ALTERED MARKERS OF TONIC INHIBITION IN THE DORSOLATERAL PREFRONTAL CORTEX OF SUBJECTS WITH SCHIZOPHRENIA

by

Jaime Gerardo Maldonado-Avilés

Bachelor of Science, Biology, University of Puerto Rico, 2001

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This dissertation was presented

by

Jaime Gerardo Maldonado-Avilés

It was defended on

January, 2008

and approved by

Richard W. Olsen, PhD, Departments of Anesthesiology, Molecular and Medical Pharmacology, University of California Los Angeles

Gregg E. Homanics, PhD, Departments of Anesthesiology and Pharmacology

Jon W. Johnson, PhD, Departments of Neuroscience and Psychiatry

Susan R. Sesack, PhD, Department of Neuroscience

Robert A. Sweet, MD, Departments of Psychiatry and Neurology

Dissertation Advisor: David A. Lewis, MD, Department of Psychiatry

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Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) appear to contribute to the impairments in working memory observed in individuals with schizophrenia. Consistent with this idea, a microarray study indicated that the mRNA levels of $GABA_A$ receptor α4 and δ subunits were lower in the DLPFC of subjects with schizophrenia. However, although α4 and δ subunits co-assemble to form functional receptors, the differences in α4 and δ mRNA expression in schizophrenia were not correlated. We assessed the mRNA levels of α4 and δ in the DLPFC of 23 subjects with schizophrenia matched to control subjects by *in situ* hybridization. The level of α4 mRNA was lower only in subjects with schizophrenia receiving medications at the time of death, whereas the level of δ mRNA was significantly lower in schizophrenia, regardless of the medications used at the time of death. We also found that across postnatal development of monkey DLPFC the level of α4 mRNA decreased with age, whereas that of δ mRNA increased in a manner similar to that previously observed for the α1 subunit. Given that α1 mRNA levels are lower in schizophrenia and α1 subunits can co-assemble with δ subunits, lower δ mRNA in schizophrenia could represent lower $GABA_A$ α1βxδ rather than α4βxδ receptors.

Studies suggest that reduced signaling through excitatory synapses, as hypothesized to be present in schizophrenia, give rise to decreased expression of δ subunit mRNA. To test this hypothesis, we measured the levels of δ subunit mRNA in the prefrontal cortex of four rodent

models of reduced cortical excitatory drive: 1) NMDAR NR1 hypomorphic mice, 2) rats with adult mediodorsal thalamic nuclei lesions, 3) rats with neonatal ventral hippocampal lesions and 4) TrkB hypomorphic mice reported to have decreased dendritic arborization. However, the mRNA levels of δ subunit were unchanged in the PFC of any of the animal models analyzed. Thus, although reduced signaling through excitatory synapses might be a pathogenetic mechanism for other abnormalities in schizophrenia, the convergence of the findings from this study do not support the hypothesis that it accounts for the lower expression of $GABA_A$ receptor δ subunit mRNA.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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1.0 GENERAL INTRODUCTION

Schizophrenia is a substantial public health problem with great personal and economic costs worldwide. It is estimated that schizophrenia affects just under 1% of the world's population, affecting individuals in all societies and geographical areas (Bromet and Fennig, 1999). Characterized by a variety of symptoms, schizophrenia affects many domains of mental function, such as language, emotion, reasoning and perception. In males, schizophrenia most frequently develops in the late second to third decade of life, between ages 17 to 27; whereas, in females typical onset occurs over five years later (Carpenter, Jr. and Buchanan, 1994). Schizophrenia produces a lifetime of disability and emotional distress for affected individuals as well as family members. Furthermore, the illness causes significant long-lasting impairments, requiring ongoing clinical care. In 2002, the overall monetary cost associated with schizophrenia in the US was estimated at $62.7 billion (Wu et al., 2005).

1.1 CLINICAL SYNDROME: COGNITIVE DEFICITS AS A CORE FEATURE

The diagnosis of schizophrenia is based on identification of the clinical syndrome (American Psychiatric Association, 1994), which includes symptoms that are usually classified into three groups: positive symptoms, negative symptoms and cognitive deficits. Positive symptoms, which represent the presence of behavioral deficits, include hallucinations, delusions

and thought disorder (Flaum and Schultz, 1996). Negative symptoms reflect the absence of normal behaviors and include flat affect, among others (Flaum and Schultz, 1996). Cognitive deficits include impairments in attention, memory and executive function, such as the ability to initiate or persist in goal-directed behaviors (Elvevag and Goldberg, 2000).

Symptoms recognized collectively as "cognitive deficits" are among the most prominent and debilitating characteristics of the disease. In particular, subjects with schizophrenia show deficits in tasks that involve working memory, a system involved in cognitive operations that uses short-term storage of information in order to guide behavior (Baddeley, 1992). While other symptoms, such as psychosis, are usually the most striking clinical features of schizophrenia, several lines of evidence suggest that cognitive deficits represent the core feature of the illness. Cognitive deficits are present throughout the course of the illness (Davidson et al., 1999; Saykin et al., 1994; Breier et al., 1991; Heaton et al., 1994) and are often observed in a milder form in unaffected relatives (Sitskoorn et al., 2004). Furthermore, cognitive deficits have been found to have a greater impact on daily activities than psychotic symptoms (Green, 1996). In addition, deficits in cognition are thought to be the best predictors of long-term outcomes (Green, 1996; Harvey et al., 1998). Given that current antipsychotic medications have limited benefits for cognitive deficits (Medalia et al., 1988; Gold and Hurt, 1990; Goldberg et al., 1993), a deeper understanding of the disease process underlying these deficits is critical to developing more effective treatments.

1.2 DORSOLATERAL PREFRONTAL CORTEX: DEFICITS IN INHIBITORY CIRCUITRY

At least some of the cognitive deficits associated with schizophrenia appear to be linked to dysfunction of the dorsolateral prefrontal cortex (DLPFC), on which certain cognitive processes such as working memory depend (Carter et al., 1999; Botvinick et al., 2001; Cohen et al., 2002). For instance, subjects with schizophrenia often perform poorly on tasks involving working memory, and exhibit reduced DLPFC activation as reflected by lower blood flow, during those tasks (Weinberger et al., 1986; Perlstein et al., 2001). These deficits do not seem to be a consequence of chronic exposure to antipsychotic medication, as both medicated and unmedicated subjects with schizophrenia show altered activation of the DLPFC when performing a working memory task (Daban et al., 2005).

The abnormal activation of the DLPFC might be due in part to deficits in GABA neurotransmission. Working memory depends on the synchronized firing of pyramidal cells in the DLPFC between the temporary presentation of a stimulus cue and the later initiation of a behavioral response (Goldman-Rakic, 1995; Lewis et al., 2005). The activity of GABA-containing interneurons appears to play a central role in the synchronization of pyramidal cells during working memory (Lewis et al., 2005). For instance, interneurons, such as fast-spiking neurons, are active during the delay period of working memory tasks (Wilson et al., 1994) and are necessary for task-related neuronal firing and for the spatial tuning of neuronal responses during working memory (Rao et al., 2000; Lewis et al., 2005). Furthermore, injection of the GABA antagonist bicuculline into the DLPFC of monkeys disrupts the performance of a working memory task (Sawaguchi et al., 1989). Human postmortem studies are consistent with the idea that altered activity of GABA-containing cells contributes to deficits in the circuitry of

the DLPFC. In particular, the mRNA for the 67 kDa isoform of glutamate decarboxylase (GAD_{67}), the principal enzyme responsible for the synthesis of GABA, is significantly reduced in the DLPFC of subjects with schizophrenia (Akbarian et al., 1995; Volk et al., 2000; Guidotti et al., 2000; Straub et al., 2007; Hashimoto et al., 2008a; Hashimoto et al., 2005).

1.3 DECREASED GAD_{67} MRNA: CAUSE OR COMPENSATION?

In addition to the lower expression of GAD_{67}, the mRNA levels of the GABA membrane transporter 1 (GAT-1) are significantly reduced in the DLPFC of subjects with schizophrenia (Volk et al., 2001). These deficits seem to represent the disease process, as no change in the mRNA levels of GAD_{67} or GAT-1 were observed in the DLPFC of monkeys chronically exposed to antipsychotic medications (Volk et al., 2000; Volk et al., 2001). Instead, these findings suggest that synthesis and re-uptake of GABA are significantly lower in subjects with schizophrenia. However, determining how a reduction in both the synthesis and re-uptake of GABA contributes to the altered circuitry of the DLPFC, and therefore cognitive deficits, depends in part on the potential cause-and-effect relationship between changes in GAD_{67} and GAT-1. For instance, the reduction in GAD_{67} mRNA could represent a primary insult, resulting in reduced presynaptic release of GABA. As a secondary compensatory response to the decreased extracellular levels of GABA, the expression of GAT-1 is decreased, reducing the re-uptake machinery in the nerve terminal. Alternatively, the more primary deficit may be a reduction in GABA uptake, represented by lower GAT-1 mRNA levels, resulting in higher extracellular levels of GABA (Pierri et al., 1999). The resulting excessive inhibition could give

rise to a compensatory reduction in the synthesis of GABA, reflected in the lower levels of GAD_{67} mRNA.

The potential relationship between altered expression of GAD_{67} and GAT-1 has been illustrated, in part, by the study of a subset of interneurons known as chandelier cells. Chandelier cells are among the subset of interneurons that express the calcium binding protein parvalbumin (PV) (Howard et al., 2005). These cells provide a linear array of axon terminals (named cartridges) that synapse along the axon initial segment of pyramidal cells (Freund et al., 1983; Lewis and Lund, 1990; Somogyi, 1977). Postmortem studies have shown that the mRNA expression levels of PV are significantly reduced in subjects with schizophrenia (Hashimoto et al., 2003). In addition, simultaneous detection of PV and GAD_{67} mRNAs revealed that in subjects with schizophrenia, 45% of PV mRNA-positive neurons did not have detectable levels of GAD_{67} mRNA (Hashimoto et al., 2003). Thus, the synthesis of GABA in chandelier cells might be lower in the DLFPC of subjects with schizophrenia. Furthermore, analysis of the density of the chandelier cell cartridges by immunereactivity for GAT-1 revealed that, consistent with the mRNA data, the density of GAT-1 labeled terminals was significantly reduced in schizophrenia. Therefore, the levels of GABA reuptake also appear to be reduced in the presynaptic terminals of chandelier cells. Interestingly, the majority of the postsynaptic $GABA_A$ receptors located in the axon initial segment of pyramidal neurons contain the α_2 subunit (Loup et al., 1998; Nusser et al., 1996; Nyíri et al., 2001). Postmortem studies have found that the density of α2 immunoreactive axon initial segments of pyramidal cells is significantly increased in the DLPFC of subjects with schizophrenia (Volk et al., 2002).

These findings suggest a model of the altered inhibitory signaling of chandelier cells in which the reduction in GAD_{67} represents a primary insult, resulting in lower synthesis of GABA

and decreasing synaptic input at the axon initial segment of pyramidal cells. As a consequence, two compensatory changes take place: a down-regulation of presynaptic GABA re-uptake (lower GAT-1 mRNA) and an upregulation of postsynaptic $GABA_A$ receptors (increased density of α2-immunolabeled axon initial segments) (Volk et al., 2002).

If the deficits in inhibitory control provided by chandelier cells represent a general abnormality of GABA neurotransmission present in other inhibitory synapses in the DLPFC of subjects with schizophrenia, it could be hypothesized that the expression of postsynaptic $GABA_A$ receptors is up-regulated in response to a reduction in presynaptic inhibitory inputs. Consistent with this hypothesis, recent studies suggest that the expression of GAD_{67} is also reduced in other subsets of interneurons, including those expressing the neuropeptide cholecystokinin (Hashimoto et al., 2008a), which primarily innervate the soma and proximal dendrites of pyramidal cells (Kawaguchi and Kondo, 2002), and those that express the neuropeptide somatostatin (Morris et al., 2008; Hashimoto et al., 2008a), which provide inhibitory inputs into dendrites of pyramidal cells (Kawaguchi and Kondo, 2002). In addition, consistent with the upregulation of α2 subunits, previous radiolabeled ligand binding studies of prefrontal $GABA_A$ receptors have reported increased muscimol binding (Benes et al., 1996; Dean et al., 1999; Hanada et al., 1987). Together, these findings suggest a working model for the disease process of schizophrenia that implicates a decrease in the synthesis and release of GABA and a compensatory upregulation of postsynaptic $GABA_A$ receptors.

1.4 ALTERED EXPRESSION OF δ-CONTAINING $GABA_A$ RECEPTORS

$GABA_A$ receptors are members of a family of ligand-gated chloride-selective ion channels formed by five subunits (Tretter et al., 1997), each consisting of an N-terminal extracellular domain, four transmembrane (TM) domains, and a large intracellular loop between TM3 and TM4 (Schofield et al., 1987). $GABA_A$ receptors are assembled by combinations of different subunits, α1-6, β1-4, γ1-3, δ, ε, μ, θ, and ρ1-3 (Farrant and Nusser, 2005). However, most receptors are composed of 2 α, 2 β and 1 γ or 1 δ subunits (Chang et al., 1996; Farrar et al., 1999). Depending on the subunit composition, these receptors exhibit distinct pharmacological and electrophysiological properties. For example, in receptors that contain α, β and γ subunits, receptors containing α1 subunit have faster deactivation kinetics than those containing α2 subunits (Farrant and Nusser, 2005).

The physiological actions of GABA can be mediated in two forms of inhibition, termed phasic and tonic inhibition. Phasic inhibition is defined by the fast and synchronous opening of $GABA_A$ receptors clustered at the postsynaptic membrane, that result from the fusion of synaptic vesicles from the presynaptic membrane at the release site (Mody and Pearce, 2004; Farrant and Nusser, 2005). This form of inhibition is mediated predominantly by synaptic γ2-containing $GABA_A$ receptors, as this subunit has been shown to be necessary for clustering of the receptors (Essrich et al., 1998).

In contrast, tonic inhibition is characterized by the unsynchronized activation of receptors that are located at peri- or extrasynaptic sites (Nusser et al., 1998; Wei et al., 2003). This form of inhibition is primarily mediated by $GABA_A$ receptors that contain δ subunits (Nusser et al., 1998; Farrant and Nusser, 2005); although α5-containing $GABA_A$ receptors, thought to co-

assemble with γ subunits, also mediate tonic inhibition (Zhang et al., 2007; Herd et al., 2007). Tonic inhibition affects the magnitude and duration of the voltage response by increasing the cell's input conductance. As a consequence, the size and duration of an excitatory postsynaptic potential are reduced, making it less likely that an action potential will be generated (Mody and Pearce, 2004; Farrant and Nusser, 2005). For instance, applications of the neuroactive steroid 3α,21-dihydroxy-5a-pregnan-20-one (THDOC), which preferentially potentiate δ-containing receptors (Brown et al., 2002; Wohlfarth et al., 2002), gradually decreased evoked excitatory field potentials recorded from dentate gyrus cells (Stell et al., 2003). In contrast to the increased density of α2 subunits in the DLPFC of subjects with schizophrenia, we recently found by microarray that the expression levels of the δ $GABA_A$ subunit is significantly reduced by 25% in the DLPFC of 14 pairs of schizophrenia and control subjects (Hashimoto et al., 2008a), consistent with a previous study (Vawter et al., 2002). These findings suggest that some $GABA_A$ receptors, like those containing δ subunit, are not up-regulated, but rather decreased in the DLPFC of subjects with schizophrenia.

Except in the cerebellum (Jechlinger et al., 1998), $GABA_A$ receptors containing δ subunits are thought to preferentially co-assemble with α4 subunits *in vivo*, as the protein and mRNA expression patterns for both subunits are similar across the rodent thalamic, hippocampal, and cortical regions (Wisden et al., 1992; Pirker et al., 2001; Sur et al., 1999; Sperk et al., 1997). Thus, a reduction in δ mRNA in subjects with schizophrenia would be expected to be paralleled by lower mRNA levels of the α4 subunit. Consistent with this idea, mRNA levels of α4 were also found by microarray analysis to be significantly lower in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2008a). However, although the mRNA levels of both δ and α4 subunits were lower, the change in α4 subunit did not correlate with that of δ ($r = 0.35$, $p = 0.22$)

(Hashimoto et al., 2008a). Thus, it is not clear whether lower expression of both δ and α4 subunits represent a reduction in α4βxδ $GABA_A$ receptors.

1.5 REDUCED ACTIVITY OF EXCITATORY SYNAPSES AS POTENTIAL PATHOGENETIC MECHANISMS

While the pathogenetic mechanisms that give rise to dysfunction of the inhibitory circuitry of the DLPFC in subjects with schizophrenia are not clearly understood, some studies suggest that a reduction in the signaling through excitatory synapses could give rise to lower expression levels of GABA-related markers in schizophrenia. For instance, low doses of ketamine, a non-competitive antagonist at *N*-methyl-D-aspartate receptors (NMDARs), resulted in significantly lower levels of both GAD_{67} and PV immunoreactivity in cultured cortical interneurons (Kinney et al., 2006). Furthermore, *in vivo* administration of the NMDAR antagonist phencyclidine (PCP) resulted in significantly lower levels of PV mRNA in the prefrontal cortex (PFC) of rats (Cochran et al., 2003).

Interestingly, studies show that changes in signaling through excitatory synapses appear to regulate the expression levels of δ subunit. For instance, the mRNA levels of δ subunit in cultured cerebellar granule cells increased significantly when grown in depolarizing medium, and decreased when the neurons were switched into a non-depolarizing medium (Gault and Siegel, 1997). Similarly, addition of NMDA to cultured cerebellar granule neurons initiated the transcript expression of δ subunit (Gault and Siegel, 1998), an effect that was abolished after addition of MK-801, a non-competitive NMDAR antagonist. Consistent with these *in vitro*

studies, the expression levels of δ subunit decreased in the rat forebrain after a week of MK-801 infusions (Kim et al., 2000). These findings are of interest as convergent lines of evidence suggest that reduced signaling through excitatory synapses represents a pathogenetic mechanism underlying some features of the clinical syndrome of schizophrenia (Coyle et al., 2003; Moghaddam, 2003). Together, these findings suggest that decreased activity of excitatory syapses might represent a pathogenetic mechanism giving rise to lower levels of δ-containing receptors. However, no experimental evidence exists to provide proof-of-concept support that a chronic reduction in excitatory levels, which is more likely to mimic the deficit in schizophrenia, results in decreased expression of δ-containing receptors, potentially α4βxδ receptors.

1.5.1 NMDAR hypofunction

Convergent lines of evidence suggest that chronic reduction in the signaling through excitatory synapses in subjects with schizophrenia could be a consequence of hypoactivity of NMDARs. For instance, normal volunteers receiving subanesthetic doses of ketamine exhibited negative symptoms and some cognitive impairments similar to those observed in subjects with schizophrenia (Krystal et al., 1994; Malhotra et al., 1996). Furthermore, subjects with schizophrenia administered NMDAR antagonists such as PCP or ketamine exhibited exacerbated positive and negative symptoms (Coyle, 2004).

NMDA receptors are tetramer proteins formed by two subunits, NR1 (required for functional receptors) and NR2A-2D (Dingledine et al., 1999). These receptors are voltage-dependent blocked by Mg^{2+} at resting membrane potentials and require binding of both glutamate and glycine/D-serine in order to open the channel (Dingledine et al., 1999). It has been proposed that hypoactivation of NMDARs in schizophrenia might arise from abnormal

activity of a number of genes (and their cognate proteins) known to regulate the activity of NMDARs (Kristiansen et al., 2007). For instance, genetic studies have shown an association of G72 with the risk of schizophrenia (Coyle, 2007). G72 encodes the protein D-Amino acid oxidase (DAAO), which catabolizes D-serine (Chumakov et al., 2002). Thus, reduced levels of D-serine, as a consequence of a G72-induced increased activity of DAAO, would lead to hypofunction of NMDARs in schizophrenia (Stevens et al., 2003). Another gene that might contribute to altered modulation of NMDARs is glutamate carboxy peptidase (GCP II). GCP II degrades N-acetylaspartylglutamate (NAAG), hypothesized to be an antagonist of NMDARs (Coyle, 2004). Postmortem studies have reported decreased activity and expression levels of GCP II in subjects with schizophrenia (Tsai et al., 1995; Hakak et al., 2001). The decreased activity of GCP II would result in higher levels of NAAG, thereby inducing hypoactivation of NMDARs. This evidence suggests that altered modulation of NMDARs contributes to reduced excitatory activity of DLPFC circuits, potentially resulting in lower levels of δ-containing receptors.

1.5.2 Reduced excitatory synaptic terminals

In addition to hypofunction of NMDARs, reduced chronic signaling through excitatory synapses might also be a consequence of reduced excitatory synaptic terminals in the DLPFC. For instance, the protein levels of synaptophysin, a marker of axon terminals, are reportedly significantly reduced in the DLPFC of subjects with schizophrenia (Glantz and Lewis, 1997; Karson et al., 1999; Perrone-Bizzozero et al., 1996). Studies suggest that reduced excitatory projections from the mediodorsal nucleus of the thalamus (MDTN), which are the main source of thalamic input to the DLPFC (Giguere and Goldman-Rakic, 1988), might represent a source of

reduced inputs. Consistent with this idea, several imaging studies have reported reduced volume of the thalamus, as well as its decreased activity in subjects with schizophrenia (Manoach et al., 1999). Furthermore, thalamic volumes were directly correlated with the volumes of prefrontal white matter in subjects with schizophrenia (Portas et al., 1998). These findings suggest that a reduction in the volume of the thalamus is associated with fewer axonal projections to the prefrontal cortex. In addition, lower MDTN axonal projections appear to originate from a loss of MDTN neurons, as several studies reported a reduction in the total number of neurons in this nucleus in schizophrenia (Broadbelt et al., 2002; Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000), although more recent studies have failed to replicate these findings (Cullen et al., 2003; Dorph-Petersen et al., 2004; Kreczmanski et al., 2007; Young et al., 2004).

Given that MDTN axons innervate DLPFC neurons located in deep layers 3 and 4 (Erickson and Lewis, 2004), neurons located in these layers might particularly reflect a reduction in excitatory inputs. Consistent with this interpretation, DLPFC deep layer 3 pyramidal cells show a significant reduction in spine density (Glantz and Lewis, 2000; Garey et al., 1998; Kalus et al., 2000) (although the decrease in dendritic spine density cannot be completely accounted for by a reduction in MDTN inputs, see section *1.5.3 Reduced Postsynaptic Targets of Excitatory Inputs*). These findings suggest that decreased excitatory synaptic inputs into the DLPFC might also represent a source of reduced signaling through excitatory synapses in subjects with schizophrenia.

Clinical evidence suggests that function of the hippocampal formation is altered in subjects with schizophrenia (Weinberger and Lipska, 1995). For instance, rats with neonatal lesions of the ventral hippocampus, which project to the PFC (Jay et al., 1989; Carr and Sesack, 1996), exhibit a post-pubertal appearance of a number of alterations, such as impairments in

working memory, hyperlocomotion, stress, and reduced social contacts, similar to those observed in schizophrenia (Lipska and Weinberger, 2000; Lipska et al., 2003). Thus, reduced excitatory synaptic terminals from the hippocampus might also contribute to reduced excitatory synaptic terminals in the DLPFC. Together, these findings suggest that lower excitatory inputs from the MDTN or hippocampus might result in lower expression of δ-containing receptors.

1.5.3 Reduced postsynaptic targets of excitatory inputs

Some, but not all, imaging studies have reported reductions in DLPFC gray matter volume in subjects with schizophrenia (McCarley et al., 1999). Consistent with these data, postmortem studies have reported decreased cortical thickness and volume and increased cell-packing density in the DLPFC of individuals with schizophrenia (Pakkenberg, 1993; Rajkowska et al., 1998; Selemon et al., 1995; Selemon et al., 1998; Selemon et al., 2002). In addition, the mean somal volume of Nissl-stained pyramidal cells in layer 3 of the DLPFC, which correlates with the size of the neuron's dendritic arbor (Jacobs et al., 1997), is reportedly significantly reduced (Pierri et al., 2001; Rajkowska et al., 1998). These findings have been interpreted as a reduction in the neocortical neuropil (Selemon and Goldman-Rakic, 1999). Indeed, multiple studies have shown reduced pyramidal cell spine density and dendritic arborization in the DLPFC of subjects with schizophrenia (Glantz and Lewis, 2000; Garey et al., 1998; Kalus et al., 2000; Black et al., 2004).

The reduction in excitatory postsynaptic targets, such as spine density, in DLPFC pyramidal cells in schizophrenia appear to be due, in part, to an intrinsic abnormality in these neurons that render them unable to support the normal complement of excitatory inputs (Lewis and Gonzalez-Burgos, 2007). While reduced presynaptic inputs from the MDTN might

contribute to a reduction in the spine density of DLPFC layer 3 pyramidal cells, thalamocortical terminals only appear to comprise a small proportion of the total excitatory inputs to cortical neurons in animals (Guillery and Sherman, 2002). Thus, if a similar proportion of thalamocortical inputs innervate the human DLPFC, a reduction in MDTN inputs would not be sufficient to account for the magnitude of the reduction in deep layer 3 spine density (Lewis and Gonzalez-Burgos, 2007). Furthermore, even though NMDARs have been shown to play a significant role across development in processes such as cell migration, cell death, survival and formation of neural circuits (Contestabile, 2000), chronic blockade of NMDARs during postnatal development did not change the density of pyramidal cells visualized by Golgi in the rat prefrontal cortex (Wedzony et al., 2005). Furthermore, rats chronically exposed to PCP, a commonly used animal model of NMDAR hypoactivation (Morris et al., 2005), actually showed increases in the spine density of prefrontal pyramidal cells (Flores et al., 2007). Thus, hypoactivation of NMDARs might not account for a reduction in postsynaptic targets (but see Akerman and Cline (2007)).

In contrast, one potential intrinsic mechanism contributing to a loss of spines in DLPFC pyramidal cells might be reduced signaling through the tyrosine kinase B (TrkB) receptor, which mediates the actions of the secreted neurotrophin brain-derived neurotrophic factor (BDNF). BDNF-TrkB signaling has been shown to enhance somatodendritic development (Horch and Katz, 2002; McAllister et al., 1995; Xu et al., 2000b). For instance, pyramidal cells of forebrain-restricted BDNF mutant mice exhibited a significant reduction in cortical thickness and somal size, and dendritic arbor complexity (Gorski et al., 2003). It should be noted, that mice genetically engineered to express low levels of BDNF do not exhibit a reduction in the morphology of basilar dendrites from prefrontal pyramidal cells (Hill et al., 2005). However, the

lack of an effect could be due to compensatory mechanisms, given that, at least in mice in which the reduction of BDNF was induced prenatally, the levels of TrkB receptor are up-regulated by 20% (Hashimoto et al., 2005).

In contrast, a reduction of TrkB receptors in populations of neocortical pyramidal cells results in a number of morphological abnormalities in neuropil such as thinner dendrites and fewer and/or shorter dendritic branches (Xu et al., 2000b). Consistent with these findings, mice genetically engineered to express low levels of TrkB receptors (*trk hypomorphic mice*, (Xu et al., 2000a)) exhibit significant reductions in the number of dendritic branches of retinal ganglionic cells (Liu et al., 2007). These findings suggest that lower signaling through TrkB receptors contributes to the reduction in postsynaptic targets of excitatory inputs in subjects with schizophrenia.

Thus, in addition to hypoactivation of NMDARs and reduced excitatory synaptic terminals, a reduction in postsynaptic excitatory targets of pyramidal cells in the DLPFC might represent another source contributing to a chronic reduction in the signaling through excitatory synapses in schizophrenia that could underlie lower expression of δ-containing $GABA_A$ receptors.

1.6 GOALS OF THE DISSERTATION RESEARCH

The evidence outlined in the preceding sections suggests that the expression levels of δ and α4 $GABA_A$ subunits are significantly reduced in the DLPFC of subjects with schizophrenia. Furthermore, lower expression of δ-containing receptors might represent a consequence of reduced signaling through excitatory synapses. As a consequence, the inhibitory signaling

through δ-receptors might be reduced, contributing to the altered inhibitory circuit in subjects with schizophrenia.

This dissertation was designed to test the following hypotheses: 1) that the expression levels of both α4 and δ mRNAs are significantly lower across cortical layers of the DLPFC of subjects with schizophrenia, 2) that lower levels of both α4 and δ subunits represent the disease process of the illness, and 3) that the expression levels of δ subunit are significantly reduced in the PFC of animal models that recapitulate deficits in the activity of excitatory synapses observed in schizophrenia.

In the studies described in Chapter 2, 1) postmortem brain human tissue and *in situ* hybridization were utilized to assess the mRNA expression levels of α4 and δ subunits in the DLPFC of subjects with schizophrenia, 2) the effect of potential confounding factors on the mRNA levels of α4 and δ subunits were assessed in both the human subject cohort and in a model of chronic exposure to antipsychotic medications, and 3) the relationship between α4 and δ subunit mRNA expression levels was assessed in several animal models.

In order to provide proof-of-concept for the role of chronic reduction in the signaling through excitatory synapses as a pathogenetic mechanism for lower levels of δ subunit mRNA, in Chapter 3 we assessed the mRNA levels of δ subunit in the PFC of several animals that model chronic reductions in the signaling through excitatory synapses in the form of 1) reduced signaling through NMDARs, 2) reduced excitatory synaptic terminals from the thalamus or hippocampus, and 3) reduced postsynaptic targets of excitatory inputs.

2.0 CHAPTER 2: ALTERED EXPRESSION OF α4 AND δ SUBUNITS ACROSS THE DLPFC OF SUBJECTS WITH SCHIZOPRHENIA

2.1 ABSTRACT

In a recent microarray study, the mRNA levels of $GABA_A$ receptor α4 and δ subunits were found to be lower in the dorsolateral prefrontal cortex (DLPFC) of subjects with schizophrenia. Although α4 and δ subunits co-assemble to form functional receptors mediating tonic inhibition in the forebrain, the differences in α4 and δ mRNA expression were not correlated in subjects with schizophrenia. In order to understand the functional significance of these findings, we assessed the mRNA levels of α4 and δ in the DLPFC of a larger cohort of subjects with schizophrenia and matched control subjects by *in situ* hybridization. The level of α4 mRNA was lower only in subjects with schizophrenia receiving benzodiazepines, mood stabilizers and/or antidepressants at the time of death. In contrast, the level of δ mRNA was significantly lower in the subjects with schizophrenia, regardless of the medications used at the time of death. In order to further examine this apparent dissociation between α4 and δ mRNA expression in schizophrenia, we measured α4 and δ mRNAs across postnatal development of monkey DLPFC. The level of α4 mRNA decreased with age, whereas that of δ mRNA increased, in a manner similar to that previously observed for the α1 subunit. Because α1 mRNA levels are lower in schizophrenia and because α1 subunits can co-assemble with δ subunits, lower δ mRNA levels could represent a reduced complement of $GABA_A$ α1βxδ receptors, rather than of α4βxδ, in

schizophrenia. Consistent with this hypothesis, δ mRNA levels were significantly reduced in the PFC of α1 knockout mice. Thus, deficits in tonic inhibition, due to a reduced number of α1βxδ receptors, could contribute to the DLPFC dysfunction characteristic of schizophrenia.

2.2 INTRODUCTION

Convergent lines of evidence suggest that deficits in certain cognitive functions, such as working memory, are the core features of schizophrenia (Weinberger et al., 1986; Perlstein et al., 2001; Silver et al., 2003). Alterations in the inhibitory circuitry of the DLPFC may contribute to impairments in working memory, as optimal levels of γ-aminobutyric acid (GABA) neurotransmission in this cortical region are essential for normal working memory performance (Sawaguchi et al., 1989; Rao et al., 2000; Kojima et al., 2007). Consistent with this idea, postmortem studies have shown that expression of the mRNA for the 67 kDa isoform of glutamate decarboxylase (GAD_{67}), the principal enzyme responsible for the synthesis of GABA, and of the GABA membrane transporter 1 (GAT-1), are significantly lower in the DLPFC of subjects with schizophrenia (Akbarian et al., 1995; Volk et al., 2001; Guidotti et al., 2000; Straub et al., 2007; Hashimoto et al., 2008a; Hashimoto et al., 2005)

Understanding the functional significance of the presynaptic alterations in GAD_{67} and GAT-1 requires knowledge of the expression levels of postsynaptic $GABA_A$ receptors. $GABA_A$ receptors are pentameric ligand-gated chloride ion channels, assembled from different subunit classes that most commonly include 2α, 2β and 1γ or 1δ subunits (Mehta and Ticku, 1999). Different combinations of subunits form $GABA_A$ receptors with unique properties. For instance, $GABA_A$ receptors containing a γ2 subunit predominantly mediate phasic inhibition, defined as the rapid and synchronous opening of synaptic receptors that result in an inhibitory postsynaptic potential (Mody and Pearce, 2004; Farrant and Nusser, 2005). In contrast, δ-containing $GABA_A$ receptors mediate tonic inhibition, defined as the constant activation of extrasynaptic receptors

that, by increasing input conductance, reduce the probability of generating an action potential (Mody and Pearce, 2004; Farrant and Nusser, 2005). Tonic inhibition mediated by δ-containing receptors has been described in many cell types including cerebellar granule cells (Brickley et al., 2001), dentate gyrus granule cells (Stell et al., 2003) and neocortical pyramidal cells (Drasbek and Jensen, 2006; Drasbek et al., 2007); but see Yamada et al., (2007).

The mRNA level of the $GABA_A$ receptor δ subunit was significantly lower in the DLPFC of subjects with schizophrenia in two microarray studies (Vawter et al., 2002; Hashimoto et al., 2008a). Because δ subunits are thought to preferentially co-assemble with α4 subunits in forebrain $GABA_A$ receptors (Peng et al., 2002; Jensen et al., 2007), lower δ mRNA levels in subjects with schizophrenia could represent a reduced complement of α4βxδ $GABA_A$ receptors in the illness. However, although the mRNA levels of α4 subunit were lower by microarray in the DLPFC of the same subjects with schizophrenia, the difference was not correlated with that of the δ subunit (Hashimoto et al., 2008a).

Thus, in order to understand the functional significance of altered expression of α4 and δ subunits in the DLPFC of subjects with schizophrenia, we 1) examined the expression patterns of α4 and δ mRNAs in the DLPFC of a larger cohort of subjects with schizophrenia, and 2) determined the relationship between α4 and δ subunit mRNA expression in several animal models.

2.3 MATERIALS AND METHODS

2.3.1 Human subjects

With the consent of the surviving next-of-kin, brain tissue specimens were obtained from the Allegheny County Medical Examiner's Office (Pittsburgh, PA) at the time of routine autopsy. Twenty-three subjects with schizophrenia (Table 1) were each matched with one control subject for sex, and as closely as possible for age and postmortem interval (PMI). Two-tailed paired *t*-tests revealed that the subjects with schizophrenia did not differ from the control subjects in age ($t_{22} = 0.16$, $p = 0.878$), PMI ($t_{22} = 0.20$, $p = 0.837$), RNA integrity number (RIN; $t_{22} = 1.84$, $p = 0.078$) or tissue storage time at -80° ($t_{22} = -0.96$, $p = 0.349$). Furthermore, the mean (±SD) pH in subjects with schizophrenia (6.8 ± 0.3) was not different ($t_{22} = 0.62$, $p = 0.541$) from control subjects (6.9 ± 0.2). An independent panel of experienced research clinicians made consensus DSM-IV diagnoses for each subject using medical records and structured interviews conducted with one or more surviving family members (Glantz and Lewis, 1997). All procedures were approved by the University of Pittsburgh's Institutional Review Board for Biomedical Research.

Table 1. Characteristics of human subjects used in this study.

Control Subjects								Subjects with Schizophrenia							
Pair	Case	Sex/Race	Age (years)	PMI [a]	Storage Time [b]	RIN	Cause of death [c]	Case	DSM IV diagnosis	Sex/Race	Age (years)	PMI [a]	Storage Time [b]	RIN	Cause of death [c]	Benzodiazepines / Mood stabilizers / Antidepressants ATOD
1	592	M/B	41	22.1	112.1	9.0	ASCVD	533	Chronic undifferentiated schizophrenia	M/W	40	29.1	122.0	8.4	Accidental asphyxiation	N/N/N
2	567	F/W	46	15.0	116.2	8.9	Mitral valve prolapse	537	Schizoaffective disorder [d]	F/W	37	14.5	121.2	8.6	Suicide by hanging	N/N/N
3	516	M/B	20	14.0	123.7	8.4	Homicide by gun shot	547	Schizoaffective disorder	M/B	27	16.5	119.8	7.4	Heat Stroke	Y/Y/Y
4	630	M/W	65	21.2	106.3	9.0	ASCVD	566	Chronic undifferentiated schizophrenia [e]	M/W	63	18.3	116.6	8.0	ASCVD	Y/N/Y
5	604	M/W	39	19.3	109.8	8.6	Hypoplastic coronary artery	581	Chronic paranoid schizophrenia [f,g]	M/W	46	28.1	114.4	7.9	Accidental combined drug overdose	Y/Y/N
6	546	F/W	37	23.5	120.1	8.6	ASCVD	587	Chronic undifferentiated schizophrenia [e]	F/B	38	17.8	113.0	9.0	Myocardial hypertrophy	Y/N/N
7	551	M/W	61	16.4	118.9	8.3	Cardiac tamponade	625	Chronic disorganized schizophrenia [h]	M/B	49	23.5	106.9	7.6	ASCVD	N/N/Y
8	685	M/W	56	14.5	99.2	8.1	Hypoplastic coronary artery	622	Chronic undifferentiated schizophrenia [d]	M/W	58	18.9	107.0	7.4	Right MCA infarction	N/N/N
9	681	M/W	51	11.6	99.8	8.9	Hypertrophic cardiomyoathy	640	Chronic paranoid schizophrenia	M/W	49	5.2	104.9	8.4	Pulmonary embolism	N/N/Y
10	806	M/W	57	24.0	78.3	7.8	Pulmonary thromboembolism	665	Chronic paranoid schizophrenia [f]	M/B	59	28.1	102.5	9.2	Intestinal hemorrhage	N/N/Y
11	822	M/B	28	25.3	75.7	8.5	ASCVD	787	Schizoaffective disorder [i]	M/B	27	19.2	82.0	8.4	Suicide by gun shot	N/N/N
12	727	M/B	19	7.0	92.8	9.2	Trauma	829	Schizoaffective disorder [d,f,j]	M/W	25	5.0	73.7	9.3	Suicide by drug overdose	Y/Y/N
13	871	M/W	28	16.5	65.1	8.5	Trauma	878	Disorganized schizophrenia [f]	M/W	33	10.8	64.2	8.9	Myocardial fibrosis	N/Y/Y
14	575	F/B	55	11.3	114.9	9.6	ASCVD	517	Disorganized schizophrenia [f]	F/W	48	3.7	123.6	9.3	Intracerebral hemorrhage	N/N/N
15	700	M/W	42	26.1	96.9	8.7	ASCVD	539	Schizoaffective disorder [k]	M/W	50	40.5	121.0	8.1	Suicide by combined drug overdose	N/Y/Y
16	988	M/W	82	22.5	43.7	8.4	Trauma	621	Chronic undifferentiated schizophrenia [d]	M/W	83	16.0	107.3	8.7	Accidental asphyxiation	N/N/N
17	686	F/W	52	22.6	98.9	8.5	ASCVD	656	Schizoaffective disorder [f]	F/B	47	20.1	103.2	9.2	Suicide by gun shot	N/N/N
18	634	M/W	52	16.2	105.7	8.5	ASCVD	722	Chronic undifferentiated schizophrenia [j,l]	M/B	45	9.1	93.2	9.2	Upper GI bleeding	N/N/N
19	852	M/W	54	8.0	68.1	9.1	Cardiac tamponade	781	Schizoaffective disorder [k]	M/B	52	8.0	83.1	7.7	Peritonitis	N/N/Y
20	987 [m]	F/W	65	21.5	43.7	9.1	ASCVD	802	Schizoaffective disorder [f,l]	F/W	63	29.0	79.0	9.2	Right ventricular dysplasia	N/Y/N
21	818	F/W	67	24.0	76.9	8.4	Anaphylactic reaction	917	Chronic undifferentiated schizophrenia	F/W	71	23.8	56.9	7.0	ASCVD	N/N/N
22	857	M/W	48	16.6	67.0	8.9	ASCVD	930	Disorganized schizophrenia [j,k]	M/W	47	15.3	53.5	8.2	ASCVD	N/Y/N
23	739	M/W	40	15.8	91.9	8.4	ASCVD	933	Disorganized schizophrenia	M/W	44	8.3	52.9	8.1	Myocarditis	N/Y/Y
		Mean	**48.0**	**18.0**	**92.4**	**8.7**					**47.9**	**17.8**	**96.6**	**8.4**		
		SD	**15.5**	**5.5**	**23.5**	**0.4**					**14.1**	**9.3**	**23.6**	**0.7**		

[a] PMI, postmortem interval in hours
[b] Storage time (months) at -80º C
[c] ASCVD, arteriosclerotic cardiovascular disease
[d] Subjects off antipsychotic medications at time of death
[e] Alcohol abuse, in remission at time of death
[f] Alcohol dependence, current at time of death
[g] Other substance abuse, current at time of death
[h] Alcohol abuse, current at time of death
[i] Other substance dependence, current at time of death
[j] Other substance abuse, in remission at time of death
[k] Alcohol dependence, in remission at time of death
[l] Other substance dependence, in remission at time of death
[m] History of post-traumatic stress disorder, in remission 39 years at time of death

2.3.2 Tissue processing

The right hemisphere of each human brain was blocked coronally, frozen and stored at -80ºC. Serial sections (20 μm) containing the superior frontal gyrus were cut at the anteroposterior level corresponding to the middle portion of the superior frontal sulcus, thaw mounted onto glass slides, and stored at -80ºC until processed. Sections containing area 9 were identified in Nissl-stained sections as previously described (Volk et al., 2000).

2.3.3 Riboprobes

Templates for the synthesis of α4 and δ subunit riboprobes were obtained by polymerase chain reaction (PCR) with specific primer sets. A 516 bp fragment for the α4 subunit corresponding to bases 1231-1746 of the human $GABA_A$ α4 gene (GenBank NM_000809) and a 607 base pair (bp) DNA fragment for the δ subunit corresponding to bases 419-1025 of the human gene (GenBank BC033801) were amplified. Nucleotide sequencing revealed 100% homologies for the amplified fragments to the previously reported sequences. DNA fragments were sub-cloned into the plasmid pSTBlue-1 (Novagen, Madison, WI). Sense and antisense riboprobes were transcribed *in vitro* in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ), using T7 or SP6 RNA polymerase, and were then digested with DNase I and purified by centrifugation through RNeasy mini spin columns (Qiagen, Valencia, CA).

2.3.4 *In situ* hybridization

For each transcript, we used three tissue sections per subject, spaced at approximately 560 μm. Sections from a given pair were always processed together; six runs were performed for each transcript. For each run, slide-mounted tissue sections were immersed in 4% paraformaldehyde in phosphate buffered saline (PBS), acetylated, dehydrated through a graded ethanol series, and de-fatted in chloroform for 10 min. The sections were then incubated with ^{35}S-labeled riboprobe in hybridization buffer containing 50% formamide, 0.75 M NaCl, 20 mM 1,4-piperazine diethane sulfonic acid, pH 6.8, 10 mM EDTA, 10% dextran sulfate, 5X Denhardt's solution, 50mM dithiothreitol, 0.2% SDS and 100 mg/ml yeast tRNA at 56°C for 16 hrs. After washing in a solution containing 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63°C, the sections were treated with RNase A (20 μg/ml) at 37°C, and washed in 0.1 SSC (150 mM NaCl and 15 mM sodium citrate) at 66.8°C. Sections were then dehydrated through a graded series of ethanol concentrations, air dried, and exposed to BioMax MR Film (Kodak, Rochester, NY). After exposure to film, sections were coated with NTB emulsion (Kodak; diluted 2:1 with water).

2.3.5 Quantification

Analyses were performed by one investigator (JGMA) without knowledge of diagnosis or subject number due to random coding of the sections. Trans-illuminated autoradiographic film images were captured by a video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID) system (Imaging Research Inc, London, Ontario, Canada). Images of the corresponding hybridized sections were captured and superimposed on

the autoradiographic images to draw contours and delineate the border between gray matter and white matter. Optical density was measured within the contours and expressed as nanocuries per gram of tissue, determined by reference to ^{14}C standards (ARC Inc., St. Louis, MO) exposed on the same film.

To assess mRNA levels across layers of the human DLPFC, three cortical traverses, 1.5 mm in width, were sampled from each section (nine traverses per subject). Each cortical traverse was located in portions of the tissue section cut perpendicular to the pial surface, as determined by the presence of pyramidal neurons with vertically oriented apical dendrites on adjacent Nissl-stained sections. The average mRNA level within each layer was determined by measuring optical density in zones located 10–20% (layer 2), 20-50% (layer 3), 50-60% (layer 4), 60-80% (layer 5) and 80–100% (layer 6) from the pial surface to the white matter border (Pierri et al., 1999). All cortical optical density measures were corrected by subtracting optical density measures in the white matter.

2.3.6 Antipsychotic-treated monkeys

Eighteen experimentally naïve, male, long-tailed macaque monkeys (*Macaca fascicularis*), 4.5–5.3 years of age, were arbitrarily divided into three groups and trained to orally ingest pellets containing either haloperidol, olanzapine or sham, twice a day (Dorph-Petersen et al., 2005). At steady state, the total daily dose of drug per animal ranged from 28 to 32 mg for haloperidol and 11.0 to 13.2 mg for olanzapine. These doses produced trough serum levels of ~1.5 ng/ml for haloperidol and ~15 ng/ml for olanzapine (Dorph-Petersen et al., 2005), which are within the therapeutic range for the treatment of schizophrenia in humans (Kapur et al., 1998; Kapur et al., 1997). After 17–27 months of drug exposure, animals (grouped into triads by body weight) were

deeply anesthetized, the brains were removed, and the right hemisphere was blocked, frozen and stored at -80°C. All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Serial cryostat sections (16 μm) were cut from fresh frozen tissue blocks containing the middle one-third of the principal sulcus. Two adjacent sections from each animal, spaced at 224 μm, were used to analyze δ mRNA expression in areas 9 and 46, identified according to cytoarchitectonic criteria in Nissl sections (Barbas and Pandya, 1989). Sections from each triad were processed together in a single *in situ* hybridization run; two runs were performed. The *in situ* hybridization procedures were performed as described above, except that the sections were not de-fatted in chloroform. Analyses of film autoradiograms were performed as described above.

2.3.7 Postnatal developmental monkeys

Thirty-one, experimentally naïve, female, rhesus macaque monkeys (*Macaca mulatta*) in seven age groups from 1 week of age to adult were used to study the postnatal development of δ and α4 mRNAs in area 46 of the DLPFC (Table 2). All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Some animals were perfused transcardially with ice-cold modified artificial cerebrospinal fluid (aCSF) at the time of euthanasia (Gonzalez-Burgos et al., 2007). Furthermore, in some animals, a small block of tissue was surgically excised from the rostral third of the principal sulcus in the left hemisphere 2-4 weeks prior to perfusion with aCSF, for electrophysiology studies (Gonzalez-Burgos et al.,

2007).

In order to control for the possible effects of neurosteroids on the expression of δ-containing receptors (Maguire et al., 2005), menstrual status was determined for the post-pubertal 42-month and adult animals by assessing serum levels of estradiol and progesterone obtained immediately prior to euthanasia in 8 of 9 animals. For the remaining adult animal, records of observed menstruation indicated that she had very stable cycles which predicted that she was in the luteal phase at the time of euthanasia (Table 2).

Serial coronal sections (16 μm) were cut from the caudal third of the principal sulcus in the right hemisphere of each monkey. Monkeys were divided into two groups, each containing an equivalent number of animals of each age. Two adjacent sections from each animal, spaced at 224 μm, were used to analyze the mRNA expression levels of δ and α4 mRNAs in area 46, identified according to cytoarchitectonic criteria in Nissl sections (Barbas and Pandya, 1989). Sections from each group were processed together in a single *in situ* hybridization run; a total of four runs were performed. In addition, a section from a control animal was included in each *in situ* hybridization run to normalize any experimental variability between runs. Analyses of α4 and δ mRNAs during postnatal development of the monkey DLPFC were performed by one investigator (AAC) without knowledge of age group, using the same procedures described above.

Table 2. Macaque monkeys used in the developmental study.

Animals	Age Group (months)	Perfusion Status	Prior Biopsy Status	Menstrual Phase
RH193	.25	–	–	NA
RH194	.25	–	–	NA
RH199	.25	–	–	NA
RH201	.25	–	–	NA
RH197	1	–	–	NA
RH200	1	–	–	NA
RH209	1	–	–	NA
RH192	3	–	–	NA
RH198	3	–	–	NA
RH203	3	–	–	NA
RH212	3	–	–	NA
RH230	3	+	–	NA
RH234	3	+	–	NA
RH241	3	+	–	NA
RH245	3	+	–	NA
RH261	9	–	–	NA
RH262	9	–	–	NA
RH240	15	+	–	NA
RH255	15	+	–	NA
RH264	15	+	–	NA
RH265	15	+	–	NA
RH236	42	+	+	luteal
RH238	42	+	+	follicular
RH239	42	+	–	luteal
RH246	42	+	+	follicular
RH249	42	+	–	follicular
RH258	42	+	–	follicular
RH248	84 (adult)	+	+	luteal
RH259	102 (adult)	+	–	luteal
RH260	132 (adult)	–	–	luteal

2.3.8 $GABA_A$ receptor α1 subunit knockout mice

Because the α1 subunit has been reported to assemble with δ subunits (Glykys et al., 2007), we utilized tissue from mice with a targeted deletion of the $GABA_A$ α1 subunit (generously provided by Dr. A. Leslie Morrow, Chapel Hill, NC) (Vicini et al., 2001). Briefly, the exon encoding nucleotides 1307 to 1509 of the α1 subunit was flanked by loxP sites and the knockout α1 allele was generated after cre-mediated recombination. Two groups of mice, wild-type and α1 knockout, were euthanized at 8 weeks of age (n = 8 for each group). Brains were frozen immediately and stored at -80°C. Serial coronal sections (12 μm) were cut from +1.98 to +1.54 bregma (Paxinos and Franklin, 2001). Three sections, spaced at 144 μm, were selected from each animal and processed for *in situ* hybridization, using riboprobes generated from a 598 bp cDNA fragment of the δ subunit corresponding to bases 235-832 of the mouse δ mRNA (GenBank NM_008070); and a 537 bp cDNA fragment of the α4 subunit corresponding to bases 655-1191 of the mouse α4 mRNA (GenBank NM_010251). For the δ subunit, two *in situ* hybridization runs, each containing sections from four wild-type and four α1 knockout mice, were performed. For α4 analysis, one *in situ* hybridization run containing sections from all 16 animals was performed. Quantification of mRNA levels was done in the prefrontal cortex (PFC), including the cingulate and prelimbic cortices (Paxinos and Franklin, 2001). Western blot studies confirmed a >95% reduction in α1 protein levels in the α1 knockout mice (data not shown).

2.3.9 Statistical analyses

Analysis of covariance (ANCOVA) models were performed to examine the expression differences in α4 and δ mRNAs between the control subjects and subjects with schizophrenia. The film optical density measures from three sections per subject can be considered to be exchangeable and correlated, and thus treated as repeated measures with a compound, symmetric, covariance structure (Neter et al., 1996). The corresponding ANCOVA models required averaging across the three sections for each dependent variable before the statistical analyses were conducted. The first ANCOVA model, used for both dependent variables, had diagnostic group as a main effect and pair as a blocking effect. The inclusion of pair reflects the matching of individual subject pairs for sex, age, and PMI. Furthermore, freezer storage time and RIN were included as covariates to control for their potential effect on mRNA quality (Stan et al., 2006).

Subject pairing may be considered an attempt to balance the two diagnostic groups with regard to the experimental factors instead of a true statistical paired design. Thus, to validate the first model, a second ANCOVA model was performed with diagnostic group as main effect and sex, age, PMI, RIN and storage time as covariates. Both models produced similar results for diagnostic group effect. However, because the effect of age on δ and α4 mRNAs expression was significant, the results of the second unpaired model are reported.

We used two-sample *t*-tests to assess the influence of sex, diagnosis of schizoaffective disorder, suicide, history of alcohol abuse and/or dependency and presence of benzodiazepines, mood stabilizers and/or antidepressants at the time of death on the within-subject pair differences in gene expression levels.

For the antipsychotic-treated monkeys, a single-factor ANOVA model was used, with triad as the blocking factor and treatment group as the main effect.

The change in levels of δ and α4 mRNAs across postnatal development were assessed by ANCOVA models, with age group as the main effect and both perfusion and prior biopsy as blocking factors. A Duncan's post hoc test was used to assess the differences between age groups for significant ANCOVAs. The mean normalized optical density levels of each animal were used as the dependent variable.

The expression of δ mRNA in the prefrontal cortex of α1 knockout mice was analyzed with an ANCOVA model using genotype as main effect, and *in situ* hybridization run as a covariate since two *in situ* hybridization runs were performed. The mRNA levels of the α4 subunit were analyzed with a two-sample t-test because only one *in situ* hybridization run was performed. All statistical tests were conducted with an α-level = 0.05.

2.4 RESULTS

2.4.1 Specificity of human riboprobes

The specificity of the riboprobes for the α4 and δ subunits was confirmed by several findings. First, clusters of silver grains were present over neurons, characterized by their faint Nissl-stain and large nuclei, in emulsion-dipped slides (Figure 1, panels A, C). In contrast, silver grains were not clustered over glia cells, characterized by their intensely Nissl-stained, small nuclei, which are known not to express these transcripts. Second, signal above background was not detected in tissue processed with sense riboprobes for α4 or δ subunits (Figure 1, panels B, D).

Third, consistent with previous reports in the rodent, primate and human cortex (Wisden et al., 1992; Huntsman et al., 1995; Petri et al., 2003), the mRNA expression levels of α4 were uniform across layers 2-5, lower in layer 6 and absent in layer 1 (Figure 2A). Similarly, the laminar distribution of δ mRNA was consistent with that previously described in the rodent and human cortex (Wisden et al., 1992; Petri et al., 2003): high and uniform across layer 2 to layer 4, low in layer 5, moderate in layer 6, and absent in layer 1 (Figure 2C).

2.4.2 mRNA expression levels of α4 and δ subunits in subjects with schizophrenia

Analysis of film autoradiograms revealed that the mean (±SD) α4 mRNA expression level (Figure 3A) in DLPFC area 9 was only 9% lower in the subjects with schizophrenia (31.2 ± 7.0 nCi/g) than in the matched control subjects (34.6 ± 8.5 nCi/g), and this difference did not achieve statistical significance ($F_{1,39}$ = 3.69, p = 0.062). In contrast, the mean expression level of δ mRNA (Figure 3B) was significantly ($F_{1,39}$ = 17.30, p < 0.0001) 19% lower in the subjects with schizophrenia (238.15 ± 63.29 nCi/g) than in the matched control subjects (295.13 ± 52.96 nCi/g).

Our analysis revealed significant negative correlations between α4 mRNA expression levels and age for both control subjects (r = -0.46, p < 0.001) and subjects with schizophrenia (r = -0.35, p = 0.003; Figure 3C). Similarly, we detected significant negative correlations between δ mRNA expression levels and age in control subjects (r = -0.48, p < 0.001) and subjects with schizophrenia (r = -0.30, p = 0.007; Figure 3D). The regression line for δ mRNA expression levels in subjects with schizophrenia was parallel to and shifted downward from that of control subjects, suggesting that the disease-related reduction in δ mRNA levels is similar in magnitude across adult life (Figure 3D). In addition, our analysis revealed that RIN was

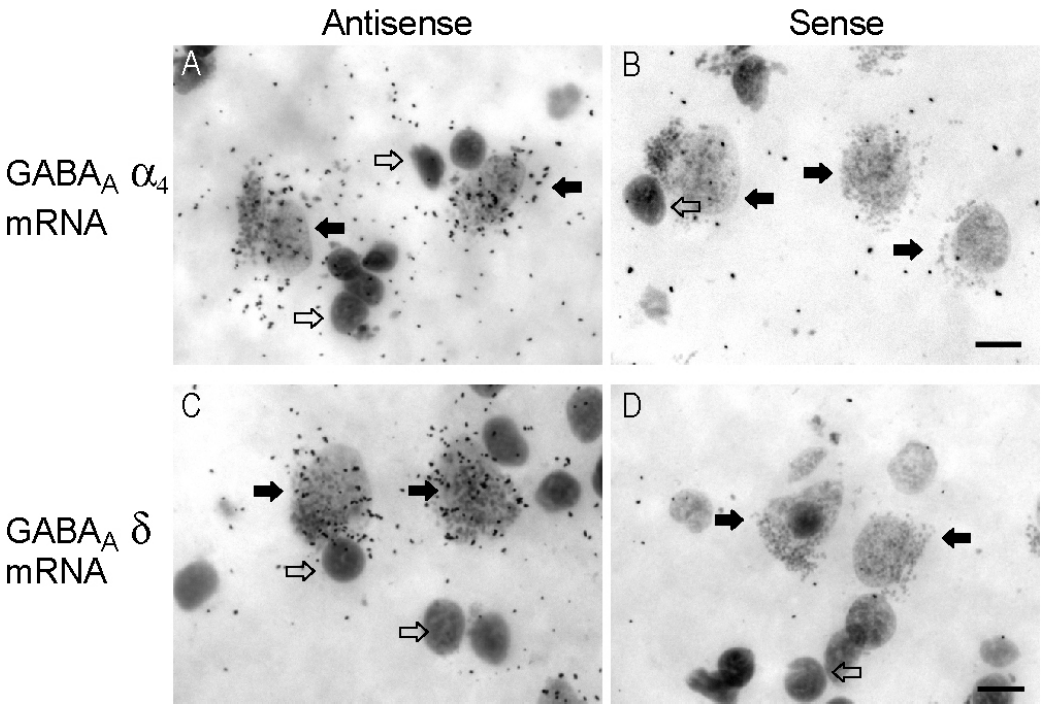

Figure 1. Photomicrographs illustrating the expression of α4 and δ mRNAs in Nissl-stained, emulsion-dipped sections.

Sections processed with antisense riboprobes for α4 (A) and δ (C) mRNAs revealed specific clustering of silver grains over neurons (solid arrows) but not over glia cells (open arrows). In addition, silver grain clustering over neurons was not observed in sections treated with α4 (B) or δ (D) sense riboprobes. Scale bars = 10 μm.

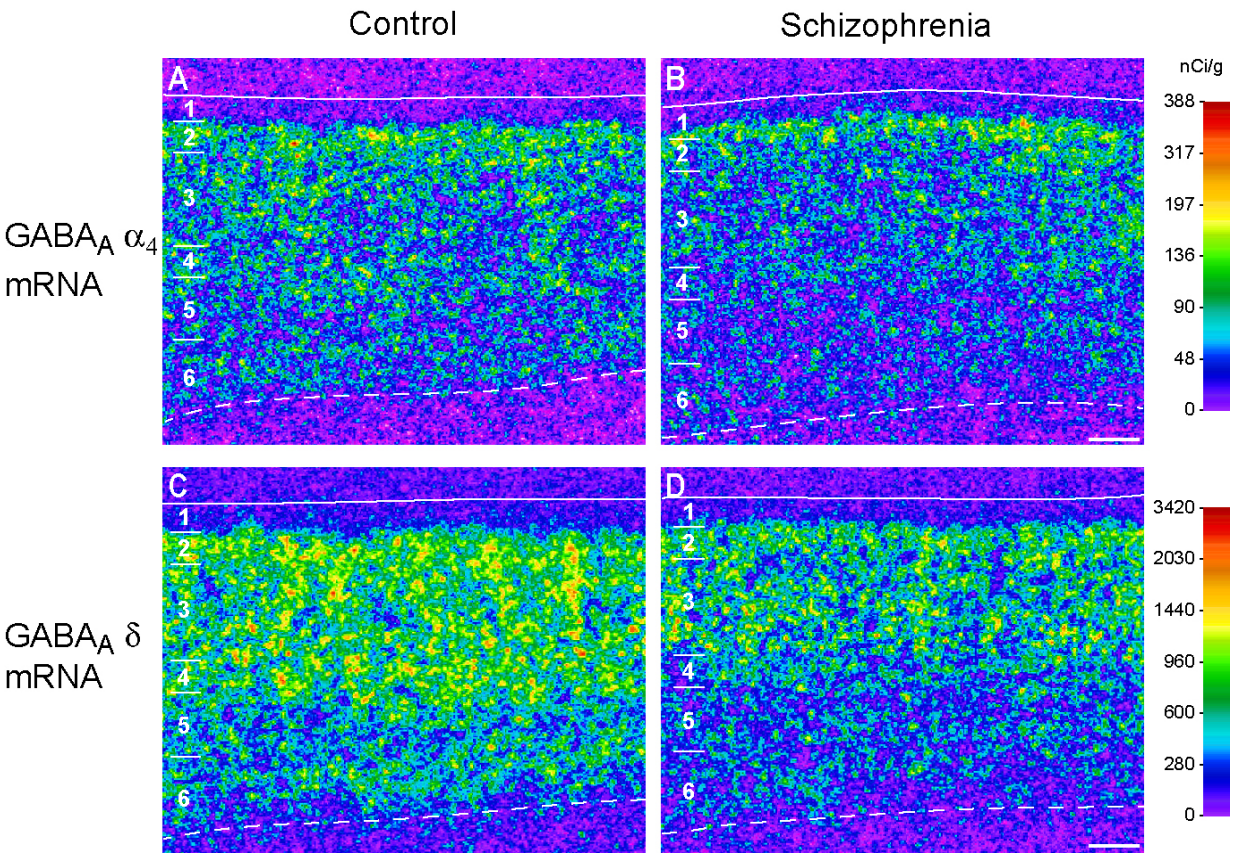

Figure 2. Representative autoradiograms of α4 and δ mRNAs across the DLPFC.

The mRNA expression levels of α4 (A, B) and δ (C, D) subunits in DLPFC area 9 of a control subject (A, C) and an age-, sex- and PMI-matched subject with schizophrenia (B, D) are illustrated. The intensity of hybridization signals is presented in a pseudocolor manner according to the calibration scales (nCi/g) for each mRNA. Solid lines represent the pial surface and dotted lines represent the gray-white matter border. The six cortical layers are identified. Scale bars = 500 μm.

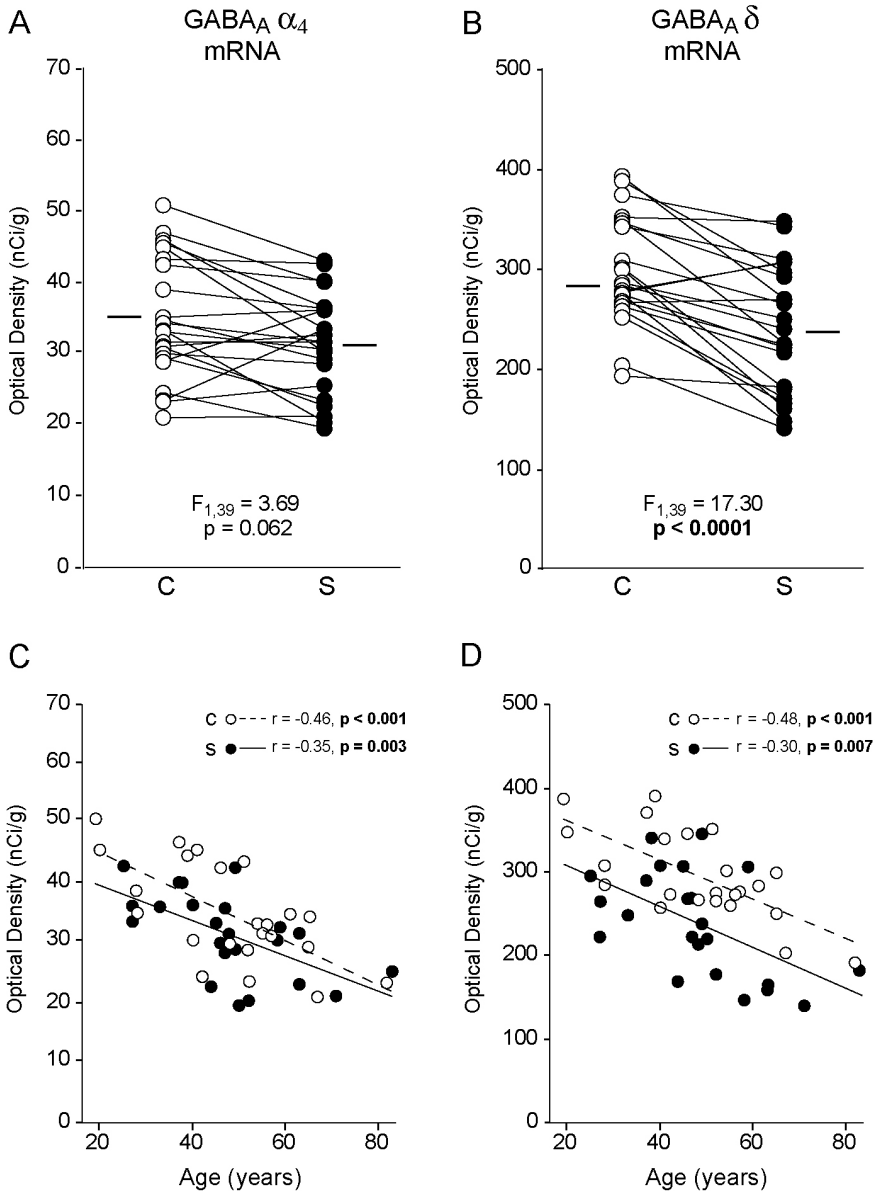

Figure 3. Film autoradiogram optical density measures for α4 and δ mRNAs in DLPFC area 9.

The mRNA expression levels for α4 (A) and δ (B) subunits in control (c) and schizophrenia subjects (s). The mean values for each subject group are represented by horizontal bars, and values for individual subject pairs are connected by lines. The mRNA expression levels of both α4 (C) and δ (D) were negatively correlated with age. The regression lines for δ mRNA in subjects with schizophrenia are parallel to and shifted downward from those for control subjects, suggesting that the decreased expression of δ mRNA in schizophrenia is similar in magnitude across adult life.

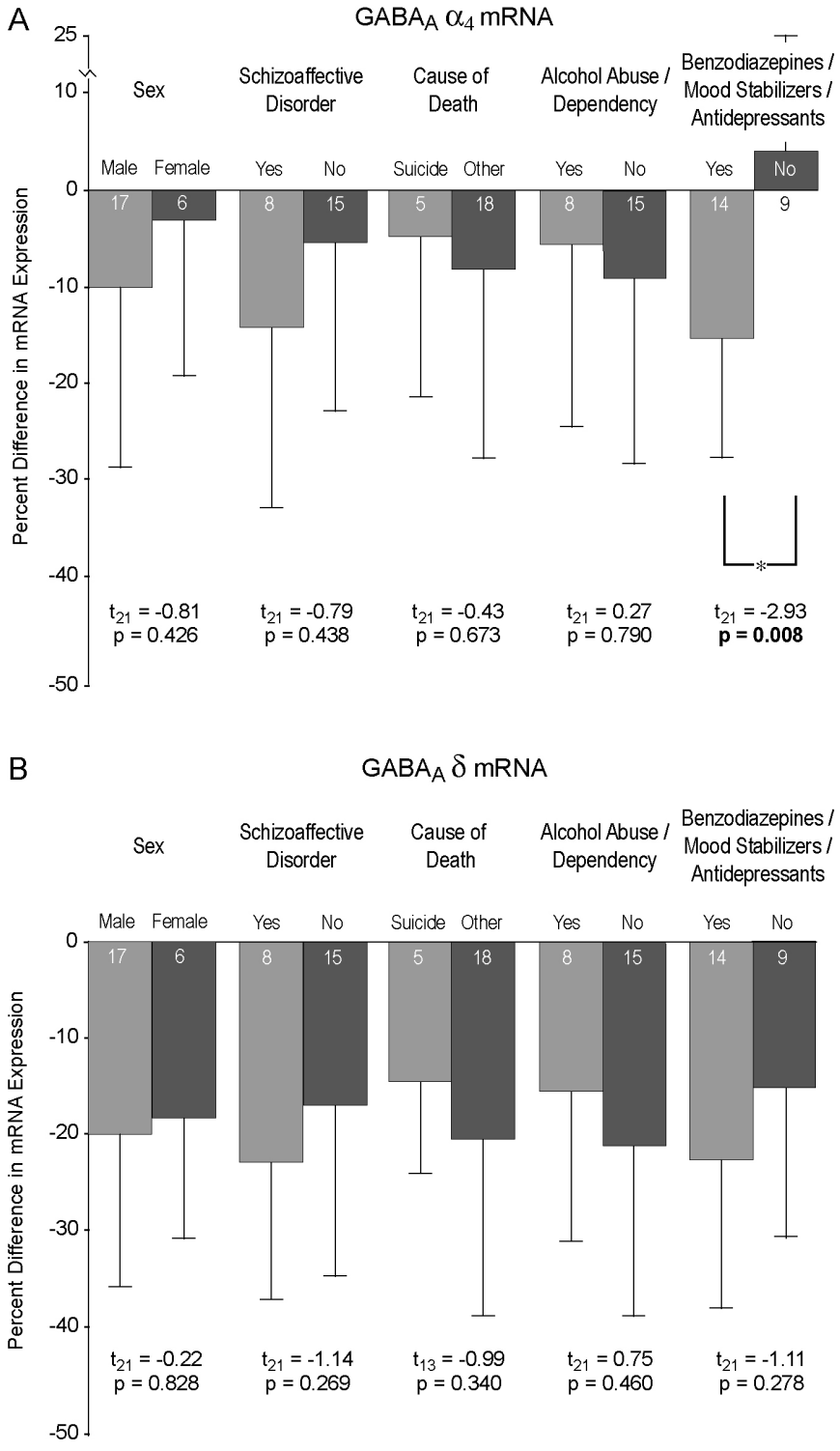

Figure 4. Effect of confounding factors on the within-subject pair percent differences in levels of α4 and δ mRNAs.

significantly correlated with α4 ($r = 0.24$, $p = 0.032$) and δ ($r = 0.50$, $p = 0.012$) mRNA levels in subjects with schizophrenia. However, no correlation was found between RIN and α4 ($r = 0.24$, $p = 0.267$) or δ ($r = 0.24$, $p = 0.273$) mRNA levels in control subjects.

2.4.3 Influence of confounding factors on the expression of α4 and δ subunit mRNAs

As shown in Figure 4A, the within-subject pair differences in α4 mRNA expression were not influenced by sex, diagnosis of schizoaffective disorder, cause of death or history of alcohol abuse and/or dependency. However, the levels of α4 mRNA were decreased only in subjects with schizophrenia receiving benzodiazepines, mood stabilizers and/or antidepressants at the time of death. In these subjects, the levels of α4 subunit mRNA were 16% lower relative to their matched controls. This within-subject pair difference was significantly different ($t_{21} = -2.93$, $p = 0.008$) from the 4% increase in α4 mRNA levels in the subjects with schizophrenia not receiving these medications at the time of death (Figure 4A). These findings suggest that the lower α4 mRNA levels in some subjects with schizophrenia represent a medication effect and not the disease process. In contrast, the mean within-subject pair difference in δ mRNA expression was not influenced by sex, diagnosis of schizoaffective disorder, cause of death, alcohol abuse and/or dependency, or use of benzodiazepines, mood stabilizers and/or antidepressants (Figure 4B).

Figure 4. Whereas the mean within-subject pair percent differences in α4 mRNA were not affected by sex, diagnosis of schizoaffective disorder, cause of death or a history of alcohol abuse and/or dependency, we found a significant effect of benzodiazepines, mood stabilizers and/or antidepressants at time of death (A). In contrast, subjects with schizophrenia showed a similar decrease in δ mRNA levels, relative to their matched control subjects, independent of the presence or absence of each factor analyzed (B).

2.4.4 δ subunit mRNA expression in the DLPFC of monkeys chronically exposed to antipsychotic medications

We further assessed whether the lower levels of δ mRNA observed in schizophrenia might reflect the effects of treatment with antipsychotic medications by analyzing the DLPFC of monkeys chronically exposed to haloperidol, olanzapine or sham (Dorph-Petersen et al., 2005). As shown in Figure 5, δ mRNA expression levels did not differ across these three groups ($F_{1,10}$ = 0.82, p = 0.468). These findings, together with the absence of effects due to other potential confounding factors described above, suggest that the reduction in δ mRNA expression in subjects with schizophrenia reflects the underlying disease process.

2.4.5 Laminar analysis of δ mRNA expression levels in subjects with schizophrenia

Because the lower levels of δ mRNA in subjects with schizophrenia did not seem to be a consequence of confounding factors, we further assessed the levels of δ mRNA across cortical layers of the DLPFC. The mean expression levels of δ mRNA were significantly lower in layers 3 ($F_{1,39}$ = 9.92, p = 0.003), 4 ($F_{1,39}$ = 10.782, p = 0.002), 5 ($F_{1,39}$ = 24.63, p < 0.00001) and 6 ($F_{1,39}$ = 11.11, p = 0.002), but not layer 2 ($F_{1,39}$ = 2.40, p = 0.130), of subjects with schizophrenia (Figure 6).

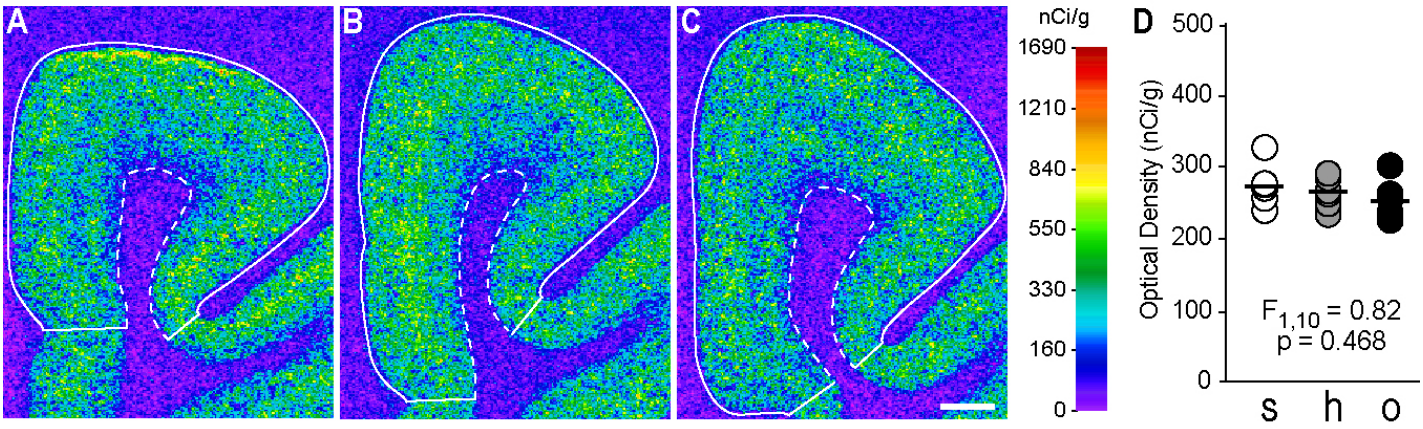

Figure 5. Representative autoradiograms illustrating the expression of δ mRNA in the DLPFC of monkeys chronically exposed to antipsychotic medications.

Monkeys exposed to sham (A), haloperidol (B) or olanzapine (C). Solid lines represent the pial surface and dotted lines represent the border between gray matter and white matter. The mean levels of δ mRNA did not differ across subject groups (D). The mean levels of δ mRNA did not differ across sham (s), haloperidol (h) or olanzapine (o) treated monkeys (D). Scale bar = 1 mm.

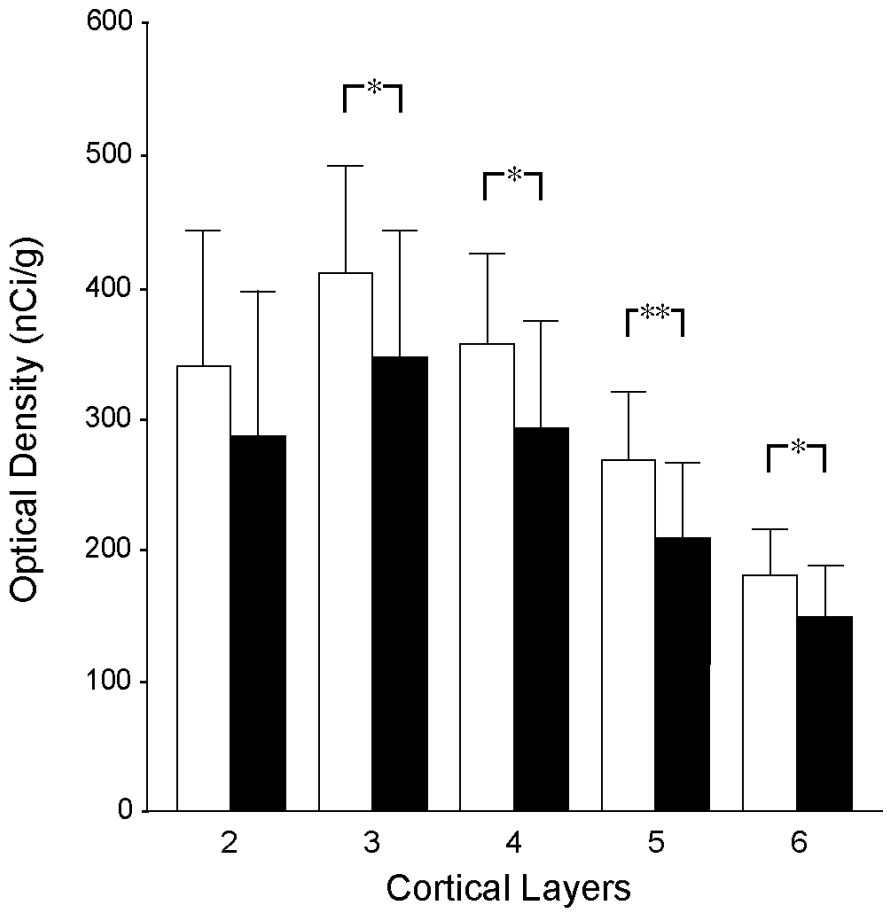

Figure 6. Mean film autoradiogram optical density measures for δ mRNA across cortical layers of the DLPFC.

The mean expression levels of δ mRNA were significantly reduced in layers 3 (16%), 4 (18%), 5 (23%) and 6 (18%) in subjects with schizophrenia (closed bars) compared to control subjects (open bars). * $p < 0.01$, ** $p < 0.0001$

2.4.6 Postnatal development of α4 and δ mRNAs in monkey DLPFC

In order to understand the dissociation between changes in expression levels of α4 and δ mRNAs in subjects with schizophrenia, we compared the postnatal developmental trajectories of these two subunits in the monkey DLPFC. The expression of δ mRNA increased during postnatal development (Figure 7A, B), with the mean overall cortical levels of δ mRNA 56% greater in the adult animals than those one week of age. However, the effect of age did not quite achieve statistical significance ($F_{6,21}$ = 2.45, p = 0.059, Figure 7C), perhaps because δ mRNA levels appeared to increase only in the deep layers (Figures 7A and B). Consistent with this interpretation, δ mRNA levels did not change with age in layer 3, but significantly ($F_{6,20}$ = 7.32, p < 0.001) increased by 116% between one week of age and adulthood in deep layer 5 (Figure 7D).

In contrast, the mRNA expression levels of α4 decreased during postnatal development across all cortical layers (Figure 7E, F). Between one week of age and adulthood, the mRNA levels of α4 decreased significantly ($F_{6,21}$ = 5.64, p = 0.0012) by 36% (Figure 7G). The opposing trajectories of α4 and δ mRNAs resulted in a marked change in the ratio of δ to α4 mRNA levels across development. Between one week of age and adulthood, the ratio of δ to α4 mRNA levels increased significantly ($F_{6,21}$ = 4.80, p = 0.003) by 199% (Figure 7H).

It should be noted that the observed mRNA expression levels of δ and α4 subunits in the sexually mature monkeys did not seem to be affected by levels of gonadal steroids and stage of menstrual cycle, since among the 42-month old monkeys, the two animals in luteal phase at the time of euthanasia had mean (± SD) normalized optical density measures of δ (0.68 ± 0.03) and

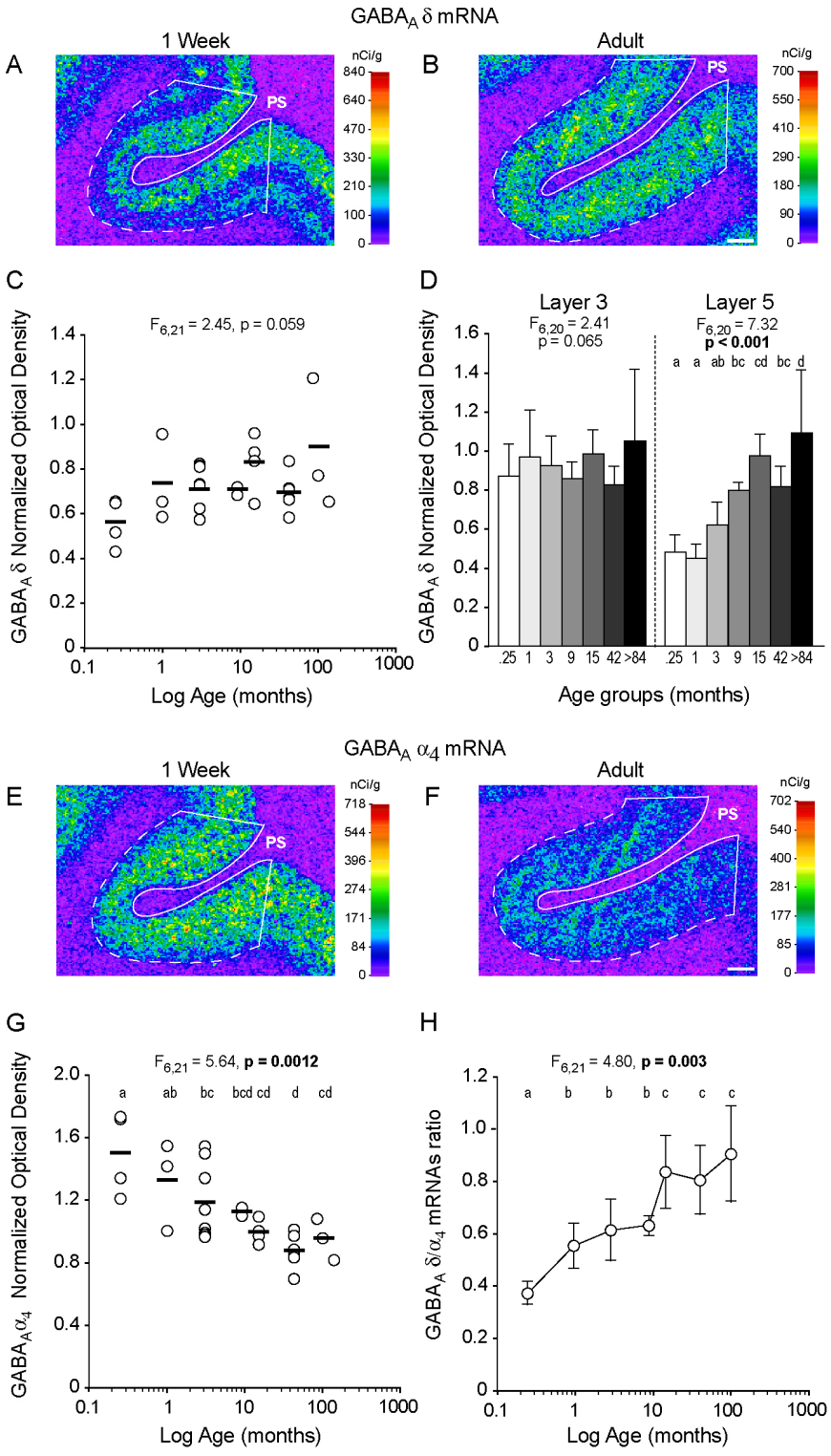

Figure 7. Expression patterns of δ and α4 mRNAs across postnatal development in monkey DLPFC.

α4 (0.83 ± 0.19) that were similar to those in the four animals in the follicular phase (δ: 0.69 ± 0.10; α4: 0.89 ± 0.08).

Figure 7. The overall gray matter levels of δ mRNA increased from 1 week of age (A) to adulthood (B), although these changes did not quite reach statistical significance (C). However, the levels significantly increased with age in layer 5, but did not change in layer 3 (D). In contrast, the expression levels of α4 decreased significantly with age (E,F,G). The opposing developmental trajectories of δ and α4 mRNAs resulted in a significant increase in the δ to α4 ratio with age (H). The density of hybridization signals are presented in a pseudocolor manner according to the calibration scales (nCi/g). Solid white lines represent the pial surface and dotted lines represent the border between gray matter and white matter (A,B,E,F). The mean values for each subject group are represented by horizontal bars (C,G). Within each panel, age groups not sharing the same letter are significantly different at $p < 0.05$. Scale bars = 1 mm.

2.4.7 Lower expression of δ subunit mRNA are associated with altered expression of $GABA_A$ α1 subunit

The dissociation between δ and α4 mRNA levels in subjects with schizophrenia, in laminar distributions and across postnatal development suggested that the δ-containing $GABA_A$ receptors altered in schizophrenia might contain another class of α subunit. Consistent with this idea, a recent study found that δ subunits assemble with α1, but not α4 subunits, in hippocampal interneurons (Glykys et al., 2007). Interestingly, the change in δ mRNA expression in schizophrenia was strongly correlated with that of α1 mRNA ($r = 0.81$, $p < 0.001$) as measured by microarray in a previous study (Hashimoto et al., 2008a). Thus, the lower expression of δ subunit mRNA in schizophrenia could represent lower levels of α1βxδ receptors.

As a proof-of-concept test of this hypothesis, we asked whether δ subunit mRNA levels were significantly reduced in the prefrontal cortex of $GABA_A$ α1 knockout mice. The mean (±SD) expression of δ mRNA (Figure 8E) was significantly ($F_{1,13} = 10.50$, $p = 0.006$) decreased by 21% in the prefrontal cortex of α1 knockout mice (104.79 ± 19.85 nCi/g) compared to wild-type mice (131.92 ± 16.75 nCi/g). In contrast, mean α4 mRNA expression (Figure 8F) was not changed ($t_{14} = 1.03$, $p = 0.321$) in knockout mice (155.50 ± 21.10 nCi/g) compared to wild-type mice (166.45 ± 21.46 nCi/g). These findings are consistent with the hypothesis that reduced expression of δ subunit mRNA in schizophrenia represents lower levels of α1βxδ $GABA_A$ receptors.

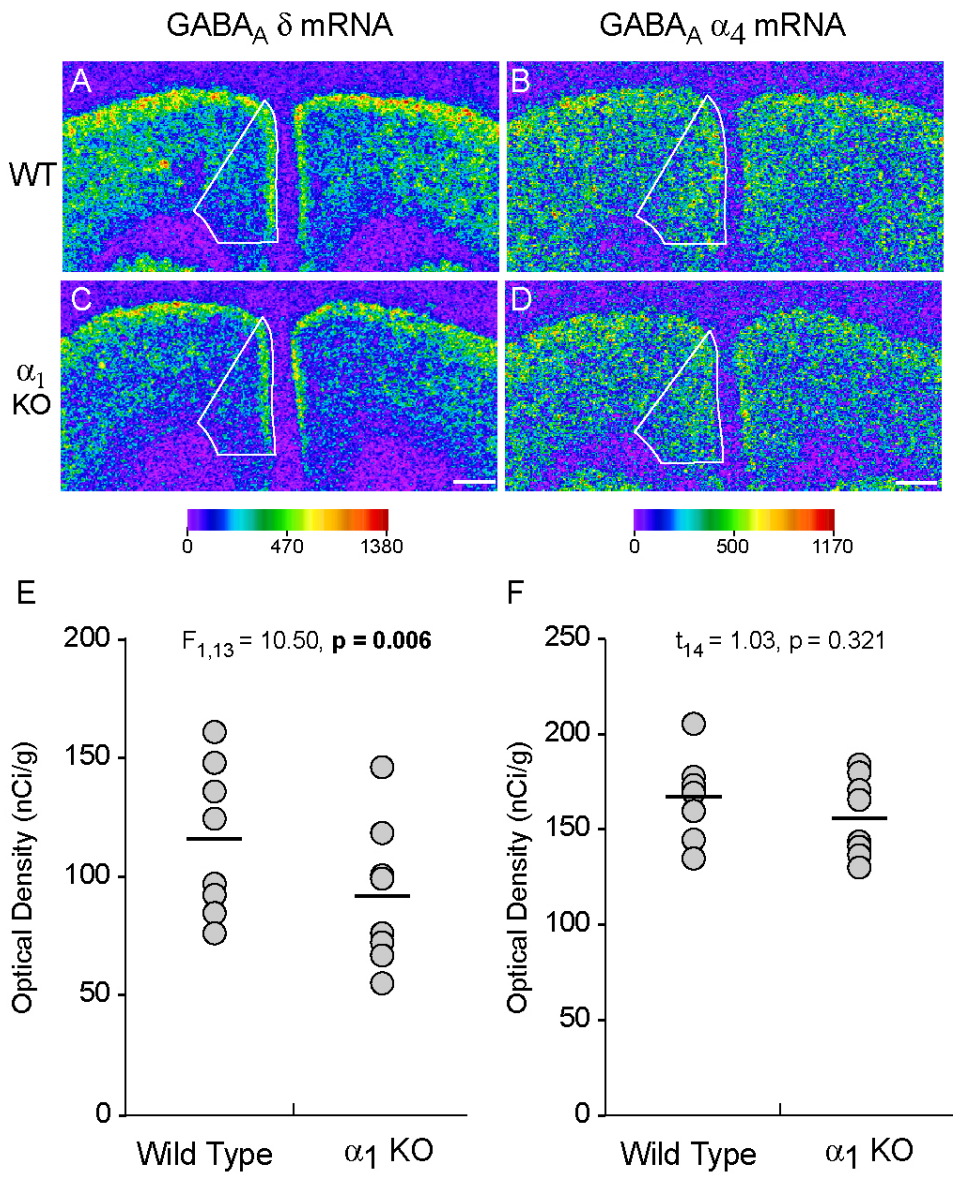

Figure 8. Expression levels of δ and α4 mRNA across the PFC of wild-type and GABA$_A$ α1 knockout (KO) mice.

The mRNA levels of δ subunit appear to be decreased in the PFC of α1KO mice (C) compared to wild-type mice (A). PFC is represented in the autoradiograms by solid lines. Consistent with these observations, mean δ mRNA levels were significantly (E) reduced by 21% in the PFC of α1 KO mice. In contrast, the mRNA expression levels of α4 subunit were not changed (F) in the PFC of α1 KO mice (D) compared to wild-type mice (B). The mean values for each subject group are represented by horizontal bars. Scale bars = 500 μm.

2.5 DISCUSSION

The findings of this study indicate that the expression level of δ mRNA, but not of α4 mRNA, is significantly lower in the DLPFC of subjects with schizophrenia. To the extent that α4 mRNA is lower in schizophrenia (Hashimoto et al., 2008a), the reduction appears to be due to an effect of benzodiazepines, mood stabilizers and/or antidepressants at the time of death; however, we cannot exclude the possibility that lower levels of α4 mRNA reflect a particular disease process in a subtype of schizophrenia with clinical features that require the prescription of these medications. In contrast, the lower level of δ mRNA in subjects with schizophrenia, which is consistent with two previous microarray studies in smaller subject cohorts (Vawter et al., 2002; Hashimoto et al., 2008a), appears to reflect the disease process of schizophrenia, and is not attributable to potential confounding factors such as sex, diagnosis of schizoaffective disorder, cause of death, alcohol abuse and/or dependency, or treatment with benzodiazepines, mood stabilizers and/or antidepressants at time of death. In addition, the lower level of δ mRNA does not appear to be a consequence of exposure to antipsychotic medication, as the levels of δ mRNA were unchanged in the DLPFC of monkeys chronically exposed to typical or atypical antipsychotics. Consistent with this interpretation, the mean percent difference in δ mRNA levels in the four subjects with schizophrenia who were off medications at the time of death (-23%) did not differ (t_{21} = -0.51, p = 0.616) from those who were receiving antipsychotic medications (-18%).

Previous studies suggest that δ subunit mRNA levels in the human cortex are higher than those of α4 subunit (Petri et al., 2003), whereas rodent neocortex levels of δ mRNA are slightly lower than those of α4 mRNA (Wisden et al., 1992). Although the probes used to assess the expression of α4 and δ in our *in situ* hybridization study do not permit a direct quantitative

comparison between α4 and δ mRNA levels, our findings are consistent with previous observations and suggest that the expression levels of the δ subunit are higher than those of α4 in the human DLPFC. For instance, in the DLPFC of normal control subjects, mean levels of δ subunit mRNA measured using probes directed at different portions of the transcript by *in situ* hybridization or microarray (Hashimoto et al., 2008) were 4-9 times higher those of α4 mRNA. These findings, along with the differential disease and developmental effects on δ versus α4 subunits are surprising, as δ subunits are thought to co-assemble preferentially with α4 subunits in forebrain $GABA_A$ receptors (Peng et al., 2002; Drasbek et al., 2007). Thus, our findings suggest that the altered δ-containing $GABA_A$ receptors in schizophrenia are not α4βxδ receptors.

Lower levels of δ subunit mRNA in subjects with schizophrenia could represent a reduced complement of cortical α1βxδ $GABA_A$ receptors. First, the magnitude of the disease-related differences in δ and α1 mRNA levels were significantly correlated ($r = 0.81$, $p < 0.001$), as measured by microarray in a previous study involving a subset of the subjects studied here, whereas the within-subject pair differences in δ and α4 mRNA levels were not correlated (Hashimoto et al., 2008a). Second, δ mRNA expression increased across postnatal development of the monkey DLPFC, paralleling the previously reported increase in α1 mRNA in the same animals (Nguyen et al., 2006), whereas levels of α4 mRNA decreased across postnatal development. Third, our findings suggest that changes in the expression levels of the δ subunit are associated with that of the α1 subunit, as δ mRNA levels were significantly reduced in the PFC of α1 knockout mice. Consistent with this hypothesis, the δ subunit has been shown to co-assemble with α1 subunits to produce functional α1β1δ, α1β1γ2Lδ and α1β3δ recombinant receptors (Saxena and Macdonald, 1994; Wohlfarth et al., 2002; Bianchi and Macdonald, 2003). In addition, immunoprecipitation studies in rat brain extracts revealed that δ subunits associate

with α1 subunits (Mertens et al., 1993). $GABA_A$ α1 subunits have also been found extrasynaptically (Baude et al., 2007; Sun et al., 2004), consistent with the typical localization of δ-containing receptors (Nusser et al., 1998; Farrant and Nusser, 2005), and interneurons in the dentate gyrus exhibit immunoreactivity for α1 and δ subunits along the cell body surface and proximal dendrites (Glykys et al., 2007). Furthermore, δ and α1 immunoreactivity levels and the ethanol-induced enhancement of tonic conductance were unchanged in interneurons of the α4 subunit knockout mice (Glykys et al., 2007).

The associated reductions in δ and α1 mRNA levels might also reveal the pathogenetic mechanism underlying the lower levels of δ-containing $GABA_A$ receptors in schizophrenia. For example, variants in the $GABA_A$ receptor α1 subunit gene have been associated with schizophrenia and with altered expression levels of $GABA_A$ receptor subunits (Petryshen et al., 2005). These findings raise the possibility that lower expression of δ mRNA is a consequence of a reduction in α1 mRNA levels. This hypothesis is supported by our findings of reduced δ mRNA in the prefrontal cortex of α1 knockout mice. However, it should be noted that the mRNA levels of the δ subunit were reported to be unchanged across the cerebellum (Ogris et al., 2006) and cerebral cortex (Ponomarev et al., 2006) of α1 knockout mice. Thus, the potential effect of lower α1 subunit on the mRNA expression levels of δ might be limited to certain cortical regions. Alternatively, lower δ mRNA levels might also be an event downstream to reduced activity of excitatory synapses (Lewis and Moghaddam, 2006). For example, infusions of MK-801, a non-competitive NMDA receptor antagonist, result in decreased mRNA levels of δ in the rat forebrain (Kim et al., 2000); however, whether these deficits are present after chronic reductions in the activity of excitatory synapses has not been determined. Under either scenario,

lower levels of δ-containing receptors, and the resulting reduction in tonic inhibition, could contribute to DLPFC dysfunction in schizophrenia.

Alternatively, lower levels of δ subunit (and potentially of tonic inhibition) might represent a compensatory response to presynaptic reductions in GABA neurotransmission. Release of GABA provides inhibitory control over postsynaptic cells via both synaptic and extrasynaptic $GABA_A$ receptors. An important functional role of synaptic receptors, which mediate phasic inhibition, is the generation of rhythmic activities in neuronal networks (Farrant and Nusser, 2005). The resulting synchronized activity gives rise to network oscillations which are thought to contribute to cognitive processes, such as working memory. For instance, the synchronized firing of neuronal networks at 30–80 Hz, known as gamma band oscillations, are induced and sustained in the human DLPFC during working memory tasks (Tallon-Baudry et al., 1998; Howard et al., 2003). Thus, lower levels of prefrontal GAD_{67} mRNA, leading to a deficit in GABA synthesis and impaired phasic inhibition, have been proposed to be a substrate for reduced frontal lobe gamma band power and working memory deficits in schizophrenia (Cho et al., 2006). Although the role of extrasynaptic receptors and tonic inhibition in the generation and/or maintenance of oscillations is less clear, a decrease in tonic inhibition could represent a compensatory response. Consistent with this idea, mutant mice with a complete loss of tonic inhibition in the hippocampus exhibit an increase in the power of gamma band oscillations (Glykys et al., 2008). Thus, the findings of this study provide additional insight into the nature of altered cortical GABA neurotransmission in schizophrenia and suggest potential molecular targets for novel therapeutic interventions. However, further studies are needed to determine how alterations in δ subunit-containing $GABA_A$ receptors are related to putative mechanisms of impaired GABA neurotransmission in schizophrenia (Huang et al., 2007; Behrens et al., 2007).

3.0 CHAPTER 3: REDUCED SIGNALING THROUGH EXCITATORY SYNAPSES AS A PATHOGENETIC MECHANISM: EFFECTS ON δ SUBUNIT EXPRESSION LEVELS

3.1 ABSTRACT

Postmortem studies indicate that expression of the $GABA_A$ receptor δ subunit mRNA is significantly reduced in the PFC of subjects with schizophrenia, potentially contributing to the altered inhibitory circuitry seen in the illness. Although the pathogenetic mechanisms upstream to lower levels of δ in schizophrenia remain unknown, some studies suggest that reduced signaling through excitatory synapses, as hypothesized to be present in schizophrenia, give rise to decreased expression of δ subunit mRNA. In order to test this hypothesis, we used *in situ* hybridization to measure the levels of δ subunit mRNA in the PFC of four rodent models of reduced cortical excitatory drive: 1) reduced signaling through NMDA receptors (NMDAR NR1 hypomorphic mice), 2) reduced excitatory synaptic terminals from the thalamus (adult mediodorsal thalamic nuclei lesioned (MDTNL) rats), 3) reduced excitatory synaptic terminals from the hippocampus (neonatal ventral hippocampal lesioned (NVHL) rats); and 4) reduced postsynaptic targets of excitatory inputs (TrkB hypomorphic mice with decreased dendritic arborization). Compared to appropriate control animals, the mRNA levels of δ subunit were not significantly different in the PFC of 1) homozygote NR1 hypomorphic mice, in which the

mRNA levels of NR1 subunit were decreased by 45%; 2) MDTNL rats with lesions ranging in size from 47 to 91% of MDTN volume; 3) NHVL rats, or 4) TrkB hypomorphic mice, in which TrkB mRNA levels were reduced by 35% and 74% in heterozygotes and homozygotes, respectively. Thus, although reduced signaling through excitatory synapses might be a pathogenetic mechanism for other abnormalities in schizophrenia, the convergence of findings from this study do not support the hypothesis that it accounts for the lower expression of $GABA_A$ receptor δ subunit mRNA.

3.2 INTRODUCTION

Several lines of evidence support the idea that abnormalities in the inhibitory circuitry of the DLPFC might underlie some of the cognitive deficits observed in subjects with schizophrenia (Lewis et al., 2005). For example, the mRNA expression levels of the GAD_{67} is significantly reduced in the DLPFC of subjects with schizophrenia (Akbarian et al., 1995; Volk et al., 2000; Guidotti et al., 2000; Straub et al., 2007; Hashimoto et al., 2008a; Hashimoto et al., 2005), suggesting that the release and extracellular levels of GABA are significantly reduced. We recently found that the mRNA expression levels of the $GABA_A$ receptor δ subunit were significantly reduced in the PFC of subjects with schizophrenia, potentially contributing to reduced inhibitory neurotransmission in the illness (*See Chapter 2*).

Previous studies suggest that expression of δ subunit mRNA is modulated by the levels of excitatory activity. For instance, δ subunit mRNA expression increases significantly in cultured cerebellar granule cells exposed to NMDA (Gault and Siegel, 1998), an effect that is abolished after addition of MK-801, a non-competitive NMDAR antagonist. In addition, δ subunit expression decreased in the rat forebrain after a week of MK-801 infusions (Kim et al., 2000). These findings are of interest as convergent lines of evidence suggest that the disease process of schizophrenia may involve a reduction in neurotransmission through NMDARs (Moghaddam, 2003; Coyle et al., 2003). Furthermore, acute reduction in signaling via NMDARs lowers expression of GAD_{67} (Kinney et al., 2006). Thus, alterations in markers of GABA neurotransmission including lower levels of δ mRNA, in schizophrenia could be a consequence of reduced excitatory activity. However, no experimental evidence exists to provide proof-of-

concept support that a chronic reduction in excitatory neurotransmission, which is more likely to mimic the nature of the hypothesized deficit in NMDA function in schizophrenia, results in decreased expression of the δ subunit of $GABA_A$ receptors.

A chronic reduction in the activity of excitatory cortical inputs in schizophrenia might be a consequence of one of the following factors. First, hypoactivation of NMDARs has been proposed to play a significant role in the pathogenesis of schizophrenia. Clinical studies have shown that subanesthetic doses of ketamine (an NMDAR antagonist) induce symptoms characteristic of schizophrenia, including the types of cognitive deficits that are dependent on the circuitry of the DLPFC (Krystal et al., 1994; Malhotra et al., 1996). Furthermore, gene variants that confer risk for schizophrenia might do so by modulating the activity of NMDARs (Coyle, 2007).

Second, reduced signaling through excitatory synapses might be a consequence of a lower number of excitatory synaptic terminals in the DLPFC. In particular, studies have shown that the volume and activity of the MDTN, the major source of excitatory thalamic input to the prefrontal cortex, are reduced in subjects with schizophrenia (Manoach et al., 1999; Byne et al., 2001; Gilbert et al., 2001), although postmortem studies reporting a reduced number of MDTN neurons in schizophrenia have not been consistently replicated (see Dorph-Petersen et al. (2004) for review). Interestingly, the mRNA levels of the δ subunit are significantly reduced in layers 3 and 4 (*see sections 2.4.5, Chapter 2*) of the DLPFC, the principal cortical layers innervated by thalamic projections (Erickson and Lewis, 2004). Thus, reduction in the number of, or activity in, excitatory projections from the MDTN might represent a pathogenetic mechanism giving rise to lower δ mRNA in subjects with schizophrenia. In addition, neonatal lesions of the ventral hippocampus in rats have replicated the post-pubertal appearance of a number of alterations in

the PFC reminiscent of those observed in schizophrenia (Lipska and Weinberger, 2000;Lipska et al., 2003). Thus, the hippocampus might also serve as a critical source of excitatory inputs to the DLPFC that could regulate δ subunit mRNA expression.

Finally, chronic reduction in the signaling through excitatory synapses can be a consequence of a reduction in the number of postsynaptic targets of excitatory inputs. Postmortem studies revealed that the density of spines and the extent of dendritic arbors of cortical pyramidal cells are reduced in subjects with schizophrenia (Glantz and Lewis, 2000; Garey et al., 1998; Kalus et al., 2000; Black et al., 2004). For instance, a lower number of spines in DLPFC pyramidal cells might reflect reduced signaling through the tyrosine kinase B (TrkB) receptor, which mediates the actions of the secreted neurotrophin brain-derived neurotrophic factor (BDNF). BDNF-TrkB signaling has been shown to enhance somatodendritic development (Gorski et al., 2003; Horch and Katz, 2002; McAllister et al., 1995; Xu et al., 2000b) and spine formation (Horch et al., 1999) of pyramidal neurons. Interestingly, mice genetically engineered to express low levels of TrkB receptors (TrkB hypomorphic mice; (Xu et al., 2000a)) exhibit significant reductions in the number of dendritic branches of retinal gangionic cells (Liu et al., 2007). Given that the mRNA levels of both BDNF and TrkB are significantly reduced in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2005; Weickert et al., 2003), these findings suggest that lower signaling through TrkB receptors might contribute to the reduction in postsynaptic targets of excitatory inputs in subjects with schizophrenia.

In order to provide experimental tests of the hypothesis that a chronic reduction in the signaling through excitatory synapses gives rise to lower levels of δ subunit mRNA, we assessed the mRNA levels of this $GABA_A$ receptor subunit in the PFC of several animals that model

chronic reductions in signaling through excitatory synapses in the form of 1) reduced signaling through NMDARs, 2) reduced excitatory synaptic terminals from the thalamus or hippocampus, and 3) reduced postsynaptic targets of excitatory inputs.

3.3 METHODS

3.3.1 NR1 hypomorphic mice

We utilized tissue from mice genetically engineered to express low levels of the NMDA receptor NR1 subunit (NR1 hypomorphic mice), generously provided by Dr. Mark Caron (Duke University, Durham, NC) (Mohn et al., 1999). Briefly, NR1 hypomorphic mice were generated using homologous recombination in embryonic stem (ES) cells, utilizing a targeting vector to insert a neomycin resistance gene into intron 20 of the Nr1 locus. ES cells carrying the targeted mutation were used to generate mice with an altered Nr1 allele ($Nr1^{neo/+}$). Three groups of mice, wild-type ($Nr1^{+/+}$), heterozygous ($Nr1^{neo/+}$) and homozygous ($Nr1^{neo/neo}$), were euthanized at 8 weeks of age (n = 4 for each group). Brains were frozen immediately and stored at -80°C. Serial coronal sections (12 μm) containing the PFC were cut (from +1.98 to +1.54 bregma (Paxinos and Franklin, 2001). Three sections (spaced at 144 μm) were selected from each animal and processed for *in situ* hybridization for each transcript, NMDA NR1 and δ, as described below. One *in situ* hybridization run containing sections from all 12 animals was performed for each transcript.

3.3.2 MDTN rats

Adult rats with lesions of the MDTN, were kindly provided by Dr. David W. Volk (University of Pittsburgh, Pittsburgh, PA) (Volk and Lewis, 2003). As previously shown, two injections of 0.10 - 0.12 µl ibotenic acid (5 µg/µl) were made into each hemisphere (anteroposterior: -2.3 mm; lateral: +0.6 mm: dorsoventral: -5.5 mm) of peripubertal Sprague–Dawley rats (n = 8) (Volk and Lewis, 2003). In sham rats (n = 8), the needle was lowered into the hippocampus, but did not penetrate the thalamus, and no injections were made. After a 4-week survival period, the animals were sacrificed by decapitation. The proportion of MDTN lesioned was previously determined (Volk and Lewis, 2003), using Nissl-stained coronal sections through the entire extent of MDTN and the Cavalieri estimator of volume (Howard and Reed, 1998). Briefly, the ibotenic acid injections resulted in neuron loss and gliosis in the MDTN (Volk and Lewis, 2003). Furthermore, minimal damage to other adjacent thalamic regions were observed, such as zones of neuron loss in the paraventricular, intermediothalamic and centromedial nuclei (Volk and Lewis, 2003). Serial coronal sections (12 µm) containing the PFC were used (from +3.7 to +2.2 mm bregma (Paxinos and Watson, 1986)). For the δ subunit, four sections (spaced at 240 µm) were selected from each animal and processed for *in situ* hybridization as described below. Two *in situ* hybridization runs, each containing sections from all 16 animals, were performed.

3.3.3 NVHL rats

Tissue from NVHL rats was generously provided by Dr. Patricio O'Donnell (University of Maryland, Baltimore, MD) (O'Donnell et al., 2002). At postnatal days 6-7, male Sprague–Dawley pups were anesthetized with hypothermia by placing them in wet ice for 10-15 min,

placed on a stereotaxic frame and administered bilateral injections of sham (n = 11) or 0.3 μL of ibotenic acid (10 μg/μL, n = 13) into the ventral hippocampus (anteroposterior: −3.0 mm; lateral: +3.5 mm; dorso-ventral: −5.0 mm). All rats were maintained on a 12-hour light/dark cycle with food and tap water available *ad libitum* until the time of the experiment. Qualitative assessments of lesion size were made for all animals with Nissl stained sections spanning the entire rostrocaudal extent of the lesion (O'Donnell et al., 2002). Out of the thirteen animals with ibotenic acid lesions, one animal exhibited an asymmetric lesion, with a very small lesion in the right hemisphere, and a second animal exhibited some damage to adjacent thalamic nuclei. Brains were frozen immediately and stored at -80°C. Serial coronal sections (12 μm) containing the PFC were cut (from +3.7 to +2.2 mm bregma (Paxinos and Watson, 1986)). Three sections (spaced at 180 μm) were selected from each animal and processed for *in situ* hybridization. One *in situ* hybridization run containing sections from all 24 animals was performed.

3.3.4 TrkB hypomorphic mice

In order to determine if a reduced number of postsynaptic targets of excitatory inputs could be an upstream factor resulting in decreased mRNA expression of δ subunits, we used tissue from *TrkB* hypomorphic mice which have a decrease in the dendritic arbor of neurons (Liu et al., 2007). In these animals, the first coding exon of the *TrkB* gene was replaced with a TrkB cDNA unit flanked by two loxP sites (fBZ locus) (Xu et al., 2000a). These mice were generated with 129 strain mice-derived embryonic stem cells and C57BL/6 mice-derived blastocytes (Xu et al., 2000a) and back-crossed into C57BL/6 mice for at least five generations. Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used as control. Homozygous (*fBZ/fBZ*) and

heterozygous (*fBZ/+*) animals were reported to express ~25% and ~62%, respectively, of the TrkB protein levels present in the wild-type animals (Xu et al., 2000a;Rohrer, 2001;Rico et al., 2002). Animals were euthanized at 8 weeks of age (n = 8 for each group). Brains were frozen immediately and stored at -80°C. Serial coronal sections (12 μm) containing the PFC were cut (from +1.98 to +1.54 bregma (Paxinos and Franklin, 2001)). Three sections (spaced at 144 μm) were selected from each animal and processed for *in situ* hybridization for each transcript, TrkB and δ, as described below. Two *in situ* hybridization runs, each containing sections from a given triad (wild-type, *fBZ/+* and *fBZ/fBZ*) were performed for each transcript.

3.3.5 *In situ* hybridization

Templates for the synthesis of δ subunit, NMDA NR1 and TrkB riboprobes were obtained by PCR with specific primer sets. A 517 bp cDNA fragment for NMDA NR1 corresponding to bases 1902-2418 of mouse NMDA Nr1 mRNA (Grin1, GenBank NM_008169); a 345 bp cDNA fragment for TrkB corresponding to bases 2567-2911 of mouse TrkB mRNA (GenBank X17647) and a 598 bp cDNA fragment corresponding to bases 235-832 of mouse δ mRNA (GenBank NM_008070) were amplified. Nucleotide sequencing revealed 100% homologies for the amplified fragments to the previously reported sequences. The mouse δ riboprobe was utilized to analyze the mRNA levels of δ in the rat brain, since the 598 bp mouse cDNA amplified had 94% homology with the rat δ subunit (GenBank NM_017289); in addition, the mRNA expression pattern of δ across the rat PFC was similar to that observed in mice. DNA fragments were sub-cloned into the plasmid pSTBlue-1 (Novagen, Madison, WI). Antisense riboprobes were transcribed *in vitro* in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ),

using T7 or SP6 RNA polymerase, which were then digested with DNase I and purified by centrifugation through RNeasy mini spin columns (Qiagen, Valencia, CA).

Slide-mounted tissue sections were immersed in 4% paraformaldehyde in phosphate buffered saline (PBS), acetylated, dehydrated through a graded ethanol series, and then incubated with ^{35}S-labeled riboprobes in hybridization buffer containing 50% formamide, 0.75 M NaCl, 20 mM 1,4-piperazine diethane sulfonic acid, pH 6.8, 10 mM EDTA, 10% dextran sulfate, 5X Denhardt's solution, 50 mM dithiothreitol, 0.2% SDS and 100 mg/ml yeast tRNA at 56°C for 16 hours. After washing in a solution containing 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63°C, the sections were treated with RNase A (20 µg/ml) at 37°C, and washed in 0.1 SSC (150 mM NaCl and 15 mM sodium citrate) at 66.8°C. Sections were then dehydrated through a graded series of ethanol concentrations, air dried, and exposed to BioMax MR Film (Kodak, Rochester, NY).

3.3.6 Quantification

Analyses were performed by one investigator (JGMA) without knowledge of genotype or experimental treatment group. Trans-illuminated autoradiographic film images were captured by a video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID) system (Imaging Research Inc, London). Images of the corresponding hybridized sections were captured and superimposed on the autoradiographic images to draw contours and to delineate the border between gray matter and white matter. Optical density was measured within contours of the PFC (including the cingulate and prelimbic cortices (Paxinos and Watson, 1986; Paxinos and Franklin, 2001)) and expressed as nanocuries per gram of tissue,

as determined by reference to ^{14}C standards (ARC Inc., St. Louis, MO) exposed on the same film.

3.3.7 Statistical analyses

To examine the expression levels of δ subunit mRNA in the NR1 and TrkB hypomorphic mice, we performed an analysis of variance (ANOVA) model, using genotype as the main effect. We used two-sample *t*-tests to examine the expression levels of δ subunit mRNA between sham and MDTNL animals, and between sham and NVHL animals. All statistical tests were conducted with an α-level = 0.05.

3.4 RESULTS

3.4.1 NR1 hypomorphic mice

NR1 Hypomorphic Mice. In order to test the hypothesis that decreased excitatory signaling through NMDARs gives rise to lower levels of δ mRNA levels, we measured the expression of δ in NR1 hypomorphic mice. Quantitative analysis of film autoradiograms revealed a significant ($F_{2,11}$ = 13.44, p = 0.002) effect of genotype on the levels of NMDA NR1 mRNA in the PFC (Figure 9A-C, G). The mean (± SD) expression levels of the NMDA NR1 subunit were significantly ($p < 0.05$) 45% lower in the PFC of mice with a *Nr1*$^{neo/neo}$ genotype (569.36 ± 50.92 nCi/g) compared to wild-type (1,043.06 ± 197.82 nCi/g) (Figure 9G). Consistent with previous data (Mohn et al., 1999), the mRNA levels of the NR1 subunit were significantly decreased in

the PFC of mice with a *Nr1*$^{neo/+}$ genotype (897.66 ± 105.99 nCi/g). NR1 genotype had no effect ($F_{2,11}$ = 0.87, p = 0.45) on the mRNA expression levels of δ mRNA (Figure 9D-F, G). The mean (± SD) levels of δ subunit were only 4% lower in the PFC of mice with a *Nr1*$^{neo/neo}$ genotype (208.60 ± 37.74 nCi/g) compared to wild-type (216.73 ± 20.71 nCi/g). Furthermore, there was no significant correlation between the mRNA levels of δ and of NMDA NR1 across all animals (r = 0.19, p = 0.54).

3.4.2 MDTNL rats

To assess whether a reduction in excitatory synaptic inputs can give rise to lower expression levels of δ subunit, we measured the mRNA levels of δ subunit in the PFC of rats with bilateral lesions of the MDTN (Volk and Lewis, 2003), the major source of excitatory thalamic input to the PFC. Analysis of the film autoradiograms revealed that the mean (± SD) mRNA levels of δ subunit in the PFC of MDTNL rats (right PFC: 76.30 ± 11.85 nCi/g; left PFC: 75.07 ± 12.88 nCi/g) did not differ (right PFC: t_{14} = -0.34, p = 0.59; left PFC: t_{14} = -0.27, p = 0.79) from those in animals with a sham lesion (right PFC: 73.84 ± 13.05 nCi/g PFC: left PFC: 72.57 ± 13.53 nCi/g) (Figure 10C). Furthermore, δ mRNA expression levels were not correlated (right PFC: r = 0.34, p = 0.428; left PFC: r = 0.19, p = 0.650) with the proportion of MDTN lesioned (Figure 11). Thus, reduced MDTN excitatory inputs into the PFC do not seem to give rise to lower mRNA levels of δ subunit.

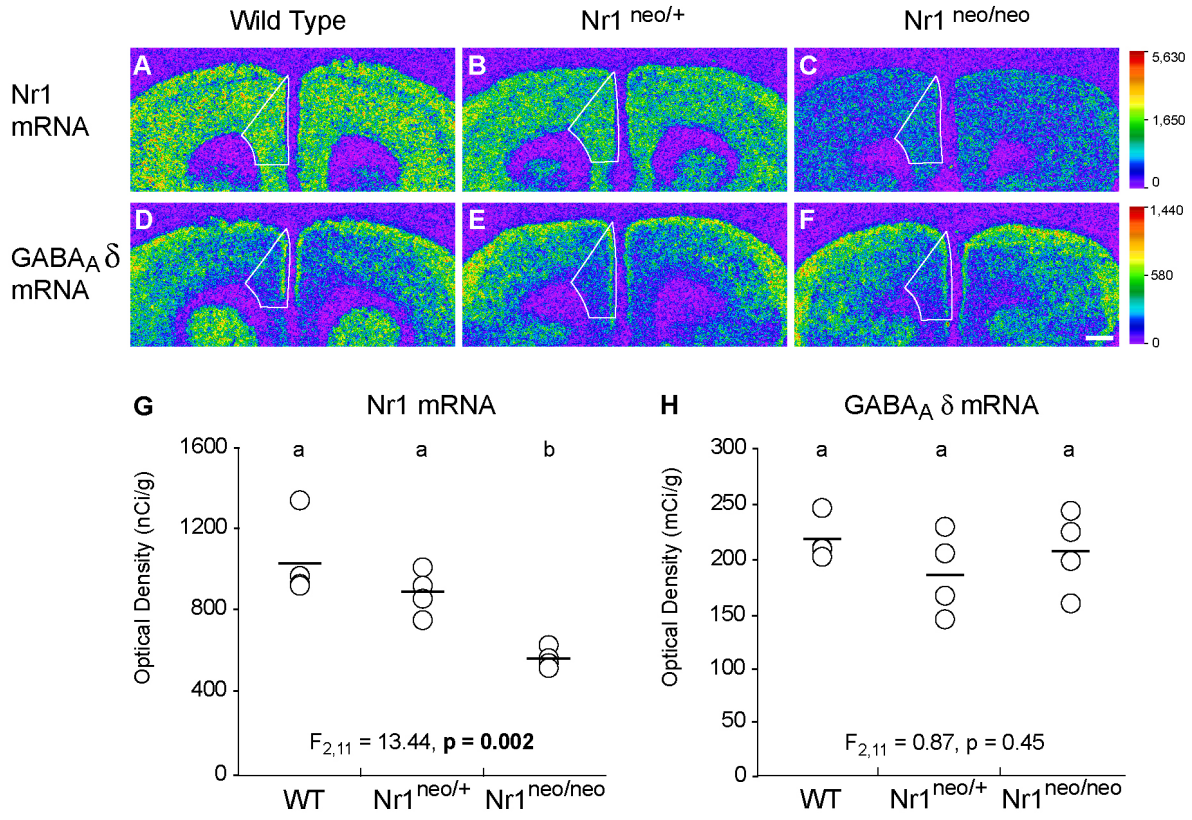

Figure 9. Representative autoradiograms and expression levels of α4 and δ mRNAs in NMDA NR1 hypomorphic mice.

Expression levels of NMDA NR1 (A-C) and $GABA_A$ δ (D-F) mRNAs in the frontal cortex of a wild-type mouse (A,D), a mouse heterozygous for the Nr1 neo locus ($Nr1^{neo/+}$; B,E) and a mouse homozygous for the Nr1 neo locus ($Nr1^{neo/neo}$; C, F). The densities of hybridization signals are presented in a pseudocolor manner according to the calibration scales (right) for each transcript. Note the progressive decrease in mRNA expression of NMDA NR1 subunit as a function of genotype. In contrast, the mRNA levels of δ subunit do not appear to differ across genotypes (D-F). PFC is represented in the autoradiograms as the area between the solid lines. Scale bar = 500 μm. Consistent with these qualitative observations, mean NMDA NR1 subunit mRNA levels (G) were significantly reduced by 45% in the PFC of $Nr1^{neo/neo}$ mice compared to wild-type mice, and the levels in the $Nr1^{neo/+}$ mice were intermediate. In contrast, mean mRNA levels of δ subunit (H) did not differ as a function of genotype. Groups not sharing the same letter are statistically different at $p<0.05$ (Duncan's post hoc test).

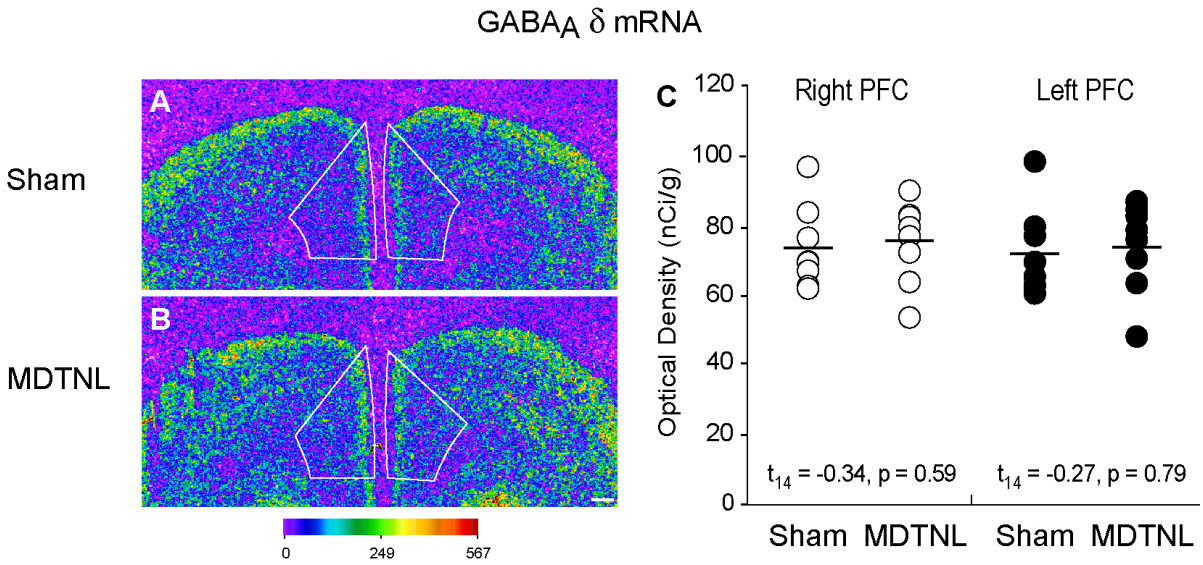

Figure 10. Representative autoradiograms and expression levels of δ mRNA in MDTNL rats.

As illustrated in panel (C), the expression levels of δ mRNA were similar between in the prefrontal cortex of sham and MDTNL rats. The densities of hybridization signals are presented in a pseudocolor manner according to the calibration scales (below). PFC is represented in the autoradiograms as the area between the solid lines. Scale bar = 500 μm. The mean mRNA levels of δ subunit did not differ between sham and MDTNL rats, in either PFC hemisphere (C).

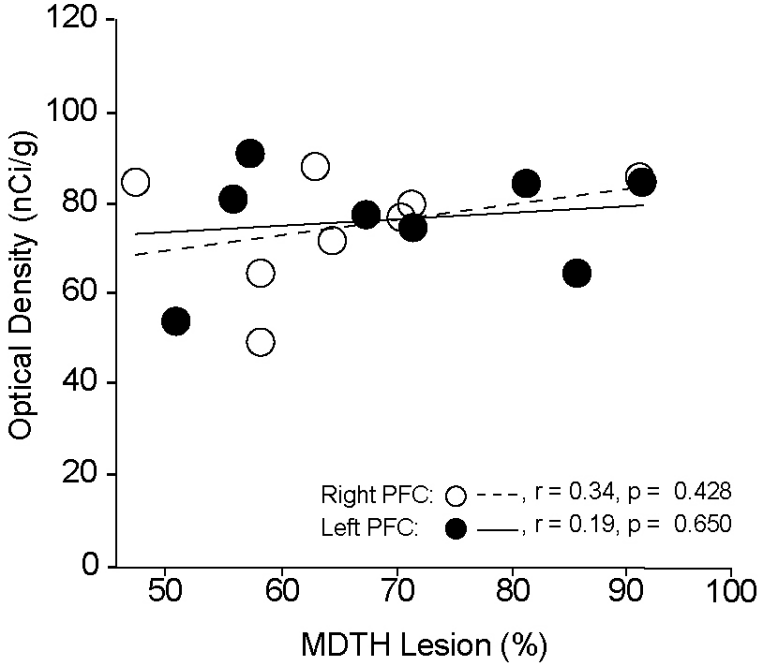

Figure 11. Scatterplots comparing mean mRNA expression levels of δ subunit in the PFC of MDTNL rats with the percent of the MDTN lesioned in each rat.

Note that the mRNA levels of δ subunit did not differ according to the extent of MDTN lesioned in either right (open circles) or left PFC (closed circles).

3.4.3 NVHL rats

In order to further assess whether a decrease in excitatory synaptic terminals in the PFC can result in lower δ subunit, we analyzed δ mRNA levels in rats with a neonatal lesion of the ventral hippocampus, which directly projects to the PFC (Jay et al., 1989; Carr and Sesack, 1996). The mean (± SD) mRNA levels of the δ subunit in the PFC of NVHL animals (right PFC: 38.59 ± 6.79 nCi/g; left PFC: 42.33 ± 8.43 nCi/g) did not differ (right PFC: $t_{22} = 0.73$, $p = 0.47$; left PFC: $t_{22} = 0.001$, $p = 0.99$) from that of animals with a sham lesion (right PFC: 40.46 ± 5.57 nCi/g PFC: left PFC: 42.34 ± 6.63 nCi/g) (Figure 12).

In addition, the levels of δ mRNA did not appear to correlate with the extent of the lesions. For instance, in the NVHL animals, the mean (± SD) mRNA levels of δ subunit in the PFC of the two animals with the smallest lesions (right PFC: 41.42 ± 4.22; left PFC: 44.03 ±0.87 nCi/g) were similar to that of five animals with the largest lesions (right PFC: 37.75 ± 6.47; left PFC: 39.36 ± 5.07 nCi/g).

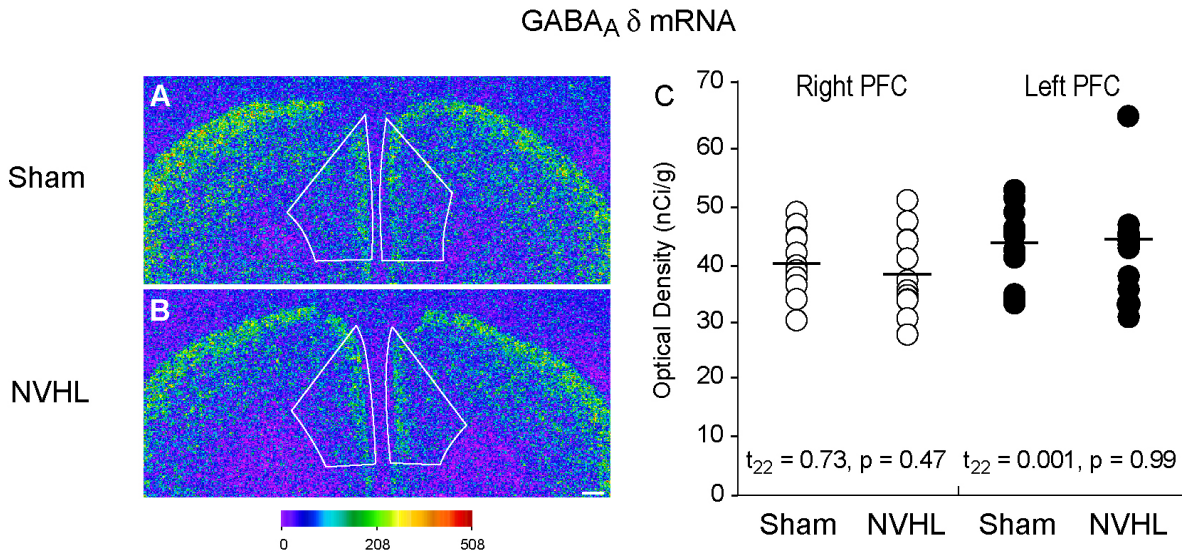

Figure 12. Representative autoradiograms and expression levels of δ mRNA in NVHL rats.

As illustrated in panel (C), the expression levels of δ mRNA levels were similar between in the prefrontal cortex of sham and NVHL rats. The densities of hybridization signals are presented in a pseudocolor manner according to the calibration scales (below). PFC is represented in the autoradiograms as the area between the solid lines. Scale bar = 500 µm. The mean mRNA levels of δ subunit did not differ between sham and NVHL rats, in either PFC hemisphere (C).

3.4.4 TrkB hypomorphic mice

As a final test of our hypothesis, we determined whether the levels of δ subunit mRNA were decreased in the PFC of TrkB hypomorphic mice, since reduced signaling through TrkB receptors results in decreased dendritic complexity due to fewer branches (Xu et al., 2000b; Liu et al., 2007). A single-factor ANOVA revealed a significant effect of genotype on the expression levels of TrkB mRNA ($F_{2,21} = 127.02$, $p < 1 \times 10^{-11}$). In particular, the mRNA levels of TrkB were significantly decreased by 35% and 74% in mice with *fBZ/+* and *fBZ/fBZ* genotypes, respectively, compared to wild-type mice (Figure 13G). In contrast, we did not find a significant ($F_{2,21} = 2.06$, $p = 0.152$) effect of genotype on the expression levels of δ mRNA. The mean mRNA levels of the δ subunit were lower by 15% in *fBZ/+*, but only by 9% in *fBZ/fBZ* mice (Figure 13H). Finally, we did not observe a significant correlation between δ and TrkB mRNA levels ($r = 0.25$, $p = 0.24$) across all animals.

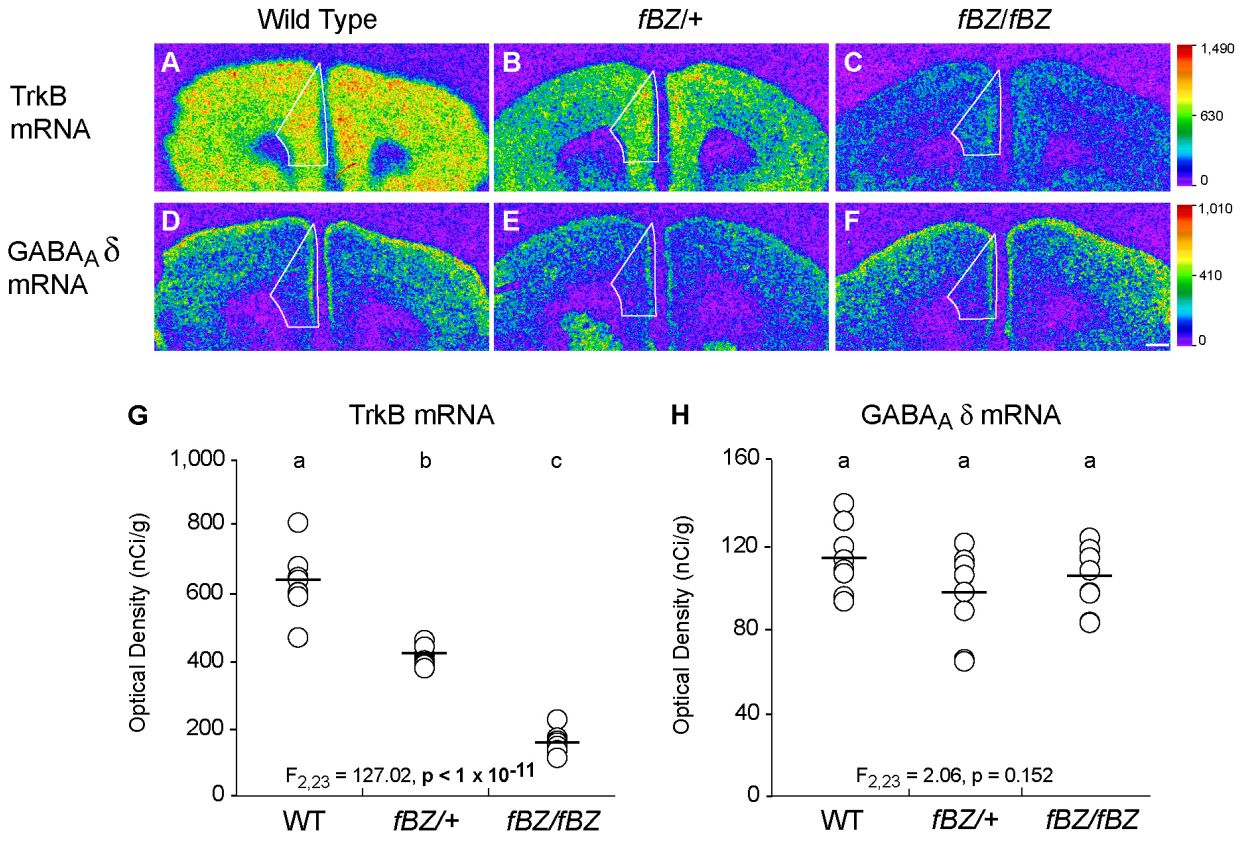

Figure 13. Representative autoradiograms and expression levels of α4 and δ mRNAs in TrkB hypomorphic mice.

Expression levels of TrkB (A-C) and GABAa δ (D-F) mRNAs in the frontal cortex of a wild-type (A,D), a mouse heterozygous for the *fBZ* locus (*fBZ*/+; B,E) and a mouse homozygous for the *fBZ* locus (*fBZ*/*fBZ*; C,F). The densities of hybridization signals are presented in a pseudocolor manner according to the calibration scales (right) for each transcript. The mRNA expression levels of TrkB receptor appear to decrease in proportion to the gene dose (A-C). In contrast, the mRNA levels of δ subunit do not appear to be changed significantly across genotypes (D-F). PFC is represented in the autoradiograms by the area between the solid lines. Scale bar = 500 μm. The mean TrkB mRNA levels in the PFC were significantly decreased by 34% and 75% in *fBZ*/+ and *fBZ*/*fBZ* mice, respectively (G). No difference was observed in the mRNA expression levels of δ subunit in *fBZ*/+ or *fBZ*/*fBZ* mice compared to wild-type mice (H). Mouse groups not sharing the same alphabetical letter are statistically different at $p<0.05$ (Duncan's post hoc test).

3.5 DISCUSSION

Based on observations that the acute disruption of signaling through NMDARs results in alterations in presynaptic markers of GABA neurotransmission similar to those seen in schizophrenia, we hypothesized that the lower expression of postsynaptic $GABA_A$ receptor δ subunit mRNA in subjects with schizophrenia was a downstream consequence of a chronic decrease in the signaling through excitatory synapses. To test this hypothesis we measured the mRNA levels of the δ subunit in the PFC of four animal models with different forms of reduced excitatory drive in the PFC: 1) reduced signaling through NMDARs (NMDA NR1 hypomorphic mice), 2) reduced excitatory synaptic terminals from the thalamus (MDTNL rats), 3) reduced excitatory synaptic terminals from the hippocampus (NVHL rats), and 4) reduced postsynaptic targets of excitatory inputs (TrkB hypomorphic). Contrary to our hypothesis, the mRNA levels of the δ subunit were not altered in any of these animal models. Furthermore, we did not find any association between the expression of δ and measures of the extent of reduced levels of markers of excitatory inputs as modeled in each animal.

Together, these findings do not support the hypothesis that chronic reduction in the signaling through excitatory synapses represents a pathogenetic mechanism resulting in lower expression of δ subunit mRNA in subjects with schizophrenia. However, this interpretation depends, in part, on the adequacy of each of the animal models utilized to replicate the type of deficits in excitatory neurotransmission observed in subjects with schizophrenia. For instance, in order to test whether hypoactivation of NMDARs could give rise to lower expression of δ subunit, we utilized tissue from NMDA NR1 hyormorphic mice, genetically engineered to express low levels of NMDARs. However, the evidence supporting the idea that the protein or mRNA expression levels of NMDARs are decreased in schizophrenia is limited and not always

replicated (Moghaddam, 2003; Lewis and Gonzalez-Burgos, 2007). Instead, it has been hypothesized that other components known to modulate the activity of NMDARs signaling are altered in schizophrenia (Kristiansen et al., 2007; Lewis and Gonzalez-Burgos, 2006).

One potential mechanism involves increased levels of *N*-acetylaspartylglutamate (NAAG) (Coyle, 2004). Postmortem studies suggest that the levels and activity of the enzyme glutamate carboxy peptidase (GCP II), which degrades *N*-acetylaspartylglutamate (NAAG), are reduced in schizophrenia (Tsai et al., 1995; Hakak et al., 2001). Because NAAG has been hypothesized to be an antagonist of NMDAR (Coyle, 2004), lower levels of GCP II could result in higher levels of NAAG, contributing to hypoactivity of NMDARs (Coyle, 2004), although recent findings indicate that inhibition of GCP II in mice reduced schizophrenia-like symptoms elicited by MK-801 or PCP, suggesting that NAAG might actually act as an agonist at NMDARs (Olszewski et al., 2008). Furthermore, it has been hypothesized that in schizophrenia, signaling through NMDA receptors might be particularly lower in GABA-containing interneurons (Coyle, 2004). Consistent with this idea, a recent study showed that in rats systemically injected with MK-801, GABA-containing interneurons exhibited a decreased firing rate, whereas pyramidal cells exhibited an increase in firing rate (Homayoun and Moghaddam, 2007; Chen et al., 2006). Together, these findings suggest that the pharmacological blockade of NMDARs may better recapitulate the pathophysiology of schizophrenia than a genetically engineered reduction in NMDARs. However, hypoactivity of NMDARs, as modeled in NMDA NR1 hypomorphic mice, results in behavioral deficits, such as hyperlocomotion, stereotypy and abnormal social interactions, which resemble the pathophysiology of schizophrenia (Chen et al., 2006). Furthermore, NMDA NR1 hypomorphic mice exhibit reduced relative C-2-deoxyglucose uptake in the PFC, suggesting reduced frontal cortical metabolic activity, similar to the reports of altered

brain metabolism in subjects with schizophrenia (Duncan et al., 2002). In addition, most, although not all, studies suggest that the behavioral and physiological consequences observed in NR1 hypomorphic mice are similar to those reported after pharmacological blockade of NMDARs (Mohn et al., 1999; Duncan et al., 2004; Miyamoto et al., 2000). Thus, both manipulations, a pharmacological blockade and a genetically-induced reduction in NMDARs, appear to have face validity for schizophrenia.

The question addressed in this study was whether a chronic reduction in signaling through NMDARs, which is more likely to mimic the deficit in schizophrenia than an acute reduction in signaling, gives rise to lower levels of the δ subunit. Although a previous study reported reduced mRNA levels of δ in the rodent PFC after pharmacological blockade of NMDARs (Kim et al., 2000), these changes appear to be transient. For instance, a single injection of MK-801 was shown to significantly reduce the mRNA levels of δ in the rat hippocampus; however, the expression of δ returned to normal levels 24 hours later (Sinkkonen et al., 2004). The NMDA NR1 hypomorphic mice provide a useful model to study the consequence of chronic and developmental reduction in the activity of NMDARs (Duncan et al., 2002). Thus, although further studies are needed to determine the effect of chronic pharmacological blockade of NMDARs on δ subunit, our findings suggest that a chronic reduction in the signaling of NMDARs, as modeled in NMDA NR1 hypomorphic mice, does not reduce the expression of δ subunit.

Furthermore, our findings suggest that reduced signaling through excitatory synapses, as a consequence of reduced presynaptic inputs to the PFC, does not contribute to lower expression of the δ subunit. In particular, we did not observe a change in the levels of δ mRNA in the PFC of adult rats peripubertal lesions of the MDTN or with neonatal lesions of the ventral

hippocampus. The latter finding is of particular interest as the neonatal lesions disrupt the development of excitatory inputs into the PFC and thus provide evidence against the idea that in the MDTNL rats we failed to observed a change in δ mRNA levels because the lesions were made after the thalamocortical projections into the PFC had already been formed (Van Eden, 1986).

Interestingly, it has been shown that pyramidal cells in the PFC of NVHL rats exhibit a significant reduction in the density of dendritic spines of prefrontal cortical neurons (Flores et al., 2005). Thus, the lack of a difference in δ mRNA in the PFC of NVHL also suggests that a reduction in postsynaptic excitatory targets does not affect the expression of δ. Consistent with this idea, we did not observe a change in δ mRNA levels in the PFC of mice with lower levels of TrkB receptors, which play a significant role in the dendritic development of pyramidal cells. However, it should be noted that no study has yet determined whether prefrontal cortical pyramidal cells of TrkB hypomorphic mice exhibit a reduction in spine density or dendritic length. Thus, further studies are needed in order to rule out the possibility that normal δ mRNA levels in TrkB hypomorphic mice reflect a lack of change in postsynaptic excitatory targets.

Although each of the experiments in this study produced negative results, it seems unlikely that these are false negatives due to a lack of sensitivity of the methods employed since we previously found reduced levels of δ mRNA in another rodent model using the same techniques *(see section 2.4.7, Chapter 1)*.

Thus, in this study, findings from several animal models that address different aspects of reduced signaling through excitatory synapses: hypoactivation of NMDARs, reduced excitatory synaptic terminals and reduced postsynaptic excitatory targets, converge on the idea that a lower expression of δ mRNA in the DLPFC of subjects with schizophrenia is not a consequence of

reduced excitatory activity. As such, these negative findings provide indirect support for the hypothesis presented in Chapter 2 that the reduction in the δ subunit in schizophrenia is a corollary of the impaired expression of α1 subunit mRNA.

4.0 GENERAL DISCUSSION

4.1 LOWER MRNA LEVELS OF THE α4 SUBUNIT IN SCHIZOPHRENIA: POTENTIAL EFFECT OF CONFOUNDING FACTORS

Our findings indicate that the mRNA levels of α4 subunit are lower only in subjects with schizophrenia receiving benzodiazepines, mood stabilizers and/or antidepressants at the time of death. These findings suggest that lower α4 mRNA is an effect of medications and does not represent the disease process.

However, several (Raol et al., 2005; Holt et al., 1997; Holt et al., 1996) but not all (Wu et al., 1994) previous studies indicate that rats exposed to benzodiazepines, such as diazepam and zolpidem, exhibit an increase in the mRNA levels of the α4 subunit, an effect that is not attributable to direct interactions between the drug and the receptors, given that α4-containing $GABA_A$ receptors are benzodiazepine-insensitive (Holt et al., 1997). Thus these studies do not support the interpretation that lower α4 mRNA levels in schizophrenia are an effect of these medications. The independent effects of mood stabilizers and antidepressants on α4 mRNA levels in experimental systems remain to be determined. Therefore, similar to our studies addressing the potential effects of exposure to antipsychotic medications, future studies should assess the effect that chronic exposure to each of these medications (benzodiazpines, mood stabilizers and antidepressants) have on α4 expression in the DLPFC of non-human primates.

However, as an alternative hypothesis, lower levels of α4 mRNA might reflect a particular disease process in a subtype of schizophrenia with clinical features that require the prescription of these medications. Of note, our findings indicate that a diagnosis of schizoaffective disorder (a diagnosis given to individuals who have had an uninterrupted period of illness during which there was a major depressive episode or manic episode concurrent with symptoms of schizophrenia (American Psychiatric Association, 1994)) does not have a significant effect on α4 mRNA levels. Thus, clinical features such as depression associated with a diagnosis of schizoaffective disorder might not be related to lower levels of α4. Further studies are needed to determine what other clinical features, if any, are common among patients with schizophrenia receiving benzodiazepines, mood stabilizers and/or antidepressants and potentially associated with lower α4 mRNA levels.

4.2 LOWER MRNA LEVELS OF THE δ SUBUNIT IN SCHIZOPHRENIA: DISEASE PROCESS OF THE ILLNESS

In contrast to α4, lower levels of δ do not appear to represent an effect of potential confounding factors. First, the mean percent difference in δ mRNA levels was not affected by sex, diagnosis of schizoaffective disorder, cause of death, alcohol abuse and/or dependency, or treatment with benzodiazepines, mood stabilizers and/or antidepressants at time of death. Second, levels of δ mRNA were unchanged in the DLPFC of monkeys chronically exposed to typical or atypical antipsychotics. Third, the mean percent difference in δ mRNA levels in the four subjects with schizophrenia who were off antipsychotic medications at time of death did not differ from those who were receiving medications.

Fourth, although the mRNA levels of δ were correlated with RIN values in subjects with schizophrenia, the observed lower level of δ mRNA expression in schizophrenia does not appear to reflect a general decline in RNA integrity in these subjects because the RIN values were in the range known to be associated with sudden death and good RNA preservation (Harrison et al., 1995). Similar studies using most of the subjects with schizophrenia used in our study demonstrated no differences between subject groups in the expression levels of a number of other mRNAs (Hashimoto et al., 2005; Hashimoto et al., 2003).

Finally, the reduction in δ mRNA is not likely to reflect a loss of neurons, given that several studies have reported either no change or an increase in neuron density (Selemon et al., 1998; Selemon et al., 1995; Glantz et al., 2000) and no change in total neuron number (Thune et al., 1998) in the DLPFC of subjects with schizophrenia. In contrast, the lower levels of δ subunit in subjects with schizophrenia appear to represent the disease process in the illness.

4.3 ALTERED EXPRESSION OF δ-CONTAINING $GABA_A$ RECEPTORS: A CONSERVED DEFICIT ACROSS NEOCORTICAL REGIONS

Similar to the reports of reduced volume and activity of the DLPFC, studies suggest that the volume of other brain regions such as the temporal lobe and medial temporal structures are reduced in subjects with schizophrenia (Lawrie and Abukmeil, 1998). Similarly, deficits in GABA-related markers also appear to be altered in brain regions outside the DLPFC (Konopaske et al., 2004). These findings raise the question of whether the observed lower levels of δ in the DLPFC of subjects with schizophrenia are present in other brain regions. Indeed, a recent study provides evidence supporting the idea that the altered expression of δ represents a conserved

deficit across neocortical regions in subjects with schizophrenia (Hashimoto et al., 2008b). Using real-time quantitative polymerase chain reaction, the expression levels of δ and other GABA-related markers were assessed in four cortical areas: DLPFC, anterior cingulate, primary motor and primary visual cortices. A cohort of 12 pairs of subjects with schizophrenia and matched controls was analyzed. Findings showed the mean mRNA expression levels of the δ subunit across cortical areas to be significantly lower by 25% across all four areas in subjects with schizophrenia (Hashimoto et al., 2008b), suggesting that a deficit in the expression of δ-containing receptors is not restricted to the DLPFC in these subjects. Such findings indicate that signaling through δ-containing receptors might be altered across multiple brain regions. Furthermore, it is possible that lower levels of δ mRNA represent a common pathological entity across cortical regions and might contribute to the pathophysiology of different domains of cortical dysfunction in schizophrenia.

In addition, it could be hypothesized that the pathogenetic mechanisms underlying deficits in δ mRNA expression might be common across the neocortical mantle in schizophrenia. Of note, the mRNA levels of the α1 subunit were also significantly lower across all four neocortical regions. This is of interest, as the findings outlined in Chapter 2 suggest that lower expression of δ mRNA in the DLPFC of subjects with schizophrenia is associated with lower levels of α1, rather than α4 *(See 4.4.1, General Discussion),* and that reduced expression of α1 might also represent an upstream pathogenetic mechanism *(See 4.5.2, General Discussion).*

4.4 LOWER δ MRNA LEVELS: REDUCED COMPLEMENT OF α4βxδ $GABA_A$ RECEPTORS

4.4.1 Co-assembly of δ and α1 subunits

Previous studies suggest that, in addition to α4, δ subunit can also co-assemble with the α1. For instance, combinations of α, β, γ2 and δ subunits have been shown to produce functional α1β1δ, α1β1γ2Lδ and α1β3δ recombinant receptors (Saxena and Macdonald, 1994; Wohlfarth et al., 2002; Bianchi and Macdonald, 2003). Consistent with these findings, the α1 subunit has been found to localized extrasynaptically (Baude et al., 2007; Sun et al., 2004), consistent with the ultrastructural localization of δ-containing receptors (Nusser et al., 1998; Farrant and Nusser, 2005). Furthermore, immunoprecipitation studies with δ-antiserum from total rat brain extracts revealed that the δ subunit associates with α1 subunits (Mertens et al., 1993). Thus, α1 and δ subunits also appear to be associated *in vivo*.

Consistent with this idea, it has recently been suggested that δ subunits form functional $GABA_A$ receptors with α1 subunits in mouse hippocampal interneurons (Glykys et al., 2007). For example, interneurons in the dentate molecular layer exhibited immunoreactivity for both α1 and δ along the cell body surface and in proximal dendrites. In addition, in $GABA_A$ α4 subunit knockout mice, the immunoreactivity levels of δ decrease significantly in dentate gyrus granule cells, consistent with the strong partnership of α4 and δ in these neurons. In contrast, the immunoreactivity levels of δ were unchanged and remained colocalized with α1 immunoreactivity in interneurons (Glykys et al., 2007). Furthermore, levels of tonic inhibition

mediated by δ-containing receptors are augmented by low concentrations of ethanol (Wallner et al., 2003; Wei et al., 2004; Hanchar et al., 2005). Interestingly, in α4 subunit knockout mice, tonic currents were potentiated by ethanol in hippocampal interneurons, whereas no potentiation was observed in dentate granule cells (Glykys et al., 2007). Together, these findings converge on the idea that α1 and δ subunits form functional α1βxδ receptors *in vivo* in at least some neurons.

4.4.2 Co-regulated expression of α1 and δ subunits in schizophrenia

The dissociation between δ and α4 mRNA levels in subjects with schizophrenia (*See Chapter 2*) raised the question of whether lower mRNA levels of the δ subunit in the DLPFC represent a decreased expression of δ-containing $GABA_A$ receptors co-assembled with α1 subunits.

While our studies do not provide direct evidence for the presence or altered expression of α1βxδ receptors in the DLPFC of subjects with schizophrenia, the following lines of evidence are consistent with the idea that lower expression of δ subunit mRNA is associated with lower levels of α1 subunit. First, across postnatal development of the non-human primate DLPFC, the levels of δ mRNA increased significantly (*See 2.4.6, Chapter 2*), paralleling the previously observed increase in the α1 subunit (Nguyen et al., 2006). In contrast, the levels of α4 decreased with development. Second, in subjects with schizophrenia, the mRNA levels of δ and α1 subunit mRNAs were reported to be significantly reduced in the DLPFC by microarray, and the magnitude of the disease-related differences in both transcripts were significantly correlated (Hashimoto et al., 2008a). In contrast, no correlation was observed between δ and α4 mRNAs. Interestingly, the mRNA levels of δ and α4 subunits were correlated in control subjects ($r = 0.94$, $p < 1.0 \times 10^{-9}$) and subjects with schizophrenia ($r = 0.78$, $p < 1 \times 10^{-4}$). However, the larger

regression coefficient in control subjects suggests that the correlation between δ and α4 mRNAs in these subjects is stronger than in subjects with schizophrenia. In fact, in control subjects the mean (± SD) values for the regression residuals between δ and α4 mRNAs in control subjects (14.7 ± 12.5) was significantly smaller ($t_{44} = -2.96$, $p < 0.01$) than in subjects with schizophrenia (31.2 ± 23.5). The weaker correlation between δ and α4 subunit in subjects with schizophrenia is consistent with the idea that, in schizophrenia lower levels of the δ subunit are not associated with lower levels of α4 subunit.

Third, a recent *in situ* hybridization study using the same 23 subject pairs used in our study, found a significant reduction in α1 mRNA levels across DLPFC layers 2-6, where the levels of δ mRNA were significantly lower (*See 2.4.5, Chapter 2*). Furthermore, the mean percent of difference for α1 in subjects with schizophrenia was significantly correlated ($r = 0.74$, $p < 0.0001$) with that of the δ subunit (Dr. Mónica Beneyto, personal communication). Finally, as previously illustrated (*see 4.3, Discussion*), the levels of both α1 and δ mRNAs are consistently lower across neocortical areas in subjects with schizophrenia (Hashimoto et al., 2008b). Together, these findings are consistent with the idea that lower mRNA levels of δ are associated with lower levels of α1 subunit in subjects with schizophrenia. Interestingly, the expression of the β3 subunit was found to be significantly lower in the DLPFC of subjects with schizophrenia, as measured by microarray (Hashimoto et al., 2008a). While which β subunit assembles with δ-containing receptors *in vivo* remains unclear, δ-containing receptors might also contain β3 (Wallner et al., 2003). Together, these findings suggest that lower levels of δ, α1 and β3 subunits in schizophrenia might represent a lower complement of α1β3δ $GABA_A$ receptors.

However, our findings require verification at the protein level, since the interpretation that schizophrenia might be associated with a lower complement of α1βxδ receptors - but not

α4βxδ - is based, in part, on the assumption that the protein levels of δ and α1 subunits are lower in schizophrenia, whereas those of α4 are not. For instance, we cannot rule out the possibility that as a result of lower δ mRNA levels, the protein levels of both δ and α4 subunits (potentially α4βxδ) are decreased significantly in schizophrenia; even though no changes in α4 mRNA levels are observed (*See 2.4.2 & 2.4.3, Chapter 2*). A similar dissociation between mRNA and protein levels for $GABA_A$ subunits have been described in α6 knockout mice (Jones et al., 1997). Unfortunately, in our experimental conditions, the antibodies against the δ subunit (generously provided by Dr. Werner Sieghart) did not detect δ subunits in the human DLPFC (unpublished work).

4.5 FUNCTIONAL SIGNIFICANCE OF LOWER δ-CONTAINING RECEPTORS

4.5.1 Lower expression of δ mRNA as a compensatory change

Lower levels of δ subunit might represent a compensatory response to presynaptic reductions in GABA neurotransmission. It has been shown that normal working memory requires the activity of GABAergic interneurons in the DLPFC (Sawaguchi et al., 1989; Rao et al., 2000), which synchronizes the activity of a local population of neurons (Klausberger et al., 2003). Networks of GABAergic inernerons, such as parvalbumin-positive cells give rise to oscillatory activity in the gamma range (30–80 Hz) (Whittington and Traub, 2003). It has been shown that gamma band oscillations in the human DLPFC increase in proportion to working memory load (Howard et al., 2003). Because synaptic $GABA_A$ receptors play a significant role in the generation and maintenance of network oscillations, as they provide postsynaptic conductance with a rapid time

course (Farrant and Nusser, 2005), the reports of decreased GAD_{67} and upregulated α2 subunits in the DLPFC of subjects with schizophrenia suggest that synaptic-phasic inhibition onto pyramidal cells is decreased and might result in altered oscillatory activity in the gamma range. Consistent with this hypothesis, the power of gamma band oscillations has been shown to be reduced in subjects with schizophrenia during a working memory task (Cho et al., 2006).

The role that δ-containing $GABA_A$ receptors play in the generation and/or maintenance of gamma oscillations is unclear. However, insight into the potential role that tonic inhibition plays in oscillations is provided by the study of α5-containing receptors, which mediate tonic inhibitory conductance in hippocampal pyramidal cells (Caraiscos et al., 2004). Using hippocampal slices of wild-type and α5 knockout mice, a previous study assessed the role of α5 subunits expressed by CA3 pyramidal cells in the generation of kainate-induced gamma oscillations (Towers et al., 2004). The study reports that the mean peak power of kainate-induced gamma oscillations in α5 knockout mice was higher than in wild-type mice. The authors proposed that tonic inhibition decreases the power of gamma oscillations because the tonic conductance reduces the influence of inhibitory postsynaptic potentials on pyramidal cells, which are essential for synchronization (Towers et al., 2004). If tonic inhibitory conductance mediated by δ-containing receptors plays a similar role in cortical pyramidal cells, lower expression of δ subunit mRNA and, consequently, a decrease in tonic inhibitory conductance would be expected to result in increased power of gamma oscillations. Consistent with this interpretation a recent study reported that mice with a genetic deletion of both δ and α5 subunit exhibit an increase in the power of gamma oscillations (Glykys et al., 2008). While the effect that a deletion of δ subunit alone might have on the generation and/or maintenance of gamma oscillations remains unclear, these findings suggest that lower levels of δ mRNA in DLPFC

pyramidal cells in subjects with schizophrenia would result in lower tonic conductance and as a consequence, increase power of gamma oscillations. Thus, the altered expression of the δ subunit might represent a compensatory response to the decreased inhibitory input associated with lower GAD_{67} mRNA.

It has been hypothesized that deficits in inhibitory neurotransmission in the DLPFC of subjects with schizophrenia are due to a reduction in the synthesis of GABA (lower GAD_{67} mRNA) that is partially compensated by an upregulation of postsynaptic α2-containing $GABA_A$ receptors (Volk et al., 2002). Interestingly, the protein levels of the α2 subunit appear to be significantly increased in the forebrain membranes of α1 knockout mice (Kralic et al., 2002) and in α1 knockout mice in which the expression of α1 subunit is reduced (Borghese et al., 2006). These findings suggest that increased levels of α2 subunits in schizophrenia might be a consequence of reduced α1 expression. As outlined in the above, lower levels of δ-containing receptors could also serve to compensate for altered inhibitory control in subjects with schizophrenia. These findings suggest that the reduction of GAD_{67} mRNA in schizophrenia, which is thought to contribute to altered oscillatory activity in the gamma range, is compensated by both: an upregulation of α2-containing receptors and a decrease in δ-containing receptors. These compensatory changes in the levels of α2- and δ-containing receptors might be mediated by a reduction in the expression levels of the α1 subunit. Further proof-of-concept studies are needed to determine whether reduced expression of GAD_{67} mRNA results in lower levels of α1, lower levels of δ and higher levels of α2, and what cellular mechanisms are involved.

Two main assumptions have been made in these interpretations that require further validation. First, the lower expression levels of δ in subjects with schizophrenia reflect lower expression levels of δ mRNA in DLPFC pyramidal cells. This is based on a previous human

postmortem study reporting that δ mRNA silver grains clustered predominantly over pyramidal cells in the motor cortex (Petri et al., 2003). However, given that lower mRNA levels of δ in schizophrenia might be associated with lower expression of α1 and that functional α1βxδ receptors have been identified in hippocampal interneurons (Glykys et al., 2007), further studies are needed to determine the type of neocortical cells that express lower levels of δ subunit in schizophrenia. Second, it has been assumed that lower mRNA levels of δ are paralleled by reductions in its cognate protein, resulting in lower δ-containing $GABA_A$ receptors. While our studies do not address whether the protein levels of δ are lower in the DLPFC of subjects with schizophrenia, previous analyses of other GABA-markers in the DLPFC of subjects with schizophrenia suggest that changes at the mRNA levels are paralleled by similar changes in their cognate proteins. For instance, both mRNA and protein levels of GAT-1 (Volk et al., 2001; Volk et al., 2002) and of the endocannabinoid receptor CB1 (Eggan et al., 2007) are reduced in schizophrenia, whereas the mRNA and protein levels of the α2 subunit appear to be increased in subjects with schizophrenia (Volk et al., 2002; Beneyto et al., 2007). However, further studies are needed to determine the protein levels of δ across the DLPFC of subjects with schizophrenia.

4.5.2 Lower expression of δ mRNA represents a consequence of lower α1 expression

Lower mRNA levels of the δ subunit in the DLPFC of subjects with schizophrenia may be a consequence of lower α1 subunit expression. Significant associations between two $GABA_A$ α1 subunit haplotypes and risk for schizophrenia in a Portuguese and German family have been described, suggesting that genetic liability in the α1 subunit might contribute to the pathogenesis of schizophrenia (Petryshen et al., 2005). In our findings, the mRNA levels of δ were significantly reduced in the PFC of α1 knockout mice (*See 2.4.7, Chapter 1*). Although

speculative, these findings suggest that, in schizophrenia a potential genetic risk in the α1 subunit could result in lower α1 expression. As a consequence, the mRNA levels of the δ subunit would be reduced, which might result in lower expression of α1βxδ receptors. However, it should be noted that in the study by Petryshen et al, no correlation was found between the α1 risk haplotypes and α1 mRNA levels. Thus, it is unclear whether the suggested genetic susceptibility leads to lower expression of α1. Furthermore, a second genetic study failed to replicate the associations between α1 haplotypes and risk for schizophrenia (Ikeda et al., 2005).

5.0 CONCLUSIONS

In summary, the studies described in this dissertation indicate that in schizophrenia, the mRNA levels of the $GABA_A$ receptor δ subunit are significantly lower, whereas those of α4 are not. Given that α1 mRNA levels are lower in schizophrenia and that α1 subunits can co-assemble with δ subunits, lower δ mRNA levels could represent a reduced complement of $GABA_A$ α1βxδ receptors, rather than α4βxδ. Thus, altered inhibitory neurotransmission in schizophrenia may be associated with deficits in tonic inhibition, due to a reduced number of α1βxδ receptors.

Furthermore, reduced signaling through excitatory synapses does not appear to represent the pathogenetic mechanism underlying the altered expression of δ-containing $GABA_A$ receptors in schizophrenia, since the mRNA levels of δ were unchanged in four rodent models of reduced cortical excitatory drive: 1) reduced signaling through NMDA receptors, 2) reduced excitatory synaptic terminals from the thalamus, 3) reduced excitatory synaptic terminals from the hippocampus, and 4) reduced postsynaptic targets of excitatory inputs. In contrast, lower levels of δ may be a consequence of reduced expression of the α1 subunit, since δ mRNA levels were significantly reduced in the prefrontal cortex of α1 knockout mice. Alternatively, given the role that reduced levels of tonic inhibition appear to play in increasing network oscillations, lower levels of α1βxδ $GABA_A$ receptors may represent a compensatory response to a reduced power of gamma oscillations in subjects with schizophrenia.

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