008). Features of schizophrenia frequently result in profound suffering, often becoming severe for the first time during late adolescence or early adulthood (Lewis and Lieberman, 2000). Schizophrenia also causes substantial emotional distress in caregivers, who are often closely related family members (Gibbons *et al.*, 1984). The schizophrenia burden also includes persistent financial problems for affected persons, caregivers and the community (Gibbons *et al.*, 1984). Direct economic costs resulting from schizophrenia include treatment provided in inpatient, outpatient and long-term care, as well as criminal justice costs and medication costs (Wu *et al.*, 2005). Indirect costs mainly arise from lost productivity suffered by individuals with the illness, their caregivers and the community. In the United States in 2002, direct and indirect costs of schizophrenia were estimated to be $62.7 billion (Wu *et al.*, 2005).

A diagnosis of schizophrenia is typically accompanied by significant societal stigma (Penn *et al.*, 1994). Many individuals experience decreased life opportunities and a loss of independent functioning beyond the impairments of schizophrenia itself, as they are the

recipients of harsh stigmatization (Penn *et al.*, 1994). Stigma can involve actual episodes of physical, verbal or emotional abuse against an individual with schizophrenia solely because he/she has the syndrome (reviewed in Jacoby *et al.*, 2005); and can also be experienced as shame due to being "schizophrenic" or fear of encountering discrimination (reviewed in Jacoby *et al.*, 2005). Although there have been recent efforts to eradicate stigma in schizophrenia, it still occurs frequently in this disease and places a substantial burden on affected people (Thornicroft *et al.*, 2009).

As a result of the above, a person with schizophrenia is more likely to suffer from unemployment, poverty, homelessness, incarceration, recurrent hospitalizations, high rates of comorbid medical illness and depression, increased suicide attempts with a suicide completion rate of 5-10%, and *a reduced life expectancy of 1-3 decades* (reviewed in Lewis and Sweet, 2009; Insel and Scolnick, 2006).

1.1.2 Epidemiology and clinical features

Today serious mental illness—mostly schizophrenia—has the same prevalence and disability as it did a century ago (Insel, 2010). A recent meta-analysis (McGrath *et al.*, 2008) found that the median (10, 90 percent quartiles) estimate per 100,000 people for the lifetime morbid risk was 720 (310, 2710) and the annual incidence of schizophrenia was 15 (8, 43). Interestingly, men have a 40% greater lifetime risk of developing schizophrenia than women (McGrath *et al.*, 2008). Importantly, the many epidemiological studies examined by meta-analysis have collected data over several decades across many countries and diverse cultures, suggesting that on average the recorded prevalence and incidence of schizophrenia were similar across time and place.

Clinically, schizophrenia is recognized as a constellation of signs and symptoms classically grouped into three domains that include positive, negative and cognitive symptoms [*Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition, Text Revision*, (DSM-IV-TR, 2000)]. Positive symptoms are abnormal behaviors that are present in schizophrenia but not in healthy individuals. They include disturbances in perception such as delusions, hallucinations and thought disorganization, and are also recognized as the "psychotic" features of schizophrenia. Delusions are fixed false beliefs that remain unchanged with a high level of conviction in the face of contradictory evidence and are present in roughly 80% of individuals with schizophrenia (Andreasen and Flaum, 1991). Hallucinations are internal sensory perceptions experienced in the absence of an external stimulus and can occur in any modality. Auditory hallucinations are most common in people with schizophrenia, with prevalence estimates between 40 to 80% (Andreasen and Flaum, 1991; Thomas *et al.*, 2007). A person suffering from schizophrenia usually hears a single or multiple voices that may be distinguishable, and often does not recognize whether the voices are being generated internally or externally.

Thought disorganization generally refers to incomprehensible thought patterns or form and often appears as loose associations, which are ideas and speech that move off track across unrelated topics. Other indicators of thought disorganization can include clang associations in which words are used together based on their sounds but not their meaning, as well as neologisms, which are invented new words. Disorganized behavior is also common and can result a variety of actions including poor maintenance of hygiene, childlike behavior and unpredictable agitation. Catatonia, a marked decrease in reactivity to the environment, is an extreme manifestation of motor abnormalities in schizophrenia. Some examples of catatonic

motor behaviors include reaching an extreme degree of unawareness, maintaining a rigid posture and resisting efforts to be moved, and the assumption of inappropriate or bizarre postures (DSM-IV-TR, 2000).

Negative (or deficit) symptoms represent an absence of typical behaviors, including poverty of speech (alogia), lack of initiative (avolition), flattened or inappropriate affect (affective dysregulation), inability to experience pleasure (anhedonia), and withdrawal from friends and family (asociality). Negative symptoms are often primary symptoms of schizophrenia, but sometimes may be influenced by positive symptoms. For example, paranoia can lead to asociality, while an unchanging facial expression interpreted as flattened affect may result from prolonged medication use (DSM-IV-TR, 2000).

Cognitive symptoms refer to abnormalities in processes that enable coordinated, purposeful thoughts and behaviors (Miller and Cohen, 2001). Cognitive dysfunction occurs across a number of domains in schizophrenia, including tests of selective attention, working memory, episodic memory, processing speed, and language production and comprehension (Elvevag and Goldberg, 2000; Green, 1996; Carter and Barch, 2007). While it is possible that people with schizophrenia have specific deficits in multiple cognitive systems, a simpler interpretation is that these many deficits reflect a disturbance in cognitive control (reviewed in (Lesh *et al.*, 2011). These debilitating symptoms can make a simple conversation or calculation difficult to handle and consequently have a profound impact on the individual's social and occupational functioning. Given recent efforts to understand cognitive symptoms in schizophrenia, I will review evidence for why they are considered to be core features of the illness in the next section.

1.1.3 Cognitive dysfunction is central to schizophrenia

Cognitive deficits are present, quite stable, and persistent in the majority of individuals with schizophrenia (Lesh *et al.*, 2011; Elvevag and Goldberg, 2000; Green, 1996). For example, individuals at their first episode of schizophrenia already perform, on average, 1-2 standard deviations below the mean of comparison subjects (Heaton *et al.*, 2001; Wilk *et al.*, 2004). These deficits often appear well before the onset of psychosis and persist independently of psychotic relapses (Niendam *et al.*, 2003; Reichenberg *et al.*, 2010) **(Figure 1)**. When cognitive dysfunction is defined based on premorbid intellectual functioning and parental education, 98% of schizophrenia subjects fall below predicted levels and are thus considered to be impaired (Keefe *et al.*, 2005). In genetically identical twins discordant for schizophrenia, the twin diagnosed with schizophrenia performed worse than the undiagnosed twin in nearly all cases (Goldberg *et al.*, 1990; Goldberg *et al.*, 1995). Cognitive symptoms are also present with milder severity but similar form in first-degree relatives (Egan *et al.*, 2001; Sitskoorn *et al.*, 2004). Importantly, cognitive impairments best predict an individual's future employment potential and social integration (Addington and Addington, 1999; Lysaker *et al.*, 1995; Harvey *et al.*, 1998; Bartels *et al.*, 1997), and a recent meta-analysis found that cognitive function, but not severity of positive symptoms, was a significant predictor of employment outcome (Tsang *et al.*, 2010). *Thus, cognitive deficits are considered to be core features of schizophrenia.*

Although positive symptoms are often most prominent in schizophrenia, they are not specific to this syndrome. In fact, no individual symptom results in a certain diagnosis of schizophrenia, which is only given once other causes of psychosis are excluded, when the time course of psychotic and accompanying negative features is recognized, and with the establishment of persistent social and occupational dysfunction (DSM-IV-TR, 2000). Despite

being extensively described in the DSM-IV-TR, cognitive features are not currently a formal part of the diagnostic criteria for schizophrenia.

1.1.4 Treatment and outcome

The treatment of psychosis was revolutionized by the astute but serendipitous discovery of antipsychotic medications over a half century ago; however, since then progress in developing novel treatments for schizophrenia has been limited (Insel and Scolnick, 2006). Positive symptoms are most responsive to, although not completely eliminated by, these antipsychotic medications. While effective against positive symptoms, a number of serious adverse events are associated with pharmacological treatment including irreversible involuntary movements, substantial weight gain, diabetes and hypertension (Fischer and Buchanan, 2011). Critically, current antipsychotic medications have little to no effect on negative symptoms (Buchanan *et al.*, 1998) and cognitive dysfunction in schizophrenia (Goldberg *et al.*, 1993; Harvey and Keefe, 2001).

Despite relative success in treating positive symptoms, long-term functional outcomes in schizophrenia vary remarkably across individuals depending upon how recovery is defined. For example, longitudinal follow-up studies have found that around 40% of patients diagnosed with schizophrenia achieve partial social or functional recovery (Crumlish *et al.*, 2009; Lambert *et al.*, 2008; Menezes *et al.*, 2006), meaning they achieve "an adequate level of social and vocational functioning that involves appropriate role functioning, capacity for independent living, and social interactions at a regular frequency" (Robinson *et al.*, 2004). However, sustained recovery occurs only in about 14% of individuals within the first five years following the initial psychotic

episode (Robinson *et al.*, 2004), and an additional 16% experience late phase recovery (Harrison *et al.*, 2001).

Although most people with schizophrenia do not experience sustained recovery, the majority of clinical evidence suggests that schizophrenia is not a classic early-onset neurodegenerative syndrome similar to the late onset neurodegenerative syndrome described by Emil Kraeplin's colleague, Alois Alzheimer (Zipursky *et al.*, 2012; Andreasen, 2010). For example, a recent study found no age by diagnosis interactions on a number of cognitive measures in schizophrenia and matched comparison subjects, suggesting that performance on cognitive tasks does not decline as a consequence of having schizophrenia (Rajji *et al.*, 2012). Instead, most clinical evidence suggests that schizophrenia has a strong neurodevelopmental component, with changes in behavior beginning during the premorbid stage (Zipursky *et al.*, 2012; Andreasen, 2010) **(Figure 1)**.

Given that many individuals with schizophrenia still have poor functional outcomes that are associated with more severe cognitive dysfunction, which in turn is mostly unresponsive to current antipsychotic medications, there is a *critical need* for the development of novel therapies that target cognitive dysfunction in schizophrenia. To address this need the National Institute of Mental Health has recently formed initiatives that include the Measurement and Treatment Research to Improve Cognition in Schizophrenia (Marder and Fenton, 2004) and the Cognitive Neuroscience Treatment to Improve Cognition in Schizophrenia (Carter and Barch, 2007).

1.1.5 Etiology

The 19th century Germ Theory of Disease postulated that acquired diseases are often caused by the presence of a single pathogenic factor that unleashes a complex syndrome (reviewed in

Whitcomb, 2012). However, multiple lines of evidence suggest that schizophrenia cannot be understood within this framework, since it is increasingly recognized as a complex syndrome caused by numerous interacting pathogenic factors that alone are neither necessary nor sufficient to cause the syndrome (Bayer *et al.*, 1999; Maynard *et al.*, 2001). Adding to its complexity, schizophrenia presents with obvious clinical heterogeneity that may result from diversity, different disease entities that exist within a population meeting DSM or International Classification of Diseases diagnostic criteria, or from variability, the variance of specific components of the disease (reviewed in Lewis and Gonzalez-Burgos, 2008).

Despite the challenges associated with clinical heterogeneity, a combination of genetic and environmental factors appears to be necessary for the expression of schizophrenia **(Figure 1)**. Evidence from twin, family and adoption studies has shown that the etiology of schizophrenia has an appreciable genetic component (reviewed in Cardno and Gottesman, 2000). For example, monozygotic "identical" twins share 100% of their genes and have a higher rate of schizophrenia, while dizygotic "fraternal" twins share 50% of their genes and have a substantially lower rate than monozygotic twins (Gottesman, 1991). However, both monozygotic twins are diagnosed with schizophrenia only 40-60% of the time, suggesting that environmental factors contribute to the disease pathogenesis (Gottesman, 1991). Taken together with first-degree sibling (Gottesman, 1991) and adoption studies (Rosenthal *et al.*, 1971), these studies have led to high heritability estimates of ~0.7 (reviewed in Cardno and Gottesman, 2000). However, there is evidence against simple Mendelian inheritance, as around 60% of individuals diagnosed with schizophrenia have neither a first- nor second-degree relative with the illness (Gottesman, 1991). Genome wide association studies have shown that as many as 37,000 single nucleotide polymorphisms (SNPs) could be contributing to schizophrenia (International

Schizophrenia *et al.*, 2009), suggesting that a large accumulation of many risk alleles at many loci can result in the schizophrenia syndrome. Rare structural variants, including 22q11 deletions and 1:11 chromosomal translocations, have also been strongly associated with schizophrenia (Karayiorgou *et al.*, 2010; Millar *et al.*, 2000). In addition, microdeletions or microduplications (copy number variants or CNVs) have recently been reported to substantially increase the risk for schizophrenia (Stefansson *et al.*, 2008; Guilmatre *et al.*, 2009). Despite these recent exciting advances in genotyping methods, the combination of the many genetic susceptibility variants still leaves the majority of schizophrenia heritability unexplained.

A number of studies have found that many environmental factors across prenatal development confer risk for schizophrenia (Brown and Derkits, 2010) **(Figure 1)**. For example, maternal immune activation due to infection and obstetrical complications are associated with an increased risk for schizophrenia (Cannon *et al.*, 2002; Cannon *et al.*, 2001). Moreover, a range of postnatal environmental factors like minority group position, childhood trauma, urbanicity and cannabis use has consistently been associated with a higher risk of developing schizophrenia (van Os *et al.*, 2010). The impact of cannabis use on GABA circuit development is an important focus of this dissertation because of its public health implications and biological plausibility in schizophrenia.

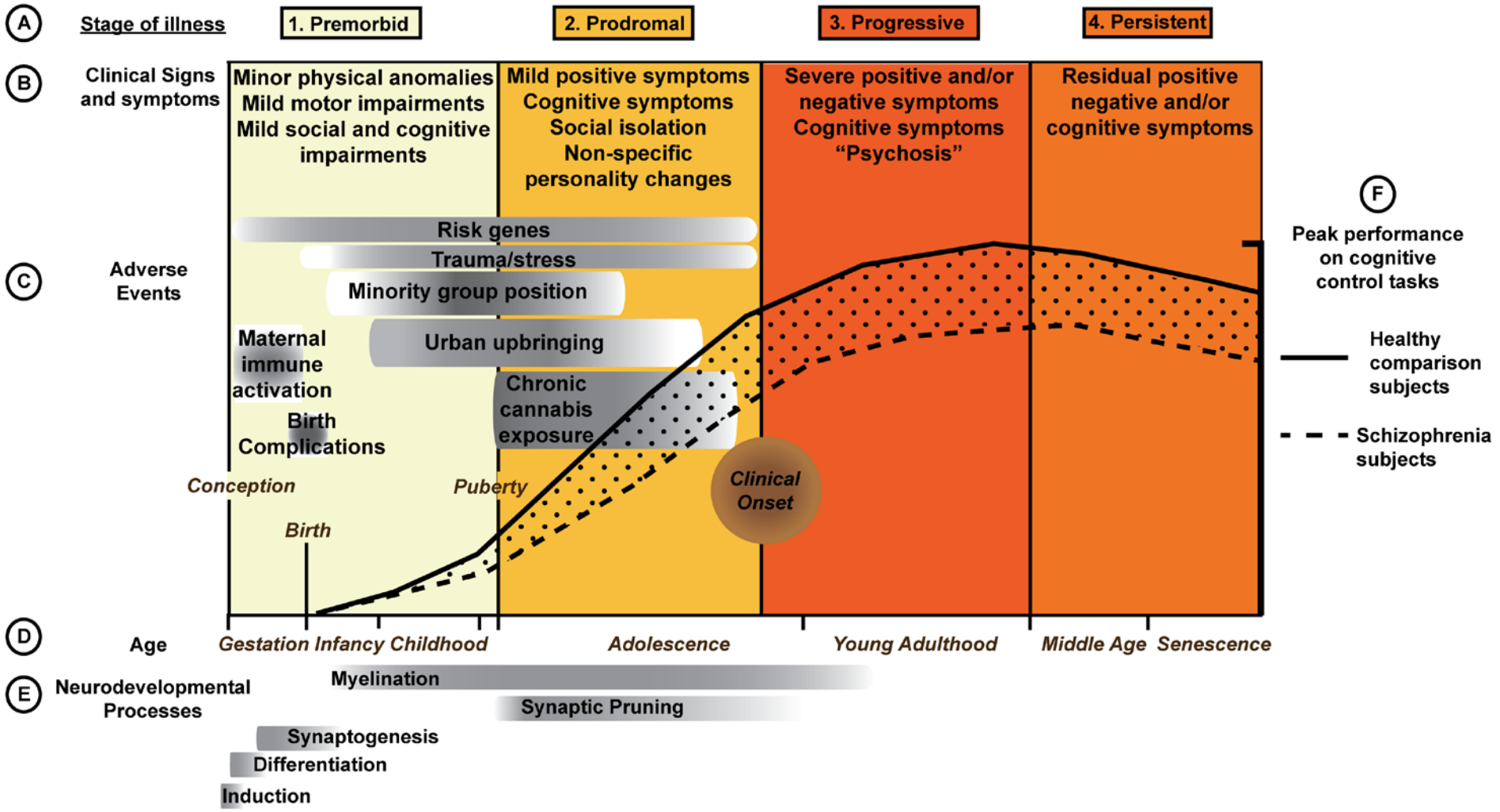

Figure 1. Summary of proposed lifetime trajectory of schizophrenia

(A) The four major stages of illness in schizophrenia. **(B)** Main groups of subclinical/clinical signs and symptoms at each stage of illness. **(C)** Key adverse genetic and environmental events associated with schizophrenia. **(D)** Age periods from gestation to senescence. **(E)** Major neurodevelopmental processes that occur from gestation through adolescence and are linked to schizophrenia. **(F)** Curves show gradual improvement of cognitive control in healthy subjects (solid line) and individuals on course to develop schizophrenia (dashed line). The dotted area between the lines represents the growing difference in cognitive control performance from childhood to early after clinical onset. Figure based on Lewis and Lieberman 2000 and Insel 2010.

1.1.5.1 Cannabis use during adolescence is associated with an increased risk of, and earlier onset for, schizophrenia

Epidemiological studies have consistently found an association between marijuana (cannabis) use and higher schizophrenia risk (Moore *et al.*, 2007; Henquet *et al.*, 2005b). Repeated cannabis use during adolescence has been associated with an elevated risk for schizophrenia relative to usage during adulthood (Arseneault *et al.*, 2002). Cannabis use at age 15 was associated with a greater likelihood of being diagnosed with schizophreniform disorder a decade later than was cannabis use at age 18 (Fergusson *et al.*, 2003), and has also been associated with an earlier age of onset for schizophrenia (Veen *et al.*, 2004; Barnes *et al.*, 2006; Large *et al.*, 2011).

The consistency and dose-dependent nature of these findings after controlling for a number of confounding factors suggest that the effects of cannabis use during early adolescence may somehow increase the liability for schizophrenia (Moore *et al.*, 2007; Henquet *et al.*, 2005b). For example, even after adjustment for factors like age, sex, social class, ethnic group, family history of psychiatric illness, city living and use of other drugs, the effect of cannabis on schizophrenia risk still persisted though the magnitude of the effect sizes was smaller (Henquet *et al.*, 2005b). The concept of reverse causality, which refers to the possibility that people who show signs of vulnerability to psychosis are more likely to start using cannabis in order to self-medicate, has also been examined closely in a number of studies. Most epidemiological studies have attempted to rule out reverse causality by excluding at baseline all individuals who ever had any psychosis-like experience (Henquet *et al.*, 2005b). In addition, cannabis use by individuals with schizophrenia is associated with an increased severity of cognitive impairments and more

frequent hospitalization, suggesting that these individuals have increased sensitivity to cannabis effects (Grech *et al.*, 2005; D'Souza *et al.*, 2005; Pencer *et al.*, 2005).

1.1.5.2 Cannabis use is associated with cognitive dysfunction

Cannabis use is correlated with deficits in cognitive control processes (Kanayama *et al.*, 2004; Block *et al.*, 2002). These cognitive impairments continue beyond the period of acute intoxication and worsen in a dose-dependent fashion (Solowij *et al.*, 2002). In healthy subjects, administration of Δ^9-tetrahydrocannabinol (THC), the principal psychoactive molecule in cannabis, produces deficits in a variety of cognitive processes including working memory, attention and executive control (D'Souza *et al.*, 2004). Also, cannabis use during adolescence is associated with more severe cognitive deficits compared to first use later in life (Pope *et al.*, 2003; Ehrenreich *et al.*, 1999). In addition, individuals who started using cannabis prior to age 17 had smaller whole brain and cortical gray matter volumes than those who started using cannabis later in life (Wilson *et al.*, 2000).

1.1.5.3 THC is bioactive at cannabinoid receptors that are enriched on cholecystokinin-containing GABA neurons

Evidence that the psychoactive effects of THC are mediated by the cannabinoid receptor 1 (CB1R) emerged from studies in healthy human subjects where the acute psychological effects of marijuana and the subjective “high experience” were blocked by pretreatment with a CB1R antagonist (Huestis *et al.*, 2001). The two most studied endogenous cannabinoids are lipophilic molecules that include anandamide, a partial agonist of CB1R, and 2-arachidonoylglycerol (2-

AG), a full agonist at CB1R that is present at much higher concentrations in the brain relative to anandamide [nanomole vs picomole per gram of tissue] (reviewed in Freund *et al.*, 2003).

Recent studies in the PFC have shown that CB1R are highly enriched at the presynaptic terminals of cholecystokinin-containing basket neurons (CCKb) (Eggan *et al.*, 2010; Marsicano and Lutz, 1999). The selective depolarization of a postsynaptic neuron induced a short-term depression of GABA release from CB1R-containing terminals innervating that same postsynaptic cell (Ohno-Shosaku *et al.*, 2001; Pistis *et al.*, 2002; Wilson and Nicoll, 2001), a phenomenon termed depolarization induced suppression of inhibition (DSI) **(Figure 2)**. Another phenomenon similar to DSI was found at excitatory synapses (depolarization induced suppression of excitation, DSE), suggesting that CB1R can also be localized to glutamatergic axon terminals and inhibit the release of glutamate (Kreitzer and Regehr, 2001).

Cannabinoids that are released from depolarized postsynaptic pyramidal neurons bind to presynaptic CB1R on CCKb cells, resulting in a reduction of perisomatic inhibitory input onto the same pyramidal cell (Wilson and Nicoll, 2002). Thus, CCKb inputs may contribute to the precise firing of PFC pyramidal neurons necessary during working memory tasks. Interestingly, THC administration to healthy adult subjects produced especially prominent deficits in the manipulation of information in working memory (D'Souza *et al.*, 2004), a feature that strongly depends on the PFC and is especially deficient in schizophrenia. These findings suggest that a potential shared biological mechanism for the effects of cannabis on cognitive function and the increased risk of schizophrenia exists via the alteration of PFC GABA circuitry.

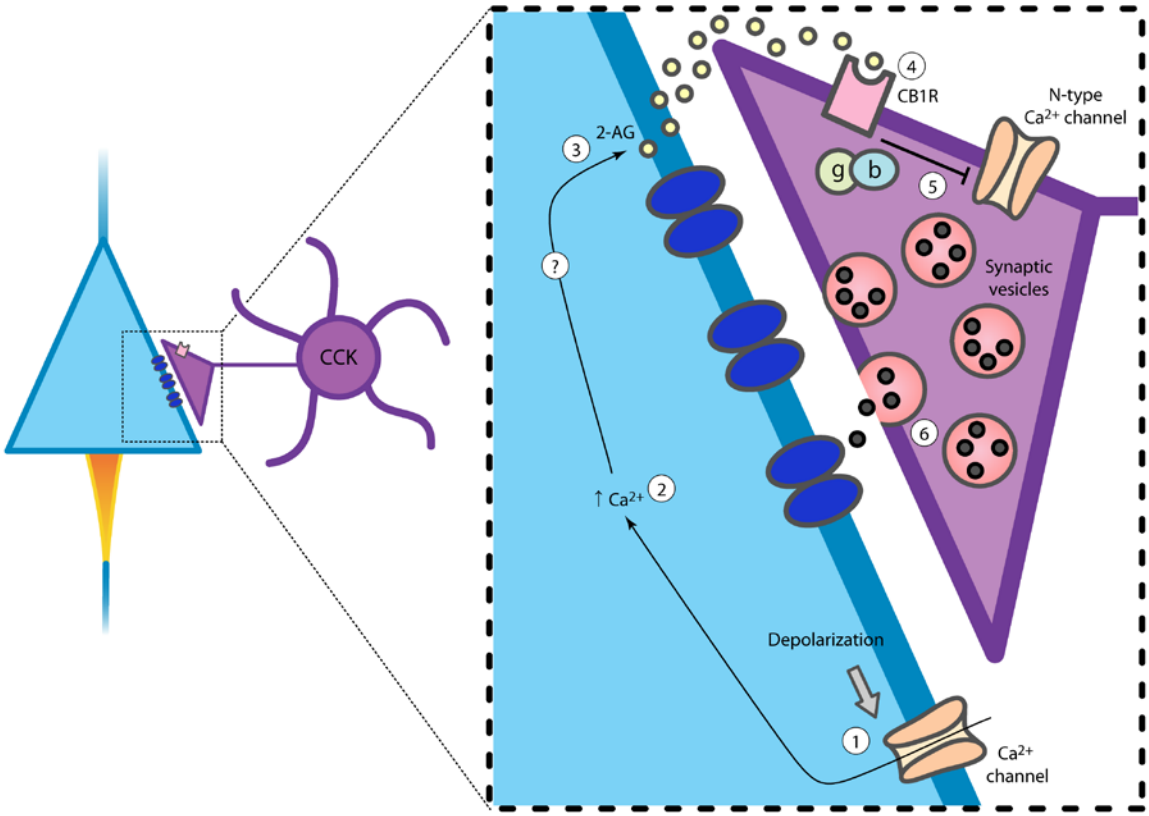

Figure 2. The endocannabinoid system and DSI

In synapse-specific DSI, membrane depolarization of the postsynaptic pyramidal neuron induces the opening of voltage-gated calcium channels **(1)**. The influx of calcium leads to an elevation of intracellular calcium **(2)**, which triggers the production of the endocannabinoid, 2-AG **(3)**. 2-AG then retrogradely activates the presynaptically located CB1R **(4)**, which inhibits the opening of presynaptic terminal N-type voltage-gated calcium channels **(5)**. The decline in presynaptic intracellular calcium concentration results in a decreased release of presynaptic GABA-containing vesicles **(6)** and a subsequent reduction of terminal-specific GABA neurotransmission. This figure was modified from Eggan et al. 2007.

1.2 PREFRONTAL CORTEX GABA NEURONS: KEY PLAYERS IN COGNITIVE IMPAIRMENT IN SCHIZOPHRENIA AND IN PROTRACTED NEURODEVELOPMENT

1.2.1 Cognitive dysfunction and the prefrontal cortex in schizophrenia

Cognitive control is dependent upon the coordinated activity of neural circuits that are distributed across many brain regions; however, dorsolateral PFC activity appears to be responsible for the maintenance of rules for action and response selection (Watanabe, 1992; Watanabe, 1990; Asaad *et al.*, 1998). Given the highly connected nature of the dorsolateral PFC to other brain regions including motor, sensory and associational areas, the PFC appears to be critical hub that integrates extrinsic and intrinsic information for top-down control of behavior (reviewed in Miller and Cohen, 2001).

Working memory, the ability to actively maintain and manipulate information for a short period of time in a goal directed fashion, is a prototypical example of a set of cognitive control processes that have been studied extensively in humans and non-human primates (Baddeley, 1992). In schizophrenia subjects, a number of regional cerebral blood flow and functional magnetic resonance imaging studies have reported dorsolateral PFC hypoactivity (Minzenberg *et al.*, 2009; Glahn *et al.*, 2005; Perlstein *et al.*, 2001; Cannon *et al.*, 2005), yet others have shown hyperactivity (Callicott *et al.*, 2000; Manoach *et al.*, 2000; Manoach *et al.*, 1999). Further study revealed that healthy individuals demonstrate an “inverted U” shape of dorsolateral PFC activation between working memory load and behavioral performance, showing low activation at low and high loads, and higher activation at intermediate loads (Callicott *et al.*, 1999). In schizophrenia, evidence suggests that this inverted U was left shifted such that these individuals

have PFC hyperactivity at lower loads and hypoactivity at moderate loads compared to healthy individuals (Callicott *et al.*, 2003; Van Snellenberg *et al.*, 2006). These findings show that working memory performance and PFC activity are disrupted in individuals with schizophrenia.

GABA neurons are critical components of coordinated PFC neural activity during working memory performance. Cortical GABA neurons comprise a remarkably diverse group of cells that can be categorized based upon distinct electrophysiological, molecular and anatomical properties (Ascoli *et al.*, 2008) **(Figure 3)**. Based on their electrophysiological properties, GABA neurons can be subdivided into fast-spiking and non-fast-spiking cells (Zaitsev *et al.*, 2009; Krimer *et al.*, 2005; Kawaguchi and Kubota, 1993). Fast-spiking neurons can produce action potentials at a high frequency with constant interspike intervals, and as a general rule, these cells contain parvalbumin (PV), do not contain neuropeptides, and target the perisomatic region (soma, proximal dendrites and axon initial segments (AIS)) of pyramidal neurons (reviewed in (Armstrong and Soltesz, 2012). PV neurons are estimated to make up ~25% of GABA neurons in the primate PFC (Conde *et al.*, 1994), and can be further divided into two major classes: basket (PVb) and chandelier (PVch) neurons that target the somata/proximal dendrites and AIS of pyramidal neurons, respectively.

Non-fast-spiking cells are more heterogeneous, including one subpopulation that contains the neuropeptide cholecystokinin (CCK), CB1R, no calcium-binding proteins, and also innervates pyramidal neuronal somata and proximal dendrites (reviewed in Armstrong and Soltesz, 2012). Both PVb and CCKb axons tend to form characteristic terminations onto pyramidal cell somata that can look like baskets under light microscopy, while PVch axons form distinctive vertical terminal arrays at pyramidal cell AIS called cartridges (Lewis and Lund, 1990). Two other non-fast-spiking GABA neuron subtypes include the population that contains

the calcium-binding protein calbindin, the neuropeptide somatostatin (SST), and mainly innervates distal dendrites of pyramidal cells; as well as the group that contains the calcium-binding protein calretinin (CR), the neuropeptide vasoactive intestinal peptide (VIP) and predominantly innervates other GABA neurons.

Fast synaptic GABA neurotransmission occurs via ionotropic $GABA_A$ receptors, which are heteropentamers most typically composed of 2α, 2β, and 1γ subunits from 7 different families (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3) (reviewed in Farrant and Nusser, 2005). Importantly, the composition of $GABA_A$ receptors can determine their functional properties, since $GABA_A$ receptors containing α1 subunits ($GABA_A$ α1) have much faster decay kinetics than currents mediated by $GABA_A$ receptors containing other α subunits (Lavoie *et al.*, 1997). In rodent hippocampus, postsynaptic $GABA_A$ receptor clusters that had a high α1/α2 subunit ratio were found to preferentially appose PVb inputs, while clusters that had a lower $GABA_A$ α1/α2 ratio because of enrichment of $GABA_A$ α2 subunits were preferentially adjacent to CCKb inputs to pyramidal cell somata/proximal dendrites and to PVch inputs to AIS (Nusser *et al.*, 1996).

Understanding which GABA neuron subtype(s) are affected in schizophrenia has important physiological implications, since different classes target specialized domains of pyramidal neurons and presumably affect pyramidal neuron activity uniquely. These GABAergic connections appear to be crucial for precisely sculpting neuronal activity both spatially and temporally (reviewed in Gonzalez-Burgos and Lewis, 2008). Given the unique morphological and molecular profile of GABA neuron subtypes, as well as evidence of their functional importance in health and disease, these neurons have become prime candidates for studying neural circuit dysfunction in schizophrenia.

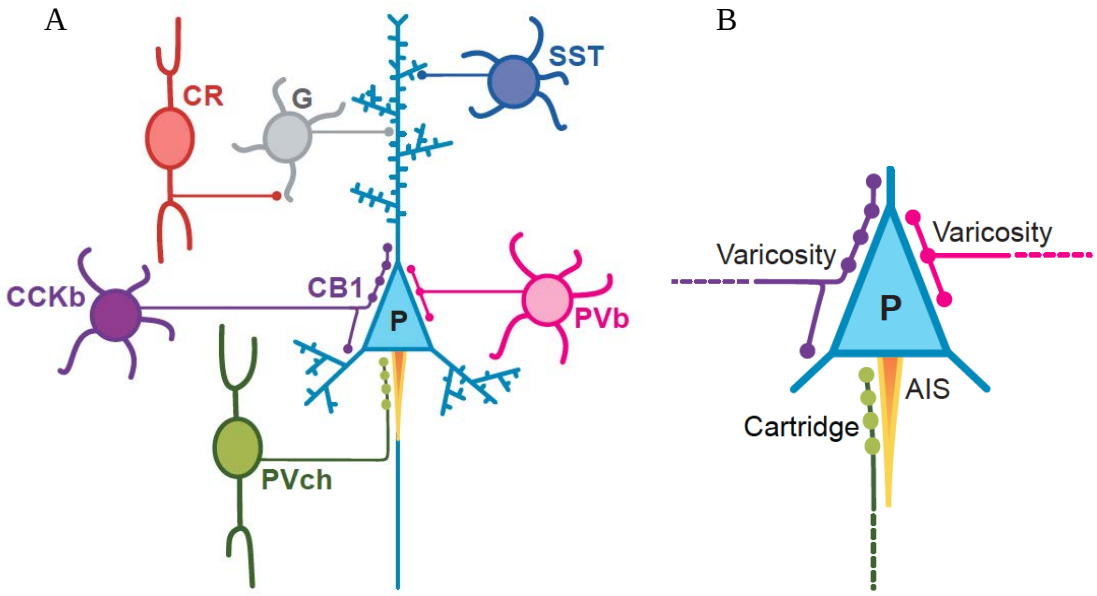

Figure 3. GABA neuron subtypes in the PFC

GABA neurons make up a remarkably heterogeneous group of neurons. Different types of GABA neurons target different subcompartments of pyramidal neurons **(A)**. Perisomatic targeting terminals arise from CCKb, PVb, and PVch neurons **(B)**. Adapted from Hoftman and Lewis 2011.

1.2.2 Alterations in GABA neurotransmission in schizophrenia

In schizophrenia, working memory dysfunction may be related to alterations in mRNA and protein levels of key regulators of GABA transmission in postmortem PFC tissue. Postmortem studies have consistently reported lower mRNA levels of the rate limiting GABA synthesizing enzyme, glutamic acid decarboxylase 67 (GAD67) (Akbarian *et al.*, 1995b; Volk *et al.*, 2000; Duncan *et al.*, 2010; Curley *et al.*, 2011; Hyde *et al.*, 2011). A recent comprehensive study showed that GAD67 protein levels were lower in the PFC of schizophrenia subjects and that lower GAD67 protein correlated with lower mRNA levels (Curley *et al.*, 2011). In contrast, mRNA levels of a second GABA synthesizing enzyme, glutamic acid decarboxylase 65 (GAD65), were only slightly reduced or unaltered in the PFC in schizophrenia (Hashimoto *et al.*,

2008a; Hashimoto *et al.*, 2008b; Guidotti *et al.*, 2000). In addition, the density of GAD65-immunoreactive (IR) puncta was also unchanged (Benes *et al.*, 2000).

Other markers of GABA transmission are also altered in schizophrenia. Transcript levels of the GABA plasma membrane transporter (GAT1), which removes GABA from the synapse, were reported lower in a subset of neurons in the PFC of subjects with schizophrenia (Volk *et al.*, 2001; Volk *et al.*, 2002). Lower mRNA levels of the postsynaptic $GABA_A$ α1 (Akbarian *et al.*, 1995a; Hashimoto *et al.*, 2008a; Hashimoto *et al.*, 2008b; Beneyto *et al.*, 2011) but see (Duncan *et al.*, 2010), β2 (Beneyto *et al.*, 2011), and γ2 (Akbarian *et al.*, 1995a; Hashimoto *et al.*, 2008a), which co-assemble together in the majority of $GABA_A$ receptors in the cortex (Mohler, 2006), have been found in the PFC in schizophrenia. Interestingly, recent findings indicate that $GABA_A$ α1 mRNA is lower in pyramidal cells but not GABA neurons in schizophrenia (Glausier and Lewis, 2011). However, not all markers of GABA transmission are lower in schizophrenia. For example, $GABA_A$ α2 mRNA levels are higher in schizophrenia (Beneyto *et al.*, 2011), while recent studies reported that mRNA and protein levels of the vesicular GABA transporter (vGAT), which loads GABA into presynaptic vesicles at axon terminals, were not significantly changed in postmortem PFC tissue from schizophrenia subjects (Fung *et al.*, 2011a; Fung *et al.*, 2011b). Some key molecular components of a characteristic GABAergic synapse are shown in **Figure 4**.

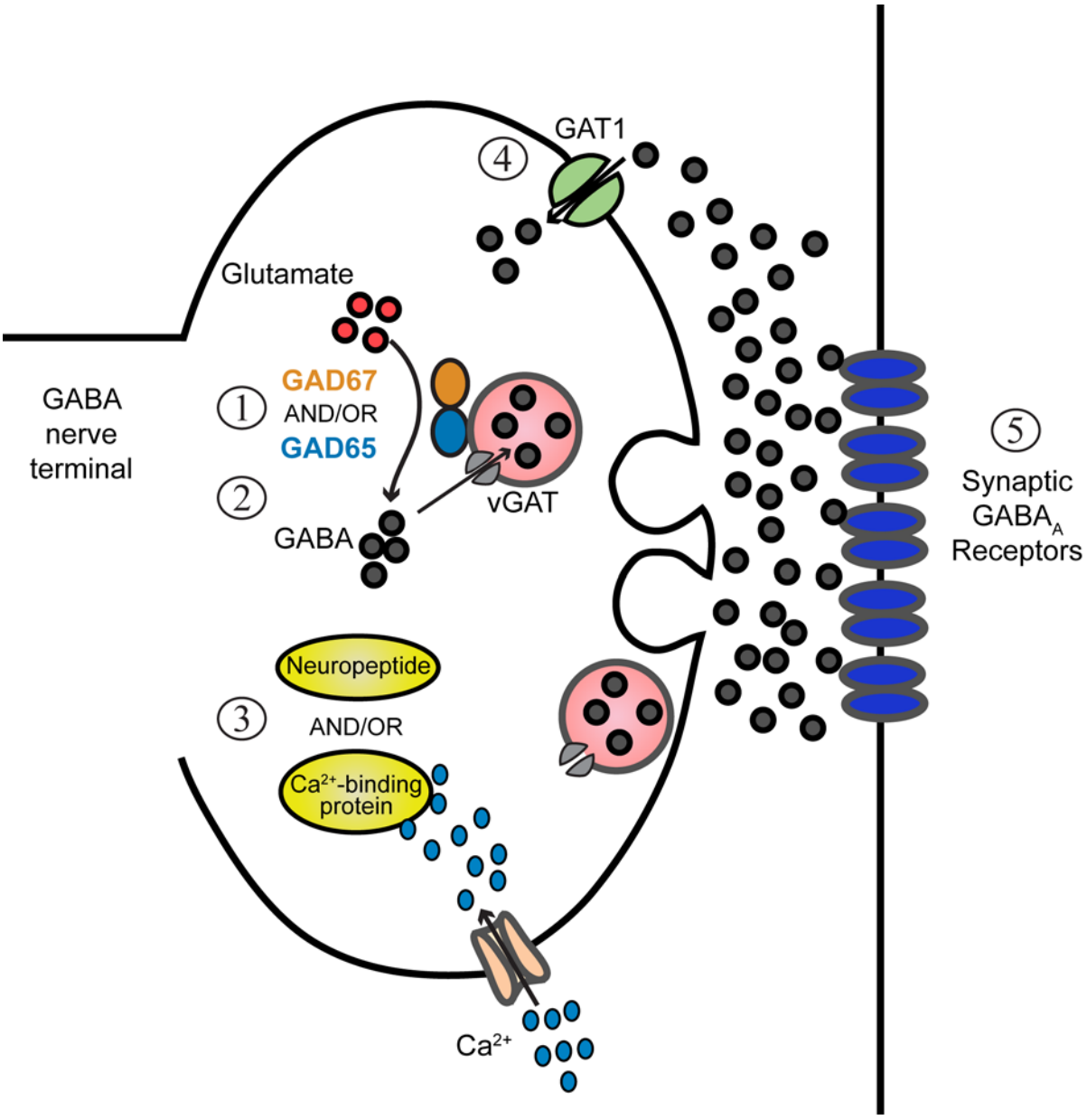

Figure 4. Synaptic GABA transmission

Typical GABA presynaptic terminal contains a vast interconnected network of machinery involved in regulating neurotransmitter release. Some key components I focus on in this dissertation include **(1)** the GABA synthesizing enzymes, GAD67 and GAD65; **(2)** the protein that loads GABA into presynaptic terminals, vGAT; **(3)** Ca^{2+}-binding proteins and neuropeptides like PV and CCK; **(4)** the protein that removes GABA from the synapse, GAT1; and **(5)** postsynaptic $GABA_A$ receptors. Adapted from Gonzalez-Burgos et al 2011.

1.2.3 Alterations in PV- and CCK-containing GABA neurons in schizophrenia

Cellular and laminar analyses in the PFC have indicated that the density of neurons with detectable levels of GAD67 mRNA was lower by 25-35% across layers 1-5 in subjects with schizophrenia (Volk *et al.*, 2000). However, the remaining GABA neurons with detectable levels of GAD67 mRNA showed no difference in the mRNA expression levels per neuron. In addition, the density of neurons expressing GAT1 mRNA was lower by 21-33% across layers 1-5 in schizophrenia and the expression level of GAT1 mRNA per neuron did not differ across subject groups (Volk *et al.*, 2001). Since the total number of PFC neurons in schizophrenia appears to be unchanged (Akbarian *et al.*, 1995b; Thune *et al.*, 2001), these findings suggest that markers of GABA transmission are impaired in a subset of GABA neurons of the PFC in schizophrenia.

In the PFC of schizophrenia subjects, ~50% of PV mRNA-positive neurons were reported to have undetectable levels of GAD67 mRNA in a dual label *in situ* hybridization study (Hashimoto *et al.*, 2003). PV expression was also lower in schizophrenia subjects, and this difference occurred in layers 3 and 4, where lower GAD67 mRNA levels were also reported in the same subjects. However, neither the density of neurons with detectable levels of PV mRNA nor the density of PV-IR neurons differed between groups. In addition, PV and GAD67 mRNA were lower within the same cells in subjects with schizophrenia (Hashimoto *et al.*, 2003). These findings suggest that GAD67 mRNA levels are reduced in PV-containing neurons that have lower, but still detectable, levels of PV mRNA. Markers of PVch inputs to pyramidal cell AIS are also affected in schizophrenia. For example, the density of cartridges detectable by GAT1-IR was 40% lower in schizophrenia (Pierri *et al.*, 1999; Woo *et al.*, 1998). The density of postsynaptic $GABA_A$ α2 immunoreactivity was higher by more than 100% in the illness (Volk *et*

al., 2002) and ankyrin-G (AnkG)-IR structures, a structural protein that is concentrated at AIS and nodes of Ranvier, was lower by 19% in the illness (Cruz *et al.*, 2009b).

Lower levels of GAD67 mRNA in the PFC are not exclusive to PV neurons in schizophrenia, since they were also reported in layers 2 and 5, where PV mRNA expression was unchanged (Hashimoto *et al.*, 2003). One class of GABA neurons whose somata are located outside of layers 3 and 4, and thus may account for lower GAD67 mRNA is the CCKb. CB1R mRNA and protein levels, as well as those for CCK, are also lower in schizophrenia (Eggan *et al.*, 2008; Hashimoto *et al.*, 2008a), and changes in GAD67, CB1R and CCK mRNA were significantly correlated in the same subject pairs (Eggan *et al.*, 2008). Finally, not all PFC GABA neurons appear to be affected in schizophrenia since CR-containing neurons, the largest population of PFC GABA neurons in this region (Conde *et al.*, 1994), did not show alterations in CR or GAD67 mRNA levels (Hashimoto *et al.*, 2003). Taken together, these findings suggest that the GABA neuron subtypes affected in schizophrenia include both PV- and CCK-containing neurons **(Figure 5)**.

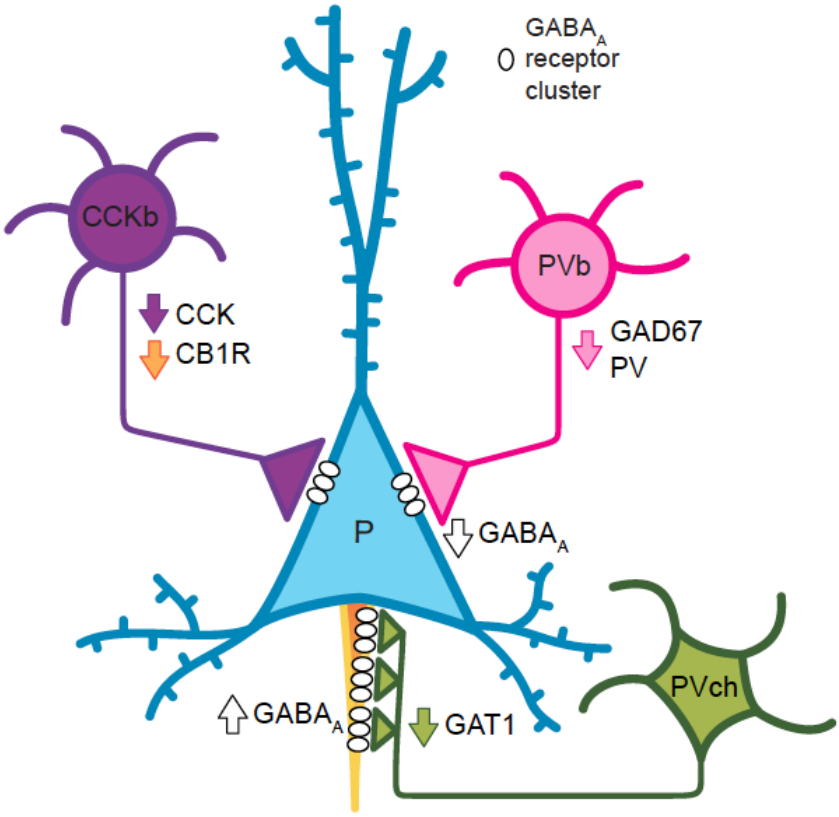

Figure 5. Changes in perisomatic GABA neurotransmission in schizophrenia

CCKb neurons have lower levels of CCK mRNA and CB1R mRNA and immunoreactivity (purple), PVb neurons have lower PV mRNA levels and GAD67 protein in their terminals (magenta), and PVch have lower GAT1-immunoreactivity in their axons (green) in the PFC from subjects with schizophrenia. The density of $GABA_A$ α1-IR receptor clusters is lower, and of $GABA_A$ α2-containing receptors at the AIS is higher, in schizophrenia (white ovals on blue pyramidal (P) cell).

1.2.4 PFC circuitry and cognitive control continue to develop during adolescence in monkeys and humans

In humans and monkeys, PFC circuitry and working memory performance continue to mature through adolescence (Luna *et al.*, 2004; Sowell *et al.*, 1999; Giedd *et al.*, 1999). In humans, adult levels of working memory performance are not reached until around age 20, an improvement that is associated with increased levels of PFC activity (Luna *et al.*, 2004). In line with these

findings, substantial changes in brain function occur during adolescence in parallel with an anatomical decrease in frontal cortical synaptic density.

As in humans, monkeys also continue to improve in working memory performance as they age through adolescence. In rhesus monkeys, the ability to perform working memory tasks first appears between 2-4 months of age and progressively improves to reach adult levels of performance after 3 years of age (Goldman-Rakic, 1987). This improvement in working memory performance appears to depend increasingly on PFC activity, since reversible cooling of PFC does not impair working memory performance in monkeys 9-16 months of age, produces modest impairment in animals between 19-31 months of age, and substantially impairs performance in animals over 3 years of age (Alexander, 1982; Alexander and Goldman, 1978). In electrophysiology studies some PFC neurons have increased firing during the delay period of working memory tasks, and the loss of this delay activity is associated with errors in performance. Between 1-3 years of age, there is a doubling in the amount of PFC neurons exhibiting delay-related activity (Goldman-Rakic, 1987).

Since opportunities to directly study the normative postnatal refinements in PFC circuitry at the cellular level in humans are limited, rhesus macaque monkeys provide an excellent model developmental system. Relative to rodents, macaque monkeys have an expanded PFC that develops over a long time period much like the human PFC (Nelson and Winslow, 2009). Primates and rodents also show developmental differences in the location of origin (Letinic and Rakic, 2001; Xu *et al.*, 2004), physiological properties (Povysheva *et al.*, 2007; Povysheva *et al.*, 2008) and proportions of GABA neuron subtypes (Gabbott and Bacon, 1996; Gabbott *et al.*, 1997) in the PFC.

The postnatal developmental trajectory of excitatory synapse density, which exhibits four distinct phases in both macaque and human PFC, has been best characterized using electron microscopy techniques (Bourgeois *et al.*, 1994; Huttenlocher, 1979; Petanjek *et al.*, 2011). In monkey PFC, the early postnatal period, between birth and ~3 months, includes a rapid increase in excitatory synaptic density; a relatively quiescent childhood period follows, between ~3 and 15 months, when the density of excitatory synapses is at a plateau; the adolescence period then lasts between ~15 to 45 months, including the period of excitatory synapse pruning; and the adult stage is reached when the density of excitatory synapses stabilizes. Although inhibitory synaptic density appears to be unchanged during adolescence in monkey PFC (Bourgeois *et al.*, 1994), dynamic changes in the expression of regulators of GABA transmission have still clearly been observed (Cruz *et al.*, 2009a; Cruz *et al.*, 2003; Erickson and Lewis, 2002). Late developmental refinements in dendritic spine densities and GABA transmission markers occur in parallel within the same cortical layers (Anderson *et al.*, 1995). While the term adolescence can be defined in many different ways, for the purposes of this dissertation adolescence is defined as the period of excitatory synaptic pruning in the PFC, which lasts between 15 and 45 months in monkeys and around 12-20 years in humans.

In monkey PFC, immunoreactivity of presynaptic cartridge markers and pyramidal cell AIS markers follow complex and protracted postnatal trajectories that coincide with the excitatory synaptic developmental changes **(Figure 6)**. For example, detectability of PV-IR cartridge density increases from around birth to 3 months of age, plateaus until around 18 months of age, and substantially declines during adolescence to reach adult levels (Cruz *et al.*, 2003). In contrast the density of puncta detectable by PV immunoreactivity progressively increases through adolescence to reach adult levels (Erickson and Lewis, 2002). Since the number of

synapses per AIS across pyramidal neurons ranges from 0 to >20 (DeFelipe and Farinas, 1992; DeFelipe *et al.*, 1985) and the proportion of AIS with detectable cartridges differs by cortical layer, age and label used to identify PVch terminals, only a small subset of pyramidal neurons has a visible cartridge associated with AIS. As a result, a plausible interpretation of the PV immunoreactivity studies is that the number of cartridges or cartridge terminals may decrease and the number of PV-IR puncta may increase during postnatal development. While the extended maturation of PV-IR puncta and cartridges is necessary for establishing adult circuits, it may also provide a window for both genetic and environmental factors to perturb these changing circuits. In contrast, CCK-IR profiles reach adult levels of stability before puberty onset in monkey PFC (Oeth and Lewis, 1993). Therefore, manipulation of CCKb inputs during adolescence may have unique developmental consequences for PVb and PVch neurons that are still in flux.

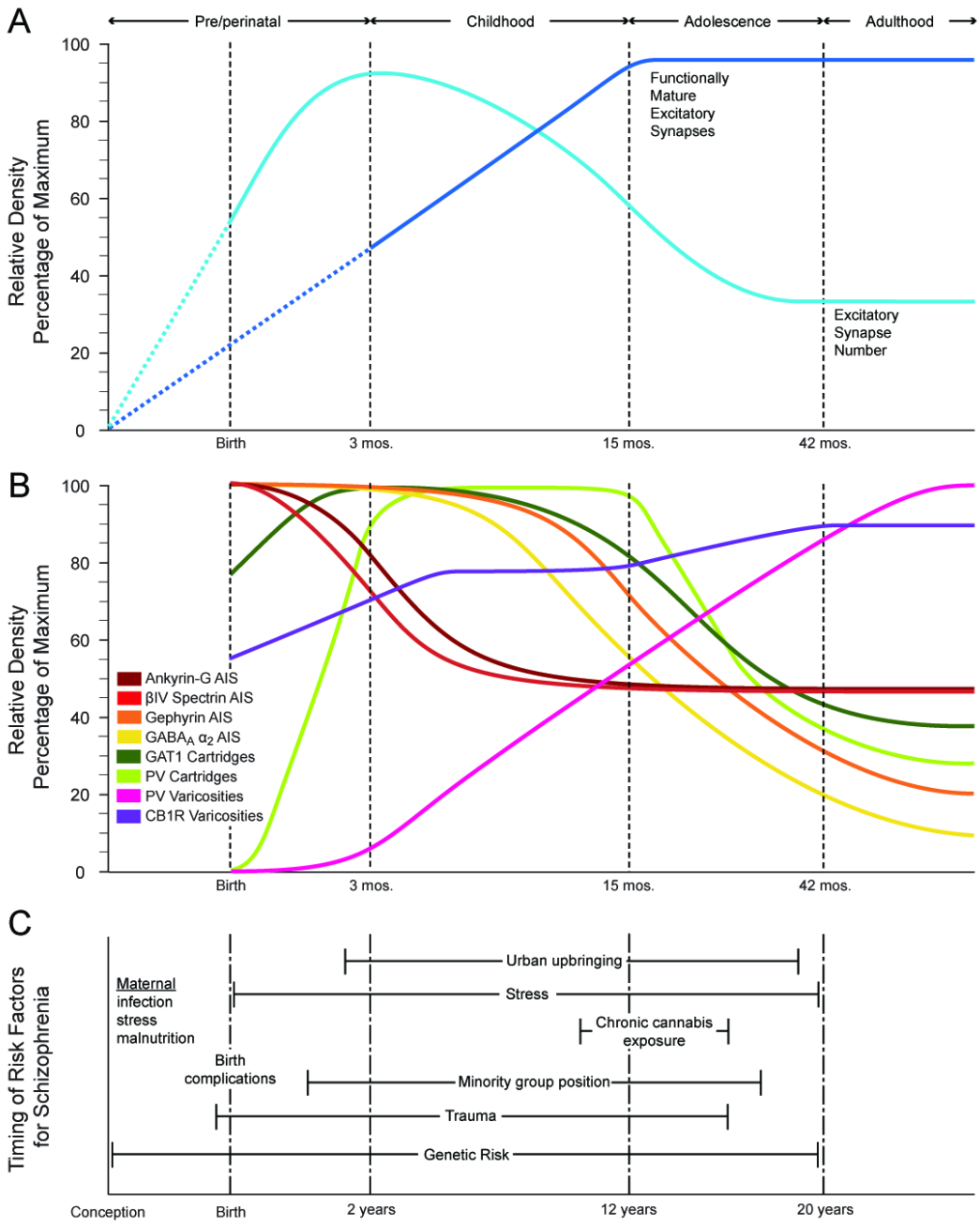

Figure 6. Developmental trajectories of PFC neural circuits and timing of risk factors for schizophrenia **(A)** Excitatory synaptic number changes and maturation continue through adolescence in monkey PFC. **(B)** Markers of perisomatic GABAergic neurotransmission have complex and protracted postnatal developmental trajectories in monkey PFC. **(C)** Genetic and environmental risk factors for schizophrenia occur from conception through late adolescence/young adulthood in human PFC, across equivalent periods of primate PFC maturation. Reproduced from Hoftman and Lewis 2011.

1.2.5 Evidence for a sensitive period of development: The effects of cannabis on PFC circuitry during adolescence

Adolescence represents a period of dynamic change in brain structure and function (Casey *et al.*, 2010). In humans, neuroimaging studies have revealed that frontal lobe cortical gray matter volume decreases while white matter shows a volumetric increase (Sowell *et al.*, 2004; Sowell *et al.*, 2002). These late changes, along with a postmortem structural developmental refinements in human and monkey PFC **(1.2.4)**, coincide with the ability to perform better on cognitive tasks such as working memory.

Since PV neurons undergo substantial refinement in parallel with improvements in working memory function during adolescence, and GABA release from CCKb inputs that target PV neurons (Karson *et al.*, 2009) is suppressed by CB1R activation; *adolescence may represent a sensitive period for the effects of cannabis exposure*. By indiscriminately suppressing GABA release from CCKb neurons, THC is poised to alter the activity of glutamatergic and GABAergic inputs to PV neurons. The resulting alterations in the regulation of PV neurons by pyramidal neuron axon collaterals may perturb the developmental refinements that normally occur during adolescence. The THC-mediated suppression of GABA release from perisomatic CCKb inputs may also affect perisomatic PV neuronal inputs to pyramidal neurons directly. In turn, these events could produce alterations in circuit mechanisms that are required for the maturation of working memory. These ideas will be tested in **3.0**.

1.3 GOALS OF THIS DISSERTATION

Given the large number of risk associations in schizophrenia, it is imperative not only to focus on the disease and attempt to treat the pathophysiology, but also to focus on health and preempting symptom expression. The classic disease model posits that the etiology "unleashes pathogenic processes that give rise to a pathological entity. This pathological entity perturbs normal physiology to produce the illness pathophysiology, which disturbs the milieu in a way that results in the emergence of the clinical syndrome" (Lewis *et al.*, 2005). Thus, interpreting neuropathological findings in schizophrenia requires knowing whether they represent an upstream cause of the illness, a consequence of pathogenic processes, a compensation for the pathophysiology induced by the pathological entity, or a confound of the illness (Lewis and Gonzalez-Burgos, 2008). Understanding their potential contribution to disease pathogenesis requires studying the normal developmental trajectories of vulnerable neural circuits. Therefore, the normal maturation of neural circuits that subserve working memory function is the focus of the particular proposal, since abnormalities in these neural circuits are thought to underlie core cognitive disturbances in schizophrenia.

In the PFC during postnatal development, substantial changes occur in the expression of mRNA and protein for key functional components of GABA neurotransmission that are implicated in schizophrenia pathology. Therefore, the purpose of this dissertation is to determine the *timing* of GABA neuron changes in the PFC **(2.0)**, the *impact* of an environmental risk factor on adolescent GABA neuron maturation **(3.0)**, and the *cell type-specific nature* of developmental changes in perisomatic targeting GABA terminals **(4.0)**. This dissertation aims to address this purpose by asking the following questions: 1) Can altered PFC expression of GABA-related genes in schizophrenia be explained by disturbances in their developmental mRNA expression

trajectories **(2.0)**?; 2) What are the consequences of chronic THC exposure during adolescence on PFC GABA circuitry **(3.0)**?; and 3) What is the postnatal developmental profile of two PV-containing axon terminal populations that target pyramidal neurons in the monkey PFC **(4.0)**?

2.0 ALTERED CORTICAL EXPRESSION OF GABA-RELATED GENES IN SCHIZOPHRENIA: EVIDENCE FOR DISRUPTED DEVELOPMENTAL TRAJECTORIES

Hoftman GD, Volk DW, Bazmi HH, Li S, Sampson AR, Lewis DA. Altered cortical expression of GABA-related genes in schizophrenia: Evidence for disrupted developmental trajectories. In preparation.

2.1 INTRODUCTION

Deficits in certain cognitive processes, such as working memory, are common in individuals with schizophrenia and have been attributed to dysfunction of the PFC (Barch and Ceaser, 2012). This dysfunction appears to reflect, at least in part, alterations in molecular markers of GABA neurotransmission (Benes and Berretta, 2001; Lewis *et al.*, 2005; Mirnics *et al.*, 2000). For example, multiple postmortem studies of schizophrenia subjects have documented lower mRNA levels for the principal enzyme responsible for cortical GABA synthesis, GAD67 (Akbarian *et al.*, 1995b; Volk *et al.*, 2000; Guidotti *et al.*, 2000; Duncan *et al.*, 2010; Hyde *et al.*, 2011; Vawter *et al.*, 2002; Straub *et al.*, 2007). Although less well-studied, mRNA levels of the presynaptic GAT1, which is responsible for the reuptake of extracellular GABA, have also been reported to be lower (Volk *et al.*, 2001), whereas mRNA levels of vGAT, which loads GABA

into presynaptic vesicles, have been reported to be unchanged (Fung *et al.*, 2011b) in the PFC of schizophrenia subjects. On the postsynaptic side, mRNA levels of two of the most common GABA ionotropic receptor subunits, $GABA_A$ $\alpha 1$ and $GABA_A$ $\alpha 2$, appear to be lower and higher, respectively, in some (Akbarian *et al.*, 1995a; Beneyto *et al.*, 2011; Volk *et al.*, 2002) but not all (Duncan *et al.*, 2010) studies of schizophrenia.

These alterations may be more common in particular subsets of cortical GABA neurons. For example, mRNA levels of the calcium-binding protein PV, which is expressed in a subset of GABA neurons, are lower in the PFC in schizophrenia (Mellios *et al.*, 2009; Fung *et al.*, 2010; Hashimoto *et al.*, 2003; Volk *et al.*, 2012a) and PV-containing neurons also exhibit lower expression of GAD67 mRNA (Hashimoto *et al.*, 2003) and contain lower levels of GAD67 protein in their axon terminals (Curley *et al.*, 2011). Importantly, these differences appear to reflect disease-related reductions in gene expression and not a deficit in the number of PV-containing neurons (Akbarian *et al.*, 1995b; Hashimoto *et al.*, 2003). In addition, mRNA levels of the neuropeptide SST, which is expressed in a separate subpopulation of GABA neurons, have also consistently been reported to be lower in the PFC of subjects with schizophrenia (Morris *et al.*, 2008; Mellios *et al.*, 2009; Fung *et al.*, 2010; Volk *et al.*, 2012a; Hashimoto *et al.*, 2008a). In contrast, mRNA levels for the calcium-binding protein CR, which is expressed in >40% of PFC GABA neurons that contain neither PV nor SST, are not lower in the PFC of individuals with schizophrenia (Hashimoto *et al.*, 2008a; Fung *et al.*, 2010; Hashimoto *et al.*, 2003; Volk *et al.*, 2012a).

Understanding the potential contributions of alterations in markers of GABA neurotransmission to working memory impairments in schizophrenia requires knowledge of whether they represent causes, consequences or confounds of the underlying disease process

(Lewis and Gonzalez-Burgos, 2008). The reported findings do not appear to be confounds due to either a non-specific down-regulation or general degradation of cortical mRNAs, since different GABA-related transcripts are lower, higher or unchanged in the illness (Duncan *et al.*, 2010; Fung *et al.*, 2010; Hashimoto *et al.*, 2008a; Hashimoto *et al.*, 2003; Benes and Berretta, 2001; Volk *et al.*, 2002). Other findings suggest that the observed transcript alterations are not attributable to confounding factors such as medications, substance use or other comorbid factors (Volk *et al.*, 2002; Curley *et al.*, 2011; Morris *et al.*, 2008; Hashimoto *et al.*, 2003).

Given that altered levels of GABA-related transcripts were observed in postmortem studies of schizophrenia subjects with varying illness duration, they could reflect the consequences of being chronically ill. If so, then the magnitude of the GABA-related transcript alterations would be expected to co-vary with illness duration. Alternatively, these alterations could be part of a causal developmental pathway leading to PFC dysfunction and working memory impairments in schizophrenia. For example, working memory performance and the associated activation of the PFC undergo a protracted maturation with adult levels of performance not achieved until late adolescence (Luna *et al.*, 2004). Interestingly, a recent longitudinal prospective cohort study reported that in individuals who were later diagnosed with schizophrenia working memory function did not differ from comparison subjects at age 7, but then failed to improve with age at the normal rate (Reichenberg *et al.*, 2010). Thus, disturbances in the developmental trajectories of GABA-related transcripts that occurred prior to the clinical onset of schizophrenia could be causal factors contributing to working memory impairments in the illness (Hoftman and Lewis, 2011).

In order to discriminate between the “chronic illness consequence” versus “developmental cause” explanations of altered GABA-related gene expression in schizophrenia,

we undertook two studies. First, we quantified levels of transcripts that either mediate GABA neurotransmission or are selectively expressed in specific subpopulations of cortical GABA neurons in the PFC from 42 matched pairs of schizophrenia and comparison subjects, and evaluated these data as a function of illness duration. Second, because it is not possible to assess the developmental trajectories of GABA-related transcripts in subjects with schizophrenia, we quantified the expression of these same transcripts in the PFC from 49 rhesus monkeys ranging in age from one week postnatal to adulthood. We then compared the developmental trajectory of each transcript with its expression status in schizophrenia to determine if a disturbance during development could account for the pattern of GABA-related transcript alterations seen in the illness.

2.2 METHODS

2.2.1 Human Studies

Brain specimens (n=84) were obtained during routine autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA) after consent was obtained from the next-of-kin. Consensus DSM-IV-TR diagnoses for each subject were made using structured interviews with family members and review of medical records (Volk *et al.*, 2012a); the absence of a psychiatric diagnosis was confirmed in healthy comparison subjects using the same approach. To control for experimental variance, subjects with schizophrenia or schizoaffective disorder (n=42) were matched individually to one healthy comparison subject for gender, and as closely as possible for age, and samples from both subjects in a pair were processed together throughout all

stages of the study. The mean age, postmortem interval (PMI), brain pH, RNA integrity number (RIN) (Agilent Bioanalyzer, Walbronn, Germany), and tissue storage time (TST) did not differ between subject groups **(Table 1; Appendix A1, Table 6)**. The University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research approved all procedures.

Table 1. Summary of demographic and postmortem characteristics of human subjects

Parameter	*Healthy comparison*	*Schizophrenia*
N	42	42
Sex	31 M/11 F	31 M/11 F
Race	34 W/8 B	29 W/13 B
Age (years)	48 ± 13	47 ± 13
Postmortem interval (hours)	17.8 ± 5.9	18.1 ± 8.7
Freezer storage time (months)	120 ± 44	121 ± 46
Brain pH	6.8 ± 0.2	6.6 ± 0.4
RIN	8.3 ± 0.6	8.2 ± 0.7

Note: M, male; F, female; W, white; B, black. For all, $t_{82}<2.0$, $P>0.05$. Values are group means ± standard deviation.

Frozen tissue blocks from each subject were confirmed to contain PFC area 9 using Nissl-stained tissue sections cut on a cryostat at 40 μm thickness (Volk *et al.*, 2000). Gray matter from adjacent sections was separately collected into a tube containing TRIzol reagent using a method that ensured minimal white matter contamination and excellent RNA preservation (Volk *et al.*, 2012a).

Levels of vGAT, GAT1, and $GABA_A$ α1 mRNAs were quantified by real-time quantitative PCR (qPCR) using previously described methods (Volk *et al.*, 2012a). The results of similar studies for GAD67, PV, CR, and SST mRNAs in the same cohort of subjects have been previously reported (Volk *et al.*, 2012a; Curley *et al.*, 2011). Briefly, qPCR was performed using

Power SYBR Green fluorescence and the StepOnePlus Real-Time PCR system (Applied Biosystems). Based on their stable relative expression levels between schizophrenia and comparison subjects (Hashimoto *et al.*, 2008a), three reference genes (β-actin, cyclophilin-A, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used to normalize the target gene expression levels. The amplification efficiency for each primer pair across a range of cDNA dilutions was greater than 96% for each of the target and reference genes, and dissociation curve analyses confirmed a single and specific amplification product. cDNA samples from both subjects in each pair were processed together in quadruplicate on the same PCR plate. The mean coefficient of variance (SD) of the replicate measures was less than 0.04 (0.02) for all transcripts. The delta CT (dCT) for each target transcript was calculated by subtracting the geometric mean of the reference genes from the CT of the target transcript. Since this dCT represents the $\log_2$-transformed expression ratio of each target transcript to the geometric mean of the reference genes, the relative expression level of the target transcript was calculated as 2^{-dCt} (Vandesompele *et al.*, 2002).

2.2.2 Monkey Studies

Forty-nine rhesus monkeys (*Macaca mulatta*) ranging in age from postnatal 1 week to 11.5 years were used. Animals were housed according to age as previously described (Erickson and Lewis, 2002). All housing and experimental procedures were conducted in accordance with the guidelines of the USDA and the NIH Guide for the Care of Animals, and with approval from the University of Pittsburgh's IACUC.

Twenty-one animals were perfused transcardially with ice-cold modified artificial cerebrospinal fluid following deep anesthesia induced with ketamine and pentobarbital; in 5 of

these animals, a small tissue block from the left principal sulcus had been surgically excised for in vitro electrophysiology studies 2-4 weeks prior to perfusion **(Table 2; Appendix A2, Table 7)**. The remaining 28 animals were experimentally naïve. After deep anesthesia with ketamine and pentobarbital was induced, the brains were removed. For all animals, the right hemisphere of each brain was blocked coronally and each block was frozen and stored at -80^{o}C.

Table 2. Summary of rhesus monkeys used in this study

Age Group	*Age Range (Mos.)*	*N*	*Female (%)*	*No. ACSF Perfused*	*No. Prior Biopsy*	*Tissue Storage Time (Mos.) Mean (SD)*
Perinatal	0.25-1	9	100	0	0	101 (30)
Childhood	3-9	13	100	5	0	62 (35)
Adolescence	15-37	17	100	10	3	24 (16)
Adult	42-138	10	90	6	2	38 (15)

Frontal pole sections (40 µm) composed entirely of gray matter were collected into tubes containing TRIzol reagent in a manner that ensured excellent RNA preservation (Hashimoto *et al.*, 2008b). Area 10 was used due to the availability of tissue from that cortical region for each animal. qPCR was conducted as described for the human study with the following differences. Primer sets were designed against the macaque sequences of the same seven target genes used in the human studies, as well as two reference genes, β-actin and cyclophilin-A, which exhibited stable relative expression levels across the postnatal developmental ages studied ($F_{7,41}$=1.594 p=0.164 in the present study, and as previously reported (Volk *et al.*, 2012b)). GAPDH was not used as a reference gene since its mRNA levels appeared to be unstable across early postnatal development (Volk *et al.*, 2012b).

2.2.3 Statistical Analyses

Two ANCOVA models were used to examine the effect of diagnosis on vGAT, $GABA_A$ α1 and GAT1 mRNA levels. The unpaired ANCOVA model included mRNA level as the dependent variable, diagnostic group as the main effect, and any relevant covariates that were significantly related to mRNA level. Since subjects were also paired to account for the parallel processing of tissue samples from a pair and to balance diagnostic groups for sex and age, a second paired ANCOVA model including subject pair as a blocking factor was also employed. Both models produced similar results for all transcripts, and results from the unpaired ANCOVA model are reported. There were no subject outliers and the differences in the reported degrees of freedom represent differences in the number of covariates used in a given analysis.

To explore whether the alterations in GABA-related transcript levels in schizophrenia subjects were a consequence of the disease process, the correlations between illness duration (the difference between a schizophrenia subject's age at death and age at clinical onset) and the age-adjusted percent difference (within-pair schizophrenia subject minus comparison subject) in expression of each mRNA transcript were computed. In addition, the age by diagnosis interactions were assessed using the unpaired ANCOVA model. If the age by diagnosis interaction was not significant, then the age effect was considered to be the same for both subject groups. If the age by diagnosis interaction was significant, the effects of age across the lifespan were examined using regression models to obtain computational estimates of age effects on GABA-related transcript expression before and after illness onset in the schizophrenia subjects. For the schizophrenia subjects, these models included the age at clinical onset, the illness duration, and relevant covariates, whereas for the comparison subjects the age effect was predicted to be constant over time. The model for schizophrenia subjects predicted the intercept

and slopes of the age effect before and after clinical onset where mRNA expression level at the age at death for schizophrenia subjects equaled the sum of the intercept β_0^s (i.e., transcript level at birth) and β_1^s*age at clinical onset and β_2^s*illness duration terms. The model for comparison subjects predicted the intercept and slope of the overall age effect, and mRNA levels for these subjects at death equaled the sum of the intercept β_0^c and β^c*age at death **(Appendix B, Figure 22)**. Hypothesis testing was conducted to determine whether the rate of transcript level decline before clinical onset was different from the rate of decline after clinical onset: H_0: $\beta_1^s=\beta_2^s$; H_A: $\beta_1^s \neq \beta_2^s$.

The relationship of the within-subject pair differences for transcript levels to each of the following potential confounds were examined by two sample t-tests: gender, diagnosis of schizoaffective disorder, death by suicide, substance use diagnosis at time of death, or antidepressant, antipsychotic, or benzodiazepine use at time of death. Medication use at time of death was determined from the results of toxicology testing and and/or records of prescriptions active at the time of death. Subjects with a documented history of medication non-adherence and negative toxicology screen were assumed to not be taking medications at time of death.

All analyses were implemented in SAS PROC GLM (Version 9.2, SAS Institute, Inc., Cary, NC), and all tests were conducted at 0.05 level.

For the monkey studies, the correlations between age and each mRNA level were determined using Pearson's correlation coefficient. A second analysis used an ANCOVA model in which animals were placed into one of four age groups based on existing data regarding probable inflection points in the maturation of primate PFC circuitry. Such inflection points have been best studied for the postnatal development trajectory of excitatory synapse density, which exhibits four distinct phases in both macaque and human PFC (Bourgeois *et al.*, 1994;

Huttenlocher, 1979; Petanjek *et al.*, 2011). Therefore, each animal in the present study was assigned to one of the following four age groups: 1) perinatal, 0.25-1 mo of age, within the period of a rapid increase in excitatory synaptic density; 2) childhood, 3-9 mo, within the period when the density of excitatory synapses is at a plateau; 3) adolescence, 15-37 mo, within the period of excitatory synapse pruning; and 4) adult, 42-138 mo, during the period when the density of excitatory synapses is at stable adult levels. Thus, for the purposes of this manuscript, a developmental trajectory refers to the pattern of change in the level of a transcript over postnatal development from 1 week of age to mid-life, the approximate age range of the available monkey cohort. The ANCOVA model for gene expression used age group as a factor and TST as a covariate. Tukey tests with $\alpha=0.05$ were conducted for post hoc comparisons between age groups.

2.3 RESULTS

2.3.1 GABA-related transcript expression in schizophrenia

In PFC area 9, levels of vGAT (-7%, $F_{1,78}$=4.13, p=0.046) and $GABA_A$ α1 mRNAs (-6%, $F_{1,78}$=8.29, p<0.01) were significantly lower in the subjects with schizophrenia, but GAT1 mRNA levels did not differ ($F_{1,78}$=0.39, p=0.53) between groups **(Figure 7A-C)**. We found that vGAT ($F_{1,78}$=5.96, p=0.02) and GAT1 ($F_{1,78}$=5.72, p=0.02) mRNA levels were negatively associated with TST. No other covariates were related to mRNA levels or significantly differed between diagnostic groups **(Table 1)**, and thus were not included in the reported statistical models. Some of these subject pairs were included in previous studies of $GABA_A$ α1 and GAT1 mRNA levels (Volk *et al.*, 2001; Beneyto *et al.*, 2011) using *in situ* hybridization **(Appendix A1, Table 6)**. In the newly studied schizophrenia subject cohort (n=19 pairs) alone, $GABA_A$ α1 mRNA levels were still significantly lower (-4.4%, $F_{1,32}$=5.38; p=0.03), and GAT1 mRNA expression still did not differ in schizophrenia subjects ($F_{1,32}$=0.04; p=0.85) from comparison subjects. Levels of vGAT, $GABA_A$ α1 and GAT1 mRNAs did not differ as a function of gender, diagnosis of schizoaffective disorder, death by suicide, substance use diagnosis at time of death, or antidepressant, antipsychotic, or benzodiazepine use at time of death (all |t|<1.96, p>0.05). In this same cohort of 42 subject pairs using the same qPCR method, mRNA levels for GAD67, PV, and SST were lower, and mRNA levels for CR were higher, in the subjects with schizophrenia (Curley *et al.*, 2011; Volk *et al.*, 2012a).

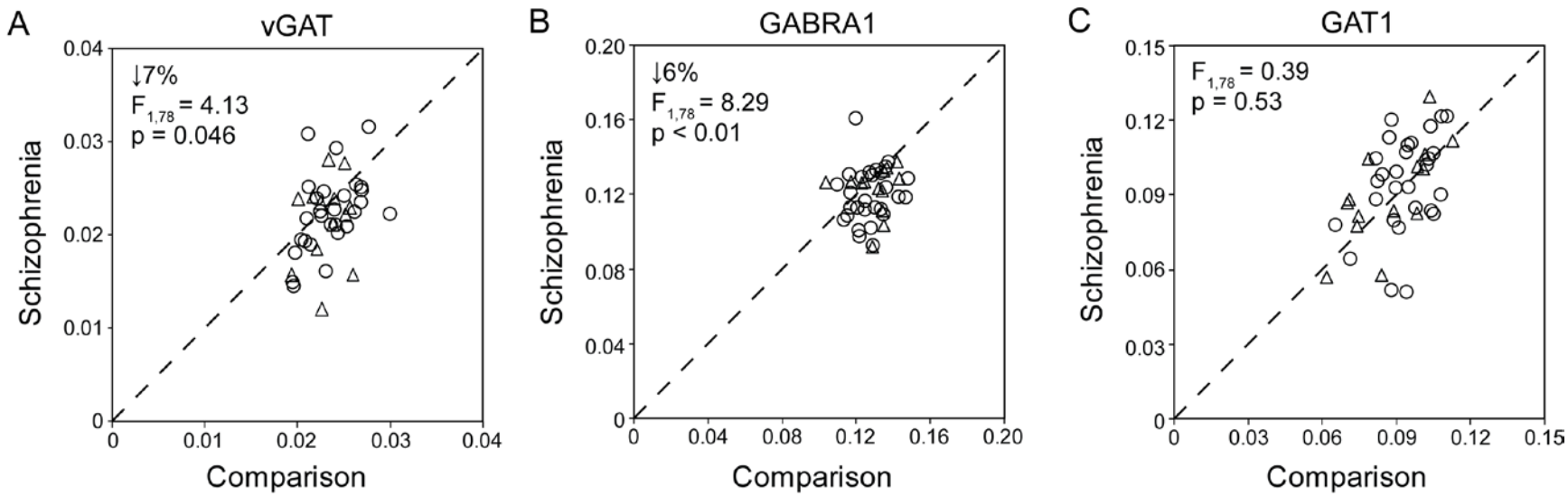

Figure 7. GABA-related mRNA levels in PFC from schizophrenia and comparison subjects.

vGAT (A), GABRA1 (B), and GAT1 (C) mRNA levels in PFC from schizophrenia and comparison subjects. Scatter plots show the transcript levels for each matched pair of a comparison and either a schizophrenia (circles) or schizoaffective disorder subject (triangles). X- and Y-axes indicate the transcript expression ratio (2^{-dCT}) for the comparison and schizophrenia (or schizoaffective) matched subject pairs. Pairs below the dashed unity line have lower transcript levels in the schizophrenia (or schizoaffective) subject relative to the comparison subject.

We next determined whether levels of GABA-related transcripts altered in schizophrenia were correlated with illness duration. The age-adjusted, within-pair, percent difference in transcript expression was plotted against illness duration for each schizophrenia subject. The age-adjusted percent differences of the expression levels of all transcripts—GAT1 excluded since it did not differ between schizophrenia and comparison subjects—did not significantly correlate with illness duration (all $|r| < 0.270$; $p > 0.08$) **(Figure 8)**. In addition, the age-adjusted percent differences of the expression levels for these same transcripts did not significantly correlate with age at illness onset (all $|r| < 0.250$; $p > 0.12$).

We further examined if any GABA-related transcripts altered in schizophrenia exhibited an age-related effect that differed between diagnostic groups. There was no significant age by diagnosis interaction on mRNA levels for all GABA-related markers studied (all $F_{1,77} < 1.60$, $p > 0.21$), with the exception of GAD67 ($F_{1,77} = 4.27$; $p = 0.04$) **(Figure 9)**.

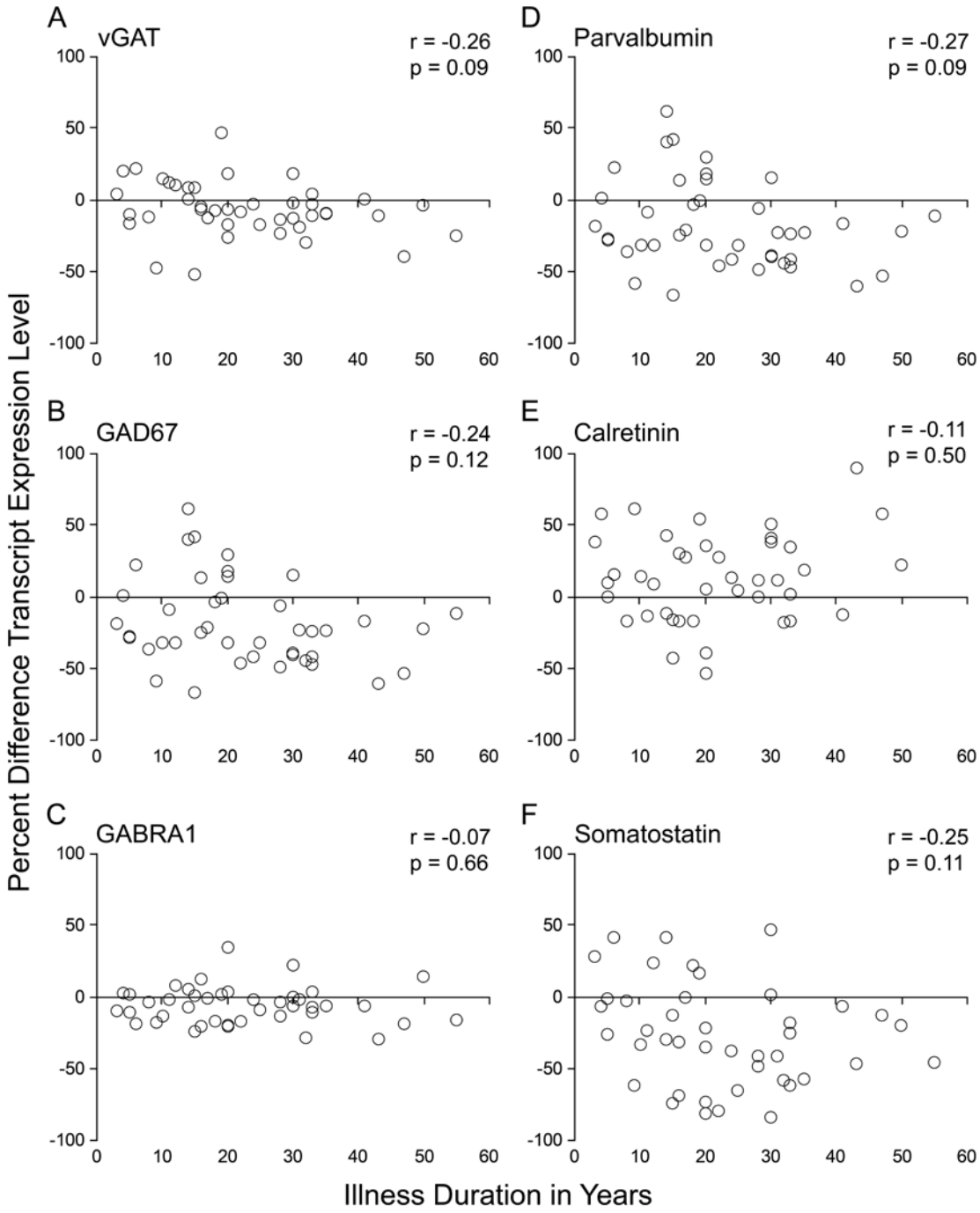

Figure 8. Within-pair percent difference (schizophrenia - comparison subject) in PFC transcript levels versus illness duration in schizophrenia subjects.

Pearson's correlation coefficient (r) was not statistically significant for any of the GABA-related mRNA levels examined that were altered in schizophrenia (A-F). For each panel, each matched pair of schizophrenia and comparison subjects is shown as an open black circle.

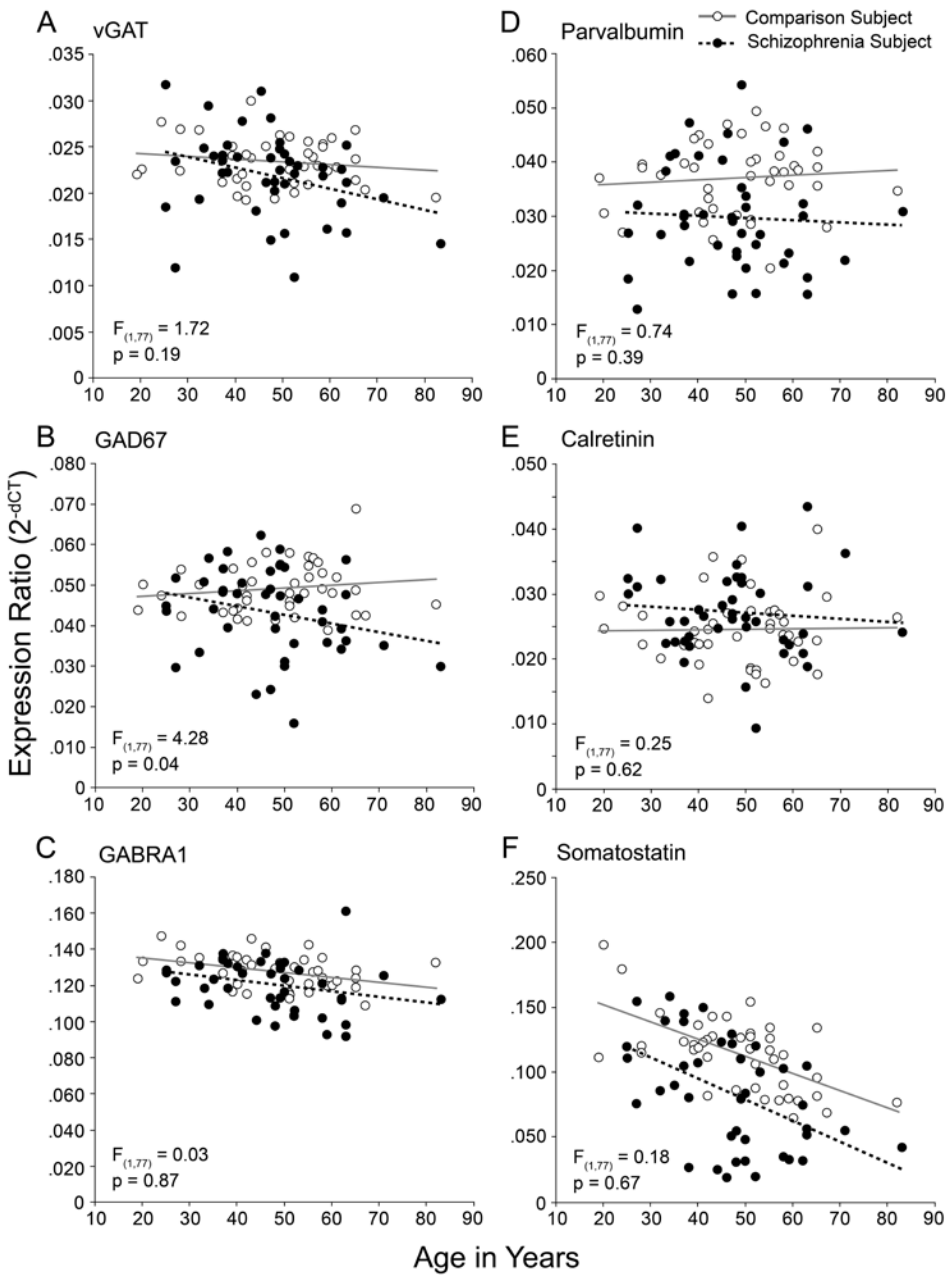

Figure 9. Interaction of age by diagnosis on GABA-related mRNA levels in the PFC of schizophrenia subjects.

Aside from GAD67 (B), the age by diagnosis interactions were not statistically significant for any of the GABA-related mRNA levels examined that were altered in schizophrenia (A, C-F). For each panel, comparison subjects are shown in open black circles and schizophrenia subjects in filled black circles. Gray solid and black dashed lines indicate lines of best fit for comparison and schizophrenia subjects, respectively. F- and p-values represent the age by diagnosis interaction statistics.

In schizophrenia subjects, the potential age by diagnosis interaction on GAD67 mRNA levels could reflect an additive effect of illness duration in the schizophrenia subjects. Thus, the effects of age on GAD67 mRNA expression were evaluated in schizophrenia subjects using a regression model to estimate the age effect on GAD67 mRNA expression before and after clinical onset **(2.2.3)**. In schizophrenia subjects, the estimated values of the slopes of the change in GAD67 mRNA with age did not differ before ($\hat{\beta}_1^S$=-0.000026) and after ($\hat{\beta}_2^S$=-0.00025) clinical onset (p=0.44), suggesting that age has a constant effect on GAD67 mRNA levels in schizophrenia subjects.

Together, our findings suggest that changes in GABA-related transcripts in schizophrenia are not a consequence of illness chronicity.

2.3.2 Postnatal trajectories of GABA-related transcripts in monkey PFC

To investigate the alternative possibility that altered cortical GABA-related mRNA levels reflect abnormalities in development that occurred before clinical illness onset, we determined the normative postnatal developmental trajectories of these GABA-related transcripts in monkey PFC. For markers of GABA neurotransmission, mRNA levels were positively correlated with age during postnatal development for vGAT (r=0.308, p=0.031), GAD67 (r=0.285, p=0.047), and $GABA_A$ α1 (r=0.592, p<0.001) mRNAs, and did not change with age for GAT1 mRNA (r=0.111, p=0.449) **(Figure 10A-D)**. The mean mRNA levels in the adult group were significantly higher than in the perinatal group for vGAT (+24%), GAD67 (+46%), $GABA_A$ α1 (+78%), but not for GAT1 **(Figure 10E-H)**. For markers of GABA neuronal populations, mRNA levels were positively correlated with age for PV (r=0.378, p=0.007), negatively correlated with

age for SST (r=-0.416, p=0.003), and did not differ with age for CR (r=-0.165, p=0.256) **(Figure 11A-C)**. The mean levels in the adult group were significantly higher than in the perinatal group for PV (+790%), lower for SST (-33%) and did not differ for CR **(Figure 11D-F)**.

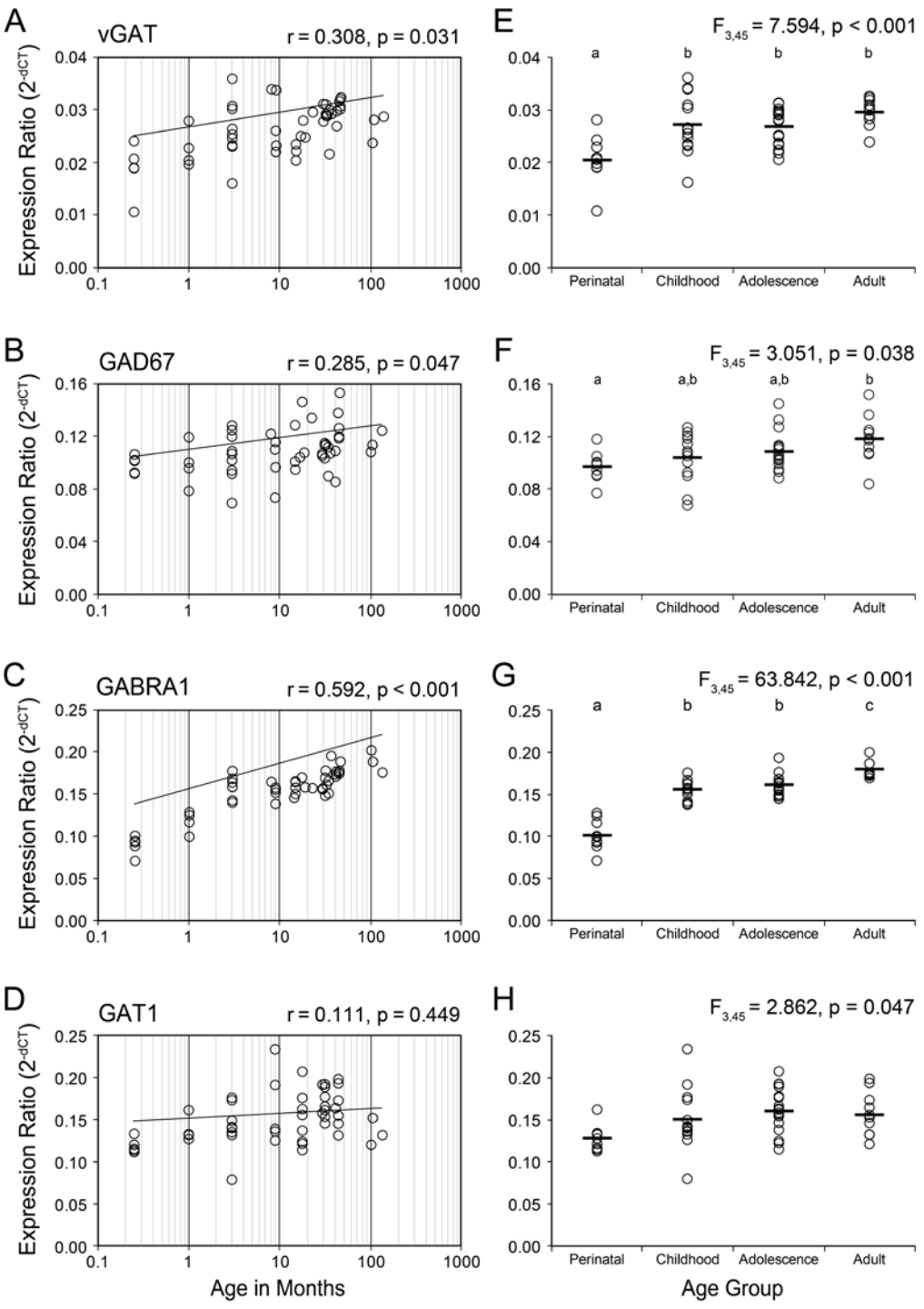

Figure 10. Postnatal developmental trajectories of transcripts regulating GABA neurotransmission in rhesus monkey PFC.

For panels A-D, the black line indicates least squares line of best fit; and Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. For panels E-H, the black bars indicate group means. Age groups that do not share the same letters are significantly different ($p<0.05$).

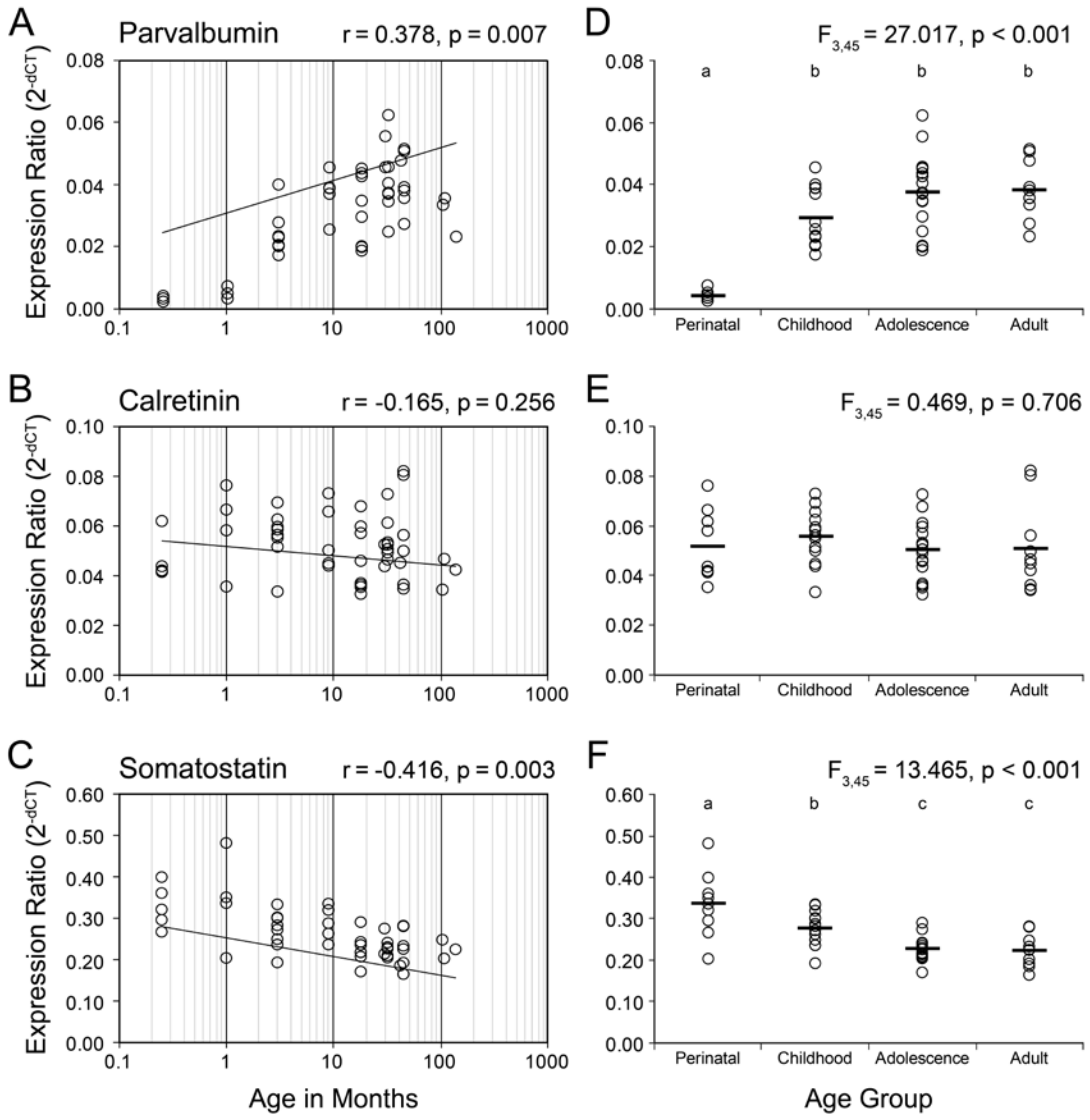

Figure 11. Postnatal developmental trajectories of GABA neuronal populations in rhesus monkey PFC.
For panels A-C, the black line indicates least squares line of best fit; and Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. For panels D-F, the black bars indicate group means. Age groups that do not share the same letters are significantly different ($p<0.05$).

Given that a number of environmental exposures from birth through adolescence are associated with increased risk for schizophrenia (van Os *et al.*, 2010), and may have a particularly strong influence on neural circuits during sensitive periods of cortical development (Hensch, 2005), we determined when during postnatal development transitions in the expression levels of GABA-related mRNAs might occur. From the perinatal to childhood period, vGAT (+32%), PV (+580%), and $GABA_A$ α1 (+54%) mRNA levels increased significantly **(Table 3)**. $GABA_A$ α1 mRNA expression also increased significantly from adolescence to adulthood (+12%), albeit more modestly than the earlier rise. GAD67 mRNA increased progressively from the perinatal to adult periods, although most of the rise occurred during the perinatal to adolescence periods (+12%). SST mRNA decreased significantly from the perinatal to childhood and childhood to adolescence periods (-17% and -18%, respectively). Taken together, the majority of GABA-related transcript expression changes were most prominent during the perinatal to childhood age periods in monkey PFC.

Table 3. Timing of the largest changes in PFC GABA mRNA levels

	Magnitude of change		
Gene	*Perinatal to childhood*	*Childhood to adolescence*	*Adolescence to adult*
vGAT	↑32%	--	--
PV	↑580%	--	--
GABRA1	↑54%	--	↑12%
SST	↓17%	↓18%	--
GAD67*	--	--	--
GAT1	--	--	--
CR	--	--	--

*GAD67 mRNA levels increased continuously during the developmental periods studied, only reaching significance between the perinatal and young adult age groups. The majority of the rise in GAD67 mRNA levels occurred from the perinatal through adolescence developmental epochs.

2.4 DISCUSSION

We report modest but significantly lower tissue levels of vGAT and $GABA_A$ α1 mRNAs in the PFC of schizophrenia subjects. Given these and other recently reported GABA-related transcript abnormalities from the same subject cohort (Curley *et al.*, 2011; Volk *et al.*, 2012a), we sought to determine whether altered expression of GABA-related mRNAs in schizophrenia could reflect the consequences of a chronic illness. Our analyses suggest that neither factors frequently comorbid with a diagnosis of schizophrenia nor duration of clinical illness accounted for the observed alterations in GABA-related gene expression.

To explore an alternative possibility that alterations in cortical GABA-related transcripts in schizophrenia might reflect developmental disturbances, we determined the postnatal developmental trajectories of GABA-related transcripts in the PFC from healthy rhesus monkeys. From birth through adulthood the levels of vGAT, GAD67, $GABA_A$ α1 and PV mRNAs significantly increased, SST mRNA significantly decreased, and GAT1 and CR mRNAs remained unchanged. These developmental patterns generally matched those previously reported in human PFC (Huang *et al.*, 2007; Duncan *et al.*, 2010; Fung *et al.*, 2010).

Interestingly, for most transcripts the change in levels with development was in the opposite direction of the difference observed between schizophrenia and comparison subjects. For example, levels of vGAT, GAD67, $GABA_A$ α1 and PV mRNAs all significantly increased during postnatal development, and subjects with schizophrenia showed lower expression of these transcripts. In contrast, CR mRNA levels decrease significantly during postnatal development in human PFC (Fung *et al.*, 2010) and were recently reported to be higher in subjects with schizophrenia (Volk *et al.*, 2012a). Finally, transcripts, such as GAT1, that did not change in expression level across postnatal development were not altered in schizophrenia. Together, these

comparisons suggest the hypothesis that expression patterns of GABA-related transcripts in schizophrenia reflect blunted or incomplete developmental trajectories, resulting in a failure to achieve normal, mature mRNA levels.

This idea of an incomplete maturation of cortical GABA-related transcripts in schizophrenia is supported by previous findings in the literature. For example, the $GABA_A$ α2 subunit declines across postnatal development in monkey PFC (Hashimoto *et al.*, 2009) and is higher in the PFC of schizophrenia subjects (Beneyto *et al.*, 2011), whereas the $GABA_A$ δ subunit shows exactly the opposite pattern in development and in schizophrenia (Maldonado-Aviles *et al.*, 2009). In addition, the μ-opioid receptor, which is selectively expressed by parvalbumin neurons (Drake and Milner, 1999; Drake and Milner, 2002; Stumm *et al.*, 2004) and suppresses GABA release when activated (Drasbek *et al.*, 2007; Drasbek and Jensen, 2006), declines with postnatal development and is higher in schizophrenia (Volk *et al.*, 2012b). Furthermore, expression patterns of alternatively-spliced transcripts of the GAD1 gene are also consistent with this hypothesis; GAD67 mRNA increases and GAD25 mRNA decreases normally during early postnatal development of human PFC, but in schizophrenia subjects, the relative levels of GAD67 and GAD25 mRNAs were lower and higher, respectively, than in matched comparison subjects (Hyde *et al.*, 2011).

However, not all GABA-related transcripts with altered expression in schizophrenia fit the pattern predicted by the incomplete maturation hypothesis. For example, SST mRNA levels are lower in the PFC of subjects with schizophrenia (Hashimoto *et al.*, 2008a; Fung *et al.*, 2010) but this transcript undergoes a pronounced decline with age that begins during early postnatal development and persists throughout adulthood both in macaques and humans (Fung *et al.*, 2010). The fact that cortical SST mRNA levels are also lower in other psychiatric disorders

(Sibille *et al.*, 2011) suggests that the altered expression of this transcript in schizophrenia may be driven by factors that are common to these disease processes and thus does not reflect early developmental events.

In the present study, four of the five cortical GABA transcripts that changed during postnatal development showed the most notable changes in expression levels during the perinatal to childhood transition **(Table 3)**. A number of perinatal and childhood environmental exposures such as birth complications and urbanicity have been associated with an increased risk for developing psychosis (van Os *et al.*, 2010; Lewis and Levitt, 2002). We also observed increases in GAD67 and $GABA_A$ α1 mRNA levels during adolescence in monkeys, and a potential sensitive period in PFC development when factors such as cannabis use might contribute to an increased liability to schizophrenia (Moore *et al.*, 2007). Interestingly, GABA neurons are involved in shaping the maturation of cortical circuits during these same developmental periods (Le Magueresse and Monyer, 2013). In concert, these data suggest that PFC development encompasses multiple sensitive periods during which adverse environmental factors could pathologically alter the developmental trajectories of GABA neurons in individuals later diagnosed with schizophrenia.

The idea that GABA-related transcripts might be particularly vulnerable is consistent with findings that GABA neurotransmission can powerfully affect critical or sensitive periods in cortical development (Hensch, 2005; Le Magueresse and Monyer, 2013). For example, the timing of the critical period for the development of binocular vision can be manipulated by the enhancement or reduction of GABA transmission in visual cortex (Hensch, 2005), especially via $GABA_A$ α1-containing neurons (Fagiolini *et al.*, 2004) postsynaptic to PV-containing GABA neurons (Nusser *et al.*, 1996). These findings, along with our results that show substantial

increases in $GABA_A$ α1 and PV mRNA levels during postnatal development, suggest that PV-containing inputs to $GABA_A$ α1-containing postsynaptic structures may be especially susceptible to environmental exposures during development. Therefore, our results of lower $GABA_A$ α1 and PV (Volk *et al.*, 2012a) mRNA levels in schizophrenia may reflect earlier disturbances in specific neural circuits during sensitive periods of postnatal development.

It is important to note that not all of the findings of the present study are in agreement with some reports in the literature. For example, recent postmortem studies (Duncan *et al.*, 2010; Fung *et al.*, 2011a) using a different subject cohort failed to find the significantly lower levels of vGAT and $GABA_A$ α1 mRNAs observed in the present study. This discrepancy could reflect differences in subject composition of the cohorts since we recently reported that altered expression of GABA-related transcripts is particularly marked in a subset of individuals with schizophrenia (Volk *et al.*, 2012a). In addition, the absence of a change during postnatal development in calretinin mRNA in monkey PFC is not consistent with the reported trajectories of calretinin mRNA and protein in human PFC (Fung *et al.*, 2010). Since CR is expressed in ~40% of GABA neurons in the macaque and human PFC, and these GABA neurons constitute a morphologically heterogeneous population of neurons, species-specific differences in the development and adult distribution of calretinin levels are possible (Hof *et al.*, 1999).

In concert, our findings, and those of other published studies, suggest that the altered expression of certain GABA-related transcripts in the PFC of subjects with schizophrenia are not the consequence of cumulative illness effects and may be due to blunted or incomplete developmental trajectories of these transcripts. Together, these findings provide another line of evidence supporting the idea that schizophrenia is a disorder of cognitive development, and they

provide clues as to what molecular systems might be targeted for preemptive interventions in at risk individuals, and when such interventions might be most effective.

3.0 REPEATED THC EXPOSURE DURING ADOLESCENCE INDUCES ALTERATIONS IN MARKERS OF GABA TRANSMISSION IN MONKEY PREFRONTAL CORTEX

Hoftman GD, Bazmi HH, Lewis DA. Alterations in markers of GABA transmission in the prefrontal cortex from THC-treated monkeys. In Preparation.

3.1 INTRODUCTION

Marijuana (and other forms cannabis) is, by far, the most commonly used illicit drug in America. In 2012, 15% of 8th graders (13-14 year olds), 34% of 10th graders (15-16 year olds), and 45% of 12th graders (17-18 year olds) reported lifetime use of marijuana (Johnston L. D., 2013). In addition, the perception that cannabis can be harmful has decreased, the accessibility increased, and use has increased among adolescents (Johnston L. D., 2013). Interestingly, the administration of Δ^9-tetrahydrocannibinol (THC), the principal psychoactive component in cannabis (Freund *et al.*, 2003), impairs working memory performance in adults (Volkow *et al.*, 1996; D'Souza *et al.*, 2004). Since performance of spatial working memory tasks becomes substantially more accurate and faster in humans during adolescence (Luna *et al.*, 2004), this growing population of cannabis users (Johnston L. D., 2013) may be particularly sensitive to the effects of THC on working memory development. Findings from epidemiological studies support

this idea showing repeatedly that cannabis use during early adolescence is associated with a dose-dependent increased risk for schizophrenia (Henquet *et al.*, 2005a; Stefanis *et al.*, 2004; Arseneault *et al.*, 2002; van Os *et al.*, 2002; Zammit *et al.*, 2002). In addition, chronic exposure of immature animals to cannabinoids results in long-lasting alterations in working memory (Schneider, 2003 #1218; O'Shea *et al.*, 2004), which is partly mediated by the PFC (Goldman-Rakic, 1995). Thus, understanding the biological mechanisms of this relationship is an important public health issue.

Adolescence is a period of development that is associated with refinements in PFC neural circuits that are correlated with improvements in the manipulation of information during working memory tasks (Luna, 2009). The neural basis for the protracted maturation of working memory performance has been attributed, at least in part, to age-related increases in dorsolateral PFC activity (Goldman-Rakic, 1995). The development of spatial working memory and PFC activity temporally parallels the structural maturation of PFC circuitry during childhood and adolescence, with dynamic changes occurring at GABA neuron connections (Cruz *et al.*, 2003; Cruz *et al.*, 2009a; Erickson and Lewis, 2002). For example, mRNA levels of GAD67 and GAD65 have been reported to increase throughout childhood and adolescence in primate PFC (Huang *et al.*, 2007; Hyde *et al.*, 2011). Levels of PV mRNA also increased during the infancy-childhood transition **(Figure 11)**. In the monkey PFC, CB1R are enriched at CCKb axon terminals (Eggan *et al.*, 2010; Freund *et al.*, 2003), which make direct synaptic connections with PVb (Karson *et al.*, 2009). In concert, these findings suggest that adolescence may be a sensitive period for the effects of chronic cannabis exposure on maturing PFC GABA circuitry.

Recently, our group reported adverse residual effects (from 7 hours to 20 days; versus acute effects within 6 hours of exposure) of repeated THC exposure on spatial working memory

in adolescent monkey PFC. Importantly, these effects occurred at doses that mimic human recreational use of cannabis (Verrico *et al.*, 2012). Therefore, we tested the hypothesis that chronic THC exposure during adolescence results in the disruption of GABA-related gene expression. Using quantitative real-time PCR from adolescent monkey PFC tissue, we report that GAD65 mRNA expression was higher by 15% in THC-treated animals relative to vehicle-treated matched comparisons, and PV mRNA was lower by 19%. In contrast, levels of GAD67, CB1R and CCK mRNAs did not differ between groups.

3.2 METHODS

3.2.1 Animals

Fourteen experimentally naïve, male rhesus macaque (*Macaca mulatta*) monkeys were acquired as a single cohort. Since spatial working memory performance increasingly becomes dependent upon PFC activity beginning at 12 months of age and continuing through ~36 months of age, animals were trained to achieve comprehension on two working memory tasks by 28 months of age (Verrico *et al.*, 2011). These animals were housed as previously described in accordance with the USDA and NIH Guidelines for Animal Care and with the approval of the University of Pittsburgh's IACUC.

Behavioral tasks and intravenous administration of THC have been described in detail elsewhere (Verrico *et al.*, 2012; Verrico *et al.*, 2011). Briefly, animals were trained to respond to computerized touchscreens and to perform a spatial delay response task and an object recall working memory task. These two tasks were chosen since spatial working memory performance

depends upon the dorsolateral PFC, which matures later than the ventrolateral PFC (Conklin *et al.*, 2007; Paus, 2005), while object recall performance depends upon the ventrolateral PFC, (Wilson *et al.*, 1994; Funahashi *et al.*, 1989; Goldman *et al.*, 1971; Goldman and Rosvold, 1970). The fourteen trained male adolescent monkeys were matched for baseline cognitive performance and assigned to a THC- or vehicle-treatment group. THC was suspended in a vehicle containing 1% Tween-80 and 0.9% saline (Verrico *et al.*, 2012). THC/vehicle administration was initiated at ~28 months of age and doses of either 120 (n=3) or 240 (n=4) μg/kg were administered daily for 5 days each week and continued until one month before the animals were sacrificed.

3.2.2 Tissue

Postpubertal animals ranging in age from 40-44 months of age **(Table 4)** were perfused transcardially with ice-cold modified artificial cerebrospinal fluid following deep anesthesia induced with ketamine and pentobarbital and the brains were removed. For all animals, the right hemisphere of each brain was blocked coronally and each block was frozen and stored at -80^{o}C.

Table 4. Adolescent male rhesus monkeys used in the THC study

Pair	Subject ID	Sex	Age at Sacrifice (Months)	Treatment
1	MJ219	M	41	Vehicle
	MJ315	M	40	THC
2	MJ401	M	41	Vehicle
	MJ320	M	41	THC
3	MJ324	M	42	Vehicle
	MJ327	M	41.5	THC
4	MJ508	M	41	Vehicle
	MJ421	M	41	THC
5	MJ407	M	42.5	Vehicle
	MJ522	M	41	THC
6	MJ607	M	42	Vehicle
	MJ512	M	42	THC
7	MJ523	M	43.5	Vehicle
	MJ505	M	44	THC

Gray matter from adjacent sections containing the principal sulcus from PFC area 46 was separately collected into a tube containing TRIzol reagent using a method that ensured minimal white matter contamination and excellent RNA preservation (Volk *et al.*, 2012a). Primer sets were designed against the macaque sequences of GAD65, PV, GAD67, CB1R and CCK, as well as three reference genes, β-actin, cyclophilin-A, GAPDH, which exhibited stable relative expression levels across in both subject groups. qPCR reactions were carried out using the SYBR 1 Green reporter as described in **(2.2.1 and 2.2.2)**.

3.2.3 Statistics

Paired t-tests were used to analyze the experimental group mean differences of the target transcript expression ratio. Pearson's correlation coefficients were calculated to determine whether a linear relationship existed between changes in GAD65 and PV mRNA levels.

3.3 RESULTS

In PFC area 46 from postpubertal animals, we found that levels of GAD65 mRNA were higher by 16% (t_6 = -3.971; p = 0.007) in experimentally matched pairs of THC-treated animals (reported as expression ratio mean ± SD; 0.0028 ± 0.0003) relative to vehicle-treated comparisons (0.0024 ± 0.0002). Levels of PV mRNA were lower by 18%, but did not reach statistical significance (t_6 = 1.12; p = 0.31) in THC-treated (0.054 ± 0.011) versus vehicle-treated (0.045 ± 0.016) subjects. However, closer examination revealed that in Pair 3, PV mRNA levels

were much higher in the THC-treated animal than its matched comparison. Furthermore in Pair 3, GAD65 mRNA levels did not differ between the THC-treated and comparison animals. An additional paired t-test analysis performed excluding pair 3 showed that GAD65 mRNA levels were higher by 19% (t_5 = -6.03; p = 0.002) and PV mRNA levels were lower by 30% (t_5 = 2.46; p = 0.058) in THC-treated animals (GAD65: THC-treated = 0.0028 ± 0.0002; vehicle-treated = 0.0023 ± 0.0003; PV: THC-treated = 0.040 ± 0.012; vehicle-treated = 0.056 ± 0.011) **(Figure 12)**. In contrast, levels of GAD67, CB1R and CCK mRNAs did not significantly differ (All t_6 < 0.53; p > 0.61) between experimental groups, with or without Pair 3 included in the analysis.

In order to determine whether a relationship existed between GAD65 and PV mRNA expression, we examined Pearson's correlations between them. The within-pair percent differences of GAD65 and PV mRNA levels were significantly negatively correlated (r=-0.768; p=0.044) **(Figure 13)**.

In chapter 2, we reported the postnatal developmental trajectories of PV and GAD67 mRNA in monkey PFC. Here in the same PFC region from the same animals, we report that GAD65 mRNA levels increase across postnatal development, while CCK mRNA levels did not differ across the ages studied **(Figure 14)**.

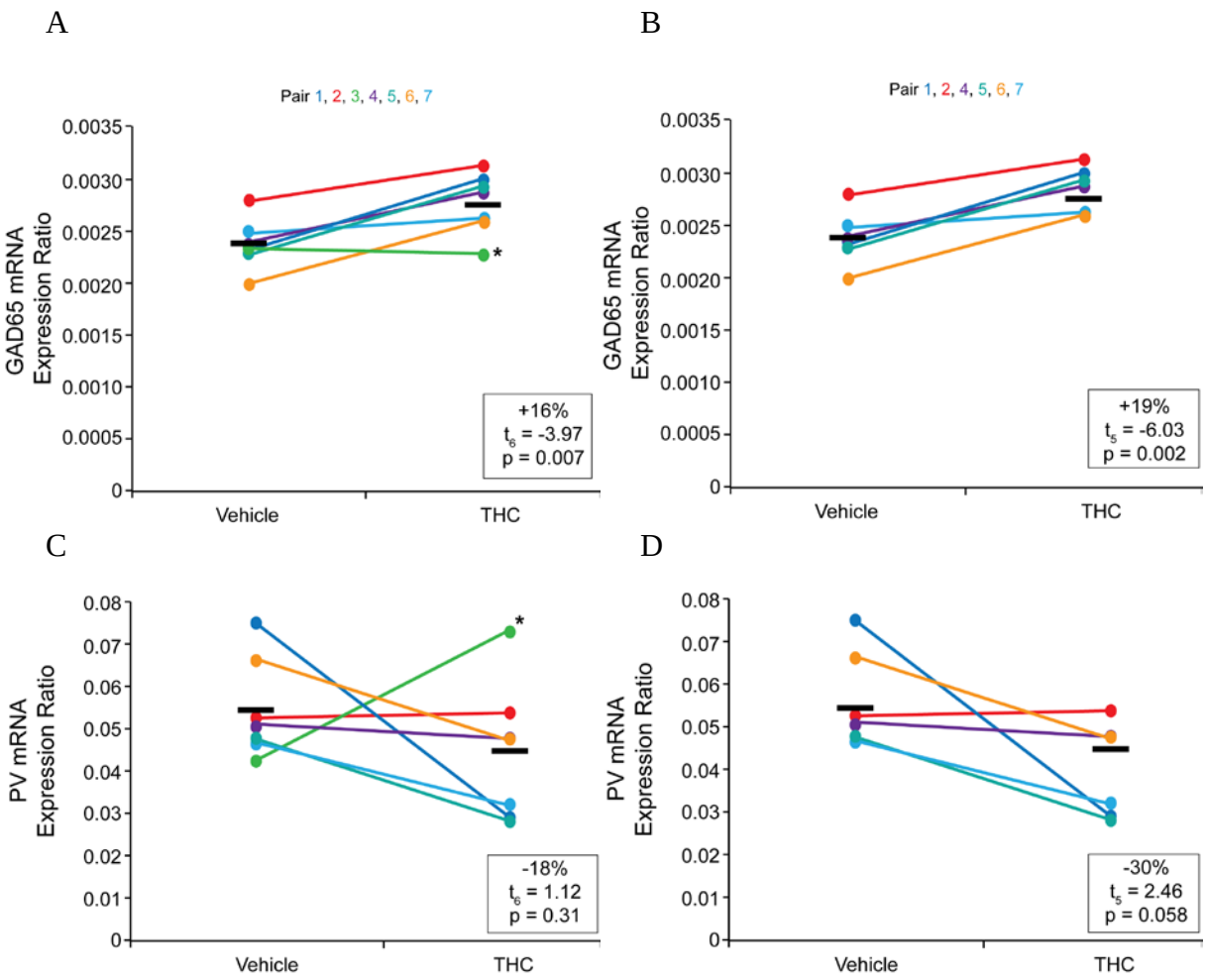

Figure 12. Higher GAD65 and lower PV mRNA levels in the PFC in THC-treated animals

Panels **(A)** and **(B)** GAD65 mRNA levels with 7 and 6 pairs, respectively. Panels **(C)** and **(D)** PV mRNA levels with 7 and 6 pairs, respectively. Paired t-tests were used to determine statistical significance. Asterisk **(*)** indicates the animal pair **(pair 3)** in which the THC-treated subject had higher PV mRNA levels by 70% and was thus excluded from the statistical analysis in panels **(B)** and **(D)**.

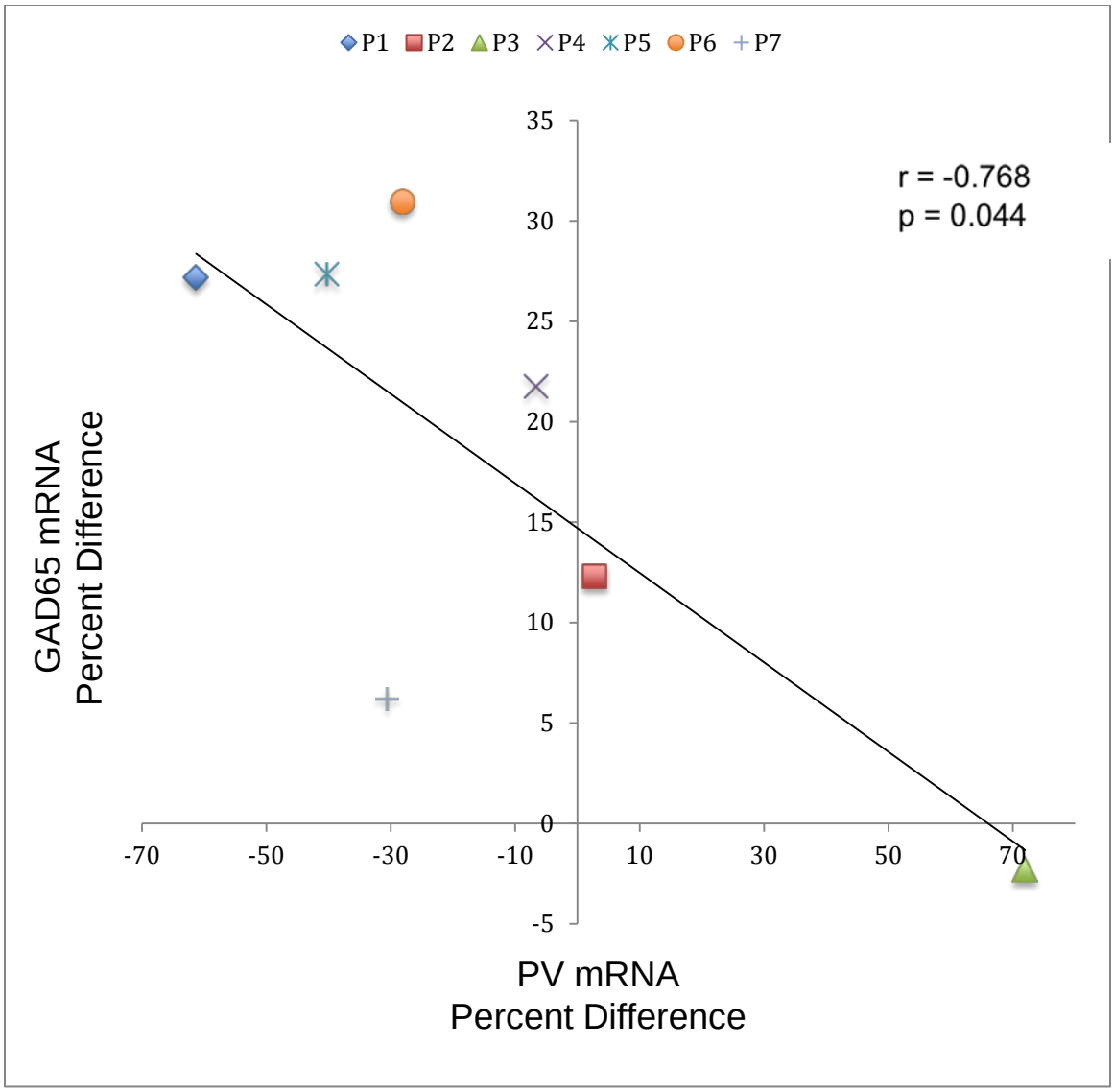

Figure 13. Within-pair percent differences in GAD65 and PV mRNA levels is significantly correlated

Pearson's correlation coefficient was used to determine the correlation. For analysis not including pair 3, r = -0.567 and p = 0.055. P1 represents an abbreviation for Pair 1 of cannabis-treated and vehicle-treated animals. Same goes for P2-P7.

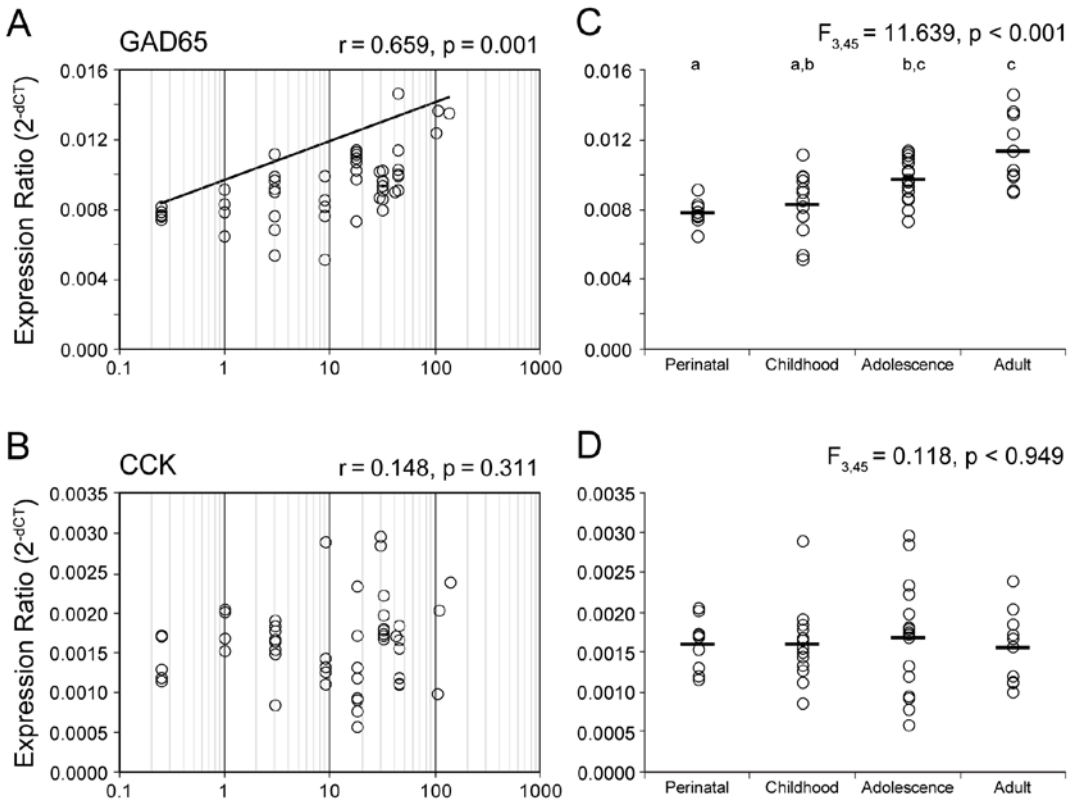

Figure 14. GAD65 mRNA levels increase and CCK mRNA levels do not significantly change during postnatal development in monkey PFC

In **(A)** and **(B)**, the black line indicates least squares line of best fit; and Pearson's correlation coefficient (r) and corresponding p-value are indicated for each panel on the left. In **(C)** and **(D)**, the black bars indicate group means. Age groups that do not share the same letters are significantly different ($p<0.05$).

3.4 DISCUSSION

In the dorsolateral PFC area 46 in post-pubertal monkeys, we found that the residual effects of repeated THC administration to adolescent monkeys resulted in a 15% increase in GAD65 mRNA levels, and a 19% decrease in PV mRNA levels. In contrast, mRNA levels of GAD67,

CB1R and CCK did not differ between THC-treated animals and their matched vehicle-treated comparison subject pairs. The within-pair percent differences in GAD65 and PV mRNA levels were correlated such that cases where the THC-treated animal had higher GAD65 mRNA levels also tended to have lower PV mRNA levels relative to the vehicle-treated animal.

These same animal pairs had performed spatial delay response and object recognition working memory tasks throughout the course of THC/vehicle-treatment (Verrico *et al.*, 2012; Verrico *et al.*, 2011). THC-treated animals were less accurate with their responses on the spatial delay response task relative to their matched comparison animals, and their developmental improvement in performance was also delayed (Verrico *et al.*, *American Journal of Psychiatry*, In Revision). In contrast, both animal groups performed similarly on the object recognition working memory task. Our findings of a change in GABA-related gene expression that accompanied a delay in accuracy on the spatial working memory task suggest that dorsolateral PFC GABA neuron alterations in response to chronic THC administration may underlie the poorer performance in these animals. However, given the large variability in behavioral performance across sessions and between monkey pairs, a direct correlational assessment of the behavioral measures with GAD65 and PV mRNA levels was not possible.

The functional consequences of increased GAD65 mRNA levels and decreased PV mRNAs in the PFC depend upon the cell type-specific effects of THC administration. The inverse correlations of GAD65 and PV mRNA levels suggest that these alterations may be occurring within the same cell type. Since CCKb are the only GABA neurons in the monkey PFC that are enriched with terminal CB1R (Eggan *et al.*, 2010), THC administration would be expected to reduce GABA release from these terminals (Freund *et al.*, 2003). Less GABA release could either reduce inhibition onto PVb receiving direct connections from CCKb, or

reduce inhibition to the perisomatic region of target pyramidal neurons. Since cortical PV neurons receive a substantial amount of excitatory input onto their dendrites (Melchitzky and Lewis, 2003), the combined reduction of inhibition directly from CCKb inputs and indirectly from excitatory inputs could result in PVb neuron hyperactivity. Given the importance of the normal development and timing of PV neuron activity for the production of gamma oscillations (Freund and Katona, 2007; Uhlhaas *et al.*, 2009), disruptions in the adolescent refinement of PFC circuits could underlie cognitive dysfunction in schizophrenia (Uhlhaas and Singer, 2010).

Interestingly, GAD65 protein is enriched in the axon terminals of both CCKb and PVb, while CB1R and PV proteins are localized to terminals of separate GABA neuron subpopulations (Fish *et al.*, 2011). In contrast, GAD67 protein is detectable by immunofluorescence in PVb but not in CCKb axon terminals. While GAD67 and GAD65 both synthesize GABA, their functions appear to be quite nuanced. Aside from their cell type-specific distributions, GAD67 and GAD65 have distinct intracellular distributions. GAD67 is a cytosolic enzyme that is found throughout the cell including at axon terminals, while GAD65 is primarily enriched at axon terminals. Both GAD isoforms are regulated by the pyridoxal 5'-phosphate cofactor that activates the enzyme when bound, although GAD67 is normally saturated with cofactor and as a result is constitutively active (Kaufman *et al.*, 1991). In contrast, GAD65 is highly regulated by this cofactor switch since it exists largely in the low bound inactive form (Kaufman *et al.*, 1991). Instead of a cofactor switch regulation, GAD67 appears to be dynamically regulated by transcription based on neural activity (Lau and Murthy, 2012; Pinal and Tobin, 1998). Germline deletion of GAD67 in mice resulted in a lethal phenotype due to severe cleft palate along with a dramatic reduction in GAD enzymatic activity and GABA levels at birth (Asada *et al.*, 1997). In contrast, deletion of the GAD65 gene altered GABA levels much

more subtly (Asada *et al.*, 1996). However, germline deletion of GAD65 results in an increased susceptibility to seizures, and GAD65 appears to be crucial for synthesizing appropriate levels of GABA during conditions of high synaptic demand, like gamma oscillations (Battaglioli *et al.*, 2003; Patel *et al.*, 2006; Tian *et al.*, 1999). Thus, if the THC-mediated reduction of GABA release occurs at CCKb terminals, then a subsequent increase in the intracellular concentration of presynaptic GABA and reduction of GAD65 protein levels could underlie the observed increase in GAD65 mRNA levels. Such a potential reduction in GAD65 protein could affect the timing of CCKb inhibition onto PVb and pyramidal neurons, thus disrupting network oscillations that require the temporally coordinated activity of these neuron populations.

Alternatively, systemic administration of THC could result in a non-specific blanket suppression of neurotransmitter release from CB1R containing axon terminals, whether these terminals produce GABA or glutamate for neurotransmission. This network level suppression of transmitter release may then alter the levels and patterns of activity necessary for the proper maturation of PVb. Since molecular markers of PV neurons, but not CCK neurons, undergo substantial changes during adolescence, PVb may be particularly vulnerable to the effects of THC on neural network refinement. Thus, the alterations in GAD65 and PV mRNA levels may actually occur in PVb cells that are actively being refined during the time period of THC administration. Alterations in GAD65 activity in PVb axon terminals would likely have an impact on gamma oscillations since GAD65 activity is thought to be important for periods of high synaptic GABA demand (Patel *et al.*, 2006; Tian *et al.*, 1999) and PV neurons are critical in determining the power of gamma oscillations (Sohal *et al.*, 2009; Cardin *et al.*, 2009). Cell type-specific protein studies will be needed to address these possibilities.

One possibility for the lack of detectable changes in GAD67 mRNA is that levels normalized during the one-month recovery period between final THC administration and sacrifice. Since GAD67 mRNA expression is particularly sensitive to neuronal activity and it is found in numerous GABA synthesizing cell types like the calretinin-containing neurons, another possibility is that a residual effect of chronic THC administration on GAD67 mRNA levels in PVb neurons may not have been large enough to detect. In addition, our observation that CB1R mRNA expression was not altered after chronic THC administration could reflect the possibility that this receptor was altered functionally by decoupling with its G-protein rather than a reduction in protein levels. Alternatively, since CB1R are located in CCKb cells, perhaps these particular neurons are not chronically altered in the face of repeated THC administration since these cells already appear to have reached functional maturity prior to adolescence (Oeth and Lewis, 1993).

4.0 PARVALBUMIN-CONTAINING CHANDELIER AND BASKET CELL BOUTONS HAVE DISTINCTIVE MODES OF MATURATION IN MONKEY PREFRONTAL CORTEX

Adapted from: *Co-first authors: Fish KN*, Hoftman GD*, Sheikh W, Kitchens M, Lewis DA (2013). Parvalbumin-containing chandelier and basket cell boutons have distinctive modes of maturation in monkey prefrontal cortex. *Journal of Neuroscience.* **33:** 8352-8358.

4.1 INTRODUCTION

The postnatal development of GABAergic circuitry in the monkey prefrontal cortex (PFC) is a protracted process that extends into early adulthood (Hoftman and Lewis, 2011). This process includes the establishment and maturation of precise connections among neuronal populations. For example, the axons of chandelier (axoaxonic) cells (PVch) exclusively target the axon initial segment (AIS) of pyramidal neurons (Szentagothai and Arbib, 1974); the convergence of axons from multiple PVch onto a single pyramidal neuron AIS forms a distinctive, vertically-oriented array of boutons termed a cartridge (Lewis and Lund, 1990). Many cortical PVch contain the calcium binding protein parvalbumin (PV) (DeFelipe *et al.*, 1989), and the density of cartridges detectable by PV immunoreactivity decreases between 3 mo and adulthood in monkey PFC (Cruz *et al.*, 2003). In contrast, the density of PV-immunoreactive (IR) boutons that do not

belong to cartridges, and which presumably arise from PV-containing basket cells (PVb), significantly increases between these same ages (Erickson and Lewis, 2002). Both of these findings appear to conflict with the results of other studies indicating that the overall densities of GABAergic synapses in monkey PFC do not undergo postnatal developmental refinements. Specifically, the density of all symmetric (putatively inhibitory) synapses does not change between 3 months and adulthood (Bourgeois *et al.*, 1994), and the density of GABA membrane transporter 1 (GAT1)-IR puncta appears to remain constant (Erickson and Lewis, 2002).

This apparent conflict might be resolved by opposite but equivalent changes in the densities of PVch and PVb boutons across postnatal development such that the overall density of inhibitory boutons and synapses does not change. Alternatively, the apparent changes with age in the densities of PVch cartridges and PVb boutons could reflect inverse developmental shifts in bouton levels of PV protein such that PVch and PVb boutons become less and more detectable with age, respectively. In order to discriminate among these alternatives, we used quantitative, multi-label, fluorescence confocal microscopy to assess the density of, and relative PV protein levels in, the boutons of PVch and PVb in the PFC of 3 month old (3 mo) and adult monkeys.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Macaque (*Macaca fascicularis*), monkeys (3 mo (3 females) and adult (156-192 mo; 2 female and 1 male)) were anesthetized and then perfused transcardially as previously described (Cruz *et al.*, 2003). Brains were immediately removed, and coronal blocks (5 to 6 mm thick) were cut and stored in a cryoprotectant solution at –30^{o}C (Cruz *et al.*, 2003). Sections (40 µm) were exhaustively cut from left hemisphere blocks containing the entire rostral-caudal extent of the principal sulcus (area 46). All experimental procedures were conducted in accordance with the NIH Guide for the Care of Animals and with the approval from the University of Pittsburgh's IACUC.

4.2.2 Antibodies and immunocytochemistry

Two sections per monkey, 1 mm apart, were taken from the middle 1/3 of the principal sulcus **(Figure 15)** and processed as follows. Sections were permeabilized with 0.3% Triton X-100 in phosphate buffered saline (PBS) for 30 min at room temperature (RT), rinsed, and blocked with 20% donkey serum for 2 hr at RT. Sections were then incubated for ~72 hr at 4ºC in PBS containing 3% donkey serum and the following antibodies: PV [sheep, R&D Systems; 1:100; (Kagi *et al.*, 1987)] and the vesicular GABA transporter [vGAT; guinea pig, Synaptic Systems; 1:500; (Guo *et al.*, 2009)], which concentrates in GABAergic boutons (Chaudhry *et al.*, 1998), to identify boutons; the $GABA_A$ receptor subunit γ2 [γ2; rabbit, Synaptic Systems; 1:400; (Fritschy and Mohler, 1995)] to identify postsynaptic sites of GABA boutons; and ankyrin-G [AnkG;

mouse, Santa Cruz; 1:100; (Wang and Sun, 2012)] and neuronal nuclei [NeuN; mouse, Millipore; 1:500; (Mullen *et al.*, 1992)] to identify the AIS and soma, respectively, of pyramidal neurons. Sections were then rinsed for 2 hr in PBS, incubated with secondary antibodies (Donkey; 1:500) conjugated to Alexa 405, 488, 568, 647 (Invitrogen), or biotin (Fitzgerald) for 24 hrs in PBS at 4ºC. Sections were then rinsed and a tertiary incubation (24 hr at 4ºC) with streptavidin 405 was performed to label the biotinylated secondary antibody. The final fluorescence detection channel assignment (excitation) was: vGAT (405nm), AnkG and NeuN (488nm), PV (568nm), and $GABA_A$ γ2 (647nm). Note that AnkG and NeuN, which were both detected using mouse primary antibodies, were visualized in the same channel.

4.2.3 Microscopy

Image stacks (512x512 pixels; 0.25 μm z-step) were collected on an Olympus IX71 microscope (Center Valley, PA) controlled by SlideBook 5.0 (3I, Denver, CO) and equipped with an Olympus spinning disk confocal, Hamamatsu C9100 EM-CCD (Bridgewater, NJ), and LEP BioPrecision2 XYZ motorized stage (Hawthorne, NY) using a 60X 1.42 N.A. objective. Sampling was confined to layers 2-4 [defined as 10-60% of the distance from pial surface to white matter (Pierri *et al.*, 1999)] of PFC area 46 **(Figure 15)** because the density of PV-IR cartridges is highest at 3 mo and lowest in adult monkeys within these layers (Cruz *et al.*, 2003); in contrast, the density of detectable PV-IR boutons was previously reported to be low in 3 mo and highest in adult monkeys in these same layers (Erickson and Lewis, 2002). Sites were systematic randomly sampled using a grid of 190 x 190 μm^2 with stacks collected from 20 randomly selected sites per section.

4.2.4 Image processing

Image stacks were taken using optimal exposures (greatest dynamic range/no saturated pixels), exposure corrected, deconvolved using the AutoQuant adaptive blind deconvolution algorithm, and segmented. Within each field, AIS were manually traced only if they were considered to be completely visualized as indicated by 1) continuity across z planes; 2) a proximal end adjacent to a clearly defined NeuN-IR soma; 3) a distal end, defined by tapering of the AnkG signal to the width of an axon; and 4) the entire AIS was contained within a virtual sampling box. The virtual sampling box started and ended 1 z-plane from the top and bottom of the image stack, respectively, and had x-y start/end coordinates that were located 20 pixels from any edge. AIS length was calculated by measuring the distance between unique points at the soma-hillock boundary, the centroid, and distal end of the AnkG-IR AIS profile.

4.2.5 Definitions of synaptic structures

Intensity/morphological segmentation was used to make object masks for each IR puncta, identified as small (0.03 to 1 μm^3), distinct fluorescing objects (Fish *et al.*, 2011). A GABAergic bouton was defined as a vGAT-IR puncta adjacent to a $GABA_A$ receptor γ2-IR cluster. Mask operations were then used to classify the different bouton populations. Specifically, a PV bouton was defined as a PV object mask that contained the center of a vGAT object mask (PV/vGAT-IR), which overlapped a $GABA_A$ receptor γ2 object mask. Importantly, the presence of vGAT excludes the possibility that it is a thalamic bouton. PV boutons adjacent to γ2-IR puncta not associated with an AnkG-labeled AIS were classified as PVb, while those adjacent to γ2-IR puncta within AnkG-labeled AIS were classified as PVch. ChC_{PVneg} boutons were defined as

those GABAergic boutons adjacent to γ2-IR puncta within AnkG-labeled AIS that could not be represented by a PV object mask. Importantly, all GABAergic boutons that overlapped with γ2-IR puncta within AnkG-labeled AIS were classified as being either a PVch or a ChC_{PVneg} bouton.

4.2.6 Statistics

Diagnostic statistics were used to confirm that the data were normally distributed. Independent t-tests were used to compare dependent measures between age groups. In all analyses, the statistics were performed on the mean values for individual monkeys (N=3 per age group) determined by first averaging data within stack, then averaging stack means within section, and finally averaging means across sections.

4.3 RESULTS

4.3.1 The density of GABAergic boutons does not differ between 3 mo and adult monkeys.

The mean (±SD) density of GABAergic boutons in layers 2-4 of the PFC neuropil did not differ (t [4]=0.731, P=0.505) between 3 mo (0.045±0.006 boutons/μm^3) and adult (0.041±0.004 boutons/μm^3) animals. Because density measures may be confounded by age-related changes in PFC volume (Erickson and Lewis, 2002), we also determined the number of GABAergic boutons per NeuN-IR neuron, which did not differ (t [4]=0.939, P=0.401) between age groups.

4.3.2 The mean number of PVch boutons per AIS, but not of PVb boutons per neuron, is lower in adult compared to 3 mo monkeys.

The mean number of PVch boutons per AIS was 32% lower (t [4]=2.853, P=0.046) in adult (10.5±1.7) relative to 3 mo (15.5±2.5) monkeys **(Figure 15)**. Additionally, the magnitude of the difference between age groups was similar for chandelier cell boutons that did not appear to contain PV (ChC_{PVneg}) **(Figure 16)**. Given that the densities of GAT1- and PV-IR cartridges were previously reported to show different patterns of change both within the same layers and across layers during postnatal development (Cruz *et al.*, 2003), we performed an additional analysis that took layer into account. Specifically, we separated sampling sites based on their laminar position into two groups: layers 2-superficial 3 and layers deep 3-4. Although the magnitude of the reduction in PVch boutons was similar across layers, there was a two-fold greater magnitude of reduction in ChC_{PVneg} boutons in layers 2-superficial 3 compared to layers deep 3-4 **(Figure 16B)**. In contrast to PVch boutons, neither the number of PVb boutons per

NeuN-IR pyramidal neuron (t [4]=-0.201, P=0.851), nor the density of PVb boutons in the neuropil (t [4]=-1.758, P=0.154), differed between 3 mo (244±84 boutons/pyramidal neuron and 0.0101±0.0013 boutons/μm^3, respectively) and adult (254±31 boutons/pyramidal neuron and 0.0117±0.0008 boutons/μm^3, respectively) monkeys.

To determine if biased sampling could have contributed to the age-related decline in the mean number of PVch boutons per AIS, the robustness of our sampling was assessed. Although the coefficient of variation for the number of PVch boutons per AIS within adult animals ranged from 75% to 129%, the coefficient of error ranged from 5% to 10%, indicating that the obtained means are robust estimates of the true population means. In addition, the difference in mean number of PVch boutons per AIS between age groups was not accompanied by any change (t [4]=-1.289, P=0.267) in PV-IR cell body density, consistent with a prior report (Conde *et al.*, 1996). These latter findings suggest that the lower number of PVch boutons per AIS in the adult animals did not result from a postnatal loss of PVch (Southwell *et al.*, 2012).

A highly stringent criterion was used to define a PVch bouton, raising the possibility that some PVch to pyramidal AIS connections were excluded from the analysis. To ensure that the age-related reduction in mean number of PVch boutons per AIS was not confounded by this possibility, the mean numbers of PV-IR, vGAT-IR, and γ2-IR puncta that overlapped AIS were compared between age groups. The mean numbers of PV-IR and vGAT-IR puncta per AIS declined (t [4]=6.028, P=0.004 and t [4]=5.327, P=0.006, respectively) between 3 mo (19.2±2.0 and 21.8±2.4, respectively) and adult (11.8±0.6 and 14.2±0.7, respectively) animals **(Figure 16)**. Likewise, the mean number of γ2-IR puncta per AIS significantly declined (t [4]=3.577, P=0.023) between 3 mo (26.7±4.8) and adult (15.3±2.7) animals. The percent declines in γ2-IR,

vGAT-IR, and PV-IR puncta per AIS, which ranged from 35%-43%, were similar to the 32% age related decline in mean number of PVch boutons per AIS.

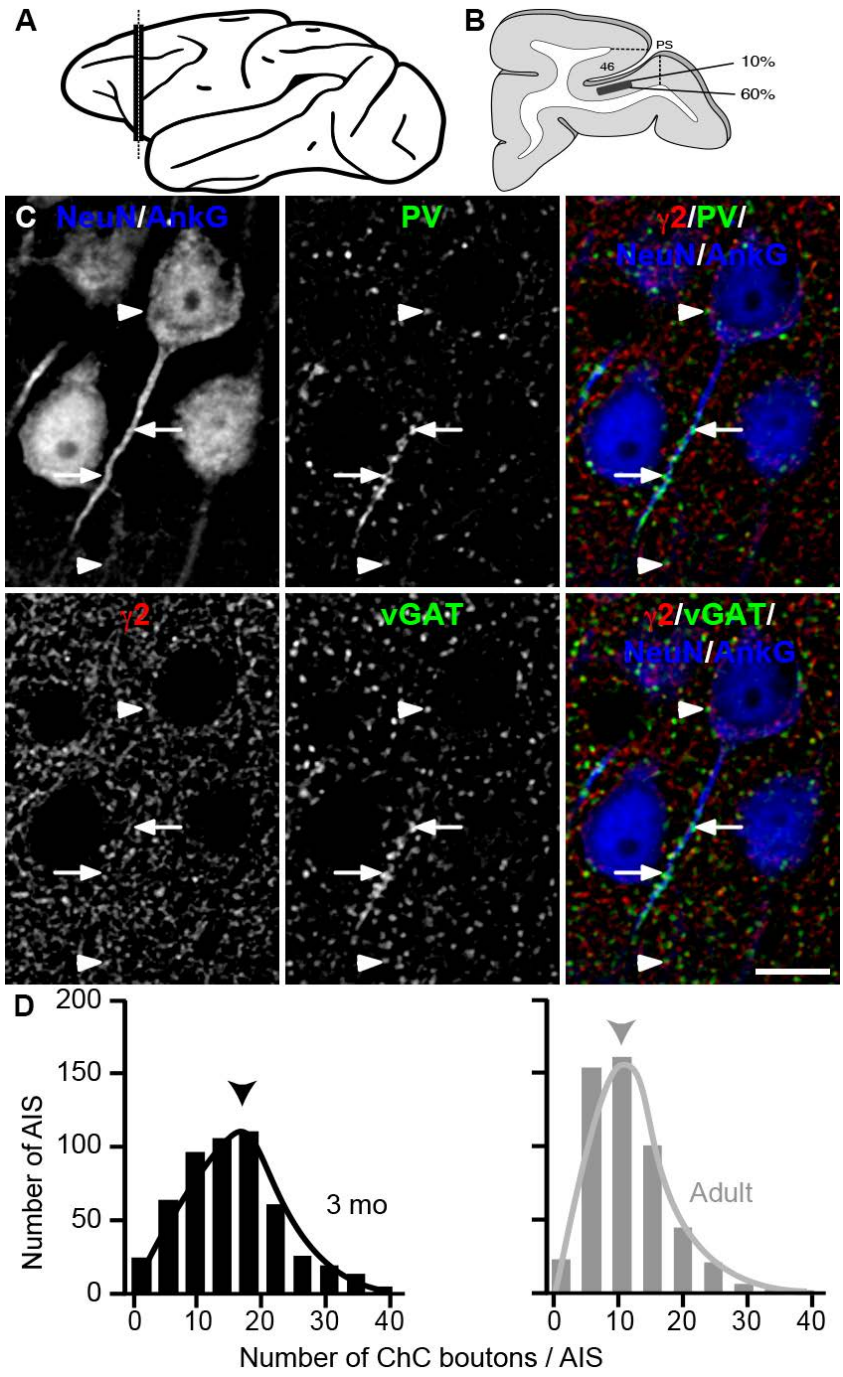

Figure 15. The mean number of PVch boutons per AIS is decreased in adult compared to 3 mo monkeys

(A) Lateral view of monkey cortex showing the approximate location (black vertical bar) of the PFC where tissue sections for this study were taken. **(B)** Schematic of a typical coronal section containing the principal sulcus (PS; area 46) at the position along the rostral-caudal axis designated by the dotted line in A. The black rectangle designates the laminar location in the ventral bank of the PS where analyses were performed. **(C)** Adult monkey section multi-labeled for NeuN/AnkG, γ2, PV, and vGAT. Single channel and merged projection images of a deconvolved image stack (5 z-planes). Arrows–PVch boutons; Arrowheads–PVb boutons. Bar=10 µm. **(D)** Histogram of PVch boutons per AIS in 3 mo (black; mean 15.5) and adult (grey; mean 10.5) monkeys. Arrowheads point to respective means. A randomly selected 500 AIS from each age group is displayed.

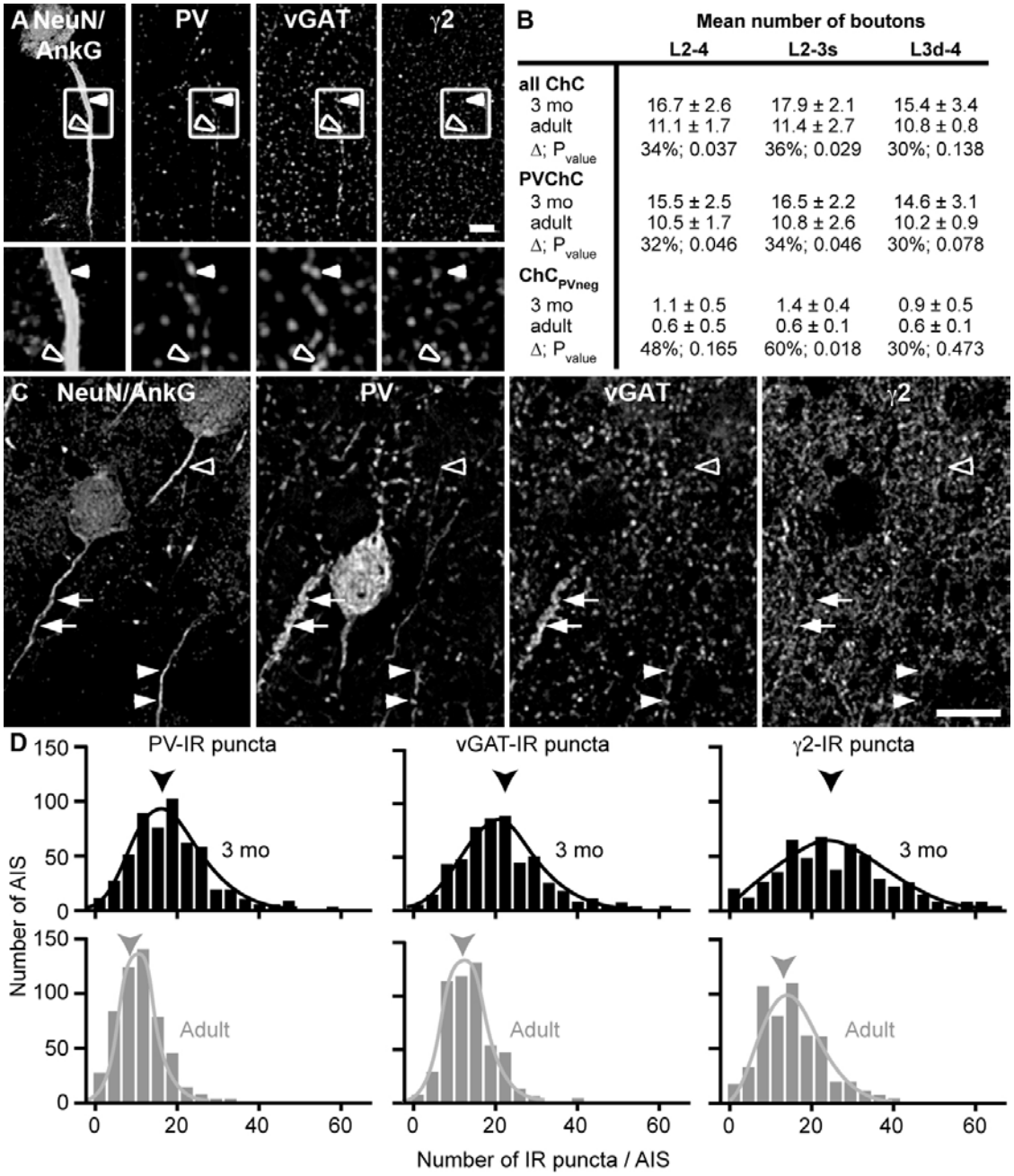

Figure 16. The mean number of puncta immunoreactive for PV, vGAT, or γ2 overlapping AIS is decreased in adult compared to 3 mo monkeys

(A) Single channel images of a deconvolved image stack (5 z-planes) of an adult monkey section multi-labeled for NeuN/AnkG, PV, vGAT, and γ2. Solid arrowhead—PVch bouton; open PVch bouton with no visually detectable PV. A 3X zoom of the boxed region is shown below each channel. Bar=10 µm. **(B)** Table showing the mean number of chandelier cell (ChC) boutons per AIS (all ChC), ChC boutons classified as containing PV (PVch), and ChC boutons classified as being PV immuno-negative (ChC_{PVneg}). **(C)** Single channel images of a deconvolved image stack (5 z-planes) of an adult monkey section multi-labeled for NeuN/AnkG, PV, vGAT, and γ2. Arrows, solid arrowheads, and the open arrowhead point to AIS differentially innervated by PVch. Bar=10 µm. **(D)** Histograms of PV- (mean 3 mo=19.2, adult=11.8), vGAT- (mean 3 mo=21.8, adult=14.2), and γ2- (mean 3 mo=26.7, adult=15.3) IR puncta per AIS in 3 mo (black) and adult monkeys (grey). Arrowheads point to respective means. A randomly selected 500 AIS from each age group is displayed.

AIS innervation by PVch was found to be highly variable in both 3 mo (0 to 42 PVch boutons per AIS) and adult (0 to 39 PVch boutons per AIS) monkeys. Considering that the mean numbers of PVch boutons per AIS in 3 mo and adult animals were 15.5 and 10.5, respectively, these findings suggest that a large number of AIS might not be associated with a morphologically detectable cartridge and that the density of detectable PVch cartridges might be significantly reduced between 3 mo and adulthood. To assess cartridge density, PV-IR cartridges were identified qualitatively by their characteristic morphology and the number of PV-IR boutons per AIS was quantified. The PV channel was used to select PV-IR cartridges, and then the other channels (vGAT, γ2, and AnkG/NeuN) were used to determine how many boutons were present in each cartridge. This approach revealed that PV-IR morphologically-distinct cartridges were composed of 12 or more PVch boutons. Of 500 randomly selected pyramidal cells from 3 mo monkeys, the AIS of 320 (64%) was innervated by ≥ 12 boutons, while in adult monkeys the AIS of only 163/500 (32.6%) pyramidal cells was innervated by ≥ 12 boutons. This 49% decrease in the number of recognizable cartridges between 3 mo and adult monkeys is similar to the previously reported 55% decline using a different method (Cruz *et al.*, 2003; Cruz *et al.*, 2009a; Anderson *et al.*, 1995).

4.3.3 The number of PVch boutons per AIS is positively correlated with AIS size.

The mean length (t [4]=6.213, P=0.003) of AIS, as determined by AnkG immunoreactivity, was ~33% shorter in adult (19.7±1.4 μm) than in 3 mo (29.1±2.3 μm) monkeys **(Figure 17A)**. In addition, the average AIS surface area determined by AnkG immunoreactivity was significantly less (t [4]=4.839, P=0.008) in adult (132±8 μm^2) than 3 mo (202±24 μm^2) monkeys **(Figure 17B)**. Furthermore, the number of PVch boutons was positively correlated (3 mo R=0.764, P <

0.001; adult R=0.735, P < 0.001) with AIS surface area within monkeys **(Figure 17C)**. Importantly, mean AnkG fluorescence intensity in AIS did not differ (t [4]=0.436, P=0.685) between adult (2494±231) and 3 mo (2554±67) monkeys, suggesting that group differences in AIS length and surface area were not confounded by changes in AnkG fluorescence.

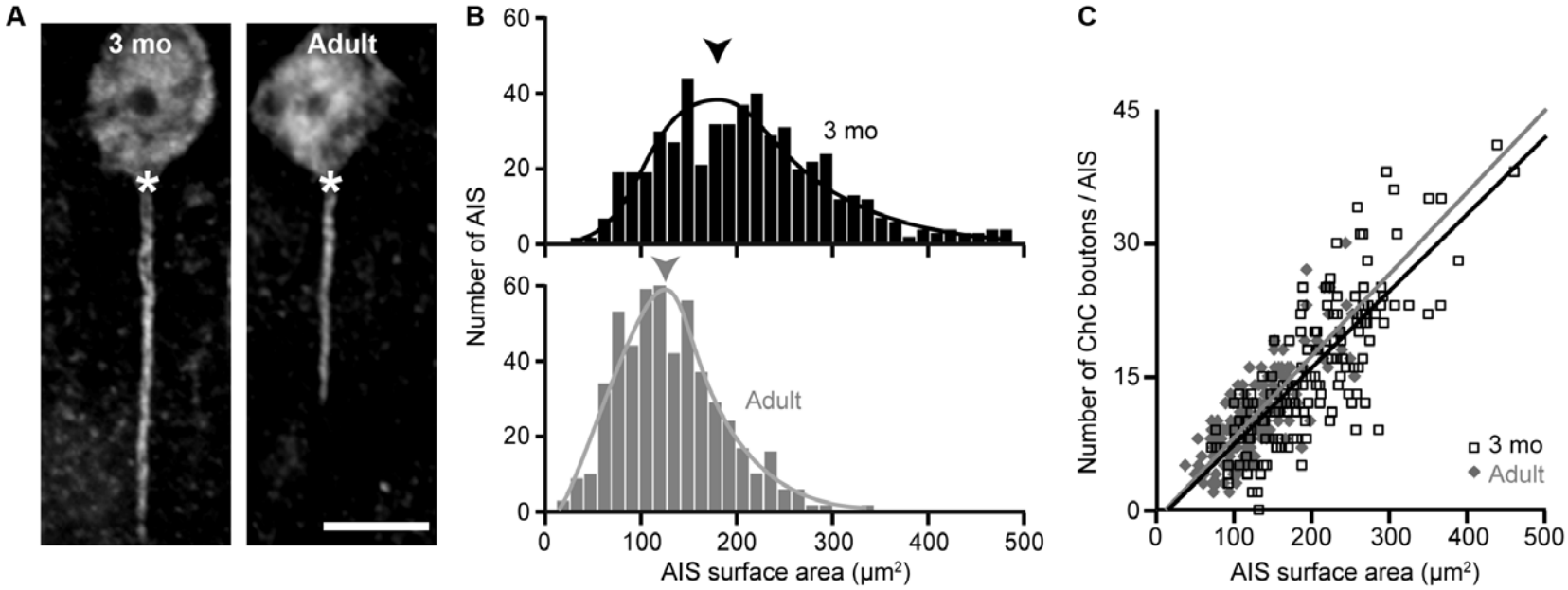

Figure 17. The number of PVch boutons per AIS is positively correlated with AIS size

(A) NeuN and AnkG labeled soma and AIS, respectively, from 3 mo and adult monkeys. The selected neurons are representative of the length differences between age groups (see main text). Asterisks designate the axon hillock. Bar=10 μm. **(B)** Histogram showing the surface area of 500 randomly selected AIS from each age group (3 mo black, mean 202; adult grey, mean 132). Arrowheads point to respective means. **(C)** Scatter plot of 150 randomly selected AIS from each age group (3 monkeys/group, 50 AIS/monkey) showing the relationship between AIS surface area and the number of PVch boutons.

4.3.4 Relative PV protein levels in PVb boutons, but not in PVch boutons, are significantly greater in adult compared to 3 mo monkeys.

The relative mean protein level of PV per PVb bouton increased significantly by 69% between 3 mo and adulthood. This increase was accompanied by an increase in the relative mean protein level of vGAT (25%) per PVb bouton and in γ2 (35%) relative protein levels in the corresponding postsynaptic clusters between 3 mo and adulthood, although neither of these changes achieved statistical significance **(Table 5)**. In contrast, relative mean protein levels of PV and vGAT per PVch bouton, and γ2 per postsynaptic cluster adjacent to a PVch bouton were similar between the two age groups **(Table 5)**.

Table 5. Relative protein levels in PVb and PVch boutons (PV and vGAT) adjacent to postsynaptic clusters (γ2) in 3 mo and adult monkeys

	PVBC		PVChC	
	3 mo	adult	3 mo	adult
PV	3582 ± 156	6050 ± 892	6261 ± 1091	6695 ± 780
	(t [2.12] = -4.72, P = 0.037)		(t [4] = -0.56, P = 0.605)	
vGAT	5058 ± 43	6324 ± 870	6176 ± 80	7102 ± 1067
	(t [4] = -2.52, P = 0.066)		(t [4] = -1.50, P = 0.208)	
γ2	1638 ± 308	2207 ± 864	1702 ± 357	2042 ± 894
	(t [4] = -1.08, P = 0.343)		(t [4] = -0.61, P = 0.574)	

4.4 DISCUSSION

Prior reports of a decreased density of PVch axon cartridges (Anderson *et al.*, 1995; Cruz *et al.*, 2009a; Cruz *et al.*, 2003) and increased density of PV-IR boutons (Erickson and Lewis, 2002) over postnatal development in monkey PFC could represent inverse changes in bouton number and/or in PV protein levels per bouton. As reported here, the mean number of PVch boutons per pyramidal neuron AIS was significantly 32% lower in adult compared to 3 mo monkeys, with no group differences in relative mean PV protein levels per PVch bouton. In contrast, the density of PVb boutons did not differ between age groups, but the relative mean PV protein levels in PVb boutons were nearly 2-fold higher in adults than in 3 mo monkeys. Thus, PVch and PVb appear to utilize fundamentally different mechanisms to achieve adult levels of innervation of their pyramidal cell targets.

The result that GABAergic bouton density in the PFC does not differ between 3 mo and adult monkeys, along with previous reports that neither the density of symmetric synapses (Bourgeois *et al.*, 1994) nor the density of GAT1-IR puncta (Erickson and Lewis, 2002) differed between these age groups suggest that GABAergic boutons are not pruned during postnatal development. In this context, our finding of an age-related reduction in PVch boutons strongly suggests that the pruning of these boutons is cell type-specific, and not detectable when all GABAergic boutons are assessed without regard to cell type.

In this study, AIS mean length and average surface area were ~33% less in adult than in 3 mo monkeys, consistent with previous reports (Cruz *et al.*, 2009a). Recent studies have shown that AIS length/location depend, at least in part, upon network activity (Grubb *et al.*, 2011). Interestingly, we found that the number of PVch boutons was positively correlated with AIS

surface area. In concert, these findings suggest that the number of PVch inputs onto a given pyramidal neuron is also regulated by network activity.

The distinctiveness of a developmental reduction in the number of PVch boutons may be related to their unique role in innervating the AIS of pyramidal neurons where they are positioned to regulate action potentials. Interestingly, we found that the number of PVch boutons associated with an AIS is highly variable across AIS in both 3 mo (0 to 42 PVch boutons per AIS) and adult (0 to 39 PVch boutons per AIS; **Figure 15D**) monkeys, similar to prior electron microscopy studies in adult primates (DeFelipe *et al.*, 1985; Inda *et al.*, 2007). These findings raise the possibility that the degree of action potential regulation by PVch differs across subtypes of pyramidal neurons. Consistent with this idea, the mean number of inputs to the AIS is greatest in callosal-projecting pyramidal cells, intermediate in pyramidal cells providing ipsilateral cortical projections, and lowest in corticothalamic pyramidal neurons in monkey neocortex (Farinas and DeFelipe, 1991). However, even among pyramidal neurons that share the same projection target, the number of PVch inputs to the AIS shows considerable variability (Farinas and DeFelipe, 1991). These findings, and those of the present study, reveal a marked heterogeneity in the need for PVch regulation of pyramidal neurons, across and within subclasses of pyramidal cells, and apparently across development for the same pyramidal cells. Given the reported roles of PVch inputs of either hyperpolarizing or depolarizing pyramidal cells based on levels of network activity (Woodruff *et al.*, 2011) or of blocking retrograde action currents (Dugladze *et al.*, 2012), why some pyramidal cells need a high level of these types of regulation and others do not is an important question for future studies.

In contrast to PVch, relative PV protein levels in PVb boutons were two-fold higher in adult compared to 3 mo monkeys. In concert with our finding that PVb bouton density did not

differ between age groups, these results suggest that the previously reported increase in PV-IR puncta density across postnatal development in monkey PFC results from an age-related rise in PV protein levels per PVb bouton and thus an increase in their detectability by standard light microscopy. Although the physiological role of Ca^{2+} buffering by PV is still unclear, an increase in bouton PV levels decreases the duration of Ca^{2+} transients that influence GABA release (Collin *et al.*, 2005). Thus, an increase in PVb bouton PV levels could result in greater bouton-specific regulation over GABA release with age.

The distinct differences in the maturation of PVb and PVch boutons may provide insight into the nature or timing of the reported alterations in these types of boutons in schizophrenia. For example, the density of PVch cartridges detectable by GAT1 immunoreactivity is 40% lower in the PFC of schizophrenia subjects relative to matched comparison subjects (Woo *et al.*, 1998; Pierri *et al.*, 1999). This difference could represent an excessive developmental elimination of PVch boutons. In addition, lower levels of PV protein in putative PVb boutons in schizophrenia (Glausier *et al.*, 2012) might represent a disease-related impairment in the normal developmental rise of PV levels in these boutons observed in the present study. Together, these alterations in perisomatic inhibitory regulation of pyramidal cell function might contribute to the emergence of PFC-mediated cognitive disturbances well before the clinical onset of schizophrenia during late adolescence or early adulthood (Reichenberg *et al.*, 2010).

5.0 GENERAL DISCUSSION

This dissertation provides evidence for understanding the *timing* of GABA neuron alterations that occur in postmortem PFC of subjects with schizophrenia, the *impact* of chronic cannabis use during adolescence on PFC GABA circuitry, and the *cell type-specific nature* of postnatal developmental changes in PV neurons. To understand timing, we investigated whether changes in cortical GABA-related mRNA levels in schizophrenia reflect developmental disturbances or chronic illness consequences; to understand impact, we explored how repeated THC exposure during adolescence alters GABA-related mRNA expression levels in monkey PFC; and to understand cell type-specific nature, we examined the maturation of PVch and PVb boutons in the monkey PFC. GABA-related mRNA levels did not change as a function of age or illness duration in subjects with schizophrenia, suggesting that chronic illness processes may not have substantially contributed to their expression profiles in schizophrenia **(2.0)**. We reported changes in some, but not all, GABA-related mRNAs in response to repeated THC exposure during adolescent development **(3.0)**. Finally, we examined cell type-specific axon terminal development in the PFC and found that PVch and PVb boutons have distinctive modes of maturation, with a decrease in number of PVch boutons per AIS and an increase in terminal protein levels within PVb boutons between infancy and adulthood **(4.0)**. In the following discussion, I will consider these three main topics to better clarify whether schizophrenia is better conceptualized as a neurodevelopmental or neurodegenerative disorder **(5.1)**, to establish

the impact of cannabis use during adolescence on PFC GABA neuron maturation **(5.2)**, and to investigate the differences between PVch and PVb maturation in monkey PFC **(5.3)**. I will then weigh the implications of this work for preemptive potential in schizophrenia **(5.4)**.

5.1 SCHIZOPHRENIA DISEASE COURSE: TIMING

5.1.1 Clinical evidence for abnormal neurodevelopmental processes in schizophrenia

Much of the evidence supporting neurodegenerative progression in schizophrenia comes from systematic studies of brain structure using computed tomography (CT) and magnetic resonance imaging (MRI) scans. CT scans showed that on average individuals with schizophrenia had larger lateral ventricles and cerebral sulci (Johnstone *et al.*, 1976; Pfefferbaum *et al.*, 1988; Reveley *et al.*, 1982; Weinberger *et al.*, 1979), and MRI scans showed widespread deficits in gray and white matter volumes (Shenton *et al.*, 2001; Harvey *et al.*, 1993; Zipursky *et al.*, 1992). However, associations between illness duration and the magnitude of ventricular and gray matter volumes changes have not been significant (Zipursky *et al.*, 1992; Gur *et al.*, 1999).

Deficits in gray and white matter and increased ventricular size in individuals with first episode schizophrenia are modest when compared to changes seen in different individuals with chronic schizophrenia (Zipursky *et al.*, 1998; Cahn *et al.*, 2002; Lim *et al.*, 1996). This finding has been interpreted to support progressive neurodegeneration resulting from the disease; however, secondary factors including prolonged use of antipsychotic medications could be the root contributors to these cortical and ventricular alterations. In fact, recent studies have demonstrated a positive association between use of antipsychotics and brain volume reductions

using longitudinal scanning in individuals with schizophrenia beginning at their first episode of psychosis, with higher doses of antipsychotic medications being associated with greater decreases in gray and white matter volumes (Ho *et al.*, 2011; Lieberman *et al.*, 2005). Importantly, haloperidol and olanzapine led to decreases in gray and white matter volume when administered in monkeys and rats (Dorph-Petersen *et al.*, 2005; Vernon *et al.*, 2011). Interestingly, the withdrawal of antipsychotic administration in rats was followed by normalization of brain volumes on MRI scans, demonstrating a direct effect of brain volume loss with antipsychotic exposure (Vernon *et al.*, 2012). Additional secondary factors associated with this profile of brain volume abnormalities include cannabis use, smoking cigarettes, and alcohol use, as well as sedentary lifestyle and high levels of stress, which are particularly relevant since individuals with schizophrenia have higher rates of these factors than the healthy population (Sapolsky, 2000; Rais *et al.*, 2008; Mathalon *et al.*, 2003; Stone *et al.*, 2012; Pajonk *et al.*, 2010). Together, these many factors confound the interpretation that progressive ventricular enlargement and cortical gray matter volume loss are due to disease-specific neurodegenerative processes in schizophrenia.

One strong finding against neurodegenerative hypotheses of schizophrenia is the absence of classic neuropathological hallmarks such as neuronal loss, senile plaques, neurofibrillary tangles, and gliosis; which are all observed in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Arnold *et al.*, 1998). However, the absence of obvious neuropathological hallmarks does not rule out the possibility of more subtle disease progression and selective cell death. For example, apoptosis is responsible for neuropil turnover, maintenance and elimination and is not accompanied by gliosis (Bredesen *et al.*, 2006); and therefore, subtle losses of neuronal subtypes due to apoptosis may have gone undetected.

This exception leaves open the possibility of subtle disease-related degenerative brain changes in schizophrenia.

Not only have there been studies showing evidence against neurodegenerative processes in schizophrenia, but also observational studies have found associations between abnormal development and schizophrenia. For example, minor physical anomalies, such as low set ears, furrowed tongue, and adherent earlobes, are observed with a higher frequency in individuals diagnosed with schizophrenia (Green *et al.*, 1994) and are considered to be the consequence of disturbed prenatal development of the ectoderm (reviewed in (Lewis and Levitt, 2002). In addition, clinicians were able to separately identify children who later developed schizophrenia from unaffected siblings, children who later developed mood disorders, and children with no family history of mental illness based solely on abnormal movements before 2 years of age (Walker *et al.*, 1994).

In light of these and many clinical reports, recent attempts to unify the concept of the etiology of schizophrenia have focused on neurodevelopmental processes. Since substantial refinement in human PFC circuitry was demonstrated during adolescence (Huttenlocher, 1979) and the age of peak clinical onset occurs during late adolescence/early adulthood (2000), one hypothesis put forth is that schizophrenia results from a disturbance in late developmental processes, such as cortical synaptic pruning during adolescence (Feinberg, 1982). Alternatively, the association of prenatal environmental factors with schizophrenia risk supports the hypothesis that schizophrenia results from a fixed brain lesion early in life that remains silent until normal developmental processes unmask the affected neural circuits during adolescence (Murray and Lewis, 1987; Weinberger, 1987). An integrated view of schizophrenia as a disorder of neurodevelopment states that schizophrenia results from a progressive, cumulative pathogenic

process that operates through adolescent development and extends through the initial phases of clinical illness (Keshavan *et al.,* 1994). This integrated view is furthered by the concept of allostatic load, which states that the expression of diagnostic features in schizophrenia appear when the homeostatic system is disrupted and can longer compensate for the cumulative effects of genetic and environmental 'hits' throughout development (Thompson and Levitt, 2010; McEwen and Wingfield, 2003). In fact, the most recent re-conceptualization of schizophrenia views psychosis onset as the end stage of a protracted pathogenic process of neurodevelopment (Insel, 2010) **(Figure 1)**.

Despite recent movement towards a neurodevelopmental model of schizophrenia evidence exists that this may not be the only explanation, as cases exist with individuals experiencing clinical onset in middle or late adult life and a more rapidly deteriorating course (reviewed in (Church *et al.*, 2002). However, these conflicting observations may be reconciled by examining subgroups of individuals diagnosed with schizophrenia or by considering that the breadth of the current diagnostic definition of schizophrenia may actually be a combination of different biological diseases. Regardless, the pathogenesis of schizophrenia appears to result in progressive, but initially subtle, alterations in brain circuitry without gliosis, raising the possibility that this progression continues throughout the lifetime course of the illness. (Andreasen, 2010).

5.1.2 Molecular evidence against illness duration related changes in GABA neurotransmission

In dorsolateral PFC area 9, we provide molecular evidence showing that changes in GABA-related mRNA levels in schizophrenia are not related to illness duration and are thus unlikely to

result from cumulative effects of illness **(Figures 8 and 9)**. In line with our findings, a recent study using a different cohort reported that declines in GAD67, $GABA_A$ α1 and $GABA_A$ α4 mRNA levels were parallel between individuals with schizophrenia and healthy comparison subjects and related to age and illness duration (Duncan *et al.*, 2010). Since age and illness duration were highly correlated with each other, the lack of an apparent interaction between age and diagnosis suggests that the parallel decline in mRNA levels across subject groups was more likely driven by age than illness duration. Importantly, medication status at the time of death did not alter the effect of age on mRNA transcript levels in the schizophrenia subject group in both studies.

5.1.3 Molecular evidence for protracted development of GABA neurotransmission

Many studies have shown that GABA neurons undergo a lengthy period of postnatal maturation in mammalian cortex, a period that is particularly prolonged in the monkey and human PFC (Erickson and Lewis, 2002; Cruz *et al.*, 2003; Hashimoto *et al.*, 2009; Hyde *et al.*, 2011; Fung *et al.*, 2010). Recent studies in the developing human PFC have shown that GAD67, vGAT, $GABA_A$ α1 and PV mRNAs all increase from birth to adolescence (Huang *et al.*, 2007; Duncan *et al.*, 2010; Fung *et al.*, 2011b; Hyde *et al.*, 2011; Fung *et al.*, 2010). These findings strengthen our approach of using rhesus monkey PFC as a developmental model of GABA-related marker expression since all four markers show a similar temporal pattern as those observed in human PFC **(Figures 10 and 11)**.

In our studies, the largest changes in mRNA levels occurred between the perinatal and childhood periods. However, GAD65 and $GABA_A$ α1 mRNA levels increased through adolescence, suggesting that multiple sensitive periods of GABA neuron development may exist.

These multiple sensitive periods can arise since diverse aspects of cortical GABA circuitry have different inflection points during development, and as a consequence may be vulnerable to unique environmental exposures (Le Magueresse and Monyer, 2013). These findings support the Keshavan integrated hypothesis and the concept of allostatic load in schizophrenia, where a series of pathogenic processes during development culminate in the clinical onset of schizophrenia during late adolescence when neural circuits can no longer compensate (Keshavan *et al.*, 1994; Thompson and Levitt, 2010; McEwen and Wingfield, 2003).

5.1.4 GABA neuron mRNA expression profile in schizophrenia may reflect disruptions earlier in development

Although it is not feasible to directly study postnatal developmental mRNA levels in schizophrenia, studies of postmortem human and monkey tissue have suggested that alterations in PFC GABA-related mRNA levels may result from an earlier disruption in the expression trajectories of these markers. For example, lower GAD67 mRNA levels were found in PFC tissue from schizophrenia subjects (Akbarian *et al.*, 1995b; Guidotti *et al.*, 2000; Curley *et al.*, 2011; Hashimoto *et al.*, 2008a; Volk *et al.*, 2000; Duncan *et al.*, 2010), along with a parallel age-related decline in GAD67 mRNA levels between schizophrenia and comparison subject groups (Duncan *et al.*, 2010) and with no significant effect of illness duration on these levels **(Figures 8 and 9)**, suggesting that alterations in GAD67 mRNA levels occurred during development before clinical onset. In addition, two independent studies have shown that GAD67 mRNA levels increase substantially from birth into late childhood/early adolescence in human PFC tissue (Huang *et al.*, 2007; Hyde *et al.*, 2011), a finding paralleled in our studies in monkey PFC **(Figure 10)**. Together these findings provide support for the idea that lower GAD67 mRNA

levels in the PFC of subjects with schizophrenia may reflect disturbances in their dynamic developmental expression trajectories.

We also found similar patterns of lowered mRNA levels in other GABA-related profiles, such as vGAT, $GABA_A$ α1 and PV in schizophrenia, which all have increasing postnatal mRNA levels **(Figures 10 and 11)**, supporting the hypothesis that these disturbances in schizophrenia may result from disrupted postnatal development. Additional support comes from investigating $GABA_A$ α2, the $GABA_A$ δ subunit, which is involved in tonic inhibition (Drake and Milner, 2002; Stumm *et al.*, 2004; Farrant and Nusser, 2005), and the μ-opioid receptor (MOR), which suppresses GABA release when activated (Drasbek *et al.*, 2007; Drasbek and Jensen, 2006). In PFC tissue from schizophrenia subjects, levels of $GABA_A$ α2 and MOR mRNAs were higher and $GABA_A$ δ mRNAs were lower, and each transcript had an inverse developmental expression trajectory (Maldonado-Aviles *et al.*, 2009; Beneyto *et al.*, 2011; Hashimoto *et al.*, 2009; Volk *et al.*, 2012b).

Cortical transcripts other than GABA-related mRNAs may also be developmentally disrupted. For example, mRNA levels of the metabotropic glutamate receptor subunit 1α (mGluR1α) and the regulator of G-protein signaling (RGS4) were higher and lower, respectively, in schizophrenia (Volk *et al.*, 2010), while their postnatal developmental trajectories were opposite in direction with mGluR1α decreasing and RGS4 increasing in monkey PFC (Volk DW, Personal Communication). In mice, a postnatal developmental increase in the NMDA receptor subunits GluN1 and GluN2A occurs, and this finding for GluN1 has been replicated in human PFC. Interestingly, GluN1 and GluN2A mRNA levels were recently reported lower in subjects with schizophrenia (Beneyto and Meador-Woodruff, 2006). Thus, in postmortem PFC tissue from schizophrenia subjects, multiple transcripts that regulate neuronal excitability appear

to have patterns of altered expression that may reflect an underlying disruption in postnatal development.

The combination of findings discussed above supports the hypothesis that the normal development of PFC GABA neurons is disrupted in schizophrenia and may, at least in part, underlie cognitive dysfunction in the illness. Recently, a longitudinal, prospective cohort study examined the development of cognitive function in children who later developed schizophrenia compared to those who remained healthy (Reichenberg *et al.*, 2010), advancing three hypotheses **(Figure 18)**: 1) Developmental lag, which predicts growth of cognitive function but at a slower rate than the growth seen in healthy individuals; 2) Developmental deficit, which predicts static cognitive impairment early in development; and 3) Developmental deterioration, which predicts a premorbid decline in cognitive functioning. In that study, the developmental lag model best predicted differences in working memory performance between the two subject groups. These findings support the possibility that the PFC neural circuits underlying working memory ability, which includes GABA neurons, undergo pathological alterations during childhood well before the onset of psychosis. Indeed, a variety of environmental insults that accumulate during childhood and adolescence may mitigate both developmental disruptions in cognitive development and in underlying GABA circuitry. Interestingly, a number of recent epidemiological studies suggest that cognitive deficits in schizophrenia subjects do not decline in excess of normal aging and there is no evidence of obvious neurodegeneration in excess of what is seen during normal aging (Rajji *et al.*, 2012; Harvey *et al.*, 2006). These findings together with the absence of cell density changes in the PFC of schizophrenia subjects (reviewed in (Lewis and Gonzalez-Burgos, 2008) suggest that alterations may occur earlier in development. The implications of understanding timing in schizophrenia will be discussed in **(5.4)**.

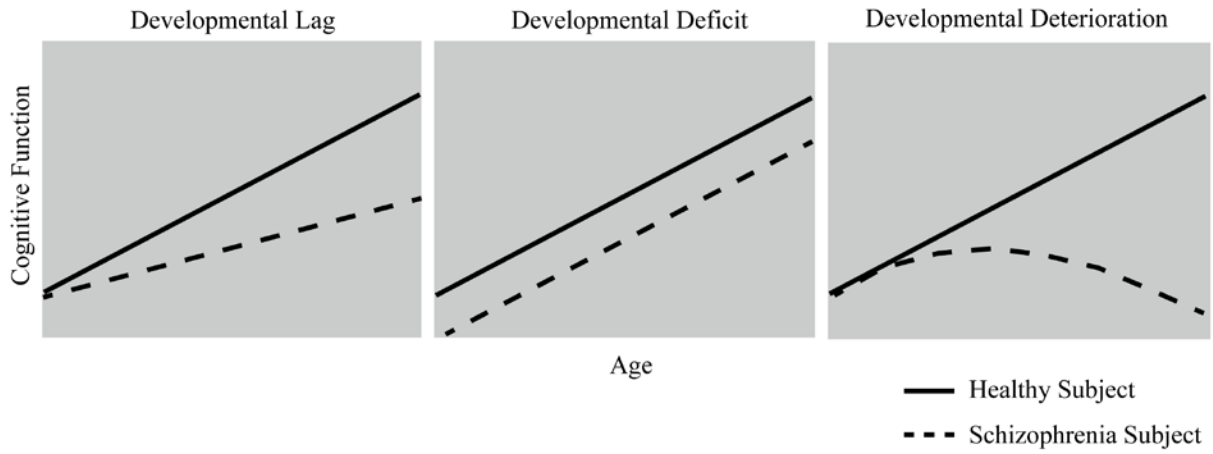

Figure 18. Hypotheses of premorbid cognitive development in schizophrenia

Left panel shows a developmental lag, where the rate of improvement in cognitive function is slower in schizophrenia subjects; center panel shows a static deficit from an early age in schizophrenia subjects; and right panel shows a deterioration of cognitive function in schizophrenia subjects. Adapted from Reichenberg *et al.*, 2010.

5.1.5 Future direction

In sections **5.1.1-5.1.4**, I have argued that schizophrenia can be viewed as a neurodevelopmental disorder and that the timing of neural circuit changes during development is critical to study in detail. One mechanistic approach for examining the effects of a developmental disruption on the maturation of GABA neurons is to manipulate interneuron specific transcription factors during different developmental time periods. For example, Lhx6, which is a LIM homeodomain transcription factor, regulates the development of PV and SST neurons during prenatal migration to the cerebral cortex (Xu *et al.*, 2004; Butt *et al.*, 2005; Cobos *et al.*, 2006) and throughout adulthood in a cell type-specific manner (Jakovcevski *et al.*, 2011). In Lhx6 germline allelic deletion mice (Lhx6$^{-/-}$), both the tangential migration to the cortex and radial migration within the cortical plate appeared to be disturbed (Zhao *et al.*, 2008; Liodis *et al.*, 2007), resulting in the abnormal distribution of GABA neurons. Lhx6$^{-/-}$ animals also had a reduction in the density of

PV and SST neurons (Zhao *et al.*, 2008; Liodis *et al.*, 2007), indicating the importance of Lhx6 for their differentiation. In PFC tissue from subjects with schizophrenia, Lhx6 mRNA levels were lower (Volk *et al.*, 2012a), and in a subset of these individuals, lower Lhx6 mRNA levels were accompanied by lower GAD67, PV and SST mRNA levels (Volk *et al.*, 2012a). These findings suggest that alterations in Lhx6 expression may affect the development or maintenance of the PV and SST phenotype in this subset of individuals with schizophrenia.

To determine the effects of a cell type-specific perturbation on PFC cortical circuit maturation, a conditional Lhx6 disruption using a cre-lox reporter system in rodent organotypic slice cultures during different stages of development can be used (Liodis *et al.*, 2007; Neves *et al.*, 2012; Chattopadhyaya *et al.*, 2007). A recent study used homologous recombination in embryonic stem cells to generate a mutant allele of Lhx6, where exons encoding the functional domain were deleted and replaced with an IRES-GFP reporter cassette. Animals heterozygous for the Lhx6^{-} mutation were generated and characterized, but Lhx6^{-} homozygous mutants died during the first two weeks after birth. To create the conditional knockout, mice that have a floxed Lhx6 mutant allele can be generated and organotypic slice cultures from these animals can be transfected with a cre-recombinase and GFP-cassette under the GAD67 promoter (Chattopadhyaya *et al.*, 2004; Chattopadhyaya *et al.*, 2007). Transfection of the GAD67 promoter cassette during different stages of slice development can be used to study its effects on markers of cortical GABA transmission, and a number of predictions can be made based on the timing of this manipulation. For example, perinatal disruption of Lhx6 may result in abnormal neural migration lower densities of PFC GABA neurons, as observed in other brain regions (Liodis *et al.*, 2007; Zhao *et al.*, 2008). However, disruption during the equivalent postnatal adolescent period in rodent organotypic culture might alter GABA-related mRNA levels without

a change in cell density, since postnatal Lhx6 levels are important for the expression of PV and SST phenotypes. Importantly, this manipulation allows us to measure the developmental trajectories of pre and postsynaptic GABA-related markers.

5.2 IMPACT OF ADOLESCENT CANNABIS USE ON PFC GABA NEURONS

As shown in **Figure 12**, GAD65 mRNA levels were higher and PV mRNA levels were lower in the PFC from THC-treated postpubertal monkeys relative to behaviorally matched, vehicle-treated comparison subjects. However GAD67, CB1R and CCK mRNA levels did not differ between groups. These findings suggest that selective alterations in markers of cortical GABA neurotransmission occur in response to chronic THC administration during adolescence.

5.2.1 THC-mediated endocannabinoid signaling alterations

Phasic endocannabinoid signaling acts “on-demand” in a synapse-specific fashion such that endocannabinoids are released when they are needed (Marsicano *et al.*, 2003) and their effect of suppressing neurotransmitter release is restricted to the associated presynaptic site (Brown *et al.*, 2003) **(Figure 19)**. Since CB1R are much more highly concentrated at GABAergic CCKb terminals than at glutamatergic terminals (Kawamura *et al.*, 2006; Uchigashima *et al.*, 2007), they are assumed to be more influential at CCKb terminals. However, a recent study in mouse hippocampus found that the minority of glutamatergic CB1R are paradoxically several fold more strongly coupled to G-protein signaling than GABAergic CB1R (Steindel *et al.*, 2013). Interestingly, the enzyme that synthesizes 2-AG, which is a critical

mediator of DSI and DSE, was present at higher amounts in neocortical glutamate synapses relative to GABA synapses (Katona and Freund, 2012). GABAergic CB1R were also not involved in seizure susceptibility (Monory *et al.*, 2006); however, they did appear to be critical for mediating THC-induced long-term memory deficits (Puighermanal *et al.*, 2009) and for protecting against age-related cognitive decline (Albayram *et al.*, 2011). Together, these properties have led to the hypotheses that the endocannabinoid system acts as a glutamatergic and GABAergic circuit breaker (Katona and Freund, 2012). However, THC exposure abolishes the "on-demand" synapse-specific nature of endocannabinoid signaling, and may alter the delicate balance between excitation and inhibition due to its "blanket of CB1R activation" at glutamate and GABA synapses.

Two cellular mechanisms by which THC administration may disrupt PV neuron maturation could involve phasic and/or tonic endocannabinoid signaling alterations in different inputs to PV neurons **(Figure 20)**. In one example, THC induces DSI at CCKb inputs to PVb and pyramidal neurons. An acute break in inhibition may serve to increase excitability in PVb and pyramidal neurons. In this scenario, the perisomatic GABAergic network may be imbalanced such that the contribution of PVb inputs becomes too large and the contribution of CCKb too small. Given that PV neurons appear to be critical time keepers and CCKb neurons appear to be integrators of information from different cortical and subcortical areas, one possibility is that

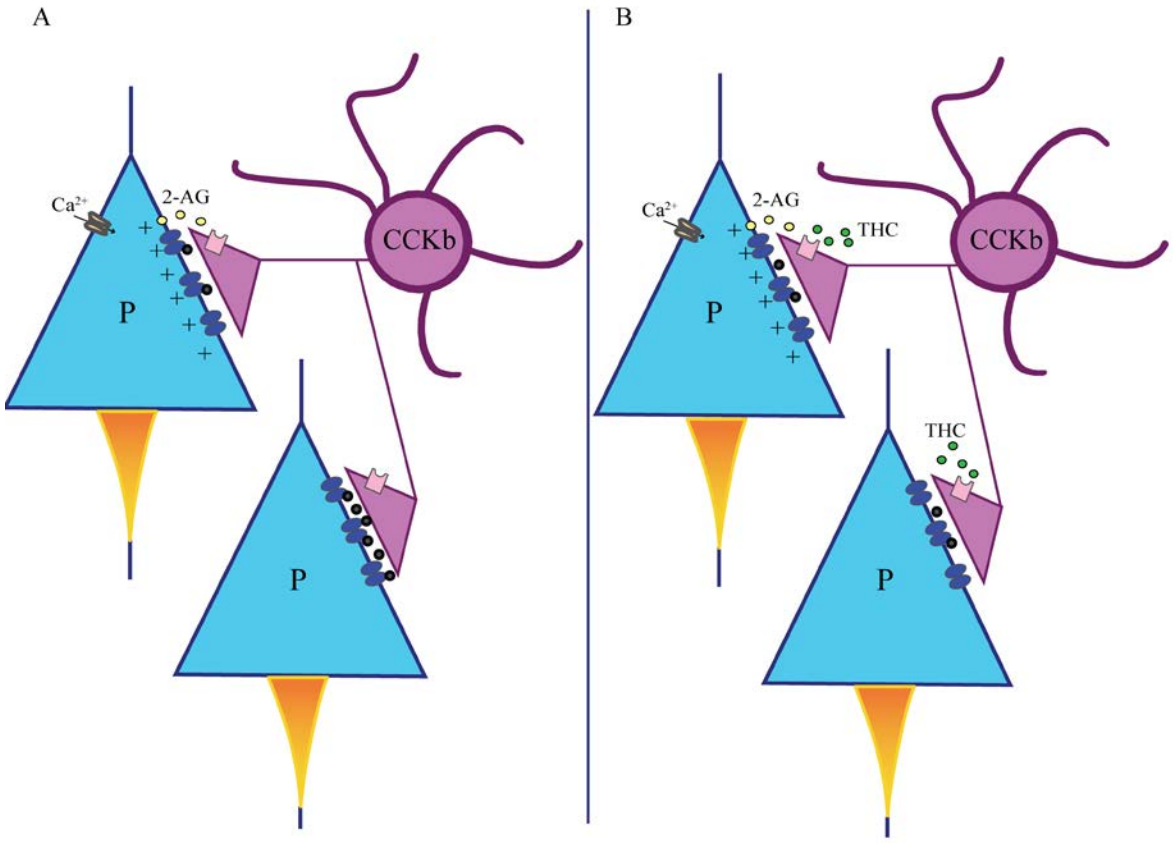

Figure 19. Synapse specific effect of endocannabinoid signaling

(A) Precise "on-demand" nature of endocannabinoid signaling. Upper pyramidal (P) cell is depolarized as indicated by (+), leading to the production and release of 2-AG which suppresses GABA release form a CCKb (purple neuron) terminal via CB1R activation. Lower pyramidal cell is quiescent resulting in normal levels of GABA release from the same CCKb neuron. **(B)** Proposed effect of THC, which abolishes the synapse-specific nature of endocannabinoid signaling by activating CB1R at all synapses regardless of the membrane polarization state of the postsynaptic neuron.

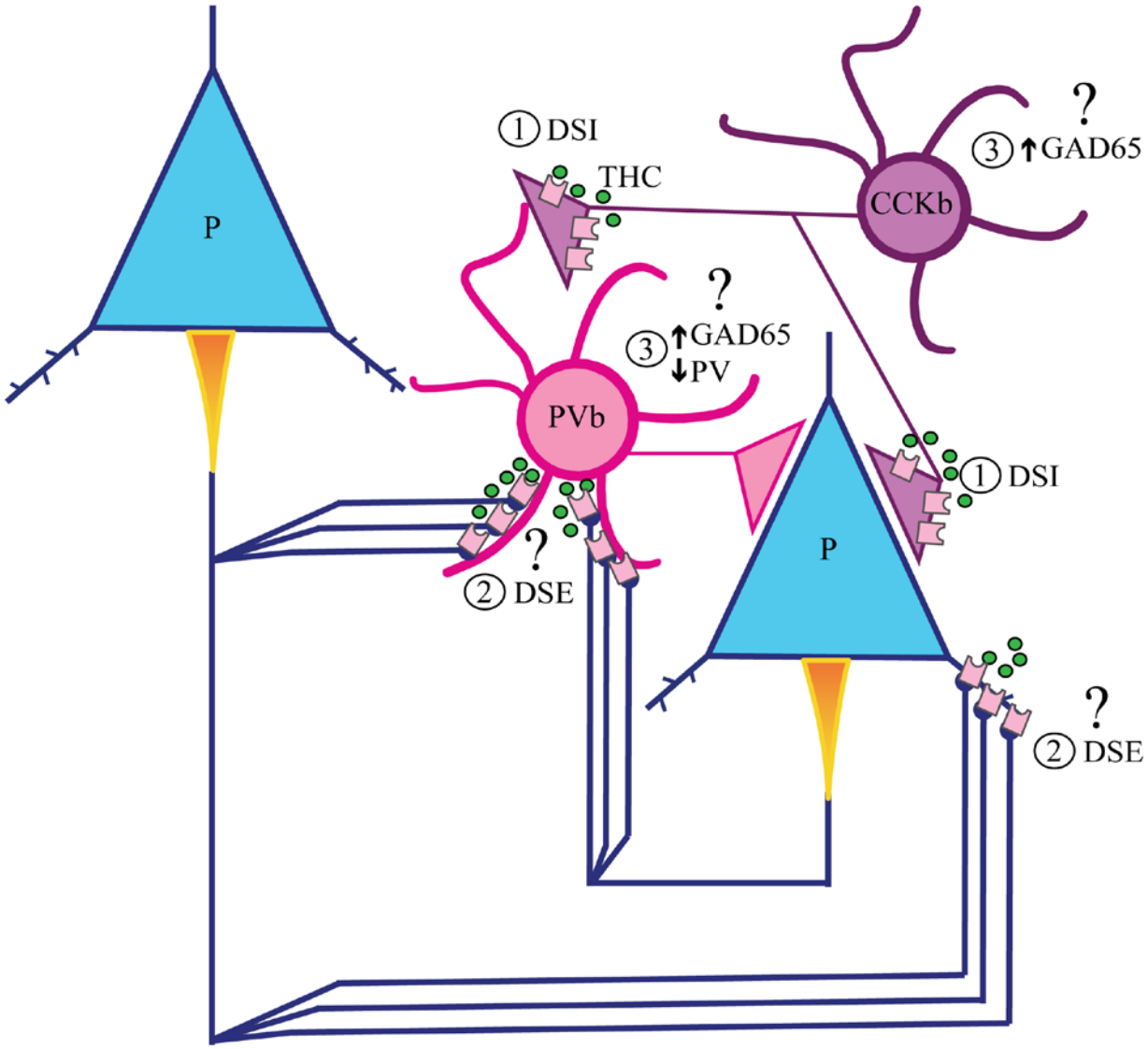

Figure 20. Potential effects of THC on PFC neural activity

THC-induced alterations of inputs to PVb neurons may disrupt adolescent PV neuron maturation. **(1)** DSI is depicted as occurring at GABA terminals from CCKb apposed to PVb and pyramidal (P) neurons. However, whether the same CCKb targets both PVb and pyramidal neurons is unknown. **(2)** DSE is shown to occur either directly at inputs to PVb neurons, or indirectly at glutamate inputs to other pyramidal neurons that also innervate PVb. Question marks indicate that the CB1R localization on glutamate inputs is unknown, especially in the primate PFC, and it is unclear what the functional impact of chronic CB1R activation on PFC neural circuit activity may be in response to THC.

excessive amounts of THC during adolescence upsets the balance between “rhythm” and “mood” (Freund, 2003; Freund and Katona, 2007). Alterations in DSE can potentially affect excitatory inputs to PVb neurons. For example, competition between direct (pyramidal inputs to PVb) and indirect (pyramidal to pyramidal to PVb) reductions in glutamate release from pyramidal cell axon terminals to PVb could also influence network excitability and potentially alter the normally precisely sculpted modulation of glutamate release from these axon terminals **(Figure 20)**. If CB1R become functionally uncoupled from their G-protein signaling pathways (Katona and Freund, 2012), then the mechanisms of protection from excitotoxicity or excessive inhibition may no longer properly function when needed. Thus, a reduction in excitatory drive of PVb neurons may lead to a reduction of PV expression, since it is linked with cell activity (Heizmann, 1984). In contrast, a decline in GABA release from CCKb may result in a reduction of GAD65 protein levels and subsequent compensatory response that increases GAD65 mRNA levels.

The consequences of chronic THC exposure on tonic endocannabinoid signaling must also be considered as a possible mechanism, since the ambient amount of extracellular cannabinoid concentration has been recently shown to affect the release of neurotransmission (Katona and Freund, 2012). For example, paired recordings in rodent hippocampus showed that tonic endocannabinoid signaling can suppress GABA release in a CB1R-dependent manner (Losonczy *et al.*, 2004). The persistence of elevated THC levels may alter the tone of endocannabinoid signaling by reducing terminal CB1R density or uncoupling CB1R from their G-protein signaling pathways (Katona and Freund, 2012). These changes raise the possibility that CCKb terminals (and possibly glutamate terminals) would no longer be sensitive to endocannabinoids released from the depolarized postsynaptic cell. Therefore, the precise “break in inhibition” mechanism would be lost and output from the postsynaptic cell would likely be

altered. Consequently, alterations in CCKb and pyramidal cell inputs to PVb during sensitive periods of postnatal development could disrupt the normal maturation of PVb neurons.

5.2.2 Implications for schizophrenia

Recent behavioral studies in the same monkeys used in **3.0** showed that repeated THC exposure during adolescence led to residual impairments in spatial working memory processes that persisted during the period of exposure and did not produce tolerance to the acute impairing cognitive effects of THC (Verrico *et al.*, *American Journal of Psychiatry*, In Revision). We reported evidence of molecular changes in dorsolateral PFC GABA circuitry from these monkeys. Together, these findings raise concerns that cannabis use during adolescence may result in poorer academic performance (Fergusson and Boden, 2008; Lynskey and Hall, 2000; Harvey *et al.*, 2007) by potentially interfering with normal maturation of PFC neural circuits. Moreover, cannabis use in individuals with a predisposition for developing schizophrenia may impact PFC circuitry in such a way that increases the likelihood, and accelerates the onset, of psychosis (Henquet *et al.*, 2005a; Large *et al.*, 2011; Moore *et al.*, 2007; Veen *et al.*, 2004).

5.2.3 Future Direction

The impacts of these many changes in response to chronic THC are currently unclear; however, one plausible approach to disentangling the effects of THC on glutamatergic and GABAergic inputs is to conduct a dose-response study in conditional glutamatergic and GABAergic CB1R knockout mice (Monory *et al.*, 2006) using THC and measure its effect on DSI, DSE, and tonic endocannabinoid signaling. A study in these conditional knockout animals that would measure

the trajectories of GABA-related mRNA level expression in the mouse medial PFC, which is thought to be the mouse homologue of the primate dorsolateral PFC, is also of great interest in order to better understand the effects of THC at glutamate or GABA terminals on GABA neuron maturation. Whether these molecular findings in rodents apply to the primate PFC is currently unclear and warrants further study.

The long period of maturation of PV neural inputs to pyramidal cells provides many opportunities, as well as cell type-specific targets, for the effects of perturbations to be amplified as they alter the trajectories of the developmental events that follow. Indeed during the same period, monkeys and humans undergo substantial improvements in emotion regulation and cognitive control. The temporal overlap of the opposing developmental changes in PVch and PVb boutons may provide the opportunity for a single environmental risk factor for schizophrenia (i.e. cannabis use during adolescence) to disrupt the maturation of both PV terminal subtypes. In a future study, we plan to measure both the density of PVb, PVch, and CCKb boutons and relative levels of GAD67, GAD65, PV and CB1R protein within these bouton populations in the same adolescent, THC-treated animals **(3.0)** using a multiple label, confocal immunofluorescence microscopy and image segmentation approach **(4.0)**. Given the terminal specific expression of GAD67, GAD65, PV and CB1R protein, we can discriminate among the three terminal populations described above as follows: PVb boutons would consist of GAD65/PV fluorescence overlap; PVch boutons would consist of GAD67/PV overlap; and CB1R would have GAD65/CB1R overlap. Therefore, in this study, we will be able to measure bouton-specific levels of GAD, PV and CB1R in response to chronic adolescent THC exposure. We will also be able to measure the comparative densities of these three bouton populations

across experimental and control groups. This future study could provide evidence that chronic THC exposure during adolescence affects PVch and PVb bouton maturation.

5.3 PV-CONTAINING CHANDELIER AND BASKET CELL BOUTONS IN THE PFC: CELL TYPE-SPECIFIC POSTNATAL DEVELOPMENT

In **4.0**, we reported that the number of PVch boutons per AIS declined between 3 months and adulthood in monkey PFC, but that relative vGAT and PV protein levels per PVch bouton and levels of adjacent γ2 receptor clusters at AIS did not differ between these two age groups. Since the detectability of two proteins involved in GABA transmission per PVch bouton and the adjacent postsynaptic receptor clusters did not change, these findings suggest that PVch boutons were pruned between infancy and adulthood in monkey PFC.

5.3.1 PVch structural plasticity – More than loss, possible rearrangement

The substantial loss of PVch boutons per AIS was accompanied by a similar magnitude decrease in AIS length, resulting in a stable density of PVch boutons per AIS in both 3 mo and adult animals. In order to determine if the reduction in AIS length altered the distribution of PVch boutons in adult animals despite no change in PVch bouton density, the data were binned into 3 groups representing the proximal, middle, and distal thirds of the total AIS length relative to the axon hillock. The distribution of boutons differed across age groups (X^2 [2] = 30.5, P < 0.001) **(Figure 21)**. In the 3 mo monkeys, the proportions of PVch boutons significantly differed ($F_{2,6}$ = 8.49, P = 0.018) between the proximal (31.5%) middle (36.8%), and distal (31.7%) portions of

the AIS. Post-hoc tests found a significant difference in the middle region compared to both the proximal and distal regions (P = 0.025 and 0.03, respectively). In adult monkeys, the proportions of PVch boutons significantly differed ($F_{2,6}$ = 48.6, P < 0.001) between the proximal (35.4%) middle (34.8%), and distal (29.8%) portions of the AIS. Post-hoc tests found a significant difference in the distal region compared to both the middle and proximal regions (P < 0.001, for both). The difference in PVch distribution between 3 mo and adult monkeys suggests that these connections are significantly rearranged between these time points.

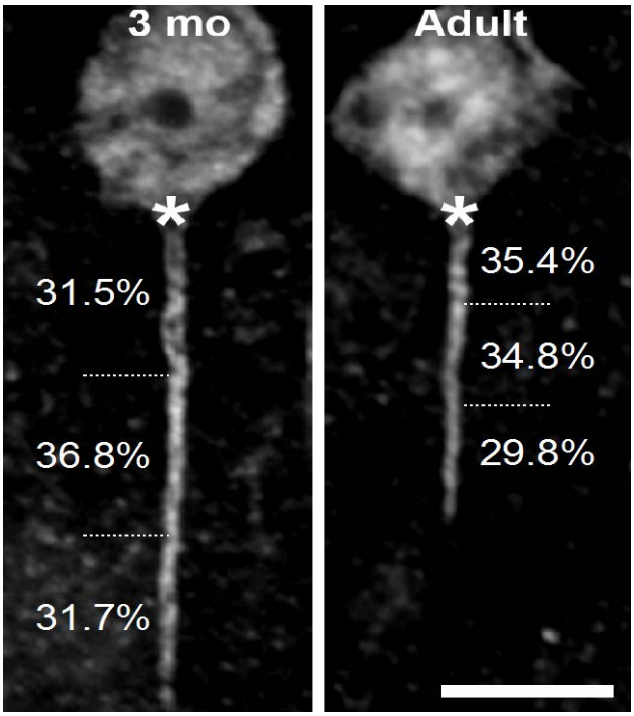

Figure 21. PVch distribution along the AIS differs between 3 mo and adult animals

Representative images of NeuN-IR somata and AnkG-IR AIS from 3 mo (left) and adult (right) animals. Values represent the mean percentage of PVch boutons per third along the AIS for each age group. Asterisks denote the axon hillock. Scale bar, 10 μm.

Previous studies have indicated that PVch boutons can be differentially distributed along AIS (DeFelipe and Farinas, 1992), and that AIS are highly plastic structures (reviewed in (Grubb

et al., 2011). These reports were made in regions and in species other than the adult monkey (DeFelipe *et al.*, 1985; Farinas and DeFelipe, 1991; Jones and Powell, 1969; Freund *et al.*, 1983). In addition, recent studies have found that the distribution of the AnkG compartment within AIS varies as a function of neuronal activity (reviewed in (Grubb *et al.*, 2011). Interestingly, removing input by unilateral cochlear ablation increased the AnkG AIS length by 1.7-fold and increased voltage gated sodium channel clustering (Kuba *et al.*, 2010). In contrast, increasing the excitatory drive in rodent dissociated hippocampal neuronal cultures using extracellular KCl or by evoking AP burst firing using optogenetics did not alter AIS length as measured by AnkG immunoreactivity, but shifted the entire structure 17 μm away from the soma (Grubb and Burrone, 2010). At the molecular level, AnkG is critical for concentrating a number of structural components at the AIS including the cell adhesion molecule, neurofascin 186 (Hedstrom *et al.*, 2007). Recent evidence suggests that the neurofascin 186 gradient is critical for the proper targeting of GABA synapses to the AIS (Ango *et al.*, 2004). Recent studies indicate that alterations in AIS length may occur *in vivo* (Baalman *et al.*, 2013; Hinman *et al.*, 2013). For example, cortical and hippocampal AIS were significantly shorter in rats exposed to a single blast wave that induced mild traumatic brain injury and behavior phenotypes (Baalman *et al.*, 2013). In concert, an activity-dependent shift in AnkG may alter the distribution of neurofascin 186 such that PVch boutons redistribute between 3 months and adulthood.

5.3.2 PFC activity as a driving force for PVch and PVb bouton maturation

The mechanisms by which these distinctive modes of maturation in PVch and PVb boutons occur may ultimately depend upon PFC neural activity. For example, as discussed in the previous section, a loss of PVch boutons may occur in response to AIS reorganization. Longer

AnkG-labeled AIS during early postnatal development could provide an initial large target for stochastic innervations by pathfinding PVch axons. Once innervated and in response to changes in PFC network activity during postnatal development, AIS would be refined, potentially along with the PVch terminals that innervate them. Shortening of AIS both in response to increase neural activity and during postnatal development has been shown in slice preparations *in vitro* (Kuba *et al.*, 2010). However, the developmental dynamics of PVch boutons and AnkG-immunoreactivity at the same AIS have not been studied.

The functional impact of changes in PVch innervation of pyramidal cell AIS will depend upon a number of factors, including understanding the effect of GABA release on AIS membrane polarization. Recent studies have found that PVch are depolarizing and can even be excitatory under certain conditions in certain brain regions (Woodruff *et al.*, 2009; Woodruff *et al.*, 2011; Szabadics *et al.*, 2006). Another study reported that PVch tonically inhibited the AIS and blocked back-propagating action currents during induced rhythmic activity *in vitro* (Dugladze *et al.*, 2012). Thus, detailed studies on the state- and region-dependent effects of PVch GABA transmission on pyramidal neuron activity are critical for understanding the dynamic structural changes observed in our studies.

While PVb boutons do not appear to be pruned like PVch, they undergo functional maturation between 3 months and adulthood in monkey PFC. We observed increases in the relative protein levels of vGAT and PV in PVb boutons **(Table 1)** without a corresponding change in the density of these boutons in the neuropil. Increases in these proteins may indicate an altered capacity for the regulation of GABA neurotransmission, since vGAT loads GABA into presynaptic vesicles (Chaudhry *et al.*, 1998) and PV regulates intracellular Ca^{2+} dynamics (Eggermann and Jonas, 2012). Increases in PV neural activity during postnatal development may

drive the increases in PV protein levels within PVb boutons, since changes in PV content have been reported to be associated with corresponding increases or decreases in neuronal activity (Heizmann, 1984). The lack of change in PV and vGAT protein levels within PVch between 3 months and adulthood may indicate that PVch boutons have already undergone increases in these proteins before 3 months of age.

5.3.3 Implications for schizophrenia

PVch cells currently represent the only known pathological entity in schizophrenia to have alterations in both pre and postsynaptic protein components. In light of our findings, the robust 40% reduction in the detectability of GAT1-IR cartridges in the PFC of schizophrenia subjects (Pierri *et al*, 1999) may reflect a structural loss of PVch boutons. Whether a structural loss of PVch boutons or a reduction of GAT1 protein levels per intact PVch bouton occurs in schizophrenia has potential implications for therapeutic intervention. For example, if presynaptic PVch can no longer release enough GABA when required, then allosteric modulators of $GABA_A$ α2 may help boost the effect of the limited amount of GABA released by keeping the postsynaptic receptors open for a longer period of time, but only when GABA is bound to the receptor. In contrast, if the presynaptic PVch structure is no longer present, then such an allosteric modulator would be ineffective, assuming that no tangible amounts of GABA would be synaptically present when needed. Recent studies found that a novel compound that enhances GABA transmission at $GABA_A$ α2-containing receptors was associated with working memory functional improvement in subjects with schizophrenia (Lewis *et al.*, 2008) and in an animal model of cognitive deficits of schizophrenia (Castner *et al.*, 2010). In contrast, a larger clinical study in chronically ill subjects with schizophrenia showed little benefit (Buchanan *et al.*, 2011).

Still, the possibility that this kind of intervention could be beneficial earlier in the illness, and possibly before psychosis onset, remains to be determined (Lewis, 2012).

Similar to the contrast between PVch and PVb during postnatal development, PVb boutons appear to have reduced levels of PV protein, without a change in bouton density in the PFC of subjects with schizophrenia (Glausier *et al*, *Molecular Psychiatry*, In Revision). These findings may reflect reduction in PFC network activity in schizophrenia, and ultimately may require interventions that alter network excitability either by boosting excitation or dampening inhibition.

5.3.4 Future direction

Studying the cell type-specific maturation of PV neurons is of special interest in the context of understanding how these findings may inform on the pathogenesis of schizophrenia. In this regard, it would be interesting to pursue a multiple label, confocal immunofluorescence microscopy study in postmortem human PFC from subjects with schizophrenia. Given our developmental findings, testing whether PVch bouton number per AIS is lower without a change in PVb bouton density in the same subjects with schizophrenia would provide valuable information at the terminal specific level. We have been able to robustly image GAD67, GAD65, PV, GAT1, βIV spectrin, an AIS marker similar to AnkG; and $GABA_A$ α1 and γ2 in postmortem human tissue (Fish KN, Personal Communication). Therefore, multiple label confocal immunofluorescence microscopy studies examining PVch and PVb are feasible in schizophrenia and comparison subjects.

Given the time-limited nature of sensitive periods during development, studying more time points during postnatal development is important for determining more precisely when

these changes in PVch and PVb occur. Thus, the more precise timing of PVch bouton loss and PV protein level increases in PVb are the foci of ongoing investigations. We are currently examining the same measures as presented in **4.0** during the perinatal period (1 week and 1 month of age) and during adolescence (18 and 45 months of age).

5.4 PREEMPTIVE POTENTIAL IN SCHIZOPHRENIA

The studies presented in this dissertation ultimately aim to provide information that could be critical for advancing preemptive and preventive strategies during preclinical illness progression or for reducing preclinical risk for schizophrenia.

5.4.1 Timing of intervention

Since the appearance of the diagnostic clinical features of schizophrenia is believed to represent a late, and potentially preventable, outcome of the illness, the development of effective preemptive treatments requires knowledge of neural circuit abnormalities that underlie the central features of the illness, when during the course of development these abnormalities arise, and how to detect these abnormalities before their functional impact becomes clinical (Insel, 2010; Lewis, 2012).

Whether key pathological events in schizophrenia occur during the perinatal period, adolescence, or some other period depending on the individual will determine when to intervene. These interventions may require pharmacological approaches to target and rescue molecular pathways that may be upstream of the inhibitory deficit in schizophrenia or that may modulate

GABA neurotransmission. For example, PFC GABA inhibition may be reduced in schizophrenia as a compensation for an upstream reduction in excitation (Lewis *et al.*, 2012). In this case, interventions might need to be delivered early in life and target molecular pathways that regulate spine formation. Alternatively, in some cases, a primary deficit in specific GABA neurons may underlie the pathogenesis of schizophrenia. In line with this possibility, allelic variation in GAD1 has been associated with schizophrenia (Straub *et al.,* 2007). Given that Lhx6 expression deficits are associated with deficits in GAD67 and PV in some individuals with schizophrenia, intervening by boosting Lhx6 levels during early development may rescue some of the phenotypic alterations in PV neurons in schizophrenia. It is possible that targeting both excitatory pathways and boosting GABA neurotransmission could have synergistic effects on restoring circuit function in schizophrenia. Importantly, understanding the normal maturation of PFC circuitry may also provide information about when interventions should be held for safety.

As previously discussed, cannabis use before the age of 16 years has been reported to increase the risk for, and decrease the age of onset of, schizophrenia in a dose-dependent manner (Moore *et al.*, 2007). In fact, a recent meta-analysis estimated that about 14% of schizophrenia cases would not occur without cannabis consumption (Moore *et al.*, 2007). Interestingly, a recent questionnaire that measured subclinical psychotic experiences found that individuals without any evidence of a predisposition for schizophrenia responded to cannabis by feeling more at ease with the world, while those who had reported previous subclinical psychotic experiences has increased feelings of suspicion and hostility as well as marked perceptual changes (Verdoux *et al.*, 2003; Konings and Maharajh, 2006). Therefore, public health measures aimed at individuals at risk for psychosis and designed to reduce or delay the use of cannabis during the early teenage years may help substantially reduce the appearance of psychosis in schizophrenia.

In those individuals who are at risk and consistently use cannabis during adolescence, interventions during adolescence or in early adulthood may ameliorate some of the circuit alterations. For example, allosteric modulators of different types of g-protein coupled receptors whose signaling cascade results in the release of endocannabinoids can be used to alter levels of these cannabinoids (Porter and Felder, 2001). In addition, a number of cannabinoids that can modulate the effect of THC exist, and detailed studies of these compounds and their effects on cannabinoid signaling, the appropriate dosage, route of administration, and timing of intervention need to be studied (Croxford, 2003) prior to understanding whether manipulation of this system will be therapeutic in schizophrenia.

5.4.2 Harnessing neural plasticity

The association of multiple genetic and environmental risk factors for schizophrenia well before the clinical onset of diagnostic features may lead to the grim conclusion that individuals who begin with high risk are on an irreversible path to developing the illness. In this view, development is an especially vulnerable period to insults that can result in the emergence of significant pathology later in life. For example, metabolic disorders like phenylketonuria can lead to profound and irreversible cognitive disability when left uncorrected during development, but in adults they seem to have milder effects (Hanley, 2004). However, there are examples of other pathologies like trauma or infection that have milder effects during development than in adults (Kolb *et al.*, 2000). One remarkable example of the human brain's ability to adapt occurs in young children who undergo functional hemispherectomy as a result of intractable seizures, a damaged hemicortex and hemiplegia. In many of these individuals, not only do they recover from seizures, but also they can improve in cognitive and motor functioning (Wiebe and Berg,

2013). These examples support the alternative view that individuals with neurodevelopmental disorders like schizophrenia are not necessarily "doomed from the womb."

Harnessing adult neural plasticity to compensate for or possibly correct specific pathologies may be a viable way to conceptualize future treatment strategies for schizophrenia. Certain genes known to be critical for normal pre and postnatal neurodevelopment and possibly disrupted during those periods [i.e. DISC1, NRG1 (Jaaro-Peled *et al.*, 2009)], and notably Lhx6, are also expressed in the adult brain. Ongoing disruptions in the expression of these genes during adulthood may contribute to cognitive dysfunction in schizophrenia. Thus, adult rescue of molecules that are critical during development but also function in adulthood may help alleviate or correct disruptions that begin during development. Recent studies suggest that the adult rodent cortex remains plastic, with the ability to produce structural changes in dendritic spines (Trachtenberg *et al.*, 2002), axons (Florence *et al.*, 1998) and dendrites (Tailby *et al.*, 2005). In rodents, substantial plasticity can occur beyond the developmental critical period in the somatosensory and visual cortices (Buonomano and Merzenich, 1998; Sawtell *et al.*, 2003). Again, whether these findings in rodents apply to the primate PFC is an open question that remains to be studied.

A more promising option may be to alter pathological plasticity as it occurs during sensitive periods of development. This approach may have a higher impact since the mechanisms of plasticity during sensitive periods appear to differ from those in adulthood. For example, dendritic spine motility decreases beyond the critical period (Holtmaat *et al.*, 2005) and thalamocortical axon plasticity and long term potentiation are reduced or eliminated (Crair and Malenka, 1995; Hubel and Freeman, 1977). In rodents, the critical period window for ocular dominance plasticity can be manipulated by altering levels of cortical inhibition (Fagiolini and

Hensch, 2000; Hensch, 2005; Hensch *et al.*, 1998; Huang *et al.*, 1999). Recent findings suggest that PV inputs to $GABA_A$ α1 containing receptor clusters are crucial for setting the timing of this critical period window (Fagiolini *et al.*, 2004) and thus may represent both a locus of cellular vulnerability and a potential therapeutic target. Another recent study showed that $GABA_A$ α1 number decreased in the soma-proximal dendrite compartment, but not on the AIS with age and sensory deprivation. Interestingly, the number of soma-proximal dendrite $GABA_A$ α1 in immature or dark-reared mice were adjusted to critical period levels by in vivo benzodiazepine treatment, triggering ocular dominance plasticity in these animals (Katagiri *et al.*, 2007). Using a GAD65 knockout animal model and subsequent rescue the critical period onset with dark-rearing in these animals, they found that an intermediate number of PV to $GABA_A$ α1 connections is required to trigger the critical period. These findings raise implications that in schizophrenia, the appropriate trigger for sensitive periods of development may be delayed or accelerated, since perisomatic PV inputs to $GABA_A$ α1 clusters appear to be altered in the disorder. The potential for manipulating the onset of sensitive periods in PFC circuits holds promise as a novel therapeutic intervention; however, determining the precise timing and nature of the onset of PFC sensitive periods is required first.

5.5 CONCLUDING REMARKS

This dissertation explored the postnatal development of PFC GABA neurons with the goals of identifying periods of vulnerability to schizophrenia, the impact of an environmental insult on developing PFC GABA neurons, and the cell type-specific maturation of PFC GABA neuron axon terminals. We found gene expression changes in key functional markers of GABA

neurotransmission during the perinatal and adolescent periods of development **(2.0)**, periods of development that are associated with a variety of risk factors for schizophrenia. The mRNA levels for these same markers did not change as a function of age or illness duration in subjects with schizophrenia, suggesting that chronic illness processes may not have substantially contributed to their expression profiles in schizophrenia. Since environmental factors are known to affect developing neural systems and contribute to the etiology of schizophrenia, we studied the impact of chronic THC administration on markers of PFC GABA neurotransmission during adolescence in monkeys. We reported changes in some, but not all, GABA-related mRNAs suggesting that repeated THC exposure selectively alters PFC GABA neurons during adolescent development **(3.0)**. Finally, we examined cell type-specific axon terminal development in the PFC and found that PVch and PVb boutons have distinctive modes of maturation, with a decrease in number of PVch boutons per AIS and an increase in terminal protein levels within PVb boutons between infancy and adulthood **(4.0)**. Each of these findings highlights the dynamic, "double-edged sword" of postnatal PFC development, which may not only open windows of susceptibility to pathological insults, but also windows of adaptability to pathological alterations in schizophrenia.

APPENDIX A

A.1 DEMOGRAPHIC, POSTMORTEM, AND CLINICAL CHARACTERISTICS OF HUMAN SUBJECTS USED IN THIS DISSERTATION

Table 6. Human subjects used in this dissertation.

Healthy Comparison Subjects								
Pair	**Case**	**Sex/ Race**	**Age**	**PMI[a]**	**Storage Time[b]**	**RIN**	**pH**	**Cause of Death**
1	592	M/B	41	22.1	174	9.0	6.7	ASCVD
2	567	F/W	46	15.0	178	8.9	6.7	Mitral valve prolapse
3	516	M/B	20	14.0	185	8.4	6.9	Homicide by gun shot
4	630	M/W	65	21.2	168	9.0	7.0	ASCVD
5	604	M/W	39	19.3	172	8.6	7.1	Hypoplastic coronary artery
6	546	F/W	37	23.5	182	8.6	6.7	ASCVD
7	551	M/W	61	16.4	181	8.3	6.6	Cardiac tamponade
8	685	M/W	56	14.5	161	8.1	6.6	Hypoplastic coronary artery

9	681	M/W	51	11.6	162	8.9	7.2	Hypertrophic cardio-myopathy
10	806	M/W	57	24.0	141	7.8	6.9	Pulmonary embolism
11	822	M/B	28	25.3	138	8.5	7.0	ASCVD
12	727	M/B	19	7.0	155	9.2	7.2	Trauma
13	871	M/W	28	16.5	128	8.5	7.1	Trauma
14	575	F/B	55	11.3	177	9.6	6.8	ASCVD
15	700	M/W	42	26.1	160	8.7	7.0	ASCVD
16	988	M/W	82	22.5	106	8.4	6.2	Trauma
17	686	F/W	52	22.6	162	8.5	7.0	ASCVD
18	634	M/W	52	16.2	168	8.5	7.0	ASCVD
19	852	M/W	54	8.0	131	9.1	6.8	Cardiac tamponade
20	987	F/W	65	21.5	107	9.1	6.8	ASCVD
21	818	F/W	67	24.0	140	8.4	7.1	Anaphylactic reaction
22	857	M/W	48	16.6	130	8.9	6.7	ASCVD
23	739	M/W	40	15.8	155	8.4	6.9	ASCVD
24	1047	M/W	43	13.8	98	9.0	6.6	ASCVD
25	1086	M/W	51	24.2	92	8.1	6.8	ASCVD
26	1092	F/B	40	16.6	91	8.0	6.8	Mitral Valve Prolapse
27	10005	M/W	42	23.5	79	7.4	6.7	Trauma
28	1336	M/W	65	18.4	56	8.0	6.8	Cardiac Tamponade
29	1122	M/W	55	15.4	88	7.9	6.7	Cardiac Tamponade

30	1284	M/W	55	6.4	67	8.7	6.8	ASCVD
31	1191	M/B	59	19.4	80	8.4	6.2	ASCVD
32	970	M/W	42	25.9	109	7.2	6.4	ASCVD
33	10003	M/W	49	21.2	80	8.4	6.5	Trauma
34	1247	F/W	58	22.7	73	8.4	6.4	ASCVD
35	1324	M/W	43	22.3	59	7.3	7	Aortic Dissection
36	1099	F/W	24	9.1	91	8.6	6.5	Cardiomyopathy
37	1307	M/B	32	4.8	62	7.6	6.7	ASCVD
38	1391	F/W	51	7.8	48	7.1	6.6	ASCVD
39	1282	F/W	39	24.5	67	7.5	6.8	ASCVD
40	1159	M/W	51	16.7	85	7.6	6.5	ASCVD
41	1326	M/W	58	16.4	59	8.0	6.7	ASCVD
42	902	M/W	60	23.6	124	7.7	6.7	ASCVD
		Mean	48.1	17.8	120.7	8.3	6.8	
		SD	13.3	5.9	43.6	0.6	0.2	

Subjects with Schizophrenia

Pair	Case	DSM IV diagnosis	Sex/ Race	Age	PMI[a]	Storage Time[b]	RIN	pH	Cause of Death	Previously Studied for vGAT, GAT1, or GABRA1 mRNA
1	533	Chronic undifferentiated schizophrenia	M/W	40	29.1	184	8.4	6.8	Accidental Asphyxiation	GABRA1, GAT1

2	537	Schizoaffective disorder	F/W	37	14.5	183	8.6	6.7	Suicide by hanging	GABRA1, GAT1
3	547	Schizoaffective disorder	M/B	27	16.5	182	7.4	7.0	Heat Stroke	GABRA1, GAT1
4	566	Chronic undifferentiated schizophrenia; AAR	M/W	63	18.3	179	8.0	6.8	ASCVD	GABRA1, GAT1
5	581	Chronic paranoid schizophrenia; ADC; OAC	M/W	46	28.1	176	7.9	7.2	Accidental combined drug overdose	GABRA1, GAT1
6	587	Chronic undifferentiated schizophrenia; AAR	F/B	38	17.8	175	9.0	7.0	Myocardial hypertrophy	GABRA1, GAT1
7	625	Chronic disorganized schizophrenia; AAC	M/B	49	23.5	169	7.6	7.3	ASCVD	GABRA1, GAT1
8	622	Chronic undifferentiated schizophrenia	M/W	58	18.9	169	7.4	6.8	Right MCA infarction	GABRA1, GAT1
9	640	Chronic paranoid schizophrenia	M/W	49	5.2	167	8.4	6.9	Pulmonary embolism	GABRA1, GAT1
10	665	Chronic paranoid schizophrenia; ADC	M/B	59	28.1	165	9.2	6.9	Intestinal hemorrhage	GABRA1, GAT1
11	787	Schizoaffective disorder; ODC	M/B	27	19.2	144	8.4	6.7	Suicide by gun shot	GABRA1, GAT1
12	829	Schizoaffective disorder; ADC; OAR	M/W	25	5.0	136	9.3	6.8	Suicide by salicylate overdose	GABRA1, GAT1
13	878	Disorganized schizophrenia; ADC	M/W	33	10.8	127	8.9	6.7	Myocardial fibrosis	GABRA1, GAT1
14	517	Disorganized schizophrenia; ADC	F/W	48	3.7	186	9.3	6.7	Intracerebral hemorrhage	GABRA1
15	539	Schizoaffective disorder; ADR	M/W	50	40.5	184	8.1	7.1	Suicide by combined drug overdose	GABRA1

16	621	Chronic undifferentiated schizophrenia	M/W	83	16.0	170	8.7	7.3	Accidental asphyxiation	GABRA1
17	656	Schizoaffective disorder; ADC	F/B	47	20.1	166	9.2	7.3	Suicide by gun shot	GABRA1
18	722	Chronic undifferentiated schizophrenia; ODR; OAR	M/B	45	9.1	156	9.2	6.7	Upper GI bleeding	GABRA1
19	781	Schizoaffective disorder; ADR	M/B	52	8.0	146	7.7	6.7	Peritonitis	GABRA1
20	802	Schizoaffective disorder; ADC; ODR	F/W	63	29.0	142	9.2	6.4	Right ventricular dysplasia	GABRA1
21	917	Chronic undifferentiated schizophrenia	F/W	71	23.8	120	7.0	6.8	ASCVD	GABRA1
22	930	Disorganized schizophrenia; ADR; OAR	M/W	47	15.3	116	8.2	6.2	ASCVD	GABRA1
23	933	Disorganized schizophrenia	M/W	44	8.3	116	8.1	5.9	Myocarditis	GABRA1
24	1209	Schizoaffective disorder	M/W	35	9.1	78	8.7	6.5	Suicide by diphenhydramine overdose	No
25	10025	Disorganized schizophrenia; OAR	M/B	52	27.1	71	7.8	6.7	ASCVD	No
26	1178	Schizoaffective disorder	F/B	37	18.9	83	8.4	6.1	Pulmonary embolism	No
27	1256	Undifferentiated schizophrenia	M/W	34	27.4	71	7.9	6.4	Suicide by hanging	No
28	1173	Disorganized schizophrenia; ADR	M/W	62	22.9	83	7.7	6.4	ASCVD	No

29	1105	Schizoaffective disorder	M/W	53	7.9	90	8.9	6.2	ASCVD	No
30	1188	Undifferentiated schizophrenia; AAR; OAR	M/W	58	7.7	81	8.4	6.2	ASCVD	No
31	1263	Undifferentiated schizophrenia; ADR	M/W	62	22.7	70	8.5	7.1	Accidental asphyxiation	No
32	1222	Undifferentiated schizophrenia; AAC	M/W	32	30.8	76	7.5	6.4	Suicide by combined drug overdose	No
33	1088	Undifferentiated schizophrenia; ADC; OAC	M/W	49	21.5	91	8.1	6.5	Accidental combined drug overdose	No
34	1240	Undifferentiated schizophrenia; ADR	F/B	50	22.9	73	7.7	6.3	ASCVD	No
35	10020	Paranoid schizophrenia; AAC; OAC	M/W	38	28.8	73	7.4	6.6	Suicide by salicylate overdose	No
36	10023	Disorganized schizophrenia	F/B	25	20.1	72	7.4	6.7	Suicide by drowning	No
37	10024	Paranoid schizophrenia	M/B	37	6.0	72	7.5	6.1	ASCVD	No
38	1189	Schizoaffective disorder; AAR	F/W	47	14.4	81	8.3	6.4	Suicide by combined drug overdose	No
39	1211	Schizoaffective disorder	F/W	41	20.1	79	7.8	6.3	Sudden unexplained death	No
40	1296	Undifferentiated schizophrenia	M/W	48	7.8	65	7.3	6.5	Pneumonia	No
41	1314	Undifferentiated schizophrenia	M/W	50	11.0	62	7.2	6.2	ASCVD	No
42	1361	Schizoaffective disorder; ODC	M/W	63	23.2	54	7.7	6.4	Cardiomyopathy	No

Mean	47.0	18.1	121.0	8.2	6.6
SD	12.8	8.7	46.1	0.7	0.4

Pair	Case	Antipsychotic ATOD	Antidepressant ATOD	Benzodiazepine/ VPA ATOD
1	533	Y	N	N
2	537	N	N	N
3	547	Y	Y	Y
4	566	Y	Y	Y
5	581	Y	N	Y
6	587	Y	N	Y
7	625	Y	Y	N
8	622	N	N	N
9	640	Y	Y	N
10	665	Y	Y	N
11	787	Y	N	N
12	829	N	N	Y
13	878	Y	Y	Y
14	517	Y	N	N
15	539	Y	Y	Y
16	621	N	N	N
17	656	Y	N	N
18	722	Y	N	N

19	781	Y	Y	N
20	802	Y	N	Y
21	917	Y	N	N
22	930	Y	N	Y
23	933	Y	Y	Y
24	1209	Y	N	N
25	10025	N	N	N
26	1178	Y	N	Y
27	1256	Y	N	N
28	1173	Y	N	N
29	1105	Y	N	N
30	1188	Y	N	N
31	1263	Y	Y	N
32	1222	Y	Y	N
33	1088	Y	Y	N
34	1240	Y	N	N
35	10020	Y	Y	Y
36	10023	Y	N	Y
37	10024	N	N	N
38	1189	Y	Y	Y

39	1211	Y	Y	N
40	1296	Y	Y	N
41	1314	Y	Y	N
42	1361	Y	N	Y
		36Y/6N	17Y/25N	15Y/27N

A.2 RHESUS MACAQUE MONKEYS USED IN THIS DISSERTATION (ALL FEMALE EXCEPT FOR SUBJECT 282)

Table 7. Rhesus macaque monkeys used in this dissertation.

Age Group (Mos.)	Subject	Age (Mos.)	Sex	Weight (kg)	Perfusion Status	Prior Biopsy Status	Storage Time
Perinatal (0.25 to 1)	193	0.25	Female	NA	-	-	120
	194	0.25	Female	NA	-	-	119
	199	0.25	Female	0.5	-	-	106
	201	0.25	Female	0.4	-	-	106
	285	0.25	Female	0.6	-	-	23
	196	1	Female	NA	-	-	115
	197	1	Female	NA	-	-	114
	200	1	Female	0.6	-	-	106
	209	1	Female	0.6	-	-	97
Childhood (3 to 9)	192	3	Female	0.8	-	-	120
	198	3	Female	0.8	-	-	112
	203	3	Female	0.9	-	-	103
	212	3	Female	1.1	-	-	94
	234	3	Female	0.9	+	-	70
	241	3	Female	1.0	+	-	63
	245	3	Female	1.2	+	-	55
	277	3	Female	1.0	+	-	31
	294	8	Female	1.8	-	-	11
	261	9	Female	1.6	-	-	42
	262	9	Female	1.8	-	-	42
	273	9	Female	2.0	+	-	36
	278	9	Female	1.6	-	-	31
Adolescence (15 to 37)	240	15	Female	2.3	+	-	63

	264	15	Female	2.5	+	-	42
	265	15	Female	2.4	+	-	44
	275	15	Female	2.4	+	+	33
	255	16	Female	2.6	+	-	44
	287	18	Female	2.4	-	-	15
	286	19	Female	2.4	-	-	15
	293	23	Female	3.2	-	-	12
	297	30	Female	4.9	-	-	7
	298	30	Female	4.2	-	-	7
	280	32	Female	3.8	+	-	27
	281	32	Female	3.7	+	-	27
	291	32	Female	3.9	-	-	13
	279	33	Female	4.5	+	-	28
	295	35	Female	4.3	+	+	10
	296	35	Female	4.4	+	+	9
	292	37	Female	5.0	-	-	12
Adulthood (42 to 138)	249	42	Female	6.2	+	-	53
	239	42	Female	5.5	+	-	63
	289	45	Female	5.7	-	-	15
	258	46	Female	6.3	+	-	43
	267	46	Female	5.7	+	+	40
	269	46	Female	4	+	+	38
	288	47	Female	5	-	-	15
	259	104	Female	6.4	+	-	43
	282	108	Male	11.7	-	-	26
	260	138	Female	9.5	-	-	43

APPENDIX B

COMPUTATIONAL ESTIMATES OF AGE EFFECTS ON GAD67 MRNA LEVELS PRIOR TO AND AFTER ILLNESS ONSET IN THE SCHIZOPHRENIA SUBJECTS

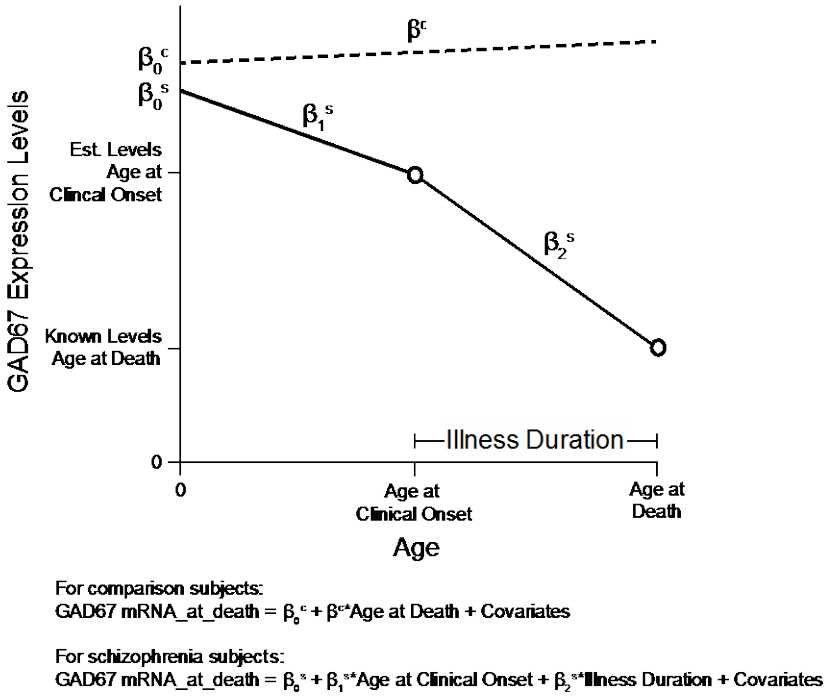

Figure 22. Computational estimate of age effects on GAD67 mRNA levels prior to and after illness onset in the schizophrenia subjects

Regression model is used for schizophrenia subjects to estimate the slopes of GAD67 mRNA expression changes before and after illness onset (solid line), where β_0^s represents intercept (i.e. transcript level at birth), β_1^s and β_2^s represent the slopes of GAD67 mRNA levels decline before and after illness onset, respectively. Rejecting the hypothesis H_0: $\beta_1^s=\beta_2^s$ versus H_A: $\beta_1^s\neq\beta_2^s$ implies that the effect of age for schizophrenia subjects is fit by a segmented line with differing slope before and after illness of onset. The slope of GAD67 mRNA expression decline over age for comparison subjects can also be obtained from a linear regression model (dashed line).

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