Alterations in GABA-related Transcripts in the Dorsolateral Prefrontal Cortex of Subjects with Schizophrenia

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Besides the financial burden upon society, families undergo a substantial emotional burden when presented with a loved one affected by schizohprenia. Elucidation of the pathophysiology underlying the core features of schizophrenia is necessary for the development of more effective treatment targets. Cognitive deficits are regarded as a core feature of schizophrenia and are thought to arise from alterations in γ-aminobutyric acid (GABA)-containing interneurons in the dorsolateral prefrontal cortex (DLPFC). Specifically, postmortem studies have demonstrated decreased levels of the mRNA encoding the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}), an enzyme that synthesizes GABA, and this alteration seems to be specific to certain subsets of GABA neurons. For example, parvalbumin and somatostatin mRNAs, which are expressed in separate subsets of GABA neurons, were decreased, whereas calretinin mRNA, expressed in a third subset of GABA neurons, was unchanged in schizophrenia. The studies in this thesis examined the compartmental and cellular expression of and the potential causal mechanisms of reductions in SST mRNA expression; furthermore, the disease and cellular specificity of and post-synaptic consequences of reductions in SST mRNA expression were examined. We found that reductions in the levels of SST mRNA appear to be restricted to SST interneurons that do not contain NPY mRNA in the gray matter and are due to reductions in expression per neuron. These alterations appear to be a consequence of impaired neurotrophin signaling through the trkB receptor. Also, the profile of alterations in GABA-related mRNA expression is specific to schizophrenia. Finally, a post-synaptic receptor of SST, SST receptor subtype 2 (SSTR2), mRNA is reduced in schizophrenia. Since the SST protein is putatively inhibitory and SST-containing interneurons target the distal dendrites of pyramidal neurons, these data suggest reduced inhibition of pyramidal neurons and may represent a compensatory mechanism to increase excitatory drive. We conclude that reductions in SST and SSTR2 mRNA

represent a downstream consequence of a neuropathological entity in the DLPFC of individuals with schizophrenia and contribute to cognitive dysfunction in schizophrenia.

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To my mother,

who began my journey

PREFACE

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1.0 OVERVIEW

1.1 SCHIZOPHRENIA OVERVIEW

1.1.1 Schizophrenia: A Devastating Illness

Approximately 1% of the world's population is affected by schizophrenia (Lewis and Lieberman, 2000). Individuals that are affected by this illness usually come to clinical attention in late adolescence through early adulthood (American Psychiatric Association, 1994; Lewis and Lieberman, 2000) and then many of these individuals experience disability for the rest of their lives. Besides the tremendous related financial burden upon society in terms of direct medical costs and lost productivity estimated to be around $62.7 billion in the US in 2002 (Wu et al., 2005), family and friends undergo a substantial emotional burden when presented with a situation in which a loved one is affected by this disorder (Moller-Leimkuhler, 2006).

1.1.2 Etiology of Schizophrenia

The specific etiology of schizophrenia remains largely unknown; however, in general, individuals with schizophrenia have increased incidence of putative susceptibility genes (Owen et al., 2004) and increased incidence of various environmental factors that occur from conception to adolescence (Lewis and Levitt, 2002) leading many to hypothesize that a combination of genetic and environmental risks contribute to the etiology of schizophrenia (Tsuang, 2000).

Family, twin, and adoption studies demonstrate that the risk for developing schizophrenia is directly associated to the degree of shared genetics (Lewis and Lieberman, 2000); however, among monozygotic twins, concordance for schizophrenia only reaches ~50% (Gottesman,

1991). Additionally, several putative susceptibility genes on various chromosomes have recently been identified (Pulver, 2000; Owen et al., 2004), but these genes confer only a small degree of risk for schizophrenia. These studies suggest that the genetic risk for schizophrenia is polygenic and not sufficient for the presentation of the illness. Consistent with this hypothesis, many studies have identified environmental insults that occur across development that are associated with increased risk for schizophrenia (Lewis and Lieberman, 2000; MacDonald et al., 2000). Therefore, it is likely that a complicated convergence of genetic risk and environmental insults during development are involved in the development of schizophrenia.

1.1.3 Clinical Manifestation of Schizophrenia: Cognitive Dysfunction as a Core Feature

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), a diagnosis of schizophrenia includes the persistent presentation of 2 or more positive symptoms (e.g. delusions, hallucinations, disorganized thought) or negative symptoms (e.g. avolition, flat affect, alogia); furthermore, these symptoms must be accompanied by social or occupational dysfunction and must be not be due to other disorders (American Psychiatric Association, 1994). Although the criterion for a diagnosis of schizophrenia as outlined by the DSM-IV was created in order to achieve a high degree of concordance of diagnoses between clinicians, it relies on a cluster of heterogeneous overt clinical symptoms and the elimination of other disorders unlike many other clinical disease models (McCormick and Flaum, 2005). This is likely necessary due to the unknown nature of the etiology of schizophrenia. The heterogeneity of clinical symptoms manifested in individuals with schizophrenia highlights the necessity of identifying features of the disease that are common and are associated with functional outcomes.

Of the clinical features of schizophrenia, positive symptoms, such as psychosis, are the most striking and most well known, but are not necessary for clinical diagnosis (Lewis et al., 2005). Instead, cognitive dysfunction is becoming regarded as the core feature of schizophrenia (Elvevag and Goldberg, 2000) and many lines of evidence support this hypothesis. First, cognitive dysfunction is likely the most prevalent clinical feature among individuals with schizophrenia (Keefe et al., 2005). Second, cognitive dysfunction is found throughout life including childhood and adolescence (Breier et al., 1991; Heaton et al., 1994; Saykin et al., 1994;

Davidson et al., 1999); furthermore, unaffected family members of individuals with schizophrenia also demonstrate milder forms of cognitive dysfunction (Sitskoorn et al., 2004) indicating that this dysfunction is found before clinical manifestation of positive symptoms, not a result of medical treatment or progressive clinical deterioration associated with illness chronicity, and is related to genetic liability. Third, the degree of cognitive deficits in individuals with schizophrenia seem to be the best predictor of long-term functional outcome (Green, 1996) suggesting that these cognitive deficits rather than psychosis have a greater negative impact on social function in affected individuals. Unfortunately, current medical treatments of schizophrenia, which have been mostly discovered by serendipity rather than design, are mainly used to treat the positive symptoms of the illness and have a rather limited impact on cognitive dysfunction (Hyman and Fenton, 2003). A more thorough understanding of the pathophysiology and, perhaps, the pathogenesis underlying the core features found in schizophrenia is necessary for the development of more effective treatment targets and integral in the movement from the "bench to bedside".

1.2 WORKING MEMORY IN SCHIZOPRHENIA: ROLE OF ALTERED INHIBITORY CIRCUITRY IN THE DORSOLATERAL PREFRONTAL CORTEX

Of the cognitive deficits found in schizophrenia, reduced working memory performance has been consistently observed in subjects with schizophrenia (Weinberger et al., 1986; Perlstein et al., 2001; Callicott et al., 2003). Working memory refers to the ability to store pieces of information for a short amount of time in order to guide future behaviors. Working memory tasks are putatively mediated by the cortical circuitry located in the dorsolateral prefrontal cortex (DLPFC) (Funahashi et al., 1989; Funahashi et al., 1993; Botvinick et al., 2001) and subjects with schizophrenia demonstrate altered DLPFC activity during working memory tasks as compared to control subjects (Weinberger et al., 1986; Perlstein et al., 2001; Callicott et al., 2003) suggesting altered DLPFC circuitry in individuals with schizophrenia. Because working memory seems to be a core feature of schizophrenia, it is important to examine the underlying pathophysiology leading to alterations in the performance of working memory tasks.

1.2.1 DLPFC Inhibitory Circuitry Underlying Working Memory

The DLPFC has been identified as a critical node in working memory by both functional imaging studies in humans and electrophysiological studies in awake behaving monkeys (Funahashi et al., 1989; Funahashi et al., 1993; Weinberger et al., 2001; Miller and Cohen, 2001). Spatial working memory has been extensively examined in monkeys performing an oculomotor delayed response (ODR) task. Spatial tuning of DLPFC neuron populations as well as coordinated and sustained firing of subsets of pyramidal neurons in the DLPFC during the delay period located between the presentation of the cue and the initiation of the behavioral response seem to be key components of the cellular basis underlying working memory (Funahashi et al., 1993; Rao et al., 1999).

Both excitatory and inhibitory neurons of the DLPFC have been demonstrated to have preferred receptive fields and fire preferentially for cues directing behavioral responses to that receptive field during the delay period of the ODR task; and, this spatial tuning is thought to be one of the neuronal substrates of working memory (Funahashi et al., 1990; Chafee and Goldman-Rakic, 1998; Rao et al., 1999; Rao et al., 2000). The generation of this spatial tuning has been suggested to be due to similar afferent inputs to adjacent neurons located in a functional microcolumn of the cortex and also due to the isodirectional inhibition of neighboring microcolumns (Rao et al., 2000). Traditional functional columns refer to a regional clustering of cells found in a column of the cortex that respond to similar features of "spots" of ~500 μm in diameter; microcolumns are considered a smaller column within these functional columns of ~50-60 μm in diameter (Rao et al., 1999). Furthermore, this local inhibition seems to be due to a $GABA_A$ receptor-mediated event since $GABA_A$ receptor blockade increases cross-directional activity leaving isodirectional activity intact, thus, reducing spatial selectivity of neurons during the delay period (Rao et al., 2000). These data suggest that disturbances in inhibitory neurotransmission could play a prominent role in the dysfunction of the DLPFC in subjects with schizophrenia and these disturbances are an integral part of the pathophysiology underlying working memory disturbances in affected individuals (Lewis et al., 2005).

1.2.2 Selective Alterations in the Inhibitory Circuitry of the DLPFC in Individuals with Schizophrenia

Several lines of evidence suggest that altered expression of markers of GABA neurotransmission is a common feature in the DLPFC of schizophrenia (Torrey et al., 2005; Akbarian and Huang, 2006). These disturbances are highlighted by a reduction in the expression of mRNA that encodes for the 67 kD isoform of glutamate decarboxylase (GAD_{67}), an enzyme involved in the synthesis of GABA in the DLPFC of individuals with schizophrenia (Akbarian et al., 1995; Guidotti et al., 2000; Volk et al., 2000; Straub et al., 2007; Hashimoto et al., 2008b). In addition, reduced GABA synthesis may be selectively mediated by the reductions in GAD_{67}, because mRNA and protein levels of GAD_{65}, an enzyme for GABA synthesis during high synaptic demand (Battaglioli et al., 2003), were found to have little to no alterations in the DLPFC of subjects with schizophrenia (Guidotti et al., 2000; Hashimoto et al., 2008c).

The reduction in GAD_{67} mRNA is thought to be restricted to a subset of GABA neurons because this decrease is due to a marked reduction in GAD_{67} mRNA expression in a minority (~25-35%) of GABA neurons in the face of conserved expression in the remaining neurons (Volk et al., 2000). Furthermore, it appears that the affected GABA neurons include, at least, a subpopulation of inhibitory neurons that comprise ~25% of GABA neurons that expresses the calcium-binding protein, parvalbumin (PV), whereas the ~50% of GABA neurons that express calretinin (CR) appear to be unaffected (Hashimoto et al., 2003). However, the reduction in PV mRNA expression was shown to be specific to layers 3 and 4 (Hashimoto et al., 2003), whereas the alterations the alterations in GAD_{67} mRNA are found in layers 2-5; these laminar differences suggest that another subpopulation of GABA neurons are affected in schizophrenia.

1.2.3 Disease Specificity of the Profile of Altered GABA-related mRNA

According to the DSM-IV, a diagnosis of bipolar I disorder includes the presentation of 1 or more manic or mixed episodes and must be not be due to other disorders(American Psychiatric Association, 1994). The etiology of bipolar disorder is unclear, but genetic studies have demonstrated that bipolar disorder and schizophrenia, but not major depressive disorder, share

many putative susceptibility genes (Potash, 2006; Potash et al., 2007). Susceptibility genes refer to gene variations found through either genetic linkage or associational studies that cosegregate (provide a statistically higher proportion of people with) a particular gene variation to the positive diagnosis of the illness across families or populations, respectively. These data suggest that bipolar disorder also has a complex polygenic etiology similar to schizophrenia. Furthermore, cognitive deficits have been extensively demonstrated in euthymic individuals with bipolar disorder and subjects with bipolar disorder have similar cognitive deficits as seen in subjects with schizophrenia as compared to control subjects (Albus et al., 1996; Zubieta et al., 2001; Martinez-Aran et al., 2004; Bora et al., 2007; Thompson et al., 2007; Martinez-Aran et al., 2008). These impairments have also been shown to occur in unaffected relatives of subjects with bipolar disorder. For example, unaffected twins (Gourovitch et al., 1999) and unaffected first degree relatives (Ferrier et al., 2004) of subjects with bipolar disorder demonstrated cognitive deficits compared to normal subjects. These studies suggest that the cognitive deficits in individuals with bipolar disorder are state independent and are reliant on a genetic component rather than a consequence of illness chronicity or treatment. Additionally, these cognitive deficits have been shown to be the best predictor of functional outcome (Green, 2006; Martinez-Aran et al., 2007). These lines of evidence are similar to studies suggesting that cognitive deficits are a core feature of schizophrenia.

Studies have also demonstrated that bipolar disorder also share neuropathology with schizophrenia. For example, alterations in inhibitory circuitry in various brain regions have been demonstrated in individuals with bipolar disorder which are similar to those found in individuals with schizophrenia (Kato et al., 2007). These alterations include reductions in the density of GAD_{67}-positive cells in the hippocampus and anterior cingulate (Heckers et al., 2002; Woo et al., 2004; Torrey et al., 2005). Furthermore, PV expression is affected in the hippocampus of individuals with bipolar disorder (Knable et al., 2004; Torrey et al., 2005) suggesting that this subset of GABA neurons are also affected in bipolar disorder. Furthermore, it has been demonstrated that GAD_{67} protein and mRNA expression is reduced in subjects with schizophrenia or bipolar disorder and a history of psychosis as compared to subjects with major depressive disorder or control subjects (Guidotti et al., 2000) suggesting that the GABA-related alterations are found in subjects with similar genetic susceptibility and are not due to common environmental factors that are associated with a chronic debilitating psychiatric illness (e.g.

social isolation and/or hospilization) often shared with individuals with major depressive disorder.

1.3 SOMATOSTATIN-CONTAINING INTERNEURONS

The preceding chapter (1.2.2) outlined severe alterations in the inhibitory circuitry in the DLPFC of individuals with schizophrenia; and, that these alterations seem to be selective for certain subsets of GABA neurons. The specificity of such alterations and the mechanisms underlying these alterations are critical in understanding the pathophysiology of and, perhaps, the pathogenetic mechanisms underlying the disease. For example, chandelier neurons in the primate PFC express PV (Williams et al., 1992; Lund and Lewis, 1993) and chandelier neurons allow powerful modulation to the temporal coordination of aggregates of pyramidal neurons (Cobb et al., 1995) because these interneurons furnish inhibitory synapses on pyramidal neurons at the axon initial segment (AIS) (Williams et al., 1992). By staining the innervation of chandelier neurons onto AISs utilyzing a presynaptic marker (i.e. GABA membrane-1 transporter), characteristic vertical arrays of axon terminals, termed "cartridges", can be visualized and subsequently quantified. The density of these cartridges have been shown to be significantly reduced in the DLPFC of subjects with schizophrenia (Pierri et al., 1999) suggesting that inhibitory modulation of pyramidal neuron AISs is severely affected and, thus, temporal coordination of aggregates of pyramidal neurons may also be affected. Examining the subset of PV-containing interneurons allowed the ability to characterize a selective alteration in the inhibitory circuitry in the DLPFC of subjects with schizophrenia which can further drive hypotheses concerning the pathophysiology underlying phenotypic characteristics of individuals with schizophrenia [i.e. reductions in working memory performance (Lewis et al., 2005)]. This chapter will examine a subset of inhibitory interneurons that contain the neuropeptide, somatostatin (SST), which is expressed in a subpopulation of GABA neurons that do not contain either PV or CR (Kubota et al., 1994; González-Albo et al., 2001; Gonchar and Burkhalter, 2003; Sugino et al., 2006) and have been implicated in schizophrenia.

1.3.1 Alterations in SST-containing Interneurons in the DLPFC of Subjects with Schizophrenia

As outlined in 1.2.2, the reduction in PV mRNA expression was shown to be specific to layers 3 and 4 (Hashimoto et al., 2003), whereas the alterations the alterations in GAD_{67} mRNA are found in layers 2-5; these laminar differences suggest that another subpopulation of GABA neurons are affected in schizophrenia. Within the gray matter of the adult primate PFC, the greatest densities of SST-containing neurons are found in layers 2-superficial 3 and layer 5 (Lewis et al., 1986; Hayes et al., 1991; Da Cunha et al., 1995) demonstrating that this subpopulation of GABA neurons are located within the layers of the cortex that did not demonstrate reductions in PV mRNA expression.

In a recent study utilizing a custom-designed microarray of GABA-related transcripts, the most robust expression difference in the DLPFC of subjects with schizophrenia was a reduction in the levels of SST mRNA (Hashimoto et al., 2008a). The reduction in SST mRNA expression in schizophrenia was confirmed by both real-time qPCR and *in situ* hybridization in the same subjects (Hashimoto et al., 2008a). These data suggest that there are robust alterations in the SST interneurons within the DLPFC of subjects with schizophrenia.

1.3.2 Properties of Somatostatin-containing interneurons

GABA neurons have been classified by many different schemes based on characteristic properties including electrophysiology, dendritic arborization, axonal targets, and expression of various calcium-binding proteins and neuropeptides; however, these classification schemes do not reveal any straight-forward correlations with each other; and, thus, it has been difficult to reliably define particular subsets of GABA neurons.

In general, SST interneurons seem to share some characteristics. In the neocortex of rats and primates, SST is exclusively localized to inhibitory GABA interneurons (Kubota et al., 1994; Kubota and Kawaguchi, 1994; Kawaguchi and Kubota, 1996; Gonzalez-Albo et al., 2001) and is expressed in a subpopulation of GABA neurons that do not contain either parvalbumin or calretinin (Kubota et al., 1994; Gonchar and Burkhalter, 1997; Gonzalez-Albo et al., 2001;

Sugino et al., 2006). Furthermore, in the rodent and monkey neocortex, SST interneurons tend to innervate the dendritic shafts of distal dendrites from pyramidal neurons (Hendry et al., 1984; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1996; Melchitzky and Lewis, 2008) suggesting they may play a role in modulating inputs to pyramidal neurons.

Other than these common characteristics, previous studies have demonstrated a large diversity of SST interneurons with respect to colocalization with other neuropeptides and calcium-binding proteins, morphological properties, and electrophysiological properties. In rat and monkey frontal cortex, NPY is present in ~30-40% of SST neurons (Hendry et al., 1984; Kubota et al., 1994); and, in the rat neocortex calbindin, a calcium-binding protein, is present in ~50-85% of SST neurons dependent on cortical layer with increasing percentages in the deeper layers (Kawaguchi and Kubota, 1997; Gonchar and Burkhalter, 1997). In the rat neocortex, SST has also been suggested to be localized to a diverse group of morphologically distinct interneurons including basket cells (Wang et al., 2002), double-bouquet cells (Cauli et al., 2000), bitufted cells (Reyes et al., 1998), and the most commonly studied layer 1-targeting Martinotti cells (Kawaguchi and Kubota, 1996; Kawaguchi and Kubota, 1997; Kawaguchi and Kubota, 1998; Kawaguchi and Kondo, 2002; Wang et al., 2004b; Ma et al., 2006). A common way in which subclasses of interneurons are electrophysiologically classified is by their firing patterns produced in response to step-current injections to the cell soma. Such studies have revealed that SST interneurons can exhibit low-threshold spikes (LTS) in at least some Martinotti cells (Kawaguchi and Kubota, 1996; Kawaguchi and Kubota, 1997; Reyes et al., 1998; Gibson et al., 1999; Ma et al., 2006) although other studies have demonstrated quite a large diversity in the electrophysiological properties of SST interneurons (Wang et al., 2004b). In addition, subsets of SST neurons with different membrane properties and morphological features tend to differ in their laminar locations (Kawaguchi and Kubota, 1996; Ma et al., 2006).

1.3.3 Neuropeptide Y-containing Subset of Somatostatin Neurons

In rat and monkey frontal cortex, ~40% of SST neurons also express neuropeptide Y (NPY) and most NPY neurons contain SST (Hendry et al., 1984; Kubota et al., 1994). Both SST (Morris et al., 2008) and NPY (Caberlotto et al., 2000) mRNAs are expressed by neurons in the gray and white matter of the human DLPFC. Furthermore, most NPY mRNA expression in the human

cortex is located in deep layer 6 and the superficial white matter (Caberlotto et al., 2000). Consistent with these observations, the co-localization of SST and NPY mRNAs in the rodent cerebral cortex is most prominent in layer 6 and is uncommon in the superficial layers (Wang et al., 2004b).

In a recent study utilizing both the gray and white matter from the DLPFC of subjects with schizophrenia or schizoaffective disorder, an expression difference was found in the levels of neuropeptide Y (NPY) mRNA (Hashimoto et al., 2008a) suggesting that the NPY-containing subclass of SST neurons is affected in these illnesses.

1.3.4 Somatostatin Receptors

As mentioned in the previous section (1.3.2), in the rodent and monkey neocortex, SST interneurons tend to innervate the dendritic shafts of distal dendrites from pyramidal neurons (Hendry et al., 1984; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1996; Melchitzky and Lewis, 2008) suggesting they may play a role in modulating inputs to pyramidal neurons. There is a robust reduction in SST mRNA expression in the DLPFC of subjects with schizophrenia that is significantly correlated to reductions in GAD67 mRNA expression (Morris et al., 2008; Hashimoto et al., 2008a) suggesting that both somatostatin and GABA signaling are altered in this interneuron subtype and examining the postsynaptic targets of SST-containing interneurons could elucidate the functional implications of altered SST and GABA in these interneurons.

There are six known G-protein coupled somatostatin receptor (SSTR) subtypes (SSTR1-5) with SSTR2 having two isoforms (SSTR2a and SSTR2b) (Moller et al., 2003). The SSTRs have a putative inhibitory effect on neuronal excitability (Vezzani and Hoyer, 1999; Baraban and Tallent, 2004). Application of SST causes hyperpolarization of rodent hippocampal pyramidal neurons *in vitro* (Pittman and Siggins, 1981) by augmenting M-currents (Moore et al., 1988) and voltage-insensitive K^+ leak current (Schweitzer et al., 1998).

Mice with selective knockouts of the receptor subtypes demonstrate that the most abundant of these receptors are SSTR1 and SSTR2 (Videau et al., 2003) suggesting that these

receptor subtypes are the major receptors for the modulation of excitatory drive to pyramidal neurons by SST. Consistent with this hypothesis both SSTR1 and SSTR2 immuno-labeled cells have a putative pyramidal morphology and have labeled ascending processes in the rat isocortex (Schindler et al., 1997; Hervieu and Emson, 1998) suggesting a postsynaptic localization of these subtypes in pyramidal neurons.

1.3.5 BDNF/TrkB signaling and SST mRNA expression

BDNF (Weickert et al., 2003; Hashimoto et al., 2005) and TrkB, the principal receptor of BDNF, mRNA expression (Hashimoto et al., 2005; Weickert et al., 2005) are reduced in the DLPFC of subjects with schizophrenia. This reduction in BDNF and trkB mRNA expression could have implications for the pathophysiology underlying alterations in the inhibitory circuitry in the DLPFC of individuals with schizophrenia since BDNF/trkB signaling seems to regulate the development of GABA interneurons. In neuron cultures, BDNF/trkB signaling induces the expression of GABA-related proteins, including GAD_{67} (Mizuno et al., 1994; Bolton et al., 2000; Marty et al., 2000; Yamada et al., 2002) and is instrumental for the functional and morphological development and can help induce neurochemical differentiation of GABA neurons (Ventimiglia et al., 1995; Arenas et al., 1996; Vicario-Abejon et al., 1998; Rutherford et al., 1998; Bolton et al., 2000; Marty et al., 2000; Yamada et al., 2002; Kohara et al., 2003). Therefore, trkB activation mediated by BDNF may be important for the development of GABA interneurons including SST interneurons.

The neurochemical expression of SST is thought to be mediated by BDNF/trkB signaling. Several lines of evidence support this hypothesis. First, approximately 50% of SST neurons express trkB (Gorba and Wahle, 1999). Furthermore, ~50% of SST neurons express trkB with predominant colocalization with trkB occurring in layers 2/3 and 5/6 (Gorba and Wahle, 1999) suggesting that many interneurons that contain SST also express trkB. Second, genetically manipulated mice that express reduced levels of BNDF demonstrated reductions in both SST protein and mRNA expression (Grosse et al., 2005; Glorioso et al., 2006). Third, previous studies have demonstrated that the addition of BDNF to cultured cortical cells or intraventricular administration of BDNF in rats increased SST mRNA and protein (Nawa et al., 1994; Villuendas et al., 2001). Furthermore, the alterations in SST expression seem to be due to a

direct activation of trkB receptors rather than a consequence of other factors that may increase BDNF release such as neuronal activity. An increase in neuronal activity by application of bicuculline in rat hippocampal slices resulted in an increase of SST; however, this effect was abolished with an application of a tyrosine kinase receptor antagonist, K252a, suggesting that increased neuronal activity increased endogenous BDNF release and through a tyrosine kinase receptor mediated event subsequently increased SST expression. Therefore, these data are consistent with the hypothesis that BDNF/trkB signaling is an "upstream" event in the expression of SST.

1.4 GOALS AND RELEVANCE OF THIS DISSERTATION

In order to improve medical treatments of schizophrenia, it is necessary to understand the pathophysiology that underlies its core feature, cognitive deficits. Therefore, the experiments in this thesis are aimed at elucidating the alterations found in a subpopulation of SST-containing inhibitory interneurons in the DLPFC of subjects with schizophrenia. The results provide evidence for the critical functional roles of these interneurons in and possible pathogenetic mechanisms of altered working memory in schizophrenia. The rationale and experiments of the following data chapters are briefly outlined:

1.4.1 Somatostatin mRNA Expression in the DLPFC of Subjects with Schizophrenia

In the DLPFC, SST mRNA expression is reduced in subjects with schizophrenia. However, the cellular substrate and causal mechanism of reduced SST mRNA expression in the DLPFC of subjects with schizophrenia had not been explored. Therefore, we utilized *in situ* hybridization and autoradiographic analyses in order to quantify SST mRNA:

1) compartmental, laminar, and cellular levels in DLPFC of subjects with schizophrenia
2) expression in a mouse model in which trkB is reduced
3) expression in a monkey model of chronic haloperidol treatment

1.4.2 Diagnostic Specificity of Alterations in GABA-related Transcript Expression

The diagnostic specificity of the expression in SST mRNA and other prominent GABA-related transcripts had not been determined. Several lines of evidence suggest that individuals with bipolar disorder have similar genetic susceptibility and GABA-related alterations as individuals with schizophrenia. In contrast, subjects with major depressive disorder do not share these alterations. However, both of these psychiatric populations share common environmental factors that are associated with having a chronic debilitating psychiatric illness. Therefore, in order to determine the contribution of shared genetic susceptibility versus common environmental factors associated with a psychiatric disorder in mediating expression of GABA-related transcripts, we utilized real time qPCR to quantify the expression of SST, PV, CR, GAD_{67}, and GAD_{65} mRNA expression in the DLPFC of subjects with bipolar disorder or major depressive disorder.

1.4.3 NPY mRNA Expression in the DLPFC of Subjects with Schizophrenia

In a custom-designed microarray of GABA-related transcripts, a robust expression difference in the DLPFC of subjects with schizophrenia was also found in the levels of neuropeptide Y (NPY) mRNA. NPY is present in ~30-40% of SST-containing interneurons suggesting that the NPY-containing subclass of SST neurons is affected in schizophrenia. Therefore, we utilized *in situ* hybridization and autoradiographic analyses to quantify the compartmental levels of NPY mRNA expression in the DLPFC of subjects with schizophrenia

1.4.4 Expression of SST receptor mRNAs in the DLPFC of Subjects with Schizophrenia

The postsynaptic consequences of alterations in SST-containing interneurons are not known in schizophrenia. Somatostatin receptor subtypes 1 and 2 are the most abundant subtypes in the rodent. Therefore, we utilized *in situ* hybridization and autoradiographic analyses to quantify the

expression of SSTR1 and SSTR2 mRNAs in the DLPFC of subjects with schizophrenia and matched control subjects. Furthermore, we quantified SSTR2 mRNA expression in monkey models of chronic antipsychotic treatment.

2.0 ALTERATIONS IN SOMATOSTATIN MRNA EXPRESSION IN THE DORSOLATERAL PREFRONTAL CORTEX OF SUBJECTS WITH SCHIZOPHRENIA

2.1 ABSTRACT

Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) appear to be a common feature of schizophrenia, and include reduced expression of the mRNA that encodes for somatostatin (SST), a neuropeptide present in a subpopulation of cortical GABA neurons. However, neither the cellular substrate nor the causal mechanisms for decreased SST mRNA levels in schizophrenia are known. Consequently, in this study we used *in situ* hybridization to quantify the compartmental, laminar, and cellular levels of SST mRNA expression in DLPFC area 9 of 23 matched pairs of schizophrenia and control subjects. In addition, we explored causal mechanisms that might contribute to decreased SST mRNA expression in schizophrenia by utilizing similar methods to analyze SST mRNA expression in several animal models. The expression levels of SST mRNA were significantly decreased in DLPFC layers 2 – superficial 6 of subjects with schizophrenia; in contrast, SST mRNA expression in layers 1, deep 6 and the white matter did not differ between subject groups. At the cellular level, both the density of cortical SST mRNA-positive neurons and the expression of SST mRNA per neuron were reduced in the subjects with schizophrenia. These alterations appear to reflect the disease process of schizophrenia and are not the consequence of potential confounds, including chronic exposure to antipsychotic medications. Observations in both individuals with schizophrenia and genetically-engineered mice suggest that these changes are a down-stream consequence of impaired neurotrophin signaling through the trkB receptor. Together, these findings support the hypothesis that a marked reduction in SST mRNA expression in a subset of GABA neurons contributes to DLPFC dysfunction in schizophrenia.

2.2 INTRODUCTION

Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) appear to be a common feature of schizophrenia (Torrey et al., 2005; Akbarian and Huang, 2006). For example, reduced levels of the mRNA that encodes for the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}), an enzyme for GABA synthesis, have been consistently found in the DLPFC of individuals with schizophrenia (Akbarian et al., 1995; Mirnics et al., 2000; Guidotti et al., 2000; Volk et al., 2000; Hashimoto et al., 2005; Straub et al., 2007). Furthermore, this decrease is due to a marked reduction in GAD_{67} mRNA expression in a minority (~25-35%) of GABA neurons, with apparently normal levels of expression in the remaining neurons (Volk et al., 2000). The affected neurons include the ~25% of GABA neurons that express the calcium-binding protein parvalbumin (PV), whereas the ~50% of GABA neurons that express calretinin (CR) appear to be unaffected (Hashimoto et al., 2003). However, the deficits in PV expression are restricted to layers 3 and 4, whereas the alterations in GAD_{67} mRNA are found in layers 2-5; these laminar differences suggest that an additional subset of GABA neurons are affected in schizophrenia.

In a recent study utilizing a custom-designed microarray of GABA-related transcripts, the most robust expression difference in the DLPFC of subjects with schizophrenia was a reduction in the levels of somatostatin (SST) mRNA (Hashimoto et al., 2008b), which is expressed in a subpopulation of GABA neurons that do not contain either PV or CR (Kubota et al., 1994; González-Albo et al., 2001; Gonchar and Burkhalter, 2003; Sugino et al., 2006). The reduction in SST mRNA expression in schizophrenia was confirmed by both real-time qPCR and *in situ* hybridization in the same subjects (Hashimoto et al., 2008a). However, neither the cellular substrate nor the causal mechanisms for decreased SST mRNA expression in schizophrenia have been explored.

SST neurons are present in all layers of the cortex as well as in the underlying white matter. SST neurons in layer 1, deep layer 6, and the white matter are generated early in development and represent residual neurons from the embryonic preplate, whereas those generated later during the development of the cortical plate reside in layers 2 – superficial 6 [(Kostovic and Rakic, 1980; Chun and Shatz, 1989b; Bayer and Altman, 1990); for review see (Allendoerfer and Shatz, 1994)]. Within the gray matter of the adult monkey and human DLPFC,

the greatest densities of SST neurons are found in layers 2-superficial 3 and layer 5 (Lewis et al., 1986; Hayes et al., 1991; Da Cunha et al., 1995). In addition, subsets of SST neurons with different membrane properties and morphological features tend to differ in their laminar locations (Kawaguchi and Kubota, 1996; Ma et al., 2006). Thus, it is important to determine if the expression deficit in SST mRNA is 1) restricted to the gray matter compartment, 2) pronounced in certain cortical layers, and 3) confined to a subset of SST neurons.

Consequently, in this study we used *in situ* hybridization and autoradiographic analyses to quantify the compartmental, laminar, and cellular levels of SST mRNA expression in DLPFC area 9 of 23 matched pairs of schizophrenia and control subjects. In addition, we explored potential causal mechanisms of decreased SST mRNA expression in schizophrenia by utilizing similar methods to analyze SST mRNA expression in several animal models.

2.3 MATERIALS AND METHODS

Human Subjects. With the consent of the surviving next-of-kin, brain tissue specimens were obtained from the Allegheny County Medical Examiner's Office 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). Subject groups did not differ in mean age, PMI, brain pH, RNA integrity number (RIN; as determined from the Agilent Bioanalyzer 2100) or tissue storage time at -80^0 C (for all $t_{(22)} < 1.84$; $p > 0.08$).

An independent committee of experienced research clinicians made consensus DSMIV (Diagnosis and Statistical Manual of Mental Disorders, 1994) diagnoses based on medical records and structured interviews conducted with family members of the deceased. One control subject (987) had a history of post-traumatic stress disorder that had been in remission for 39 years at the time of death. For the subjects with schizophrenia, the mean (SD) age of illness onset was 25.2 (8.0) years and the mean duration of illness was 23.3 (13.3) years. Fifteen subjects with schizophrenia had a history of substance (including alcohol) abuse and/or dependence disorder, although only 8 met criteria for dependence at time of death. Four subjects with schizophrenia (537, 622, 621, and 829) were free of antipsychotic medications at time of death for 9.6 months,

1.2 months, 8.2 years, and unknown length of time, respectively. Toxicology of all subjects detected positive plasma alcohol levels (0.01 and 0.06 %) in two control subjects (516 and 685) and one subject with schizophrenia (0.09 %; 656). All procedures were approved by the University of Pittsburgh's Institutional Review Board for Biomedical Research and Committee for Oversight of Research Involving the Dead.

Tissue Preparation. The right frontal cortex from each brain was blocked coronally, immediately frozen, and stored at -80°C. Serial sections with a thickness of 20 μm containing the superior frontal gyrus were cut, thaw-mounted onto glass slides and stored at -80°C until processed. The location of DLPFC area 9 was identified by cytoarchitectonic criteria in Nissl-stained sections as previously described (Glantz et al., 2000; Volk et al., 2000). Three sections per subject, at intervals of approximately 300 μm, were matched for anterior-posterior location within subject pairs, and used to assess SST mRNA expression.

In situ *hybridization.* Templates for the synthesis of the antisense and sense riboprobes for human and mouse SST mRNA were first generated by polymerase chain reaction (PCR). The specific primers amplified a 337 and 439 base pair (bp) fragment of human and mouse SST, respectively. These fragments corresponded to bases 112-448 of the human (GenBank NM_001048) and mouse (GenBank NM_009215) SST gene. Nucleotide sequencing confirmed 100% homology of the amplified fragment to the previously reported sequence. The fragment was then subcloned into a plasmid (pSTBlue-1, Novagen, Madison, WI). The antisense and sense riboprobes were transcribed in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNeasy mini spin columns (Qiagen, Valencia, CA). One section from each pair was processed during a single run with the sections from each pair processed side by side. Prior to the hybridization reaction, tissue sections were fixed with 4% paraformaldehyde in PBS solution, acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine/0.9% NaCl for 10 minutes, dehydrated with a graded alcohol series, and then defatted in chloroform for 10 minutes. The sections were then hybridized with ^{35}S-labeled riboprobes (1.0 X 10^6 cpm/μl) in hybridization buffer at 56°C for 16 hours. The hybridization

Table 1 Characteristics of Subjects

Pair	Control Subjects							Schizophrenia Subjects							
	Case	Sex/race	Age	PMI[a]	RIN	Storage Time[b]	Cause of death[c]	Case	DSM IV diagnosis	Sex/race	Age	PMI[a]	RIN	Storage time[b]	Cause of death[c]	Antipsychotic medication[d]
1	592	M/B	41	22.1	9.0	100	ASCVD	533	Chronic undifferentiated schizophrenia	M/W	40	29.1	8.4	110	Accidental asphyxiation	Typical
2	567	F/W	46	15.0	8.9	104	Mitral Valve prolapse	537	Schizoaffective disorder	F/W	37	14.5	8.6	109	Suicide by hanging	None
3	516	M/B	20	14.0	8.4	111	Homicide by gun shot	547	Schizoaffective disorder	M/B	27	16.5	7.4	107	Heat Stroke	Typical
4	630	M/W	65	21.2	9.0	94	ASCVD	566	Chronic undifferentiated schizophrenia[e]	M/W	63	18.3	8.0	104	ASCVD	Atypical
5	604	M/W	39	19.3	8.6	97	Hypoplastic coronary artery	581	Chronic paranoid schizophrenia[f,g]	M/W	46	28.1	7.9	102	Accidental combined drug overdose	Typical
6	546	F/W	37	23.5	8.6	108	ASCVD	587	Chronic undifferentiated schizophrenia[e]	F/B	38	17.8	9.0	101	Myocardial hypertrophy	Both
7	551	M/W	61	16.4	8.3	107	Cardiac Tamponade	625	Chronic disorganized schizophrenia[h]	M/B	49	23.5	7.6	95	ASCVD	Typical
8	685	M/W	56	14.5	8.1	87	Hypoplastic coronary artery	622	Chronic undifferentiated schizophrenia	M/W	58	18.9	7.4	95	Right MCA infarction	None
9	681	M/W	51	11.6	8.9	87	Hypertrophic cardio-myopathy	640	Chronic paranoid schizophrenia	M/W	49	5.2	8.4	93	Pulmonary embolism	Atypical
10	806	M/W	57	24.0	7.8	66	Pulmonary thromboembolism	665	Chronic paranoid schizophrenia[f]	M/B	59	28.1	9.2	90	Intestinal hemorrhage	Typical
11	822	M/B	28	25.3	8.5	63	ASCVD	787	Schizoaffective disorder[i]	M/B	27	19.2	8.4	70	Suicide by gun shot	Typical
12	727	M/B	19	7.0	9.2	80	Trauma	829	Schizoaffective disorder[f,j]	M/W	25	5.0	9.3	61	Suicide by drug overdose	None
13	871	M/W	28	16.5	8.5	53	Trauma	878	Disorganized schizophrenia[f]	M/W	33	10.8	8.9	52	Myocardial fibrosis	Atypical
14	575	F/B	55	11.3	9.6	102	ASCVD	517	Chronic disorganized schizophrenia[f]	F/W	48	3.7	9.3	111	Intracerebral hemorrhage	Atypical
15	700	M/W	42	26.1	8.7	84	ASCVD	539	Schizoaffective disorder[k]	M/W	50	40.5	8.1	109	Suicide by combined drug overdose	Atypical
16	988	M/W	82	22.5	8.4	31	Trauma	621	Chronic undifferentiated schizophrenia	M/W	83	16.0	8.7	95	Accidental asphyxiation	None
17	686	F/W	52	22.6	8.5	87	ASCVD	656	Schizoaffective disorder[f]	F/B	47	20.1	9.2	91	Suicide by gun shot	Atypical
18	634	M/W	52	16.2	8.5	93	ASCVD	722	Undifferentiated schizophrenia[j,l]	M/B	45	9.1	9.2	81	Upper GI bleeding	Typical
19	852	M/W	54	8.0	9.1	56	Cardiac tamponade	781	Schizoaffective disorder[k]	M/B	52	8.0	7.7	71	Peritonitis	Typical
20	987[m]	F/W	65	21.5	9.1	31	ASCVD	802	Schizoaffective disorder[f,l]	F/W	63	29.0	9.2	67	Right ventricular dysplasia	Both
21	818	F/W	67	24.0	8.4	64	Anaphylactic reaction	917	Chronic undifferentiated schizophrenia	F/W	71	23.8	7.0	44	ASCVD	Typical
22	857	M/W	48	16.6	8.9	55	ASCVD	930	Disorganized schizophrenia[j,k]	M/W	47	15.3	8.2	41	ASCVD	Typical
23	739	M/W	40	15.8	8.4	79	ASCVD	933	Disorganized schizophrenia	M/W	44	8.3	8.1	40	Myocarditis	Atypical
	Mean		48.0	18.0	8.7	80.0					47.9	17.8	8.4	84.3		
	SD		15.5	5.5	0.4	23.6					14.1	9.3	0.7	23.7		

[a]PMI indicates postmortem interval in hours; [b]storage time (months) at -80°C; [c]ASCVD indicates arteriosclerotic cardiovascular disease; [d]indicates prescribed 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

buffer contained 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 (0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA), 50 mM dithiothreitol, 0.2% SDS, and 100 μg/ml yeast tRNA. Following the hybridization reaction, sections were washed in a solution of 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63°C, treated with RNase A (20 μg/ml) at 37°C, washed in 0.1 X SSC (1.5 mM NaCl, 150 μM sodium citrate) at 67°C, dehydrated through a graded ethanol series, and air dried. The sections, as well as carbon-14 radioactive standards, were exposed on the same BioMax MR film (Kodak, Rochester, NY) for 3 days. Afterward, sections were coated with NTB2 emulsion (Kodak) diluted 2:1 with water. The consistency of the thickness of the emulsion was maintained with use of a mechanical dipper (Auto-dip Emulsion Coater, Ted Pella, Redding, CA) at a constant withdrawal speed (64 mm/min) and temperature (43°C). The emulsion was exposed for 18 days at a constant temperature of 4°C. The slides were developed with D-19 (Kodak) and counterstained with Cresyl-violet.

Quantification of mRNA expression levels. Each section was randomly coded, so that subject number and diagnosis were unknown to the single rater (HMM). Autoradiographic films were trans-illuminated and captured on video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID; Imaging Research Inc., London, Ontario, Canada). Digitized images of adjacent sections stained with cresyl violet were superimposed onto autoradiographic images to draw contours of the full cortical thickness of the zones of area 9 that were cut perpendicular to the pial surface. Optical density measures within each sampled area were calibrated to radioactive carbon-14 standards (ARC Inc., St. Louis, MO), exposed on the same autoradiographic film, and expressed as nanocuries per gram (nCi/g) of tissue. The mean (SD) total area of gray matter sampled in each subject was 381 (141) mm^2 for control subjects and 353 (101) mm^2 for subjects with schizophrenia. Optical density measures in the superficial white matter were determined in a zone 800 μm below, and with a contour that followed, the layer VI/white matter border of the previously sampled gray matter zones. The mean total areas of sampled superficial white matter per subject were 32 (14) mm^2 for control subjects and 30 (10) mm^2 for subjects with schizophrenia. Total white matter was determined by outlining the gray matter/white matter border and including all white matter on the section. The

mean total areas of the sampled total white matter per subject were 280 (117) mm^2 for control subjects and 277 (105) mm^2 for subjects with schizophrenia.

SST mRNA expression as a function of cortical layer was determined in a series of cortical traverses (1-2 mm in width) extending from the pial surface to the white matter. Three cortical traverses were sampled for each section (nine traverses per subject) (Figure 1A). Each traverse was divided into 50 equal bins parallel to the pial surface and the optical density was determined for each bin. These bins were then combined into zones that approximated laminar boundaries based on previous studies (Akbarian et al., 1995; Pierri et al., 1999). These zones (i.e., bins 1-5, 6-15, 16-25, 26-30, 31-40, and 41-50) corresponded to layers 1, 2/superficial 3 (2/3s), deep 3 (3d), 4, 5, and 6, respectively (Figure 1B). The mean OD was calculated for each zone. Background measures were sampled from deep white matter where no specific expression of SST mRNA was observed. All sampled areas were corrected by subtracting the corresponding background measure from the same slide.

Evaluation of mRNA expression at the cellular level was performed by determining silver grain accumulation on emulsion-dipped, Nissl counter-stained sections. Using the MCID system coupled to a microscope equipped with a motor-driven stage, two regions of interest (ROI) (Figure 1B**'**) were defined in 1-mm-wide cortical traverses (3/section; 9/subject). The superficial ROI extended from 10-30% of the distance from the pial surface to the white matter (corresponding to layer 2-superficial 3), and the deep ROI extended from 60-80% (corresponding to layer 5). Four sampling frames (120 X 170 μm) were placed in each ROI such that the edges of the frames were equidistant from the border of the ROI and the edge of the next sampling frame. Within each frame, a circle with a fixed diameter of 22 μm (380 μm^2) was placed over each Nissl-stained nucleus under brightfield illumination using an unbiased inclusion and exclusion rule (Figure 1C)**,** and then the number of grains within the circle were counted in the corresponding dark-field image (Figure 1C'). Because RNase A treatment during the *in situ* hybridization procedure degrades Nissl-stainable substances within the cytoplasm, it was not possible to draw contours around the soma of neurons. In a previous study, we determined that the largest cross-sectional area of human DLPFC GABA neurons is ~400 μm^2 (Volk et al., 2000). The use of fixed diameter sampling does not account for potential differences in somal size across subject groups. However, this confound is unlikely because the somal size of GAD_{67} mRNA-positive neurons has been reported to be unchanged in subjects with schizophrenia

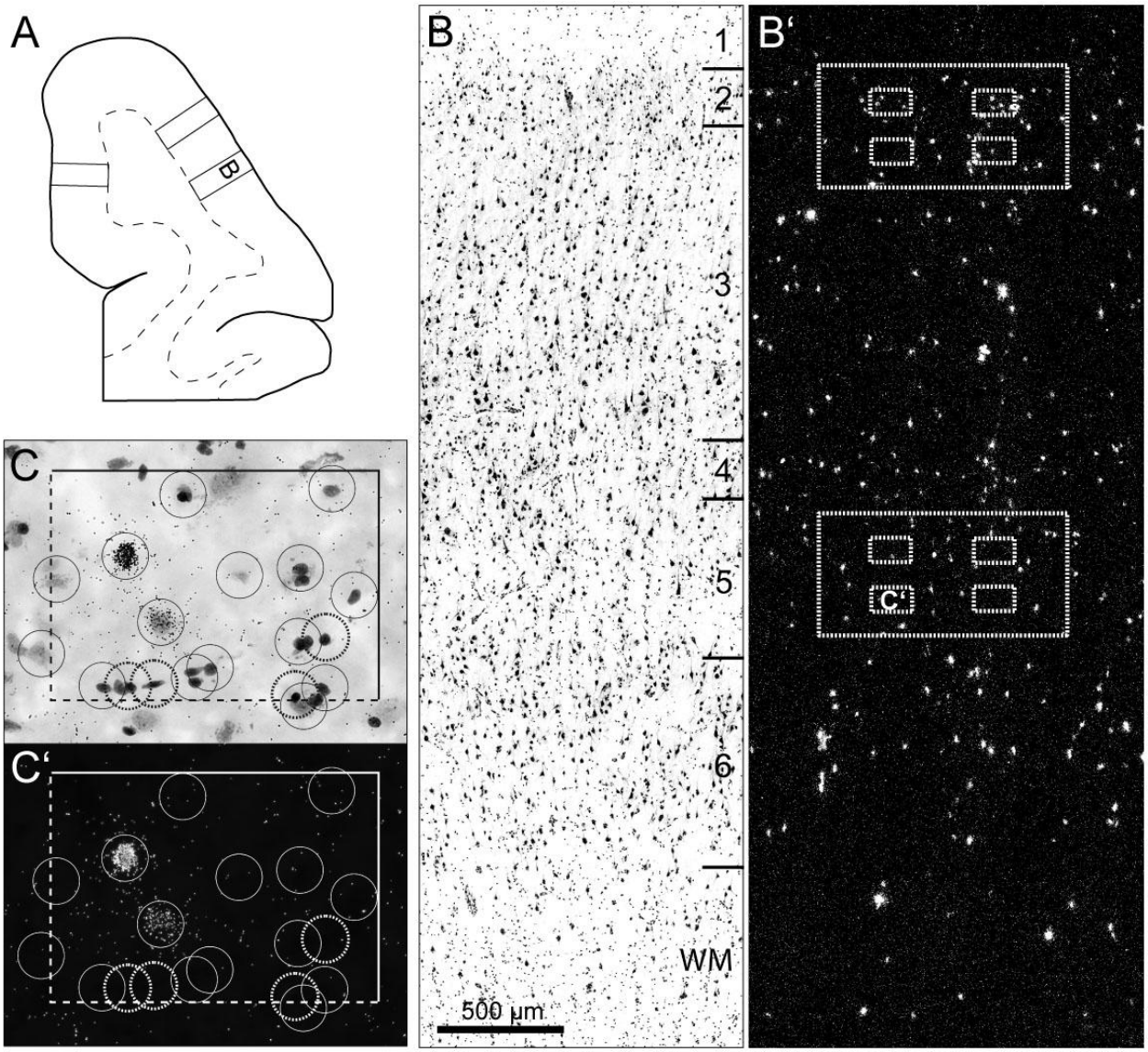

Figure 1 Schematic representation of the sampling strategy for grain analysis of SST mRNA expression.

A, Camera lucida drawing of the portion of DLPFC containing area 9, with the dotted line indicating the grey/white matter border. Three cortical traverses from the pial surface to white matter were placed in each section. B, Brightfield photomicrograph of a representative traverse from a Nissl-stained section. B', Darkfield photomicrograph of an adjacent emulsion-dipped section illustrating silver grain accumulation over neuronal nuclei. Two ROIs (large dashed rectangles) were placed in layers 2/3s and 5. Four sampling frames (smaller dashed rectangles) were placed in each zone. C, Representative brightfield image in which Nissl-stained neuronal nuclei were identified and sampled based upon unbiased inclusion and exclusion rules. Each 120

X 170 μm counting frame had broken and solid lines indicating inclusion and exclusion boundaries, respectively. Circles with a 22 μm diameter were centered over all neuronal nuclei (solid circles) and four glia nuclei (dashed circles) in every counting frame and the number of grains in each circle were counted in the corresponding darkfield image (C').

(Akbarian et al., 1995; Volk et al., 2000). Background grain density was measured in each sampling frame by using the same sampling circle to count grains over four glial nuclei in each sampling frame. The smaller size and intense cresyl violet staining of glial nuclei distinguished them from the larger, more faintly stained neuronal nuclei (Figure 1C). Total neuron numbers sampled in the superficial ROI were 13,791 and 14,593 for control and subjects with schizophrenia, respectively. Deep ROI total neuron numbers sampled were 13,981 and 14,356 for control and subjects with schizophrenia, respectively.

Grain density per neuron (i.e., number of grains within the 22 μm diameter circle) was calculated for all neurons. Specifically-labeled neurons were determined by creating a threshold of grains per neuron. For both subject groups, histograms (natural log transformed) of the grain density of all sampled neurons had a bimodal distribution representing nonspecifically- and specifically-labeled neuron populations (Gerfen et al., 1991). A point of rarity between these populations was used to calculate the threshold above background needed to distinguish the labeled neuron population. Using this threshold, which was equal to 7X background grain density, histograms (natural log transformed) of the grain density of all sampled neurons appeared unimodal and normal in both subject groups. Therefore, grain densities of ≥ 7X background were considered to be specifically-labeled and are referred to as SST mRNA+ cells.

Haloperidol Exposed Monkeys. To mimic the treatment of schizophrenia with high doses of haloperidol, four pairs of young adult, male macaque monkeys (*Macaca fascicularis*), matched for age and weight, were chronically exposed to haloperidol decanoate [mean (SD) trough plasma level, 4.3 (1.1) ng/ml] and benztropine mesylate (1 mg b.i.d.) to treat extrapyramidal symptoms for 9 – 12 months, as previously described (Pierri et al., 1999).

Processing of monkey brain tissue was conducted as previously described (Hashimoto et al., 2003). Briefly, coronal sections with a thickness of 16 μm were cut from fresh-frozen tissue blocks containing the middle one-third of the principal sulcus. Two serial sections from each animal were processed for SST mRNA expression utilizing the ^{35}S-labeled riboprobe as described above. The optical density of the cortex was determined for the full cortical thickness of areas 9 and 46 cut perpendicular to the pial surface similar to the methods in the human study. Background measures were sampled from deep white matter where no specific expression of SST mRNA was observed. All sampled areas were corrected by subtracting corresponding background measures from the same section.

Genetically Engineered Mice. To test the effect of decreased expression of the neurotrophin receptor tyrosine kinase B (trkB) on the expression of SST mRNA, we used *trkB* hypomorphic mice (Xu et al., 2000) in which the first coding exon of the *trkB* gene is replaced with a trkB cDNA unit flanked by two loxP sites (fBZ locus). These mice were generated with 129 strain mice-derived embryonic stem cells and C57BL/6 mice-derived blastocytes (Xu et al., 2000) 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., 2000; Rohrer, 2001; Rico et al., 2002). Animals with the *fBZ/fBZ* genotype do not express the truncated isoforms of trkB (Xu et al., 2000). Heterozygous and homozygous mice for the *fBZ* locus and wild-type controls (n=3 for each group) were euthanized at 8 weeks of age.

The optical density from the autoradiographic film was measured for SST mRNA expression of the cortex in the PFC, including the cingulate and prelimbic cortices, as described for the human study. All density measures were corrected by subtracting background measures in the corpus callosum.

Statistical Analyses. Analyses were performed on SPSS (SPSS Inc., Chicago, IL). Analysis of covariance (ANCOVA) models were used to test differences in SST mRNA expression between control subjects and subjects with schizophrenia. The data were averaged across the three sections for each subject before statistical analysis. The first ANCOVA model used diagnostic group as the main effect, pair as a blocking effect, and storage time and RIN as covariates. The pair effect reflects the matching of individual subject pairs for sex, age and PMI. RIN was included as a covariate because it reflects mRNA integrity (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 a main effect of diagnostic group and covariates of sex, age, PMI, RIN, and storage time. Storage time as a covariate was not significant in either model and, thus was excluded in the reported analyses. Both models produced comparable

results for diagnostic group effect; however, because the effect of age on SST mRNA expression was significant, the results of the second model are reported.

In order to correct for multiple comparisons in the laminar analyses, significance of the diagnostic group effect was determined for individual layers using the Holm simultaneous inference procedure (Holm, 1979) as previously described (Volk et al., 2002). To maintain consistency, the reported p-values for each laminar comparison have been adjusted to correspond to the family-wise error rate of 0.05.

The potential influence of sex, diagnosis of schizoaffective disorder, history of substance abuse/dependency, use of antidepressant medication at time of death, use of benzodiazepines/valproate at time of death, or suicide on the within-pair percentage of differences in mRNA expression was assessed by two-sample t-test analyses. Correlations between age and mRNA expression were assessed by Pearson's correlation analyses.

For the haloperidol-treated monkeys, paired t-test analyses were used to assess the effects of treatment group on SST mRNA expression.

For the *trkB* hypomorphic mice, the effects of genotype on SST mRNA expression were determined by a single-factor ANOVA. Tukey's multiple comparison test was used in post-hoc comparisons across genotypes.

2.4 RESULTS

Specificity of SST riboprobe

Several lines of evidence confirm the specificity of the riboprobe for SST mRNA used in this study. First, the distinctive laminar distribution of SST mRNA expression is very similar to that previously reported for both SST-immunoreactive cell bodies in monkey and human prefrontal cortex (Lewis et al., 1986; Hayes et al., 1991) and SST mRNA expression in monkey prefrontal cortex (Da Cunha et al., 1995). Specifically, the density of SST mRNA+ neurons was lowest in layer 1, highest in layers 2 and superficial 3, moderate in deep layer 3 and layer 4, high in layer

5, and moderate in layer 6 (Figure 1B', Figure 2A). Second, the presence of intensely SST mRNA+ neurons in the superficial white matter (Figure 1B', Figure 2A) is consistent with previous studies of SST-immunoreactive cell bodies in human and monkey prefrontal cortex (Lewis et al., 1986; Hayes et al., 1991) and SST mRNA expression in the monkey prefrontal cortex (Da Cunha et al., 1995). Third, sense riboprobes for SST mRNA showed an absence of signal above background (data not shown).

Compartmental expression of SST in DLPFC area 9 of control subjects and individuals with schizophrenia

We previously reported reduced SST mRNA expression by microarrary, qPCR, and *in situ* hybridization in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2008a). However, whether this expression deficit was specific to the gray matter or also present in the white matter was not examined. Consistent with qualitative impressions (Figure 2), quantitative measures of SST mRNA expression in the gray matter of DLPFC area 9 confirmed that the mean (±SD) optical density of gray matter was significantly ($F_{(1,40)} = 18.07$; $p < 0.001$) decreased by 36% in subjects with schizophrenia (55.8 ± 31.9 nCi/g) compared to control subjects (87.5 ± 22.9 nCi/g). Optical density measurements in the gray matter were lower in the subjects with schizophrenia for 19 of the 23 subject pairs (Figure 3A). In contrast, mean film OD from the total white matter did not significantly differ ($F_{(1,40)} = .72$; $p = .40$) between control subjects (18.8 ± 5.6 nCi/g) and subjects with schizophrenia (17.0 ± 5.3 nCi/g) (Figure 3B). Because SST mRNA expression was most dense in the superficial white matter (Figure 2), the OD was also determined from contours in the 800 μm of white matter immediately below the layer 6-white matter border. These measures also did not differ ($F_{(1,40)} = 1.74$; $p = .20$) between control subjects (33.8 ± 10.9 nCi/g) and subjects with schizophrenia (28.3 ± 10.1 nCi/g) (Figure 3C).

Examination of factors that may affect cortical expression of SST mRNA

In the second ANCOVA model, age was a significant ($F_{(1,40)} = 19.24$; $p < 0.0001$) determinant of SST mRNA levels in the gray matter. Indeed, OD measures in the gray matter contours were negatively correlated with age in both control subjects ($r = -0.74$; $p < 0.0001$) and subjects with schizophrenia ($r = -0.51$; $p = 0.01$) (Figure 4). However, the within-subject pair percent differences in SST mRNA expression in the gray matter did not differ as a function of sex,

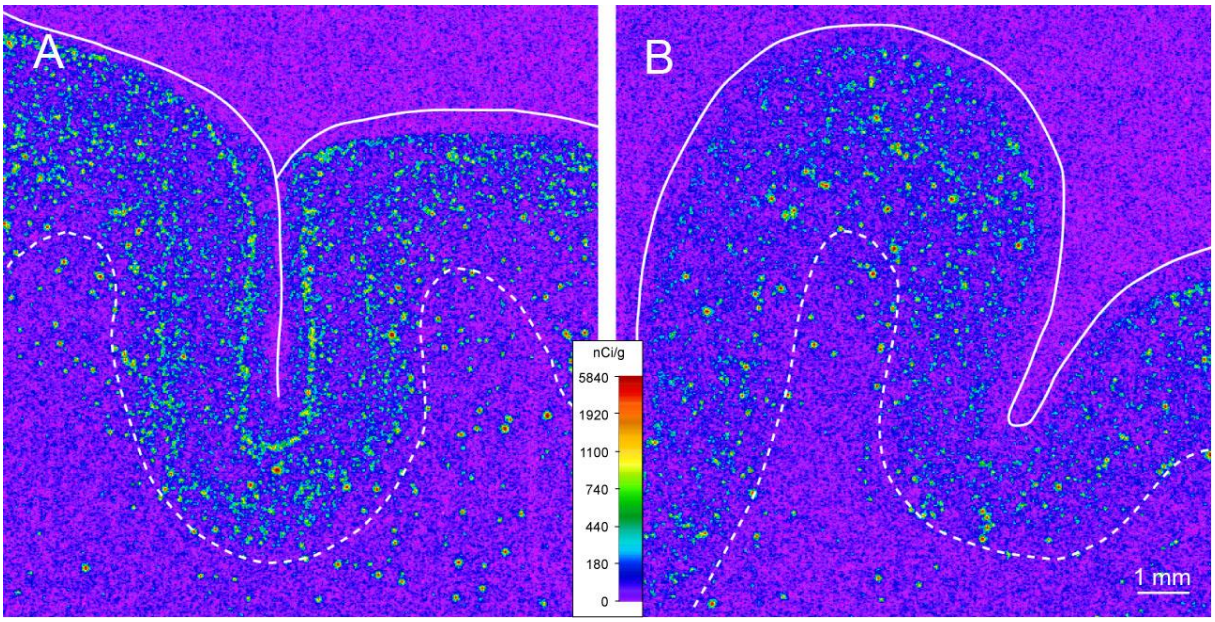

Figure 2 Representative film autoradiograms from one pair of subjects illustrating SST mRNA expression in human DLPFC.

The densities of hybridization signals are represented in a pseudocolor manner according to the calibration bar (center). The solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. Expression of SST mRNA in the cortical gray matter is reduced in the subject with schizophrenia (B) relative to the matched control subject (A).

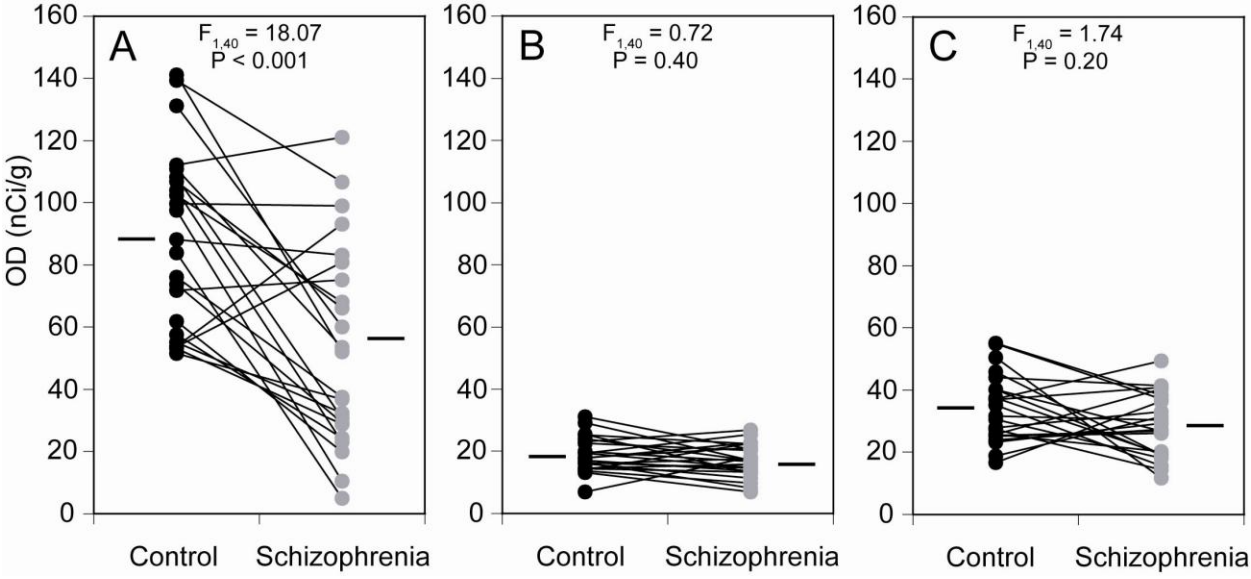

Figure 3 SST mRNA expression levels assessed by autoradiographic film optical density (OD) measures in DLPFC area 9 of subjects with schizophrenia (gray circles) and control subjects (black circles).

Subjects in each pair are connected by black lines and the mean expression levels for each subject group are represented by a horizontal line. The expression of SST mRNA is significantly reduced in the gray matter (A), but not in the total white matter (B) or the superficial white matter (C) of the schizophrenia subjects.

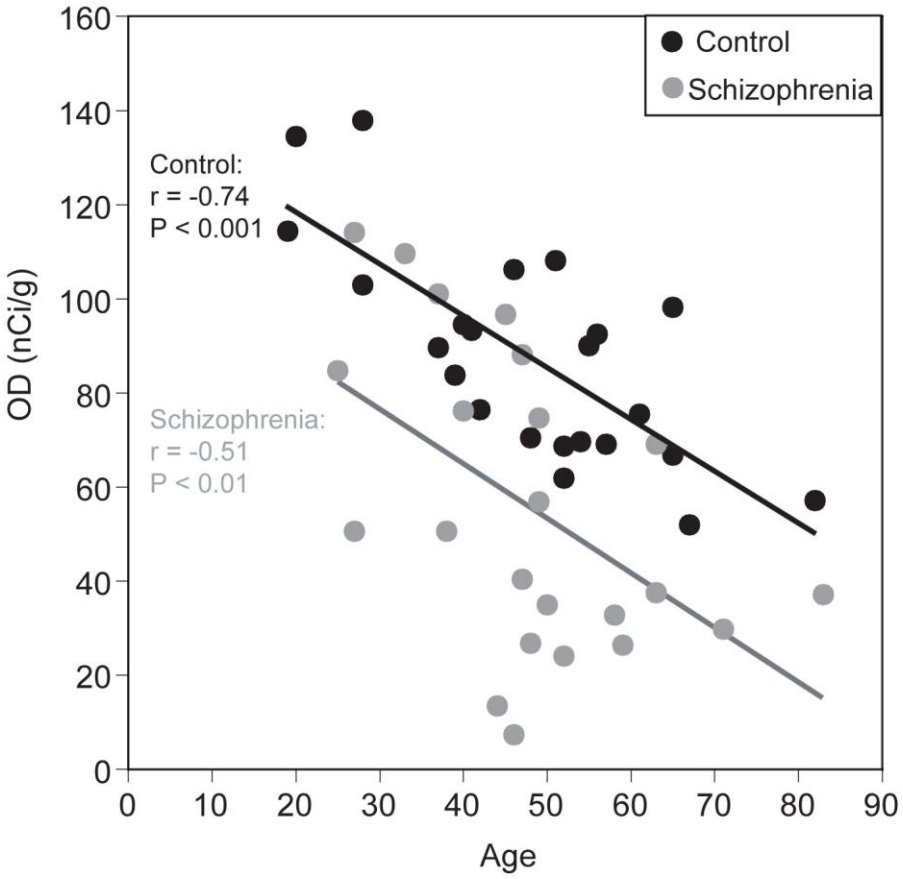

Figure 4 Optical density (OD) measures of SST mRNA expression in human area 9 as a function of age.

SST mRNA expression levels are significantly negatively correlated with age in both subject groups. The regression line for subjects with schizophrenia (gray line) are parallel to and shifted downward from those for control subjects (black line), suggesting that the decreased expression of SST mRNA is similar in magnitude across adult life.

diagnosis of schizoaffective disorder, history of substance abuse/dependency, use of antidepressant medication at time of death, use of benzodiazepines/valproate at time of death, or suicide (all $t_{(21)} < 1.52$, all $p > 0.14$) (Figure 5).

In a previous study, the expression of SST mRNA in DLPFC areas 9 and 46 of monkeys did not differ among monkeys chronically exposed to haloperidol, olanzapine, or sham (Hashimoto et al., 2008a). However, the steady-state trough plasma levels (~1.5 ng/ml) in the haloperidol-treated monkey group might be considered relatively low compared to levels likely achieved in at least some of the subjects with schizophrenia included in this study. Thus, in order to determine if chronic exposure to higher levels of haloperidol could affect SST mRNA expression, we examined the gray matter of DLPFC areas 9 and 46 from monkeys that received haloperidol decanoate for one year with mean trough plasma levels >4 ng/ml (Figure 6). Mean (SD) SST mRNA expression did not differ ($t_{(3)} = 1.08$, $p = 0.36$) between these haloperidol-exposed monkeys (275.3 ± 40.3) and their sex-, age-, and weight-matched controls (238.4 ± 56.1).

Laminar expression patterns of SST mRNA in DLPFC area 9

In order to determine if the reduction in SST mRNA expression in schizophrenia was selective for certain cortical layers, we examined the OD measures by layer (Figure 7). Because of the high density of SST mRNA+ neurons in layers 2-superficial 3 (2/3s), these layers were combined and distinguished from deep layer 3 (3d). SST mRNA expression was significantly decreased in all layers ($F_{(1,40)} > 4.34$; $p < 0.04$, for all layers), with the exception of layer 1 ($F_{(1,40)} = 0.64$; $p = 0.23$). Of the layers with a significant diagnostic effect, the largest percentage difference was in layer 2/3s (36.6%) and the smallest difference in layer 3d (29.6%).

Cellular levels of SST mRNA expression

In order to determine if all or a subset of SST cortical neurons were affected, we determined the expression level per neuron by counting the silver grains deposited over Nissl-stained neurons in layers 2/3s and 5. The mean (± SD) number of grains per positive neuron in layers 2/3s was significantly ($F_{(1,40)} = 9.72$, $p = .003$) 31% lower in the subjects with schizophrenia (104.4 ± 49.1) than in the control subjects (150.4 ± 53.4). Similarly, the mean number of grains per positive neuron in layer 5 was significantly ($F_{(1,40)} = 6.96$, $p = .012$) decreased by 25% in

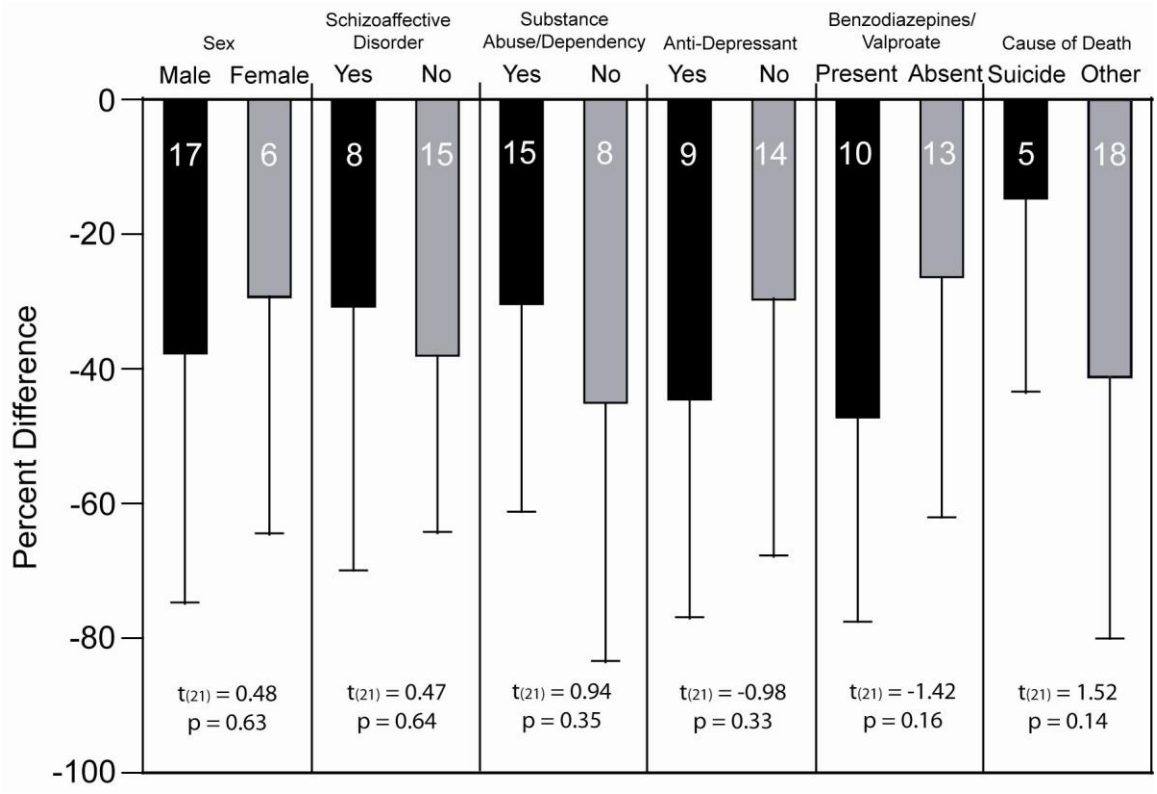

Figure 5 The effects of potential confounding factors on the expression changes in SST mRNA in subjects with schizophrenia.

The bars represent the mean (SD) percent differences from control subjects for SST mRNA within subject pairs, and the numbers within each bar indicate the number of subject pairs. Neither sex, diagnosis of schizoaffective disorder, history of substance abuse/dependence, history of antidepressant medications, the presence of benzodiazepines/valproate at time of death, nor cause of death significantly affected the expression changes in SST mRNA.

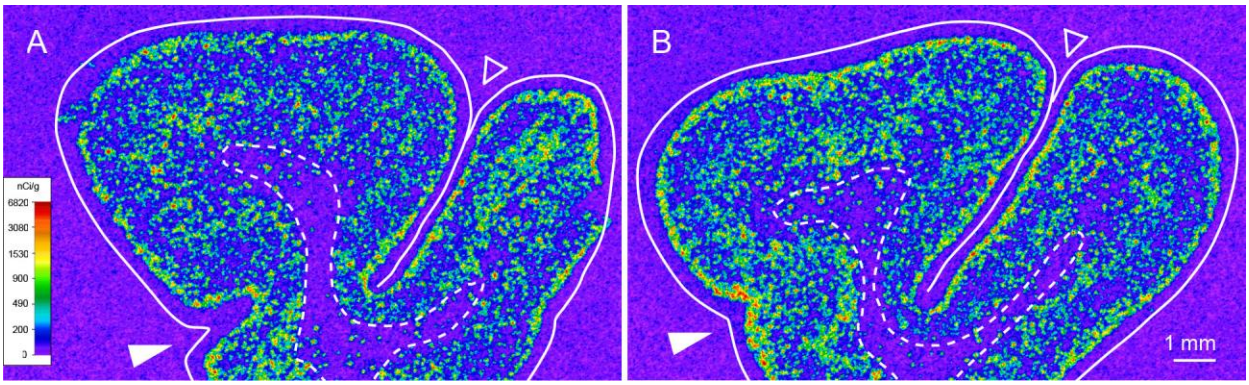

Figure 6 Representative film autoradiograms illustrating the expression of SST mRNA in the dorsal PFC of a control monkey (A) and an age-, sex-, and body weight-matched monkey chronically exposed to haloperidol (B).

The densities of the hybridization signal are presented in a pseudocolor manner according to the calibration scale (in A). The signal density appears to be unchanged in the haloperidol-exposed monkey (B) relative to the control monkey (A). Solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. Open and filled arrowheads indicate the principal and cingulate sulci, respectively.

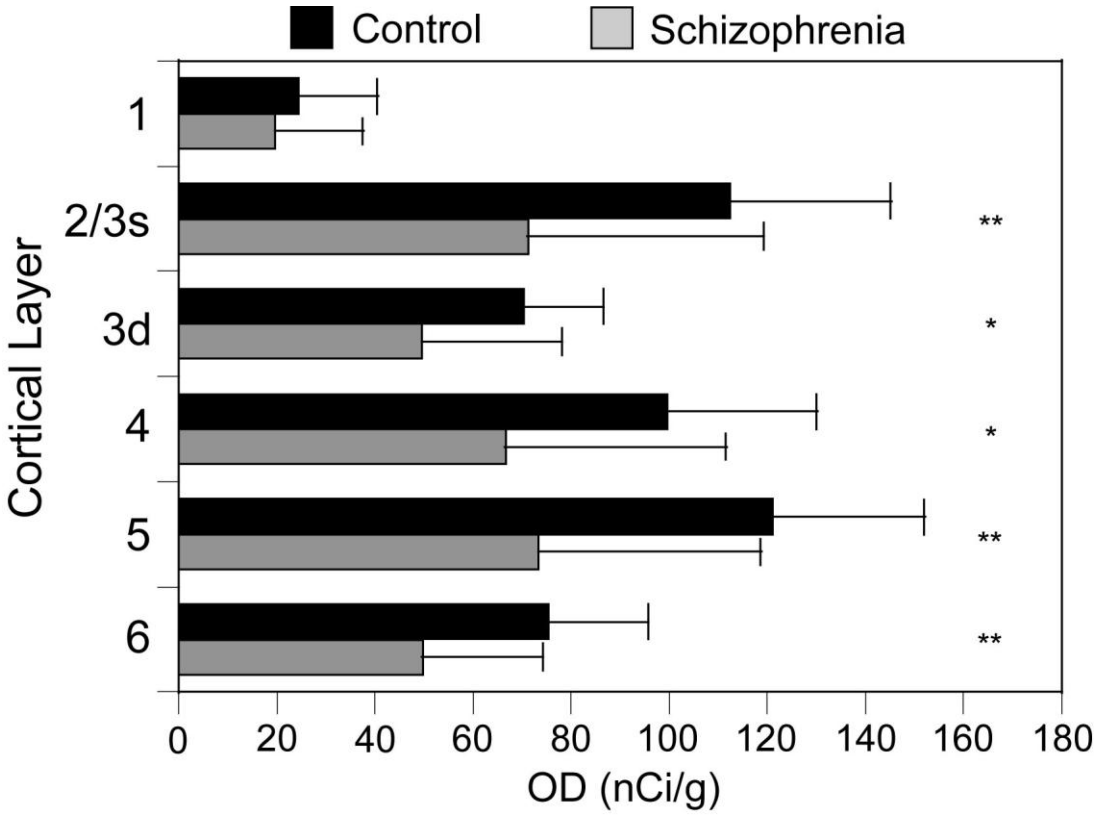

Figure 7 Mean (SD) expression levels of SST mRNA by layer as assessed by film autoradiography optical density (OD) measures in each cortical layer.

* $p < 0.05$, after Holm's correction. ** $p < 0.01$, after Holm's correction.

subjects with schizophrenia (121.5 ± 67.3) compared to controls (162.7 ± 45.7) (Figure 8A,B). Furthermore, the mean density of SST mRNA+ neurons in layers 2/3s was significantly ($F_{(1,40)}$ = 10.79, p = .002) decreased by 26% in subjects with schizophrenia (63.3 ± 23.7 neurons/mm^2) relative to control subjects (85.3 ± 16.2 neurons/mm^2), and in layer 5 was decreased by 23% ($F_{(1,40)}$ = 3.93, p = .054) in subjects with schizophrenia (52.5 ± 27.2 neurons/mm^2) compared to control subjects (67.7 ± 15.4 neurons/mm^2) (Figure 8C,D).

Correlation of altered expression of SST mRNA with changes in other transcripts

Within-pair percentage differences in SST mRNA expression were significantly correlated (r = 0.72, p < 0.001; Figure 9A) with those found for GAD_{67} mRNA expression in a previous study of the same subjects (Hashimoto et al., 2005). These reductions in GAD_{67} mRNA expression in schizophrenia were also strongly correlated with changes in the expression of receptor tyrosine kinase B (trkB) mRNA, the principal receptor for brain-derived neurotrophic factor (BDNF), and to a lesser extent with those in BDNF mRNA expression (Hashimoto et al., 2005). Thus, because trkB is expressed in ~50% of SST neurons (Gorba and Wahle, 1999), we examined whether reduced BDNF-trkB signaling might be associated with the reduced SST mRNA expression in schizophrenia. The within-pair percentage differences in SST mRNA were significantly correlated with within-pair percentage differences in both BDNF mRNA expression (r = 0.77, p < 0.001; Figure 9B), and trkB mRNA expression (r = 0.74, p < 0.001; Figure 9C).

SST mRNA expression in genetically engineered mice

We have previously shown that mice with reduced expression of BDNF mRNA exhibit decreased SST mRNA expression (Glorioso et al., 2006), suggesting that the correlations observed in the human subjects might represent cause and effect. In order to determine if the level of trkB mRNA expression also regulates SST mRNA expression, we examined SST mRNA expression in the PFC of *trkB* hypomorphic mice (Xu et al., 2000). Levels of trkB mRNA in the frontal cortex of *fBZ/+* and *fBZ/fBZ* genotype mice were decreased by 42% and 75%, respectively, as compared to wild-type animals (Hashimoto et al., 2005). TrkB genotype was significantly ($F_{(2,6)}$ = 6.03, p = 0.037) related to the expression level of SST mRNA (Figure 10). SST mRNA levels in wildtype mice (596.9 ± 31.4 nCi/g) were significantly greater (p = 0.045) than in the *fBZ/fBZ* genotype (406.3 ± 55.6 nCi/g), and SST mRNA expression in the

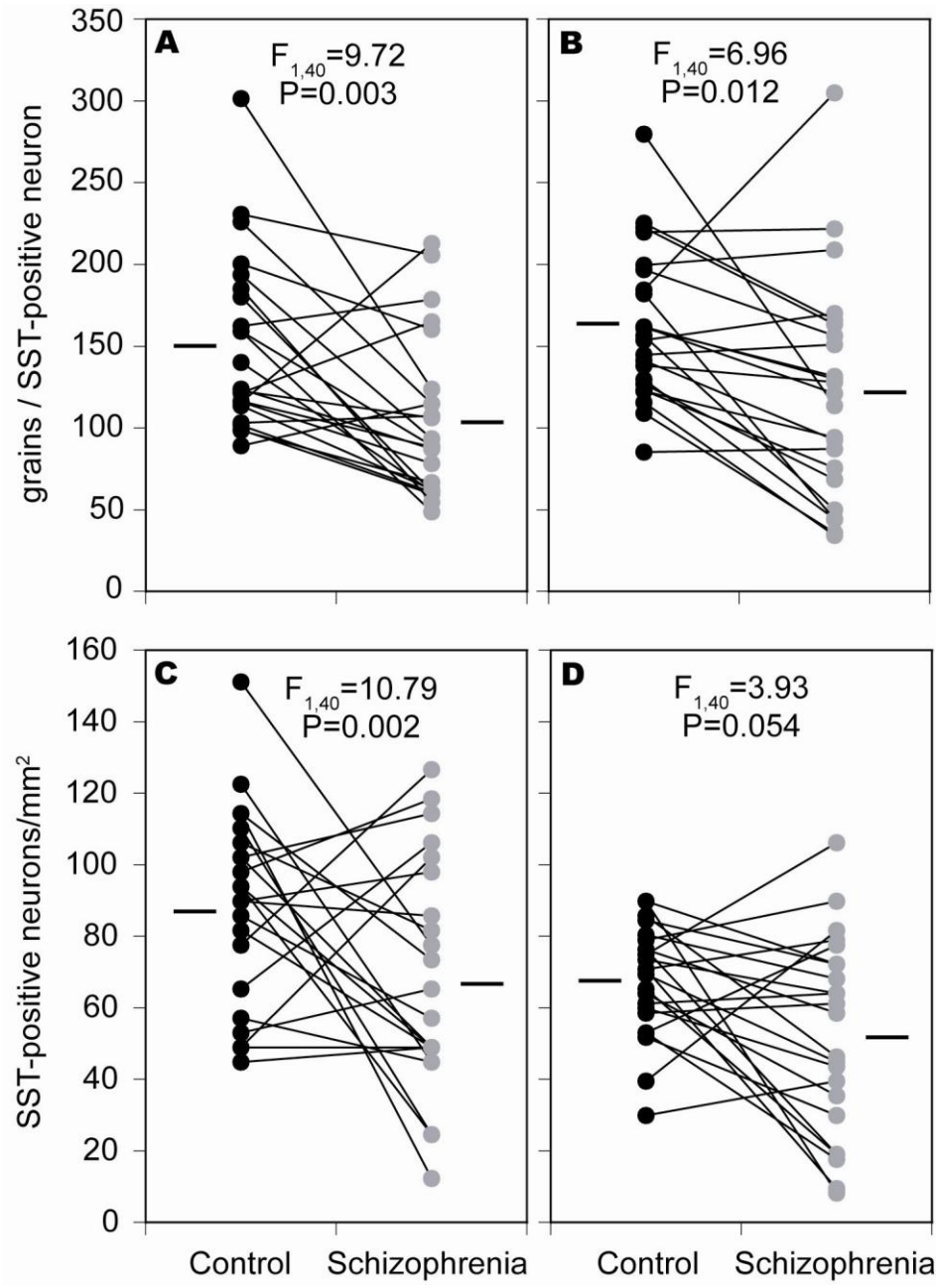

Figure 8 Cellular SST mRNA measurements in the DLPFC area 9

of subjects with schizophrenia (gray circles) and control subjects (black circles) as expressed by grains per positive neuron (A,B) in layer 2/3s (A) and layer 5 (B) and positive neuron density (C,D) in layer 2/3s (C) and layer 5 (D). Subjects in each pair are connected by black lines and the mean values for each subject group are indicated by a horizontal line.

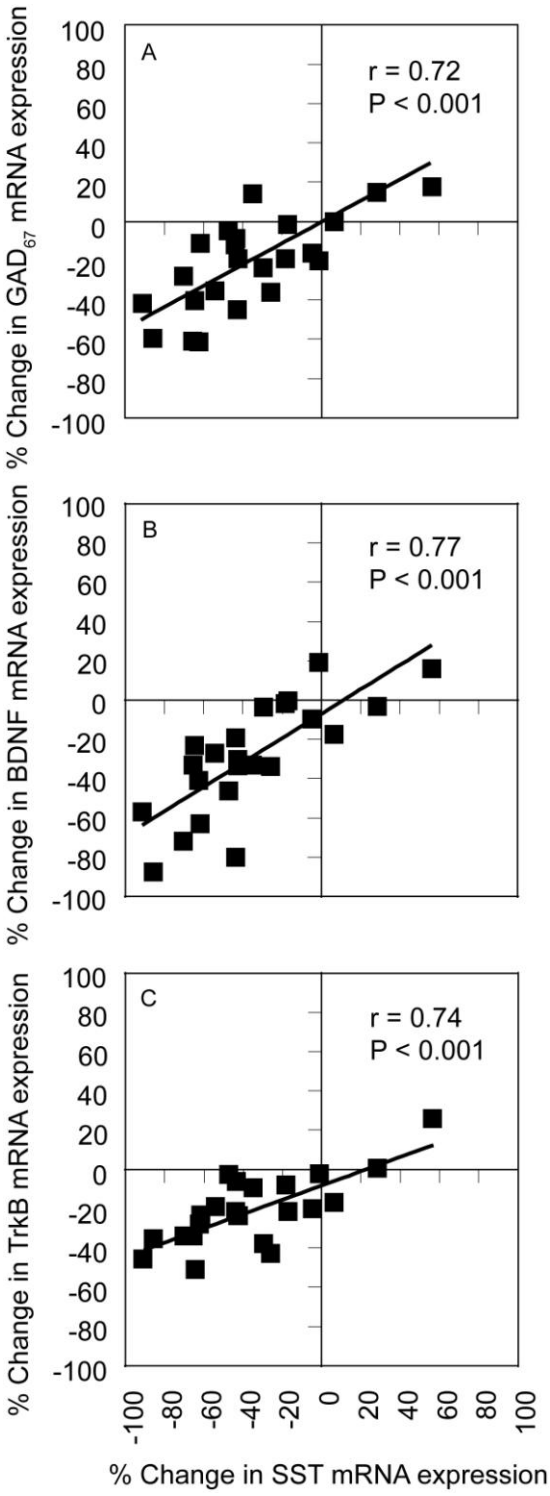

Figure 9 Correlations of within-subject pair percent differences in SST mRNA expression with GAD67 (A), BDNF (B), and TrkB (C) mRNA expression.

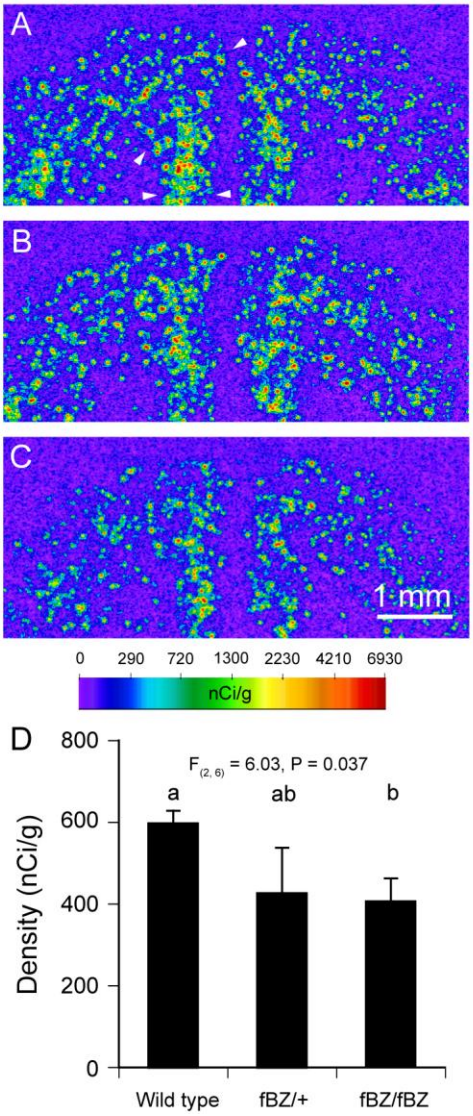

Figure 10 Representative film autoradiograms illustrating SST mRNA expression in a wild-type mouse (A), a mouse heterozygous for the fBZ locus (fBZ/+) (B), and a mouse homozygous for the fBZ locus (fBZ/fBZ) (C).

The arrowheads (apply to all sections) in A indicate the quantified regions in the frontal cortex. The densities of hybridization signals are presented in a pseudocolor manner according to the calibration scale (below C). D, Bar graph representing mean ± SD of SST mRNA in the PFC in each genotype. Bars not sharing the same letter are significantly different ($p < .05$; post hoc Tukey's multiple comparison).

fBZ/+ genotype (426.6 ± 110.5 nCi/g) was intermediate, showing a trend to lower values as compared to wildtype mice ($p = 0.068$).

2.5 DISCUSSION

The expression levels of SST mRNA were significantly decreased in layers 2 – 6 of DLPFC area 9 in subjects with schizophrenia compared to matched controls; in contrast, SST mRNA expression in layer 1 and the white matter did not differ between subject groups. At the cellular level, both the density of cortical SST mRNA+ neurons and the expression of SST mRNA per neuron were reduced in the subjects with schizophrenia. These alterations appear to reflect the disease process of schizophrenia and not to be the consequence of potential confounds. Observations in both humans and genetically-engineered mice suggest that these changes are a down-stream consequence of impaired neurotrophin signaling in schizophrenia. Together, these findings indicate that reduced SST mRNA expression in the DLPFC of subjects with schizophrenia is restricted to the gray matter, confined to a subset of SST neurons, and the consequence of a plausible pathogenetic mechanism.

Altered SST mRNA expression in schizophrenia is not due to confounds

Several lines of evidence indicate that the reduction in SST mRNA expression is due to the disease process of schizophrenia and is not the result of other factors commonly associated with the illness. First, all four subjects with schizophrenia off antipsychotic medications at the time of death demonstrated a decrease in SST mRNA expression relative to their matched controls. Second, the gray matter expression of SST mRNA was not altered in monkeys chronically exposed to plasma levels of haloperidol that produced marked extrapyramidal symptoms requiring treatment with benztropine mesylate. Third, SST mRNA expression was unaltered in the DLPFC of monkeys chronically exposed to either haloperidol or olanzapine with trough plasma levels in the therapeutic range for humans (Hashimoto et al., 2008a). Fourth, neither SST mRNA (Marcus et al., 1997) nor protein (Sakai et al., 1995) levels were decreased in the frontal

cortex of rats exposed to haloperidol. Furthermore, neither sex, schizoaffective diagnosis, history of substance abuse/dependency, treatment with antidepressant medications or benzodiazepines/valproate, nor suicide accounted for the decreased expression of SST mRNA in the subjects with schizophrenia (see Figure 5).

A significant negative correlation between age and SST mRNA gray matter expression was seen in both controls and individuals with schizophrenia, consistent with previous findings of an inverse relationship between age and SST mRNA or protein levels in rat hippocampus and monkey frontal cortex (Hayashi et al., 1997; Vela et al., 2003). The regression line for the subjects with schizophrenia was parallel to and shifted downward from that of the control subjects (Figure 4), indicating that the decreased expression of SST mRNA in the subjects with schizophrenia was present across adult life and is, thus unlikely to be a consequence of illness chronicity. Furthermore, this observation suggests that the SST mRNA expression deficit is present early in the course of the illness and, thus could contribute to the pathophysiology underlying the clinical features of the illness.

A subset of SST neurons is affected in schizophrenia

Our results demonstrate that SST mRNA expression in layers 2 – 6, but not in layer 1 or the white matter, was reduced in subjects with schizophrenia. During development of the cerebral cortex, early germinal zones proliferate over successive rounds of cell division and give rise to post-mitotic migratory neurons. ^{3}H-thymidine birth-dating and autoradiographic analyses have demonstrated that the earliest generated cells comprise the preplate which, later in development, is split into the marginal zone (adult layer 1) and the subplate (adult deep layer 6 and superficial white matter) by the later born neurons of the cortical plate (adult layers 2 – superficial 6) (Kostovic and Rakic, 1980); (Luskin and Shatz, 1985; Chun and Shatz, 1989a; Bayer and Altman, 1990). The combination of birth-dating techniques and immunohistochemistry revealed that a subpopulation of the early generated preplate neurons expresses SST (Chun and Shatz, 1989a). Our data suggest that the early generated SST mRNA+ neurons which reside in layer 1 and the superficial white matter are not affected in schizophrenia, whereas the later developing SST mRNA+ neurons which migrate to the cortical plate are affected. Additional analyses support this interpretation. For example, in the rat cortex sublayer 6b also contains residual neurons of the embryonic preplate (Valverde et al., 1989), suggesting that SST+ neurons in layer

6b might be less affected in schizophrenia than those present in the more superficial layer 6a. Consistent with this prediction, the mean decrease in OD measures of SST mRNA in subjects with schizophrenia was significantly greater ($t_{(22)}$ = -5.08; $p < 0.0001$) in the superficial (-37.4 nCi/g) than in the deep (-14.0 nCi/g) half of layer 6. Given that the cortical plate forms during the second trimester of gestation, these findings raise the possibility that the alterations in SST neurons reflect the effect of adverse environmental events during that time frame [e.g., maternal influenza (Brown, 2006)] that have been associated with an increased risk for schizophrenia. However, given how common the alterations in SST neurons appear to be from the present study (i.e., 20/23 pairs), other causal factors must also be contributory. For example, both SST and parvalbumin-containing cortical GABA neurons are affected in schizophrenia, whereas those that contain calretinin appear to be unaffected. Thus, factors shared by SST and parvalbumin neurons that differ from calretinin neurons [e.g., place and timing of neuron birth, transcription factors regulating cell fate, etc; see (Wonders and Anderson, 2006) for review] might also contribute to cell type-specific vulnerability. Of course, other features intrinsic to, or associated with the connectivity of, adult SST neurons in layers 2-superficial 6 might contribute to their greater vulnerability relative to other SST neurons.

In both layers 2-superficial 3 and layer 5, we observed a significant reduction in the mean density of SST mRNA+ neurons. Similarly, the mean expression level of SST mRNA per neuron was significantly reduced. In subjects with schizophrenia, total neuron number in the frontal lobe is unchanged (Thune et al., 2001) and the density of nonpyramidal neurons in the DLPFC is slightly increased (Selemon et al., 1995) or unchanged (Akbarian et al., 1995). Thus, it appears that SST neurons are still present in the DLPFC of subjects with schizophrenia, but that the expression of SST mRNA per neuron is reduced, with the reduction so great in some neurons that SST mRNA levels fall below the threshold of detection. This pattern of change in SST mRNA expression contrasts with the alterations in GAD_{67} and PV mRNA expression in the DLPFC of subjects with schizophrenia. The expression levels of GAD_{67} mRNA were reduced below detectable levels in ~25-35% of GABA neurons, whereas the remaining GABA neurons expressed normal levels of GAD_{67} mRNA, suggesting that a subpopulation of GABA neurons is affected in the illness (Volk et al., 2000). The reduced expression of PV mRNA was found to be due to a decrease in expression level per neuron rather than to a decrease in the density of PV mRNA+ neurons suggesting that most PV mRNA+ neurons are affected (Hashimoto et al.,

2003). Because the reduction in SST mRNA expression was due to both a decrease in positive neuron density and in expression per neuron, these findings suggest that a majority of SST neurons express reduced levels of SST mRNA and that a subset of severely affected SST neurons express undetectable levels of SST mRNA.

The severely affected neurons might include the approximately 50% of SST+ neurons that express trkB (Gorba and Wahle, 1999), the principal receptor of BDNF. Consistent with this interpretation, mice with genetically engineered reductions in the expression of BDNF mRNA have significantly lower levels of cortical SST mRNA and protein (Grosse et al., 2005; Glorioso et al., 2006). In addition, the significant reduction in the expression of SST mRNA in the PFC of homozygote *trkB* hypomorphic mice indicates that signaling via trkB is also involved in regulating the expression of SST mRNA, even in the face of conserved levels of BDNF. Given that the expression levels of BDNF (Weickert et al., 2003; Hashimoto et al., 2005) and trkB mRNAs (Hashimoto et al., 2005; Weickert et al., 2005) are reduced in the DLPFC of subjects with schizophrenia, our findings imply that reduced BDNF-trkB signaling in schizophrenia is an "upstream" event that contributes to reduced SST mRNA expression. This interpretation is consistent with studies demonstrating that the addition of BDNF to cultured cortical cells or the intraventricular administration of BDNF in rats increased SST mRNA and protein levels (Nawa et al., 1994; Villuendas et al., 2001). Also, previous studies have reported that BDNF can influence the expression of GAD_{67} (Yamada et al., 2002; Cotrufo et al., 2003; Wirth et al., 2003; Patz et al., 2004; Palizvan et al., 2004). Therefore, altered BDNF-trkB signaling in schizophrenia may be a conserved mechanism driving the highly correlated reductions in SST and GAD_{67} mRNA expression in schizophrenia.

Functional implications of alterations in SST neurons in schizophrenia

In rat and monkey frontal cortex, ~40% of SST neurons also express neuropeptide Y (NPY) (Hendry et al., 1984; Kubota et al., 1994). Interestingly, expression deficits in SST and NPY mRNAs are strongly correlated in schizophrenia ($r = 0.81$; $p < 0.001$) (Hashimoto et al., 2008a) and both are similarly reduced in BDNF knockout mice (Glorioso et al., 2006) suggesting that the NPY-containing subclass of SST neurons are affected in schizophrenia. Interneurons that contain both SST and NPY include the Martinotti cells (Kawaguchi and Kubota, 1997; Reyes et al., 1998; Gibson et al., 1999; Ma et al., 2006). The axons of Martinotti cells project to layer 1

where they synapse on the apical dendrites of pyramidal neurons and in rodent and monkey neocortex, SST interneurons predominately innervate the dendrites of pyramidal neurons (Hendry et al., 1984; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1996; Melchitzky and Lewis, 2005). Thus, disturbances in the SST/NPY-containing Martinotti class of GABA neuron could contribute to the dysfunction of DLPFC circuitry associated with working memory impairments in schizophrenia (Weinberger et al., 1986; Goldman-Rakic, 1994; Tan et al., 2005; Cannon et al., 2005) through several mechanisms.

First, in individuals with schizophrenia, disturbances in sensory-gating have been correlated with reductions in working memory performance (Silver and Feldman, 2005), suggesting that the inability to filter distracting stimuli disrupts working memory. Interestingly, in computational modeling of cortical microcircuits and working memory, inhibitory interneurons that target the dendritic domain of pyramidal neurons provide resistance against distracting stimuli by sending enhanced inhibition to dendrites of nearby pyramidal neurons that are selective for other stimuli (Wang et al., 2004a). Furthermore, in rats, high frequency trains from pyramidal neurons produce facilitating excitatory inputs to Martinotti cells that, via synapses onto the dendrites of neighboring pyramidal neurons, cause disynaptic inhibition (Silberberg and Markram, 2007). Therefore, Martinotti interneurons, by mediating the disynaptic inhibition of neighboring pyramidal neurons selective for other stimuli, may filter distracting stimuli during working memory tasks. Thus, alterations in SST/NPY-containing Martinotti cells in schizophrenia may contribute to altered working memory performance.

Second, Martinotti interneurons also exhibit low threshold-spiking membrane properties (Kawaguchi and Kubota, 1997; Reyes et al., 1998; Gibson et al., 1999; Ma et al., 2006). These low threshold-spiking, SST-containing interneurons are extensively electrically coupled into networks that robustly synchronize their spiking activity (Gibson et al., 1999; Gibson et al., 2005) in the theta range (4-7 Hz) producing synchronized inhibitory post-synaptic potentials in neighboring pyramidal neurons (Beierlein et al., 2000). Although a direct connection between the synchronized theta spiking frequency in these neuronal networks and theta band EEG oscillations has not been demonstrated, EEG studies in humans and monkeys have demonstrated that theta band EEG oscillations increase in power during working memory tasks (Krause et al., 2000; Raghavachari et al., 2001; Lee et al., 2005). Furthermore, subjects with schizophrenia demonstrate altered frontal theta oscillations during working memory tasks (Schmiedt et al.,

2005). Thus, disturbances in SST/NPY-containing, low threshold-spiking Martinotti interneurons might contribute to alterations in theta oscillations and ultimately to impaired working memory performance in subjects with schizophrenia.

3.0 DISTINCTIVE GABA-RELATED MRNA EXPRESSION PROFILES IN THE DORSOLATERAL PREFRONTAL CORTEX OF SUBJECTS WITH BIPOLAR OR MAJOR DEPRESSIVE DISORDER

3.1 ABSTRACT

Background. Dysfunction of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia is associated with abnormalities in γ-aminobutyric acid (GABA)-containing interneurons. The mRNAs for the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}), an enzyme of GABA synthesis, and for somatostatin (SST) and parvalbumin (PV) which are expressed in separate subsets of GABA-containing interneurons, exhibit lower expression, whereas the mRNAS for the 65 kDa isoform of GAD (GAD_{65}) and calretinin (CR), which is expressed in a third subset of interneurons, are unchanged. However, the expression profile of these transcripts have not been examined in bipolar disorder (BPD), which shares genetic risk with schizophrenia, and major depressive disorder (MDD), which shares environmental consequences of a debilitating psychiatric disorder.

Methods. We used real-time quantitative polymerase chain reaction to assess the levels of 5 GABA-related transcripts in the DLPFC of 19 matched triads of subjects with BPD or MDD and normal comparison subjects. Results were compared to those from a previous study in comparable subjects with schizophrenia.

Results. Subjects with BPD demonstrated a reduction in PV mRNA expression with no alteration in GAD_{67}, GAD_{65}, CR, or SST mRNA expressions; subjects with MDD demonstrated a reduction in SST mRNA expression with no alteration in GAD_{67}, GAD_{65}, CR, or PV mRNA expressions.

Conclusion. These findings suggest that in the DLPFC the profile of alterations in these GABA-related transcripts is distinct for each psychiatric population (schizophrenia, BPD, and MDD). These profiles may used to explore unique pathogenic mechanisms that may distinguish between schizophrenia, BPD, and MDD.

3.2 INTRODUCTION

One of the core features in schizophrenia appears to be impairments in cognitive functions associated with the dorsolateral prefrontal cortex (DLPFC) and alterations to the circuitry in the DLPFC may contribute to these impairments (Weinberger et al., 1986; Goldman-Rakic, 1994; Lewis and Lieberman, 2000). Specifically, converging lines of evidence suggest that there are disturbances in γ-aminobutyric acid (GABA)-containing interneurons (Lewis et al., 2005). For example, reduced amounts of the mRNA and protein for the 67 kDa isoform of glutamate decarboxylase (GAD_{67}), an enzyme for GABA synthesis, have been consistently found in the DLPFC of individuals with schizophrenia (Akbarian et al., 1995; Mirnics et al., 2000; Guidotti et al., 2000; Volk et al., 2000; Straub et al., 2007; Hashimoto et al., 2008c). Furthermore, the alterations in inhibitory circuitry appear to implicate specific subsets of GABA neurons. The transcripts encoding for parvalbumin (PV) and somatostatin (SST), each of which is expressed in separate subsets of GABA neurons, have been found to be robustly decreased, whereas the transcript encoding calretinin (CR), which is expressed in a third subset of GABA neurons was unchanged in subjects with schizophrenia (Hashimoto et al., 2003; Morris et al., 2008; Hashimoto et al., 2008b). In addition, reduced GABA synthesis may be selectively mediated by the reductions in GAD_{67}, because mRNA and protein levels of GAD_{65}, an enzyme for GABA synthesis during high synaptic demand (Battaglioli et al., 2003), were found to have little to no alterations in the DLPFC of subjects with schizophrenia (Guidotti et al., 2000; Hashimoto et al., 2008c).

Subjects with bipolar disorder (BPD) (Potash, 2006), but not major depressive disorder (MDD), seem to share genetic risks with subjects with schizophrenia. Furthermore, subjects with BPD and MDD most likely share several environmental consequences associated with a

debilitating, persistent psychiatric disorder (e.g. frequent hospitalizations, social isolation). Therefore, in order to determine the extent to which the profile of GABA-related mRNA expression alterations in schizophrenia is shared with other major psychiatric disorder, we utilized real-time qPCR to examine the expression of GAD_{67}, GAD_{65}, CR, PV, and SST mRNAs in the DLPFC area 9 of subjects with BPD or MDD.

3.3 MATERIALS AND METHODS

Human Subjects

After consent was obtained from the next of kin, brain specimens were obtained during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA, USA). Nineteen subjects triads, each composed of one subject with bipolar disorder, or major depressive disorder and a normal comparison subject matched for sex and as closely as possible for age and post-mortem interval (PMI) were used in this study **(Table 2)**. Subject groups did not differ in mean age, PMI, RNA integrity number (RIN), brain pH, or tissue storage time (for all, $F_{2,54} > 1.04$; $p > 0.36$)

An independent committee of experienced research clinicians made consensus DSM IV (Diagnosis and Statistical Manual of Mental Disorders) (American Psychiatric Association, 1994)) diagnoses for each subject based on medical records and the results of structured interviews conducted with family members of the deceased as previously described{Glantz, 2000 3533 /id. All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

Tissue Preparation

As previously described{Volk, 2000 5752 /id}, the right hemisphere of each brain was blocked coronally, immediately frozen and stored at -80°C. Cortical gray matter was dissected from cryostat sections (20 μm) from the anterior-posterior level corresponding to the middle portion of the superior frontal sulcus were cut serially and collected into tubes containing Trizol reagent

Table 2 Characteristics of Subjects

	Control Subjects								Bipolar Disorder Subjects								
Triad	Case	Sex/race	Age	PMI[a]	pH	RIN	Storage Time[b]	Cause of death[c]	Case	DSM IV diagnosis	Sex/race	Age	PMI[a]	pH	RIN	Storage time[b]	Cause of death[c]	Medications[d]
1	10003	M/W	49	21.2	6.5	8.4	51	Trauma	1102	Bipolar Disorder NOS[g,i,j,l]	M/W	50	12.1	6.7	8.3	61	ASCVD	AD, BV, SI
2	1374	M/W	48	21.7	6.6	7.2	23	Coronary atherosclerotic disease	1121	Bipolar I Disorder[g,m]	M/W	40	18.5	6.4	8.3	59	Pulmonary Thromboembolism	BV, AP
3	1282	F/W	39	24.5	6.8	7.5	39	ASCVD	957	Bipolar I Disorder	F/W	39	22.0	6.7	8.4	83	Drowning	AD, SI
4	1298	M/W	48	24.5	6.5	7.9	36	ASCVD	886	Bipolar I Disorder[h]	M/W	45	27.3	7.1	8.3	99	Gunshot to chest	
5	1047	M/W	43	13.8	6.6	9	70	ASCVD	1020	Bipolar I Disorder[h,l,m]	M/W	42	6.0	6.7	8.5	74	Combined drug toxicity	AD, BV
6	795	M/W	68	12.0	6.8	8.2	115	Ruptured abdominal aortic aneurysm	1130	Bipolar I Disorder[e,m]	M/W	65	8.9	6.7	8	59	Carcinoma of esophagus	AD, BV, AP, SI
7	1318	F/W	58	18.8	6.6	7.4	33	ASCVD	1048	Bipolar I Disorder[m]	F/W	51	21.4	6.7	7.7	69	Asphyxiation	AD
8	1324	M/W	43	22.3	6.7	7.3	31	Dissection of the ascending thoracic aorta	697	Bipolar I Disorder[m]	M/W	39	25.9	6.6	7.8	133	Exsanguination	BV
9	1444	M/W	46	22.0	6.3	8.4	10	Pulmonary Thromboembolism	1069	Bipolar I Disorder[e]	M/W	48	27.2	6.9	8.1	66	ASCVD	
10	1086	M/W	51	24.2	6.6	8.1	64	ASCVD	1244	Bipolar I Disorder[e,j,l,m]	M/W	52	23.5	6.7	8	45	Asphyxiation	AD, BV, AP
11	1391	F/W	51	7.8	6.6	7.1	20	ASCVD	10004	Bipolar I Disorder[l]	F/W	50	11.7	6.4	8.5	52	Combined drug toxicity	AP
12	1196	F/W	36	14.5	6.4	8.2	52	Positional asphyxia	1180	Bipolar I Disorder[m]	F/W	28	22.3	6.3	7.5	55	Trauma (jump)	AD, SI
13	1293	F/W	65	18.5	6.5	7.0	38	Trauma	10006	Bipolar I Disorder[g,m]	F/W	55	19.0	6.4	8.1	51	Gunshot to head	
14	1153	M/W	55	28.0	6.1	8	57	Atherosclerotic & Hypertensive heart disease	716	Bipolar I Disorder[h,j]	M/W	58	27.5	6.8	8.3	130	Gunshot to head	
15	789	M/W	22	20.0	6.8	7.8	117	Accidental Asphyxiation	1181	Bipolar I Disorder[i,l,m]	M/W	28	27.4	6.2	8	55	Morphine toxicity	AD, BV, SI
16	686	F/W	52	22.6	7.1	8.5	135	ASCVD	1328	Bipolar Disorder NOS	F/W	49	21.5	6.7	7.5	31	Arrhythmogenic sudden cardiac arrest	AD, BV, SI
17	1247	F/W	58	22.7	6.4	8.4	45	ASCVD	1044	Bipolar I Disorder[m]	F/W	56	24.5	6.1	7.1	71	Cardiac arrhythmia	AD, BV, AP, SI
18	1092	F/B	40	16.6	6.8	8.0	64	Mitral Valve Prolapse	984	Bipolar I Disorder[m]	F/W	42	30.8	6.5	8	80	Combined drug overdose	AD, AP, SI
19	840[e]	F/W	41	15.4	6.6	9.1	106	ASCVD	945	Bipolar I Disorder[f]	F/W	43	31.8	6.7	7.2	86	Asphyxiation	AD, BV, AP, SI
	Mean		48.1	19.5	6.6	8.0	58					46.3	21.5	6.6	8.0	72		
	SD		10.6	5.1	0.2	0.6	36					9.5	7.3	0.2	0.4	26		

Table 2 cont. Characteristics of Subjects

	Major Depressive Disorder Subjects								
Triad	Case	DSM IV diagnosis	Age	PMI[a]	pH	RIN	Storage Time[b]	Cause of death[c]	Medications[d]
1	10010	Major Depressive Disorder[e,m]	42	14.3	6.4	7.6	48	Amitriptyline overdose	AD, BV
2	1226	Major Depressive Disorder[i,j,k,l,m]	44	19.3	6.5	7.5	47	ASCVD	
3	967	Major Depressive Disorder[h]	40	22.2	6.6	7.4	81	ASCVD	
4	1053	Major Depressive Disorder	47	24.0	6.6	8.1	68	ASCVD	
5	1215	Major Depressive Disorder[g]	44	11.0	6.5	7.9	50	ASCVD	BV
6	698	Major Depressive Disorder[m]	59	13.0	6.6	9.0	132	Hanging	AD, AP
7	1190	Major Depressive Disorder[h]	47	22.3	6.6	8.0	53	Asphyxiation	
8	668	Major Depressive Disorder[e,m]	34	24.3	6.6	8.1	137	Hanging	
9	863	Major Depressive Disorder	51	28.3	7.3	8.4	101	ASCVD	
10	1312	Major Depressive Disorder[g,l]	51	24.6	6.5	8.1	33	Combined drug toxicity	
11	986	Major Depressive Disorder	53	11.9	6.7	8.7	79	Bronchial asthma	AD, SI
12	1157	Major Depressive Disorder	26	13.4	6.4	7.8	57	Hanging	AD, SI
13	1041	Major Depressive Disorder[f,l,m]	52	10.3	6.5	8.4	71	Combined drug toxicity	AD, BV, AP, SI
14	1071	Major Depressive Disorder[e]	62	25.6	6.5	8.1	66	Gunshot to trunk	
15	1131	Major Depressive Disorder	29	26.6	6.9	8.5	59	Gunshot	
16	1143	Major Depressive Disorder[g,l]	49	23.4	6.4	8.1	58	Combined drug toxicity	AD, BV, SI
17	934	Major Depressive Disorder[m]	54	17.9	6.2	8.2	89	ASCVD	AD, SI
18	1289	Major Depressive Disorder	46	25.0	6.3	7.3	39	ASCVD	
19	1221	Major Depressive Disorder	28	24.8	6.6	7.2	49	Pulmonary thrombosis	AD
	Mean		45.2	20.1	6.6	8.0	69		
	SD		10.1	6.0	0.2	0.5	29		

[a]PMI indicates postmortem interval in hours
[b]storage time (months) at -80°C;
[c]ASCVD indicates arteriosclerotic cardiovascular disease;
[d]indicates prescribed medications at time of death;
[e]alcohol abuse, in remission at time of death;
[f]alcohol abuse, current at time of death;
[g]alcohol dependence, in remission at time of death;
[h]alcohol dependence, current at time of death;
[i]other substance abuse, in remission at time of death;
[j]other substance abuse, current at time of death;
[k]other substance dependence, in remission at time of death;
[l]other substance dependence, current at time of death;
[m]history of psychotic features;
AD = antidepressants; BV = Benzodiazines/Sodium Valproate; AP = antipsychotics; SI = SSRIs

(Invitrogen, Carlsbad, CA, USA) for RNA isolation or mounted on Super frost plus glass slides (VWR International, West Chester, PA, USA) for Nissl-staining. The location of DLPFC area 9 was determined from the Nissl-stained sections using cytoarchitectonic criteria as previously described (Volk et al., 2000). Total RNA was isolated from Trizol homogenates of sections, further purified by RNeasy columns (Qiagen, Valencia, CA, USA) and RNA integrity was assessed by measuring RIN (Imbeaud et al., 2005) using the Bioanalyzer 2100 (Agilent Technologies, Walbronn, Germany). For all subjects used in this study, RIN was ≥ 7.0.

Real-time qPCR

For the five GABA-related transcripts, real-time qPCR analyses (Glorioso et al., 2006) were performed on DLPFC samples from the subject triads. Using 50 ng of total RNA, cDNA synthesis by random primers and SuperScript II reverse transcriptase (Invitrogen) was conducted. All primer pairs used **(Table 3)** exhibited high amplification efficiency (> 97%) in the standard curve analysis and specific single products in dissociation curve analysis. After primer validation, the comparative threshold cycle (Ct) measurement was performed for quantification using SYBR Green I Dye (Applied Biosystems, Foster City, CA, USA) and Stepone Plus Real-time PCR instrument (Applied Biosystems) according to the manufacturer's instructions. Each qPCR run included the three subjects in a triad and amplified all 8 transcripts of interest in quadruplicate using a plate with 96 wells (3 subjects x 8 transcripts x 4 replications). Three internal control transcripts encoding for beta-actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase were amplified for each subject. These internal control transcripts were selected based on their stable expression across subjects with schizophrenia as previously described(Hashimoto et al., 2008c). Furthermore, the internal control transcripts were stably expressed across our subject groups regardless of diagnosis **(Table 4)**. The difference in cycle threshold for each GABA-related transcript was calculated by subtracting the mean cycle threshold for the three internal controls from the cycle threshold of each GABA-related transcript. This difference in cycle threshold (dCt) represents the $\log_2$-transformed expression ratio of each GABA-related transcript to the geometric mean of the three internal control transcripts (Vandesompele et al., 2002); therefore, the relative expression level of each GABA-related transcript was determined as 2^{-dCt}.

Table 3 Primer pairs and efficiency of amplified gene products

Gene	Accession	Size (position)	Forward primer	Reverse primer	Ex
Beta actin (ACTB)	NM_001101	73 bp (23-95)	AGCCTCGCCTTTGCCGA	GCGCGGCGATATATCATCATC	0.97
Cyclophilin (CYC)	NM_021130	126 bp (159-284)	GCAGACAAGGTCCCAAAG	GAAGTCACCACCCTGACAAC	0.98
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	87 bp (556-642)	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	0.97
67 kDa glutamate decarboxylase (GAD67)	NM_000817	86 bp (2495-2580)	GTTTCCCGCTCCAAGAGAAT	TGGAGTTGTTGGACAAGCTG	1.09
65 kDa glutamate decarboxylase (GAD65)	NM_000818	105 bp (177-281)	GTCGGAAGATGGCTCTGG	AGGGCGCACAGTTTGTTT	0.98
Parvalbumin (PV)	NM_002854	140 bp (111-250)	GCTACCGACTCCTTCGAC	ATGAATCCCAGCTCATCC	1.00
Somatostatin (SST)	NM_001048	93 bp (319-411)	ATGCCCTGGAACCTGAAGAT	CCATAGCCGGGTTTGAGTTA	1.07
Calretinin (CR)	NM_001740	145 bp (395-539)	AGCGCCGAGTTTATGGAG	GGGTGTATTCCTGGAGCTTG	1.05

Ex (primer efficiency): Ex is defined by $Rn = Ro*(1+Ex)_n$. Rn = amplification signal of target molecules at cycle n, Ro = initial signal, n = number of cycles. For all primer sets, PCR thermal cycling was 10 min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec and 62°C for 1 min.

Table 4 Internal Control Ratios

	Control (mean ± SD)*	BPD (mean ± SD)*	MDD (mean ± SD)*	$F_{2,49}$ value	p-value
AB/GAP	0.81 ± 0.091	0.80 ± 0.086	0.81 ± 0.056	0.016	0.98
Cyc/AB	0.48 ± 0.054	0.47 ± 0.040	0.47 ± 0.043	0.17	0.85
GAP/Cyc	2.62 ± 0.38	2.67 ± 0.23	2.65 ± 0.27	0.032	0.97

*The mean 2^{-dCt} relative expression values for beta actin (AB), glyceraldehyde-3-phosphate dehydrogenase (GAP), and Cyclophilin (Cyc)

Statistical Analysis

To determine the diagnosis-related expression differences of each GABA-related transcript, we utilized an analysis of covariance (ANCOVA) model with SPSS (SPSS, Inc., Chicago, IL). The data were averaged across the four replicates and transformed into relative expression levels (2^{-dCt}) in order for intuitive plots of data (i.e. higher values represent larger relative expression). Confirmation of the 2^{-dCt} values for normal distribution within each subject group was determined before statistical analyses were performed. The ANCOVA model was performed with a main effect of diagnostic group and covariates of sex, age, PMI, RIN, brain pH, and storage time. Storage time as a covariate was not significant the model and, thus, was excluded in the reported analyses. Multiple comparisons were controlled by adjusting for simultaneous inference of significance levels using the Bonferroni-Holm method (Volk et al., 2000) in which p values are ordered from the smallest (i = 1) to the largest (i = N) among multiple comparisons; the significance level for each comparison is defined as $\alpha=0.05/([N+1]-i)$. For PV mRNA analysis, a Tukey's multiple comparison test was used in post-hoc comparisons across diagnostic groups. However, because the effect of age on SST mRNA expression was significant and post-hoc analyses do not take cofactors such as age into account, we performed 2 separate ANCOVAs in which control subjects and subjects with BPD or subjects with MDD were included.

The influences of potential confounding variables on the relative expression values in each diagnostic group were assessed with ANCOVA models using each confounding variable as the main effect and sex, age, postmortem interval, pH, and RIN as covariates. Correlations between age and mRNA expression were assessed by Pearson's correlation analyses.

3.4 RESULTS

Alterations in GABA-related transcripts across subject groups

In order to determine the profile of expression in 5 different GABA-related transcripts in the DLPFC of subjects with BPD or MDD, the expression levels of GAD_{67}, GAD_{65}, CR, PV, and

SST were quantified by real-time qPCR in 19 subject triads. The primer sets for all the GABA-related and internal control transcripts have been previously used (Hashimoto et al., 2008c) with the exception of CR **(Table 3)**. The rank order of the mean relative expression levels of each of the GABA-related transcripts in normal control subjects closely matched previously reported rank order of mean expression levels of these mRNAs (Hashimoto et al., 2008c) with SST and GAD_{67} having the highest levels of expression, CR and PV having the lowest levels of expression, and GAD_{65} having an intermediate level of expression **(Figure 11)**. Furthermore, in normal control subjects SST mRNA expression was robustly and significantly correlated with age ($r = -0.86$; $p < 0.00001$) **(Figure 12)**.

The expression profile of the GABA-related transcripts for each subject group is shown in Figure 11. Across the three subject groups, the mean levels of GAD_{67}, GAD_{65}, and CR mRNA were comparable and, consequently, diagnosis did not significantly effect the expression of these transcripts ($F_{2,49} = 0.56$, $p = 0.57$; $F_{2,49} = 0.91$, $p = 0.82$; $F_{2,49} = 2.15$, $p = 0.38$, respectively.

The expression level of PV mRNA was significantly reduced in subjects with BPD. There was a significant effect of diagnosis on the mean expression levels of PV mRNA which remained after adjustment for multiple comparisons ($F_{2,49} = 10.73$; adjusted $p < 0.001$). Post-hoc analysis revealed a significant reduction in mean PV mRNA expression in subjects with BPD as compared to normal control subjects (18%; $p=0.004$) or to subjects with MDD (20%, $p = 0.001$), but no significant difference between normal control subjects and subjects with MDD ($p = 0.88$).

Qualitative examination of the data suggested that, at least in subjects with MDD, there was significant reduction in SST mRNA expression. However, there was only a trending effect of diagnosis on mean expression levels of SST mRNA ($F_{2,49} = 2.76$; $p = 0.07$) that did not remain after correction for multiple comparisons (adjusted $p = 0.30$). However, because of the large standard deviation in SST mRNA expression due to correlation with age **(Figure 12)** and the inability of post-hoc analyses to correct for covariates (i.e. age), we also performed 3 separate ANCOVA comparisons with normal control subjects and subjects with BPD or MDD. When compared to normal comparison subjects, there was a trending effect of diagnosis on the mean expression level of SST mRNA in subjects with bipolar disorder (-14%; $F_{1,31} = 3.34$; $p = 0.08$) and a significant effect of diagnosis on the mean expression level of SST mRNA in subjects with MDD (-18%; $F_{1,31} = 9.31$; $p = 0.005$); however, there was no significant effect of diagnosis on

SST mRNA expression when subjects with BPD were compared to subjects with MDD ($F_{1,31}$ = 0.604; p = 0.85).

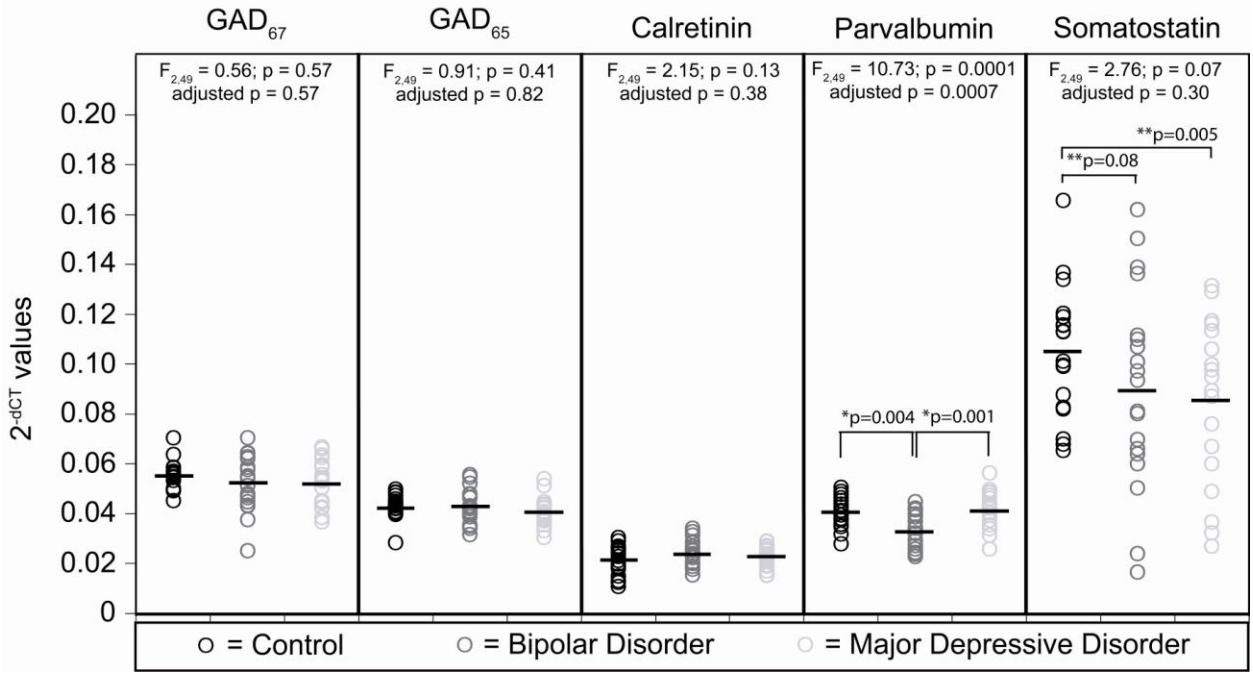

Figure 11 GABA-related Transcript Expression Levels Across Three Subject Groups.

The 2^{-dCt} relative expression values are shown for each subject (circles). For each transcript, the mean 2^{-dCt} relative expression values are shown for each subject group (black hash marks). The statistics (top of graph) represent the effect of diagnosis for each GABA-related transcript. For correction of multiple comparisons, the Bonferroni-Holm p-values are given as "adjusted" p-values (top of graph). PV transcript expression was significantly reduced in subjects with BPD before and after correction for multiple comparisons. Effect of diagnosis for SST transcript expression reached trending expression that did not remain after correction for multiple comparisons.

*post-hoc Tukey's multiple comparison test p-values

**p-values for the effect of diagnosis from one-way ANCOVAs between the subject groups indicated

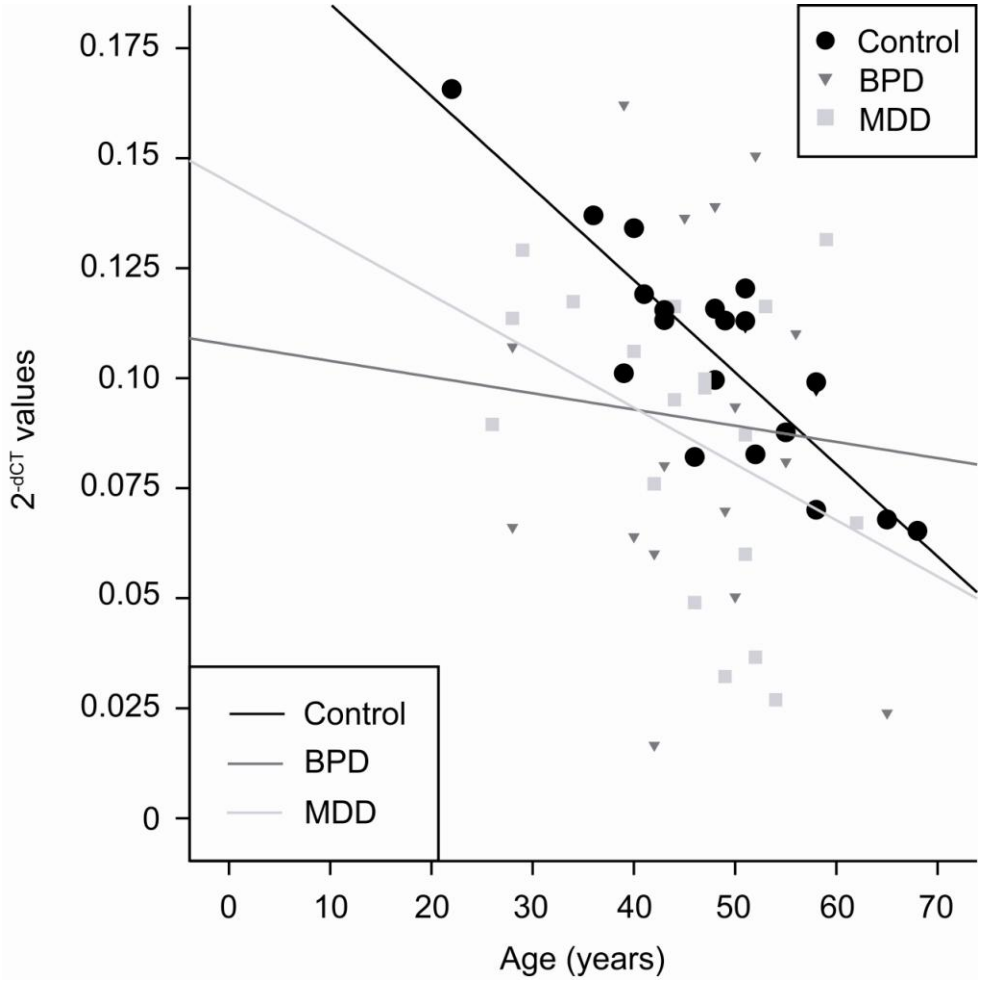

Figure 12 The 2^{-dCt} Relative Expression Values of SST mRNA in Human Area 9 as a Function of Age.

Expression levels of SST mRNA are significantly correlated with age in normal control subjects (r=-0.86; p=<0.00001), but not in subjects with BPD (r=-0.09; p=0.72) or MDD (r=-0.39; p=0.10).

Potential Effect of other Factors on GABA-related Transcript Expression

In the first ANCOVA model of SST mRNA expression which included all three subject groups, age was a significant ($F_{1,49}$ = 10.69; p <0.01) determinant of mean expression levels. Further examination revealed that normal control subjects had a significant correlation between age and SST mRNA expression (r = -0.86; p < 0.00001); in contrast, both subjects with BPD (r=-0.09; p=0.72) and MDD (r = -0.39; p = 0.10) did not have significant correlations between age and SST mRNA expression **(Figure 12)**.

The effects of potential confounding factors on trending or significantly altered GABA-related transcript expression are shown in Figure 13. We compared the mean expression of PV mRNA in subjects with BPD **(Figure 13A)** and the mean expression of SST mRNA in subjects with BPD **(Figure 13B)** or MDD **(Figure 13C)** as a function of sex, death by suicide, antidepressant medication use at time of death (ATOD), use of benzodiazepines or sodium valproate ATOD, antipsychotic medication use ATOD, diagnosis of substance abuse or dependence ATOD, use of selective serotonin reuptake inhibitors (SSRIs) ATOD, or history of psychosis.

A majority of the potential confounding factors did not significantly affect transcript expression in the relevant subject group. However, perhaps by chance due to the large number of comparisons without multiple comparison corrections, a few of the potential confounds had a statistically significant effect on transcript level. In order to determine if these confounding effects were driving the reductions we saw in transcript levels, we performed ANCOVAs in which we restricted our examination to diagnostic subjects that did not have the relevant confound and their matched normal comparison subject. These comparisons are shown in Figure 14. Even with the reduced power of these comparisons, all but one transcript alteration remained either trending or significant. When we restricted our examination of SST mRNA expression to subjects with BPD that did not take SSRIs ATOD and their matched normal comparison subjects (n = 10 pairs), there was no significant effect of diagnosis on mean SST mRNA levels **(Figure 14C)** suggesting the trending reduction of SST mRNA expression in subjects with BPD is due to usage of SSRI medication.

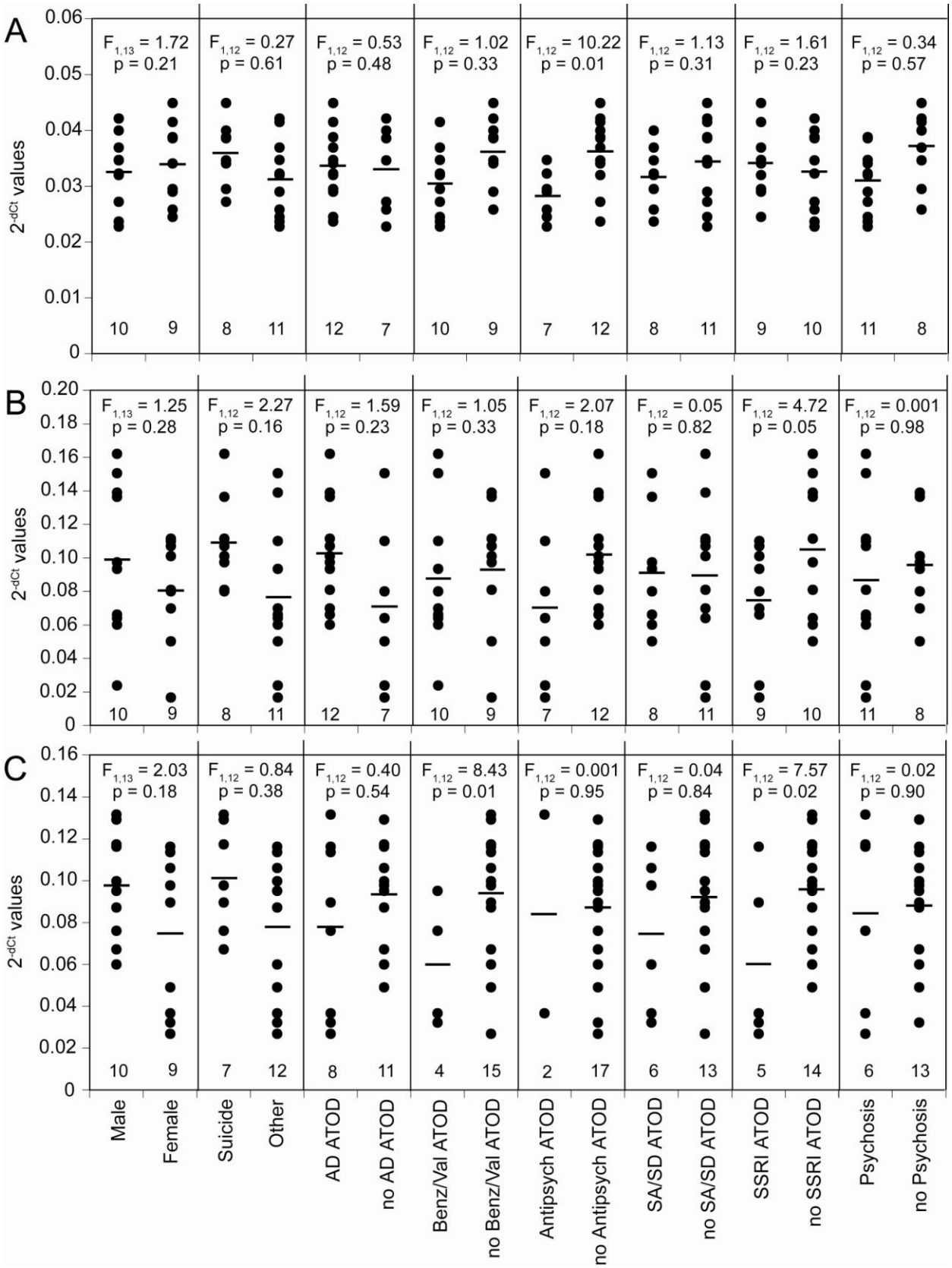
A
B
C
2-dCt values
F1,13 = 1.72 p = 0.21
F1,12 = 0.27 p = 0.61
F1,12 = 0.53 p = 0.48
F1,12 = 1.02 p = 0.33
F1,12 = 10.22 p = 0.01
F1,12 = 1.13 p = 0.31
F1,12 = 1.61 p = 0.23
F1,12 = 0.34 p = 0.57
F1,13 = 1.25 p = 0.28
F1,12 = 2.27 p = 0.16
F1,12 = 1.59 p = 0.23
F1,12 = 1.05 p = 0.33
F1,12 = 2.07 p = 0.18
F1,12 = 0.05 p = 0.82
F1,12 = 4.72 p = 0.05
F1,12 = 0.001 p = 0.98
F1,13 = 2.03 p = 0.18
F1,12 = 0.84 p = 0.38
F1,12 = 0.40 p = 0.54
F1,12 = 8.43 p = 0.01
F1,12 = 0.001 p = 0.95
F1,12 = 0.04 p = 0.84
F1,12 = 7.57 p = 0.02
F1,12 = 0.02 p = 0.90
10 9 8 11 12 7 10 9 7 12 8 11 9 10 11 8
10 9 7 12 8 11 4 15 2 17 6 13 5 14 6 13
Male
Female
Suicide
Other
AD ATOD
no AD ATOD
Benz/Val ATOD
no Benz/Val ATOD
Antipsych ATOD
no Antipsych ATOD
SA/SD ATOD
no SA/SD ATOD
SSRI ATOD
no SSRI ATOD
Psychosis
no Psychosis

Figure 13 The Effects of Confounding Factors in Diagnostic Groups with Trending or Significantly Altered GABA-related Transcripts.

The effects of sex, suicide, antidepressant medication use at the time of death (AD ATOD), use of benzodiazepines or sodium valproate at the time of death (Benz/Val ATOD), antipsychotic medication use at the time of death (Antipsych ATOD), diagnosis of substance abuse or dependence at the time of death (SA/SD ATOD), use of selective serotonin reuptake inhibitors at the time of death (SSRI ATOD), and a history of psychosis are evaluated for PV mRNA expression in subjects with BPD (A), SST mRNA expression in subjects with BPD (B), and SST mRNA expression in subjects with MDD (C). Mean (hash mark) and individual (circle) 2^{-dCt} relative expression values are grouped by potential confounding factors. Note the varying scales for the Y-axis between (A) and (B or C). Numbers below circles indicate the number of subjects in each diagnostic group for each category.

3.5 DISCUSSION

In this study, we examined the profile of expression alterations in 5 GABA-related mRNA in the DLPFC area 9 of two psychiatric populations (BPD and MDD) which we have previously characterized in subjects with schizophrenia (Hashimoto et al., 2008c). With the exception of CR, our primer sets have been used in a previous study (Hashimoto et al., 2008c) examining the expression levels of these GABA-related transcripts in subjects with schizophenia; the rank-order of expression levels for each GABA-related mRNA in the normal control subject group was comparable to previously reported rank-order of expression levels in a separate normal control subject group (Hashimoto et al., 2008c). In addition, in normal control subjects SST mRNA expression was robustly correlated with age **(Figure 12)** confirming previously reported correlations between age and SST mRNA or protein expression in the rat (Vela et al., 2003), monkey (Hayashi et al., 1997), and human (Morris et al., 2008). These data suggest that the expression levels of the GABA-related transcripts in this study are reliable and comparable to previously reported expression levels validating our decision to measure mRNA expression with reverse transcriptase qPCR.

In subjects with BPD, the expression of GAD_{67}, GAD_{65}, and CR mRNAs were not significantly altered in DLPFC area 9. In addition, PV mRNA expression was significantly decreased in subjects with BPD when compared to either normal control subjects or subjects with MDD. There was a trending reduction in SST mRNA expression in subjects with BPD as compared to normal control subjects; however, this alteration is likely due to an effect of SSRI usage at time of death **(Figure 14C)**. In subjects with MDD, the expression of GAD_{67}, GAD_{65}, CR, and PV mRNAs were not significantly altered in DLPFC area 9. Furthermore, in subjects with MDD SST mRNA expression was significantly reduced when compared to normal controls subjects. In contrast to these results, our previous study (Hashimoto et al., 2008c) demonstrated a distinctly different profile of GABA-related alterations in the DLPFC area 9 of subjects with schizophrenia in which the expression of CR mRNA was not significantly altered; however, there were robust reductions in GAD_{67}, PV, and SST mRNAs and a smaller reduction in GAD_{65}

mRNA. Together, these findings indicate that the profiles of alterations in GABA-related transcripts found in the DLPFC of subjects with BPD, MDD, or schizophrenia are distinct from one another.

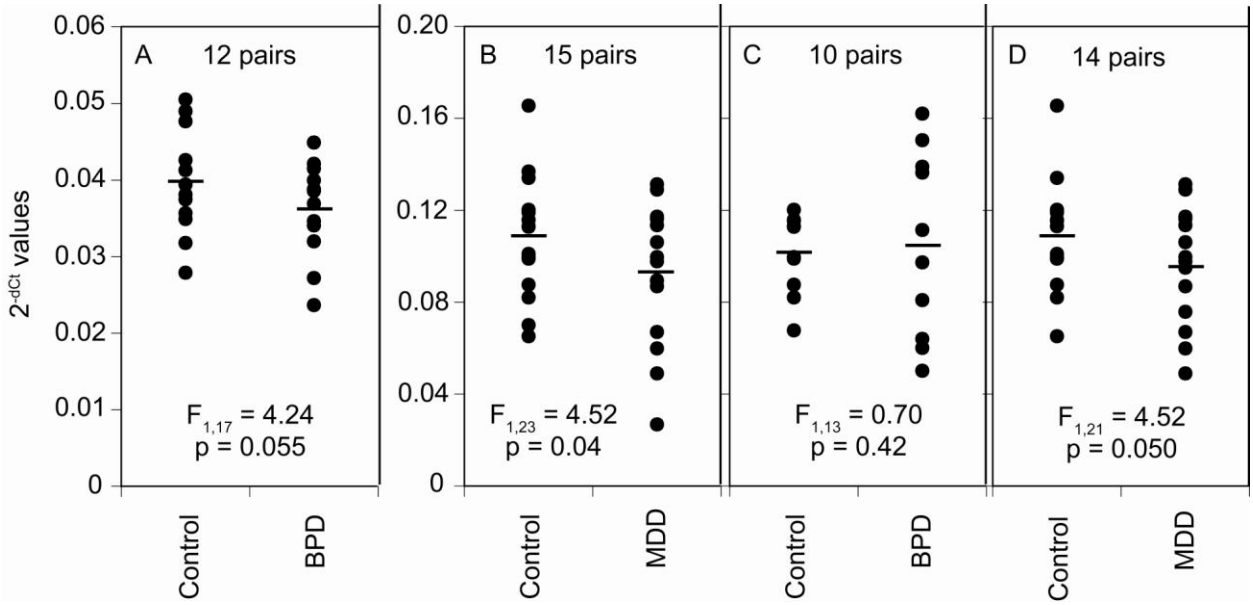

Figure 14 GABA-related Transcript Expression Levels after Removal of Subject Pairs with Potential Confounding Factors.

The mean (hash mark) and individual (circle) 2^{-dCt} relative expression values are plotted by diagnostic group for PV mRNA (A) and SST mRNA (B, C, and D). Note the varying scales for the Y-axis between (A) and (B, C, and D). Subjects with BPD that had antipsychotic medication use at the time of death and their matched controls were removed and the subjects with BPD (n = 12) demonstrated a trending reduction in PV mRNA expression (A). Subjects with MDD that were using benzodiazepines or sodium valproate at the time of death and their matched control subjects were removed and the subjects with MDD (n = 15) demonstrated a significant reduction of SST mRNA expression (B). Subjects with BPD (C) or MDD (D) that were using selective serotonin reuptake inhibitors at the time of death and their matched control subjects were removed and the subjects with MDD (n = 14) demonstrated a significant reduction of SST mRNA expression (D) while the subjects with BPD (n = 10) did not demonstrate a significant alteration in SST mRNA expression (C).

Several lines of evidence indicate that the reduction in PV mRNA expression in subjects with BPD is not a consequence of factors frequently associated with the illness. PV expression is not altered by chronic antipsychotic treatment in several animal models. For example, PV mRNA expression was not altered in the PFC of monkeys with long-term exposure to high plasma levels of haloperidol, which produced marked extrapyramidal symptoms and required treatment with benztropine mesylate (Hashimoto et al., 2003); furthermore, PV expression was reported to be increased (Scruggs and Deutch, 1999) or not altered (Cahir et al., 2005) in the frontal cortex of mice that were chronically treated with either haloperidol or clozapine. Additionally, when we compared subjects with BPD that were not medicated with antipsychotics ATOD to their matched controls there remained a trending reduction in PV mRNA expression **(Figure 14A)**. Furthermore, alterations in PV mRNA expression was not associated with death by suicide, use of antidepressant medication, benzodiazepines, valproate, or SSRIs at time of death, diagnosis of substance abuse or dependency at time of death, or a history of psychosis **(Figure 13A)**. These data suggest that the reduction in PV mRNA is associated with the disease process of BPD, or at least not a consequence of other factors commonly associated with the illness.

Converging lines of evidence suggest that the significant reduction in the expression of SST mRNA in subjects with MDD is related to the disease process. The use of benzodiazepines or valproate at time of death in subjects with MDD was associated with lower expression of SST mRNA **(Figure 13C)**. However, benzodiazepine or valproate use at time of death is not associated with significant alterations SST mRNA expression in the DLPFC of subjects with schizophrenia (Morris et al., 2008) or subjects with BPD **(Figure 13A)**. Consistent with these data, chronic administration of diazepam did not significantly alter SST protein levels in rat frontoparietal cortex (Martinez-Ferrer et al., 2000). Furthermore, there was a significant reduction in SST mRNA in subjects with MDD that were not using benzodiazepines or valproate at time of death compared to their matched control subjects **(Figure 14B)**. The use of SSRIs at time of death in subjects with MDD was associated with lower expression of SST mRNA. This association is also seen in subjects with BPD **(Figure 13B)** suggesting that SSRIs may influence SST mRNA expression. However, when subjects with MDD that were not using SSRIs at time of death were compared to their matched control subjects there was a significant reduction in SST mRNA expression **(Figure 14D)**. Additionally, the alterations in SST mRNA expression in subjects with MDD were not associated with death by suicide, use of antidepressant medication

at time of death, diagnosis of substance abuse or dependency at time of death, or a history of psychosis. Together, these data suggest that the reduction in SST mRNA expression is associated with the disease process of MDD and not due to other common factors associated with the illness.

Effect of SSRIs on SST mRNA Expression

The trending reduction in SST mRNA expression in subjects with BPD was associated with the use of SSRIs at time of death **(Figure 13B)**. Further examination of subjects with bipolar disorder that were not using SSRIs at time of death did not demonstrate a significant alteration in SST mRNA expression when compared to their matched control subjects **(Figure 14C)** suggesting that the trending alteration in SST mRNA expression in subjects with BPD is associated with SSRI use. Subjects with MDD also demonstrated a significant interaction of SSRI use on the expression of SST mRNA expression **(Figure 13C)** consistent with the hypothesis that SSRI use alters SST mRNA expression. Interestingly, in normal control subjects the expression of SST mRNA expression was robustly correlated with age **(Figure 12)**; this correlation was not apparent in either subject group suggesting that a third factor such as SSRI usage may be influencing SST mRNA expression. These data suggest an interaction between SSRI usage and levels of SST mRNA in the DLPFC; however, further verification in animal models is needed to confirm this hypothesis.

GAD_{67} Transcript Expression in BPD

We did not observe an alteration in the expression of GAD_{67} mRNA in the DLPFC of subjects with BPD. In contrast, previous studies have demonstrated reduced GAD_{67} protein in the DLPFC of subjects with BPD (Guidotti et al., 2000) and reduced GAD_{67} mRNA+ neuron density in the DLPFC area 9 in subjects with BPD (Woo et al., 2008). In both of the studies, the reduction in GAD_{67} was found only in subjects with BPD that had a history of psychosis. When examination of the effect of a history of psychosis in subjects with BPD (n=11) did not reveal any effect on GAD_{67} mRNA expression; however, it is possible that we did not have enough power to detect any significant effect.

The distinct GABA-related neuropathologies found in each of these subject groups may arise from disparate upstream mechanisms; alternatively, the GABA-related neuropathology found in schizophrenia is a culmination of one or more upstream mechanisms common to either BPD and/or MDD.

In subjects with BPD, there was a reduction in PV mRNA expression with no change in GAD_{67} mRNA expression suggesting that it is not due to a common factor that may induce both a decrease in PV and GAD_{67} mRNA. Instead, this reduction could reflect alterations in transcriptional regulation secondary to the genetic liability for the illness. For example, the PV gene lies in the vicinity of the marker D22S278 (GenBank NT_011520) a putative susceptibility gene for bipolar disorder as well as schizophrenia (Schwab and Wildenauer, 2000).

4.0 NPY MRNA EXPRESSION IN THE PREFRONTAL CORTEX: SELECTIVE REDUCTION IN THE SUPERFICIAL WHITE MATTER OF SUBJECTS WITH SCHIZOAFFECTIVE DISORDER

4.1 ABSTRACT

Background. Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia include reduced expression of the messenger RNA (mRNA) for somatostatin (SST), a neuropeptide present in a subpopulation of γ-aminobutyric acid (GABA) neurons. Neuropeptide Y (NPY) is expressed in a subset of SST-containing interneurons and lower levels of NPY mRNA have also been reported in schizophrenia spectrum disorders. However, whether the alterations in these two transcripts identify the same, particularly vulnerable, subset of GABA neurons has not been examined.

Methods. We used in situ hybridization to quantify NPY mRNA levels in DLPFC gray and white matter from 23 pairs of subjects with schizophrenia or schizoaffective disorder and matched normal control subjects; results were compared to those from a previous study of SST mRNA expression in the same subjects.

Results. In contrast to SST mRNA, NPY mRNA levels were not significantly lower in the gray matter of subjects with schizophrenia or schizoaffective disorder. However, NPY, but not SST, mRNA expression was significantly lower in the superficial white matter of subjects with schizoaffective disorder.

Conclusion. These findings suggest that the alterations in SST-containing interneurons in schizophrenia and schizoaffective disorder are selective for the subset that do not express NPY mRNA, and that lower NPY mRNA expression in the superficial white matter may distinguish subjects with schizoaffective disorder from those with schizophrenia.

Keywords: GABA, interneurons, mood disorder, somatostatin, suicide

4.2 INTRODUCTION

Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) appear to be a common feature of schizophrenia (Torrey et al., 2005; Akbarian and Huang, 2006). For example, lower levels of the mRNA that encodes for the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}), an enzyme for GABA synthesis, have been consistently found in the DLPFC of individuals with schizophrenia (Akbarian et al., 1995; Mirnics et al., 2000; Guidotti et al., 2000; Volk et al., 2000; Hashimoto et al., 2005; Straub et al., 2007); this decrease is due to a marked reduction in GAD_{67} mRNA expression in a minority (~25-35%) of GABA neurons, with apparently normal levels of expression in the remaining neurons (Akbarian et al., 1995; Volk et al., 2000). The affected neurons include the GABA neurons that express the calcium-binding protein, parvalbumin (PV) (Hashimoto et al., 2003), or the neuropeptide, somatostatin (SST) (Morris et al., 2008), whereas the ~50% of GABA neurons that express calretinin (CR) appear to be unaffected (Woo et al., 1998; Hashimoto et al., 2003; Sakai et al., 2008).

In the frontal cortex of rodents, ~40% of SST neurons also express NPY and most NPY neurons contain SST (Hendry et al., 1984; Kubota et al., 1994). Both SST (Morris et al., 2008) and NPY (Caberlotto et al., 2000) mRNAs are expressed by neurons in the gray and white matter of the human DLPFC. Some studies have found lower levels of NPY mRNA (Mellios et al., 2008; Hashimoto et al., 2008a) or protein (Gabriel et al., 1996) in homogenates containing both gray and white matter from the DLPFC of subjects with schizophrenia or schizoaffective disorder, suggesting that the NPY-containing subclass of SST neurons is preferentially affected in these illnesses (Morris et al., 2008). However, we found that lower SST mRNA expression in schizophrenia was restricted to cortical layers 2-superficial layer 6 (Morris et al., 2008), whereas most NPY mRNA expression in the human cortex is located in deep layer 6 and the superficial

white matter (Caberlotto et al., 2000). Consistent with these observations, the co-localization of SST and NPY mRNAs in the rodent cerebral cortex is most prominent in layer 6 and is uncommon in the superficial layers (Wang et al., 2004b).

Consequently, in order to determine if the levels of NPY and SST mRNAs are altered in the same or different populations of DLPFC neurons, we used in situ hybridization and autoradiographic analyses to quantify NPY mRNA expression in the gray and white matter compartments of DLPFC area 9 from 23 pairs of subjects with schizophrenia or schizoaffective disorder and matched normal control subjects in which we had previously measured SST mRNA expression using an identical approach (Morris et al., 2008).

4.3 MATERIALS AND METHODS

Human subjects. Brain tissue specimens were obtained from the Allegheny County Medical Examiner's Office at the time of autopsy with the consent of the next-of-kin. Subjects with schizophrenia (n = 15) or schizoaffective disorder (n = 8) were each matched with one normal control subject for sex, and as closely as possible for age and postmortem interval (PMI) (Table 2). Subject groups did not differ in mean age, PMI, brain pH, RNA integrity number (RIN), or tissue storage time at -80^0 C (for all $t < 1.61$; $p > 0.11$). Additional demographic and clinical details are provided in our previous study of SST mRNA expression in this subject cohort (Morris et al., 2008). An independent committee of experienced research clinicians made consensus DSMIV (Diagnosis and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994)) diagnoses based on structured interviews conducted with family members of the deceased and a review of medical records. All procedures were approved by the University of Pittsburgh's Institutional Review Board for Biomedical Research and Committee for Oversight of Research Involving the Dead.

Tissue preparation. For each brain specimen, coronal blocks from the right frontal cortex were immediately frozen and stored at -80°C. Serial sections (20 μm) containing the superior frontal

gyrus were cut on a cryostat, thaw-mounted onto glass slides and stored at -80°C until processed. Adjacent sections were collected into tubes containing Trizol (Invitrogen Corp, Carlsbad, CA) in order to obtain RNA for RIN measures using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol as previously described (Hashimoto et al., 2008a). The location of DLPFC area 9 was identified by cytoarchitectonic criteria in Nissl-stained sections as previously described (Glantz et al., 2000; Volk et al., 2000; Volk et al., 2001). Three sections per subject, at intervals of approximately 300 µm, were matched for anterior-posterior location within subject pairs, and used to assess NPY mRNA expression.

In situ hybridization. Templates for the synthesis of the antisense and sense riboprobes for human NPY mRNA were first generated by polymerase chain reaction (PCR). The specific primers amplified a 430 base pair fragment of human NPY. These fragments corresponded to bases 34-463 of the human (GenBank NM_000905) NPY gene. Nucleotide sequencing confirmed 100% homology of the amplified fragment to the previously reported sequence. The fragment was then subcloned into a plasmid (pSTBlue-1, Novagen, Madison, WI). The antisense and sense riboprobes were transcribed in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNeasy mini spin columns (Qiagen, Valencia, CA).

For each matched subject pair, one section from each pair was processed side-by-side in three separate runs. Prior to the hybridization reaction, tissue sections were fixed with 4% paraformaldehyde in PBS solution, acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine/0.9% NaCl for 10 minutes, dehydrated with a graded alcohol series, and then defatted in chloroform for 10 minutes. The sections were then hybridized with ^{35}S-labeled riboprobes (2.0 X 10^6 cpm/µl) in hybridization buffer at 56°C for 16 hours. The hybridization buffer contained 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 (0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA), 50 mM dithiothreitol, 0.2% SDS, and 100 µg/ml yeast tRNA. Following the hybridization reaction, sections were washed in a solution of 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63°C, treated with

RNase A (20 μg/ml) at 37°C, washed in 0.1 X SSC (1.5 mM NaCl, 150 μM sodium citrate) at 67°C, dehydrated through a graded ethanol series, and air dried. Sections from both subjects in a pair were exposed on the same BioMax MR film (Kodak, Rochester, NY) for 4 days, and then coated with NTB2 emulsion (Kodak) diluted 2:1 with water. The emulsion was exposed for 9 days at a constant temperature of 4°C. The slides were developed with D-19 (Kodak) and counterstained with Cresyl-violet.

Quantification of mRNA expression levels. Each section was randomly coded, so that subject number and diagnosis were unknown to the single rater (RS). Autoradiographic films were trans-illuminated and captured on video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID; Imaging Research Inc., London, Ontario, Canada) as previously described (Eggan et al., 2008). Digitized images of adjacent Nissl-stained sections were superimposed onto autoradiographic images to draw contours of the full cortical thickness of the locations in area 9 that were cut perpendicular to the pial surface. Optical density measures within each sampled area were calibrated to radioactive Carbon-14 standards (ARC Inc., St. Louis, MO), exposed on the same autoradiographic film, and expressed as nanocuries per gram (nCi/g) of tissue. The mean (SD) total areas of gray matter sampled for each control and schizophrenia subject were 114 (55) mm^2 and 115 (48) mm^2, respectively. Optical density measures in the superficial white matter were determined in a zone extending 800 μm below, and with a contour that followed, the layer 6/white matter border of the sampled gray matter zones. The mean (SD) total areas of superficial white matter sampled were 10.7 (5.7) mm^2 for control subjects and 12.9 (5.5) mm^2 for subjects with schizophrenia. Total white matter was sampled by outlining the gray matter/white matter border and including all white matter on the section. The mean total areas (SD) of white matter sampled were 95 (39) mm^2 for control subjects and 105 (49) mm^2 for schizophrenia subjects.

***Statistical analyses*.** Two analyses of covariance (ANCOVA) models were used to test differences in NPY mRNA expression between control subjects and subjects with schizophrenia. The data were averaged across the three sections per subject before statistical analyses. The first ANCOVA model used diagnostic group as the main effect, pair as a blocking effect, and storage time, brain pH, and RIN as covariates. Brain pH and RIN were included as covariates because

they may influence mRNA quantity and integrity (Harrison et al., 1995; Stan et al., 2006). The pair effect reflects the matching of individual subject pairs for sex, age and PMI. 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 a main effect of diagnostic group and covariates of sex, age, PMI, brain pH, RIN, and storage time. Storage time as a covariate was not significant in either model and, was excluded in the reported analyses. Both models produced comparable results for diagnostic group effect; because age showed trend level effects, the results of the second model are reported.

The potential influence of history of substance abuse/dependence, diagnosis of schizoaffective disorder, medications at time of death, or death by suicide on the within-pair percentage of differences in mRNA expression was assessed by two-sample t-test analyses. Correlations were assessed by Pearson's correlation analyses.

4.4 RESULTS

Specificity of NPY riboprobe. Several lines of evidence confirm the specificity of the riboprobe for NPY mRNA used in this study. First, NPY mRNA expression had the distinctive distribution previously reported for NPY-containing interneurons in the human frontal cortex (Caberlotto et al., 2000). Specifically, NPY mRNA levels were lowest in layer 1, moderate in layer 2, low in layers 3-5, and high in layer 6 and the white matter (Figure 15A). Second, the presence of intensely NPY mRNA-positive neurons in the superficial and deep white matter (Figure 15A) is consistent with previous descriptions of NPY mRNA-positive and immunopositive cell bodies in human frontal cortex (Caberlotto et al., 2000). Third, emulsion-coated sections demonstrated silver grain clusters over large, faintly Nissl-stained neuronal nuclei, whereas the smaller and

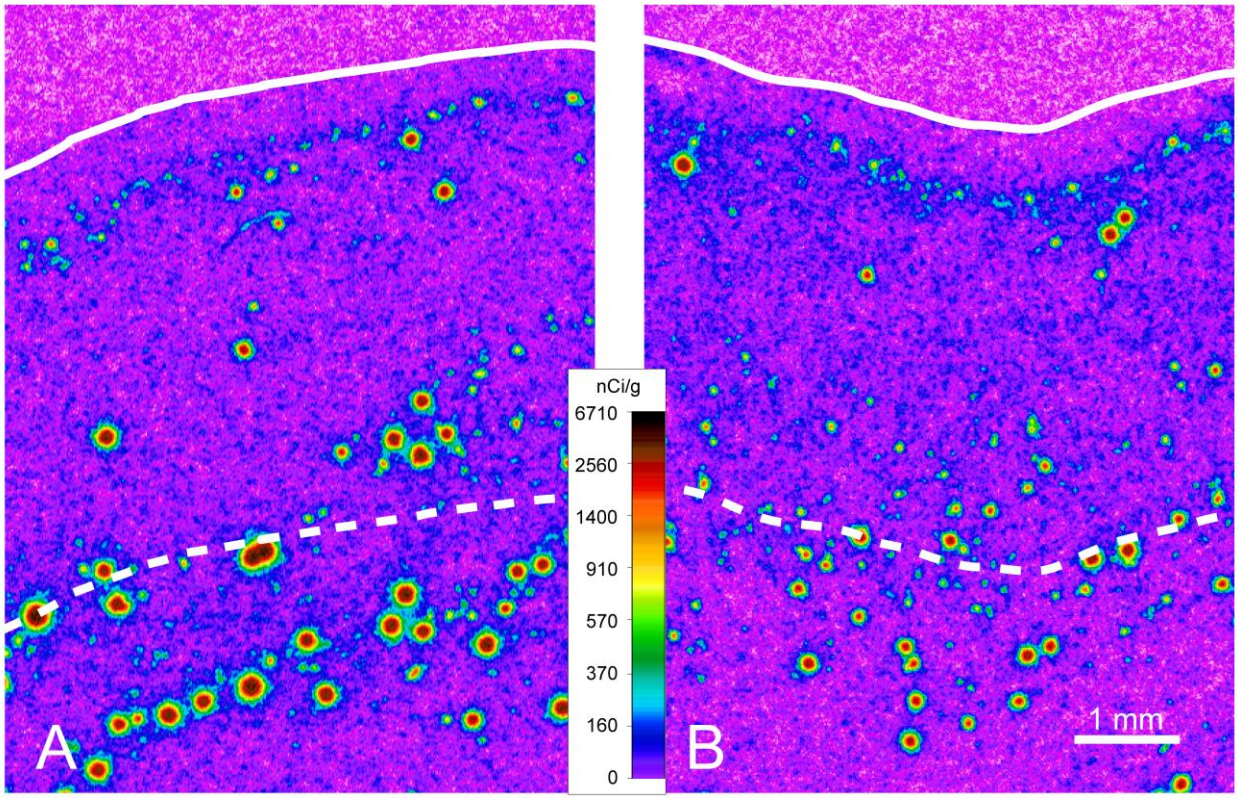

Figure 15 Representative film autoradiograms from a normal control subject (A) and matched subject with schizoaffective disorder (B).

The densities of hybridization signals are presented in a pseudocolor manner according to the calibration bar. The solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. The calibration bar applies to both panels. The expression of NPY mRNA in the superficial white matter appears to be lower in the subject with schizoaffective disorder (B) relative to the matched control subject (A).

more intensely stained glial nuclei lacked silver grains (data not shown). Fourth, sense riboprobes for NPY mRNA showed an absence of signal above background (data not shown).

Expression of NPY in area 9 gray and white matter. The mean (±SD) optical density (OD) of NPY mRNA expression in the gray matter did not significantly ($F_{(1,39)}$ = 0.67; p = 0.42) differ between the schizophrenia (31.8 ± 9.0 nCi/g) and control (35.0 ± 8.9 nCi/g) groups (Figure 16A). Similarly, the mean OD in the total white matter was not significantly different ($F_{(1,39)}$ = 1.41; p = 0.242) between the schizophrenia (43.1 ± 13.0 nCi/g) and control (49.3 ± 13.3 nCi/g) groups (Figure 16C). In contrast, mean OD in the superficial white matter was significantly reduced ($F_{(1,39)}$ = 6.14; p = 0.018) by 19% in the schizophrenia groups (68.6 ± 15.4 nCi/g) relative to the control groups (84.3 ± 25.3 nCi/g) (Figure 16B).

The effect of age on NPY mRNA levels in the superficial white matter showed a trend ($F_{(1,39)}$ = 3.42; p = 0.072) level of significance. Further analyses revealed that OD measures from superficial white matter contours in the schizophrenia groups were not significantly correlated with age (r = -0.12; p = 0.58); in contrast, NPY mRNA expression did significantly decline with age in control subjects (r = -0.51; p = 0.013). The within-subject pair percent differences in NPY mRNA expression in the superficial white matter did not differ as a function of sex, history of substance abuse/dependence, or the use of antidepressant medications or benzodiazepines/valproate at time of death (all $t_{(21)}$ > -1.68, all p > 0.11) (Figure 17). However, the mean within-subject pair percent difference in NPY mRNA expression in the superficial white matter was significantly larger for subject pairs with a diagnosis of schizoaffective disorder or death by suicide ($t_{(21)}$ = -2.80, p = 0.01 and $t_{(21)}$ = -2.73, p = 0.01, respectively) (Figure 17).

Consistent with the absence of a group difference in SST mRNA levels in the superficial white matter (Morris et al., 2008), the within-pair percent differences in NPY mRNA expression in the superficial white matter were not significantly correlated (r = 0.006, p = 0.98) with those for SST mRNA in the same subjects.

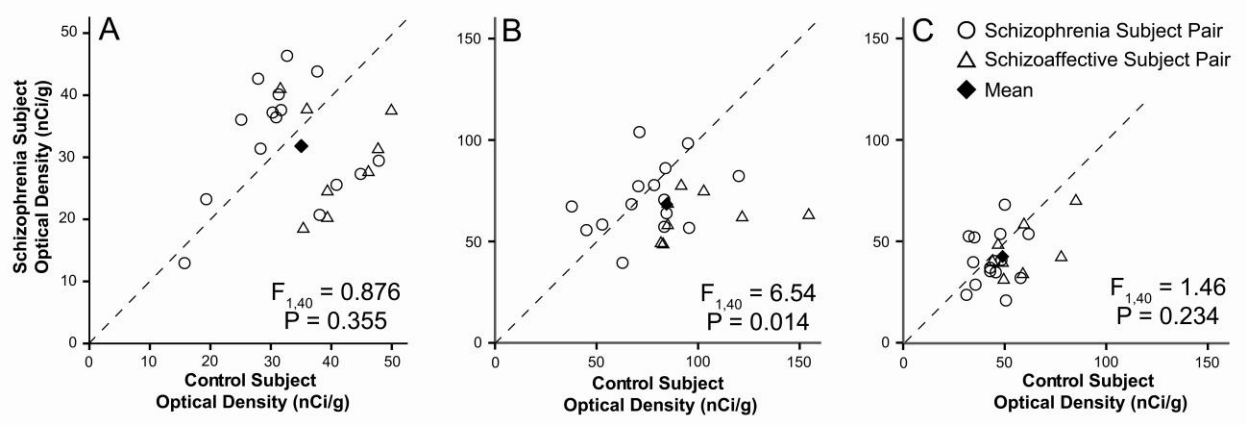

Figure 16 NPY mRNA expression levels assessed by autoradiographic film optical density (OD) measures in DLPFC area 9 of matched subject pairs for the gray matter (A), superficial white matter (B), and total white matter (C).

Values below the dashed unity line indicate a lower level of NPY mRNA expression in the subjects with schizophrenia or schizoaffective disorder relative to their matched control subject. The key in (C) applies to all graphs.

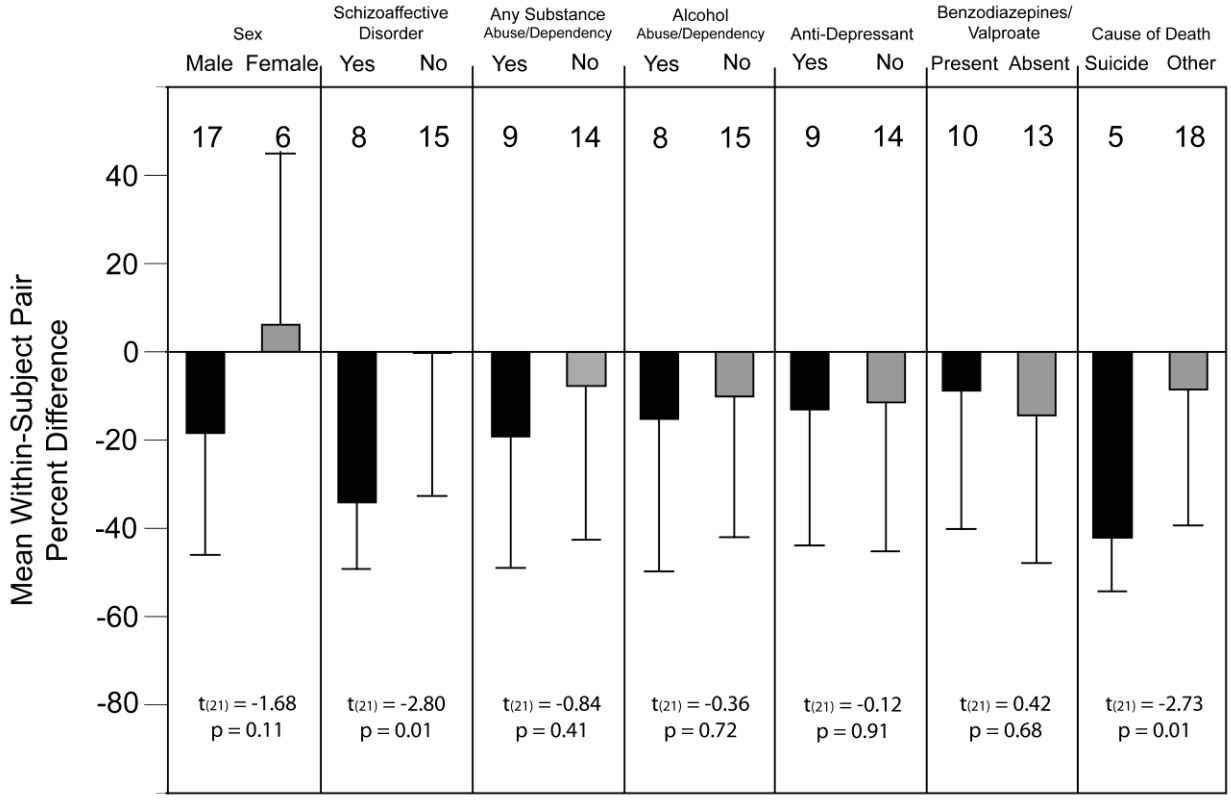

Figure 17 The effects of potential confounding factors on the expression differences of NPY mRNA in the superficial white matter.

Bars represent the mean (SD) percent differences from control subjects for NPY mRNA within subject pairs, and the numbers above each bar indicate the number of subject pairs. Neither sex, substance abuse/dependence at time of death, use of antidepressant medications at time of death, or use of benzodiazepines/valproate at time of death affected the expression differences. However, within-subject pair differences in NPY mRNA expression were significantly greater in subjects with a diagnosis of schizoaffective disorder or death by suicide.

4.5 DISCUSSION

The expression of NPY mRNA in the DLPFC of normal control subjects was lowest in layer 1, moderate in layer 2, low in layers 3-5, and high in layer 6 and the white matter; this pattern was distinctively different from the high levels of SST mRNA in layers 2, superficial 3 and 5 in these subjects (Morris et al., 2008). In addition, in contrast to SST mRNA, the expression of NPY mRNA in gray matter and total white matter of DLPFC area 9 did not differ between the schizophrenia and normal control groups. However, NPY (but not SST) mRNA levels were significantly lower in the superficial white matter of the schizophrenia group, a finding that was due to markedly lower levels in subjects with a diagnosis of schizoaffective disorder or death by suicide. Because suicide was not associated with altered NPY mRNA or protein levels in previous studies of the frontal cortex of subjects with psychiatric disorders (Caberlotto and Hurd, 1999; Klempan et al., 2009), it seems likely that a diagnosis of schizoaffective disorder is the primary cause of the lower NPY expression. Finally, the NPY mRNA expression differences in the superficial white matter were not correlated with those for SST mRNA in the same subjects. Together, these findings indicate that alterations in NPY and SST mRNA expression in schizophrenia occur 1) in separate populations of neurons, 2) in distinct compartments of the DLPFC, and 3) in different subsets of subjects.

The finding of lower NPY mRNA levels does not appear to be attributable to antipsychotic medications since both subjects with schizoaffective disorder (537 and 829) who were off antipsychotic medications at time of death had lower levels of NPY mRNA expression in the superficial white matter. Consistent with this interpretation, chronic administration of either olanzapine, clozapine, or haloperidol to rats was not associated with reduced NPY mRNA expression in the cingulate cortex (Huang et al., 2006).

Although some studies have reported lower levels of NPY protein and mRNA in the frontal cortex of subjects with schizophrenia, these studies evaluated tissue homogenates that included both gray and white matter (Gabriel et al., 1996; Mellios et al., 2008; Hashimoto et al., 2008a) and/or included subjects with schizoaffective disorder (Hashimoto et al., 2008a). Our

results clarify these findings by demonstrating that NPY mRNA expression is not altered in either the gray or white matter compartments in the DLPFC of subjects with "pure" schizophrenia, consistent with another in situ hybridization study of prefrontal gray matter NPY expression (Caberlotto and Hurd, 1999), but is reduced in the superficial white matter of subjects with schizoaffective disorder. Interestingly, NPY mRNA expression was previously reported to be lower in the prefrontal cortex of subjects with bipolar disorder, a mood disorder frequently accompanied by psychosis, but not in subjects with schizophrenia or major depressive disorder (Caberlotto and Hurd, 1999). These findings, in concert with the results of the present study, suggest that NPY mRNA neurons in the superficial white matter may be preferentially vulnerable in individuals with severe disruptions in both reality testing and mood regulation.

The affected NPY-containing neurons are likely the remnants of the earliest born neurons in the neocortex. During development of the cerebral cortex, the earliest born cells form the preplate which is subsequently split into the marginal zone (adult layer 1) and the subplate (adult deep layer 6 and superficial white matter) by later born neurons which migrate to become the cortical plate (adult layers 2 – superficial 6) (Kostovic and Rakic, 1980). Interestingly, NPY protein is present very early in human development in the preplate neurons underlying DLPFC areas 9 and 46; and in adult human prefrontal cortex, NPY protein is most strongly expressed by neurons located in the residual preplate (layer 1, deep layer 6, and the underlying white matter) (Uylings and Delalle, 1997; Delalle et al., 1997). Thus, the vulnerable neurons in subjects with schizoaffective disorder, that is those with concurrent psychotic symptoms and a mood disorder, appear to be residual preplate neurons. The relatively low expression of NPY mRNA in layer 1 likely precluded the ability to detect any differences between subjects groups in the residual preplate neurons present in this location. However, other markers of layer 1 interneurons that are co-localized with NPY do suggest that these neurons are affected in individuals with both psychosis and mood alterations. For example, most NPY-containing neurons in layer 1 also express reelin mRNA, at least in the adult mouse brain (Alcantara et al., 1998), and both reelin protein and mRNA levels are lower in subjects with bipolar disorder with a history of psychosis (Guidotti et al., 2000), although the laminar specificity of this finding was not examined.

In contrast, the deficits in SST mRNA expression in the DLPFC of subjects with schizophrenia were restricted to cortical layers that arise from the cortical plate, and were not present in residual preplate neurons (Morris et al., 2008). Together, these findings raise the

hypothesis that the phenotypic differences between "pure" schizophrenia and schizoaffective disorder may reflect differences in the types of, or timing of exposure to, environmental factors that are associated with increased risk for a psychotic illness (Lewis and Levitt, 2002). That is, although the liability to schizophrenia and schizoaffective disorder (and psychotic bipolar disorder) may arise from shared genetic factors (Potash, 2006), the resulting clinical phenotype may reflect the impact of adverse environmental events during development that preferentially affect NPY-containing preplate neurons or SST-containing cortical plate neurons. Testing of this hypothesis might include the determination of whether the nature of the reported altered density [increased in some studies and decreased in others (Akbarian et al., 1996; Eastwood and Harrison, 2003; Eastwood and Harrison, 2005)], of neurons in the superficial white matter of a subset of subjects with schizophrenia, is associated with a particular clustering of clinical features.

5.0 SOMATOSTATIN RECEPTOR SUBTYPE 1 AND 2 MRNA EXPRESSION IN THE PREFRONTAL CORTEX OF SUBJECTS WITH SCHIZOPHRENIA

5.1 ABSTRACT

Background. Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia include reduced expression of the messenger RNA (mRNA) for somatostatin (SST), a neuropeptide present in a subpopulation of γ-aminobutyric acid (GABA) neurons. However, whether the alterations in SST-containing interneurons affect post-synaptic receptors for SST has not been examined in the DLPFC of subjects with schizophrenia. In mice, SST receptor subtype 1 (SSTR1) and SSTR subtype 2 (SSTR2) are the most abundant of the receptor subtypes in the frontal cortex.

Methods. We used *in situ* hybridization to quantify SSTR1 and SSTR2 mRNA levels in DLPFC area 9 from 23 pairs of subjects with schizophrenia or schizoaffective disorder and matched normal control subjects; results were compared to those from a previous study of SST mRNA expression in the same subjects. Using *in situ* hybridization we quantified SSTR2 mRNA expression in 2 cohorts of monkeys chronically treated with typical or atypical antipsychotics.

Results. SSTR1 mRNA levels were not significantly lower in the DLPFC of subjects with schizophrenia or schizoaffective disorder. However, SSTR2 mRNA expression was significantly lower in the DLPFC of subjects with schizophrenia or schizoaffective disorder. Furthermore, there was an effect of chronic haloperidol exposure on SSTR2 mRNA expression in monkey frontal cortex that did affect our results.

Conclusion. These findings suggest that the reduction in both SST and SSTR2 mRNA expression in schizophrenia and schizoaffective disorder provide converging reductions in

inhibitory drive which may be a result of a compensation for a common "upstream" event such as reductions in excitatory drive at the dendritic domain of pyramidal neurons.

Keywords: GABA, interneurons, typical, antipsychotic, dendrite

5.2 INTRODUCTION

Many converging lines of evidence suggest that alterations in specific subpopulations of inhibitory neurons of the dorsolateral prefrontal cortex (DLPFC) is a common feature of schizophrenia (Torrey et al., 2005; Akbarian and Huang, 2006). Evidence of this is demonstrated by lower levels of the mRNA that encodes for the 67 kDa isoform of glutamic acid decarboxylase (GAD_{67}) in a minority (~25-35%) of GABA neurons, with apparently normal levels of expression in the remaining neurons (Akbarian et al., 1995; Volk et al., 2000). The affected neurons include the GABA neurons that express the calcium-binding protein, parvalbumin (PV) (Hashimoto et al., 2003), or the neuropeptide, somatostatin (SST) (Morris et al., 2008), whereas the ~50% of GABA neurons that express calretinin (CR) appear to be unaffected (Woo et al., 1998; Hashimoto et al., 2003; Sakai et al., 2008).

In a recent microarray study of GABA-related transcripts, the most robust difference in the DLPFC of subjects with schizophrenia was reduction in the expression of SST mRNA (Hashimoto et al., 2008b) which has subsequently been confirmed by real-time qPCR and in situ hybridization (Morris et al., 2008; Hashimoto et al., 2008b). SST is expressed in a subpopulation of GABA neurons that do not contain either PV or CR (Kubota et al., 1994; Gonzalez-Albo et al., 2001; Gonchar and Burkhalter, 2003; Sugino et al., 2006) and target distal dendritic shafts of pyramidal cells (Hendry et al., 1984; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1996; Melchitzky and Lewis, 2008). However, the post-synaptic receptors for SST have not been examined in schizophrenia.

There are six known G-protein coupled somatostatin receptor (SSTR) subtypes (SSTR1-5) with SSTR2 having two isoforms (SSTR2a and SSTR2b) (Moller et al., 2003). Mice with selective knockouts of the receptor subtypes demonstrate that the most abundant of these

receptors are SSTR1 and SSTR2 (Videau et al., 2003); furthermore, SSTRs have a putative inhibitory effect on neuronal excitability (Vezzani and Hoyer, 1999; Baraban and Tallent, 2004) suggesting that SSTR1 and SSTR2 are the major receptor subtypes for the modulation of excitability of pyramidal neurons by SST. Consistent with this hypothesis SSTR1 immuno-labeled cells have a putative pyramidal morphology and have labeled ascending processes in the rat isocortex (Hervieu and Emson, 1998); similarly, SSTR2-labeled cells in the rat isocortex have a putative pyramidal morphology, and have labeled ascending processes (Schindler et al., 1997) suggesting a postsynaptic localization of these subtypes in pyramidal neurons.

Consequently, in order to determine if the levels of SSTR1 and SSTR2 mRNAs are altered, we used *in situ* hybridization and autoradiographic analyses to quantify SSTR1 and SSTR2 mRNA expression in the DLPFC area 9 from 23 pairs of subjects with schizophrenia or schizoaffective disorder and matched normal control subjects in which we had previously measured SST mRNA expression using an identical approach (Morris et al., 2008).

5.3 MATERIAL AND METHODS

Human Subjects. With the consent of next-of-kin, brain tissue specimens were obtained from the Allegheny County Medical Examiner's Office at the time of autopsy. Twenty-three subjects with schizophrenia (n = 15) or schizoaffective disorder (n = 8) were each matched to 1 normal control subject for sex, and as closely as possible for age and postmortem interval (PMI) (Table 1). Subject groups did not differ in mean age, PMI, brain pH, RNA integrity number (RIN), or tissue storage time at -80° C (for all $t_{(44)} < 1.61$; $p > 0.11$). Additional demographic and clinical details are provided in our previous study of SST mRNA expression in this subject cohort (Morris et al., 2008). An independent committee of experienced research clinicians made consensus DSMIV (Diagnosis and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994)) diagnoses based on structured interviews conducted with family members of the deceased and a review of medical records. All procedures were approved by the University of

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Tissue preparation. For each brain specimen, coronal blocks from the right frontal cortex were immediately frozen and stored at -80°C. Serial sections (20 μm) containing the superior frontal gyrus were cut on a cryostat, thaw-mounted onto glass slides and stored at -80°C until processed. Adjacent sections were collected into tubes containing Trizol (Invitrogen Corp, Carlsbad, CA) in order to obtain RNA for RIN measures using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's protocol as previously described (Hashimoto et al., 2008a). The location of DLPFC area 9 was identified by cytoarchitectonic criteria in Nissl-stained sections as previously described (Glantz et al., 2000; Volk et al., 2000; Volk et al., 2001). Three sections per subject for each transcript, at intervals of approximately 300 μm, were matched for anterior-posterior location within subject pairs, and used to assess SSTR1 and SSTR2 mRNA expression.

In situ hybridization. Templates for the synthesis of the antisense and sense riboprobes for human SSTR1 and SSTR2 mRNAs were first generated by polymerase chain reaction (PCR). The specific primers amplified 465 and 247 base pair fragments of human SSTR1 and SSTR2, respectively. These fragments corresponded to bases 886-1370 of the human SSTR1 (GenBank NM_001049) and 1018-1264 of the human SSTR2 (GenBank NM_001050) genes. Nucleotide sequencing confirmed 100% homology of the amplified fragment to the previously reported sequences. The fragments were then subcloned into a plasmid (pSTBlue-1, Novagen, Madison, WI). The antisense and sense riboprobes were transcribed in the presence of ^{35}S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNeasy mini spin columns (Qiagen, Valencia, CA).

For each matched subject pair, one section from each pair was processed side-by-side in three separate runs for each gene of interest. Prior to the hybridization reaction, tissue sections were fixed with 4% paraformaldehyde in PBS solution, acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine/0.9% NaCl for 10 minutes, dehydrated with a graded alcohol series, and then defatted in chloroform for 10 minutes. The sections were then hybridized with ^{35}S-labeled

riboprobes (1.0 X 10^6 cpm/μl) in hybridization buffer at 56°C for 16 hours. The hybridization buffer contained 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 (0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA), 50 mM dithiothreitol, 0.2% SDS, and 100 μg/ml yeast tRNA. Following the hybridization reaction, sections were washed in a solution of 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63°C, treated with RNase A (20 μg/ml) at 37°C, washed in 0.1 X SSC (1.5 mM NaCl, 150 μM sodium citrate) at 67°C, dehydrated through a graded ethanol series, and air dried. Sections from both subjects in a pair were exposed on the same BioMax MR film (Kodak, Rochester, NY) for 7 days, and then coated with undiluted NTB2 emulsion (Kodak) for SSTR2 and NTB2 emulsion diluted 2:1 with water for SSTR1. Utilizing DLPFC sections from control subjects, different emulsion exposure times were systematically evaluated in order to achieve an optimal signal to noise ratio. The emulsion was exposed for 77 and 71 days at a constant temperature of 4°C for SSTR1 and SSTR2, respectively. The slides were developed with D-19 (Kodak) and counterstained with Cresyl-violet.

Quantification of mRNA expression levels. Each section was randomly coded, so that subject number and diagnosis were unknown to the single rater (HMM). Autoradiographic films were trans-illuminated and captured on video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID; Imaging Research Inc., London, Ontario, Canada). Digitized images of adjacent sections stained with cresyl violet were superimposed onto autoradiographic images to draw contours of the full cortical thickness of the zones of area 9 that were cut perpendicular to the pial surface. Optical density (OD) measures within each sampled area were calibrated to radioactive carbon-14 standards (ARC Inc., St. Louis, MO), exposed on the same autoradiographic film, and expressed as nanocuries per gram (nCi/g) of tissue. The mean (SD) total area of gray matter sampled in control subjects and subjects with schizophrenia was 360 (178) and 344 (117) mm^2 for the SSTR1 riboprobe, respectively, and 358 (185) mm^2 and 304 (91) mm^2 for the SSTR2 riboprobe, respectively.

SSTR2 mRNA expression as a function of cortical layer was determined in a series of cortical traverses (1-2 mm in width) extending from the pial surface to the white matter. Three cortical traverses were sampled for each section (nine traverses per subject). Each traverse was divided

into 50 equal bins parallel to the pial surface and the optical density was determined for each bin. These bins were then combined into zones that approximated laminar boundaries based on previous studies (Akbarian et al., 1995; Pierri et al., 1999). These zones (i.e., bins 1-5, 6-15, 16-25, 26-30, 31-40, and 41-50) corresponded to layers 1, 2/superficial 3 (2/3s), deep 3 (3d), 4, 5, and 6, respectively **(Figure 20)**. The mean OD was calculated for each zone. Background measures were sampled from deep white matter where no specific expression of SSTR 2 mRNA was observed. All sampled areas were corrected by subtracting the corresponding background measure from the same slide.

Typical and Atypical Antipsychotic-exposed Monkey Triads. As described previously (Dorph-Petersen et al., 2005), experimentally naive, male macaque monkeys (Macaca fasicularis), 4.5 – 5.3 years of age, were chronically exposed to twice daily oral doses of haloperidol, olanzapine or placebo (n = 6 monkeys per group) for 17–27 months. The doses of haloperidol and olanzapine were gradually increased until the monkeys were receiving 24–28 mg of haloperidol or 11.0 – 13.2 mg of olanzapine per day. The final trough drug plasma levels were within the range associated with clinical efficacy in humans (~1.5 ng/ml for haloperidol and ~15 ng/ml for olanzapine) (Dorph-Petersen et al., 2005). Matched by terminal body weight, monkeys were euthanized in triads (one animal from each group) on the same day. Brains were rapidly removed, and the right frontal lobe was cut into coronal blocks, frozen in isopentane on dry ice and stored at –80°C. Serial coronal sections (16 mm) were cut from the slabs containing the anterior one-third of the principal sulcus and mounted on glass slides. The antisense and sense riboprobes were transcribed in the presence of ^{35}S-CTP (PerkinElmer, Waltham, MA, USA) using T7 and SP6 RNA polymerase, respectively. Two sections evenly spaced at 224 mm were hybridized with an antisense RNA probe against human SSTR2 mRNA as described above in the human study. The optical density for SSTR2 mRNA was measured in the gray matter of the DLPFC bordered by the cingulate and principal sulci and corrected by subtracting the white matter density measures. Density values of two sections were averaged before statistical analysis. In addition, since SSTR2 mRNA is expressed at relatively lower levels in the human DLPFC and monkey PFC, ^{33}P-CTP (PerkinElmer, Waltham, MA, USA), a relatively stronger emitter, was used for radiolabeling in a separate experiment in order to increase detectability and confirm the results from the ^{35}S-labeled riboprobe. Two sections evenly spaced at 224 mm were hybridized

with the antisense RNA probe against human SSTR2 mRNA as described above. Optical density was measured and averaged for statistical analyses as described above. All studies 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.

Haloperidol-Exposed Monkeys. In order to confirm our results from the monkey triad experiment and to mimic the treatment of schizophrenia with high doses of haloperidol, 4 pairs of young adult, male macaque monkeys (Macaca fascicularis), matched for age and weight, were chronically exposed to haloperidol decanoate (mean [±SD] trough plasma level, 4.3 ± 1.1 ng/mL) and benztropine mesylate (1 mg b.i.d.) to treat extrapyramidal symptoms for 9-12 months, as previously described (Pierri et al., 1999). Processing of monkey brain tissue was conducted as previously described (Hashimoto et al., 2003). Briefly, coronal sections with a thickness of 16 µm were cut from fresh-frozen tissue blocks containing the middle one-third of the principal sulcus. Three pairs of serial sections spaced ~160 µm apart from each other for each monkey (6 sections/monkey) were processed for SSTR2 mRNA expression utilizing the ^{35}S-labeled riboprobe as described above. The optical density was measured and averaged for statistical analyses as described in the above monkey study. All sampled areas were corrected by subtracting corresponding background measures from the same section.

***Statistical analyses*.** Analyses were performed on SPSS (SPSS Inc., Chicago, IL). Analysis of covariance (ANCOVA) models were used to test differences in SSTR1 and SSTR2 mRNA expression between control subjects and subjects with schizophrenia. The data were averaged across the three sections for each subject before statistical analysis. The first ANCOVA model used diagnostic group as the main effect, pair as a blocking effect, and storage time and RIN as covariates. The pair effect reflects the matching of individual subject pairs for sex, age, and PMI. RIN was included as a covariate because it reflects mRNA integrity (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 a main effect of diagnostic group and covariates of sex, age, PMI, RIN, and storage time. Storage time as a covariate was not significant in either model and, thus was excluded in the reported analyses. Both models produced comparable

results for diagnostic group effect; however, because the effect of age on SSTR1 and SSTR2 mRNA expression was significant, the results of the second model are reported.

For the laminar analysis, multiple comparisons were controlled by adjusting for simultaneous inference of significance levels using the Bonferroni-Holm method (Volk et al., 2000) in which p values are ordered from the smallest (i=1) to the largest (i=N) among multiple comparisons; the significance level for each comparison is defined as $\alpha=0.05/([N+1]-i)$.

The potential influence of sex, diagnosis of schizoaffective disorder, history of substance abuse/dependence, medications at time of death, or death by suicide on the within-pair percentage of differences in mRNA expression was assessed by two-sample t-test analyses. Correlations were assessed by Pearson's correlation analyses.

For the antipsychotic-treated monkey triads, the effects of drug exposure on mRNA expression levels were evaluated by a one-way analysis of variance (ANOVA). Tukey's multiple comparison test was used in post-hoc comparisons across treatment groups. For the haloperidol-treated monkey pairs, paired t-test analyses were used to assess the effects of treatment group on SSTR2 mRNA expression.

5.4 RESULTS

***Specificity of SSTR1 and SSTR2 Riboprobes*.** Several lines of evidence confirm the specificity of the riboprobe for SSTR1 and SSTR2 mRNAs used in this study. First, The laminar distribution of SSTR1 mRNA is similar to that previously reported for SSTR1 mRNA in the human frontal cortex (Thoss et al., 1996). Specifically, the density of SSTR1 mRNA was high in superficial and middle layers and intermediate in the deep layers **(Figure 18A)**. Similarly, the laminar distribution of SSTR2 mRNA is similar to reports in previous studies for SSTR2 mRNA in the human frontal cortex and immuno-positive cell bodies in the human temporal cortex (Thoss et al., 1996; Schindler et al., 1997). SSTR2 mRNA expression was primarily expressed in the deep layers (5 and 6) **(Figure 18B)**. Second, emulsion-coated sections demonstrated silver

grain clusters over large, faintly Nissl-stained neuronal nuclei, whereas the smaller and more intensely stained glial nuclei lacked silver grains (data not shown). Third, sense riboprobes for SSTR1 and SSTR2 mRNA showed an absence of signal above background (data not shown).

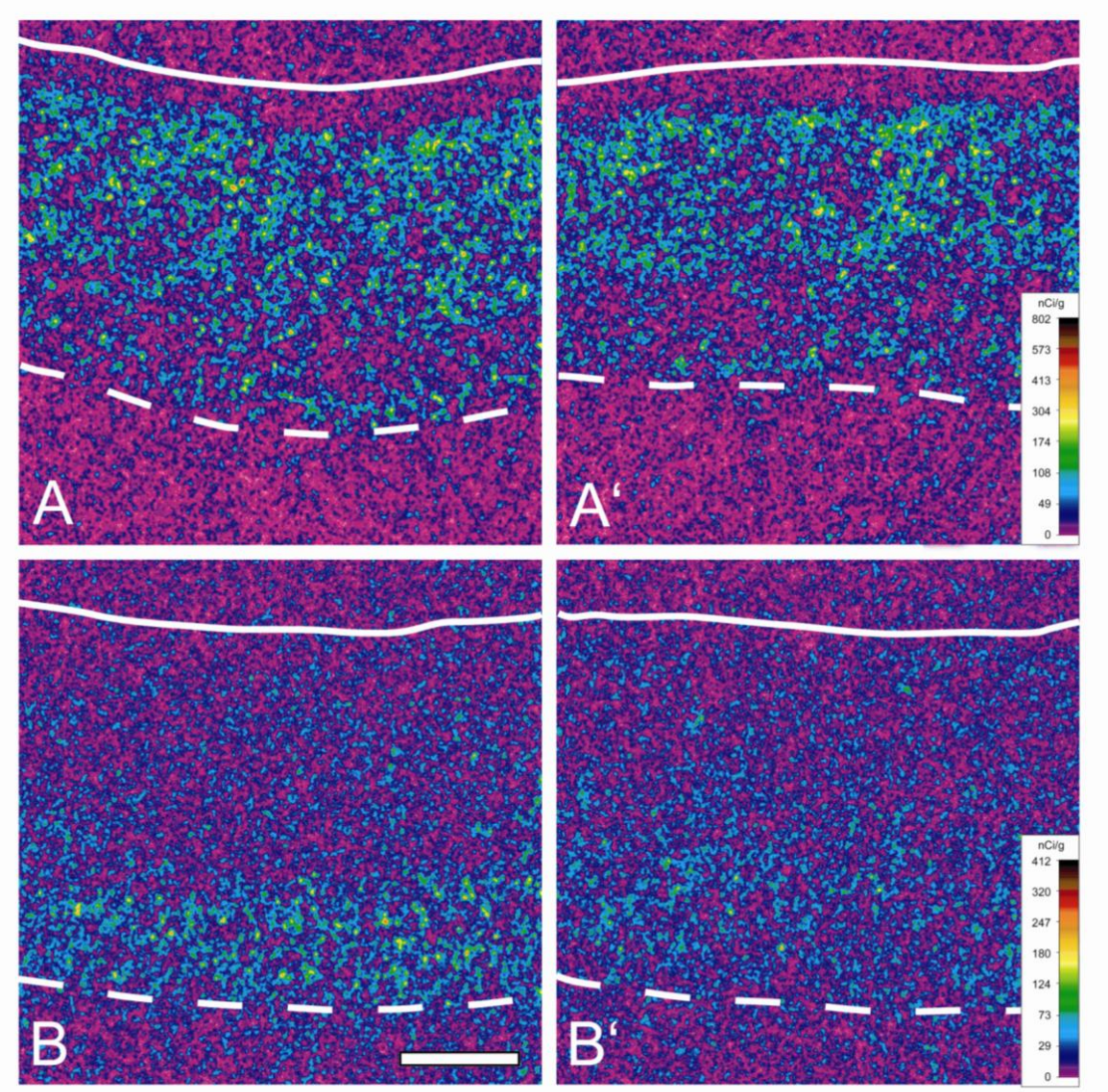

Figure 18 Representative film autoradiograms from pairs of control subjects (A, B) and matched subjects with schizophrenia (A', B').

The densities of hybridization signals are represented in a pseudocolor manner according to the calibration bar (right side; applies to both panels in a row). The solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. The white bar (in B) represents 1 mm and applies to all panels. Expression of SSTR1 mRNA is not significantly altered in the subject with schizophrenia (A') as compared to the control subject (A). Expression of SSTR2 mRNA is reduced in the deep layers of the subject with schizophrenia (B') as compared to the control subject (B).

Expression of SSTR1 and SSTR2 in DLPFC Area 9. We previously reported reduced SST mRNA expression by microarray, qPCR, and *in situ* hybridization in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2008a). This change in SST mRNA expression could affect the expression of postsynaptic receptors; therefore, we assessed the expression levels of the transcripts that encode for the highest expressing SST receptors (Videau et al., 2003), SSTR1 and SSTR2, in the DLPFC area 9 of subjects with schizophrenia and matched control subjects. Quantitative measures of SSTR1 mRNA expression revealed that the mean (±SD) optical density of gray matter was not significantly ($F_{(1,39)}$ = 2.85; p = 0.10) altered in subjects with schizophrenia (45.4 ± 9.2 nCi/g) compared to control subjects (49.6 ± 8.9 nCi/g) **(Figure 19A)**. In contrast, quantitative measures of SSTR2 mRNA expression revealed a significant reduction ($F_{(1,39)}$ = 5.32; p = 0.03) of 19.1% in mean (±SD) OD in subjects with schizophrenia (19.0 ± 6.4 nCi/g) as compared to control subjects (23.5 ± 6.4 nCi/g) **(Figure 19B)**.

Qualitative impressions of SSTR2 mRNA expression **(Figure 18B)** suggested predominant SSTR2 mRNA distribution in the deeper layers; therefore, we examined the OD measures by layer **(Figure 19)**. Because of the high density of SST mRNA+ neurons in layers 2-superficial 3 (2/3s) (Morris et al., 2008), these layers were combined and distinguished from deep layer 3 (3d). SSTR2 mRNA expression was significantly decreased by 20.4% in layer 5 and by 28.7% in layer 6 ($F_{(1,39)}$ = 7.33, p = 0.01; $F_{(1,39)}$ = 22.75, p < 0.0001, respectively).

Consistent with the absence of a group difference in the expression of SSTR1 mRNA, the within-pair percent differences in SSTR1 mRNA expression were not significantly correlated (r = 0.17, p = 0.43) with those for SST mRNA in the same subjects. In contrast, within-pair percentage differences in SSTR2 mRNA expression were significantly correlated with those found for SST mRNA gray matter expression (r = 0.77, p < 0.0001).

Examination of Factors that May Affect Expression of SSTR2 mRNA. In the second ANCOVA model, age was a significant ($F_{(1,39)}$ = 4.72; p < 0.036) determinant of SSTR2 mRNA expression. Further analyses demonstrated that OD measures were negatively correlated with age in control subjects (r = -0.42; p < 0.044), but was only trending in subjects with schizophrenia (r = -0.39; p = 0.067). However, the within-subject pair percent differences in SSTR2 mRNA expression did not differ as a function of sex, diagnosis of schizoaffective disorder, diagnosis of substance or alcohol abuse at time of death (ATOD), use of antidepressant medication ATOD,

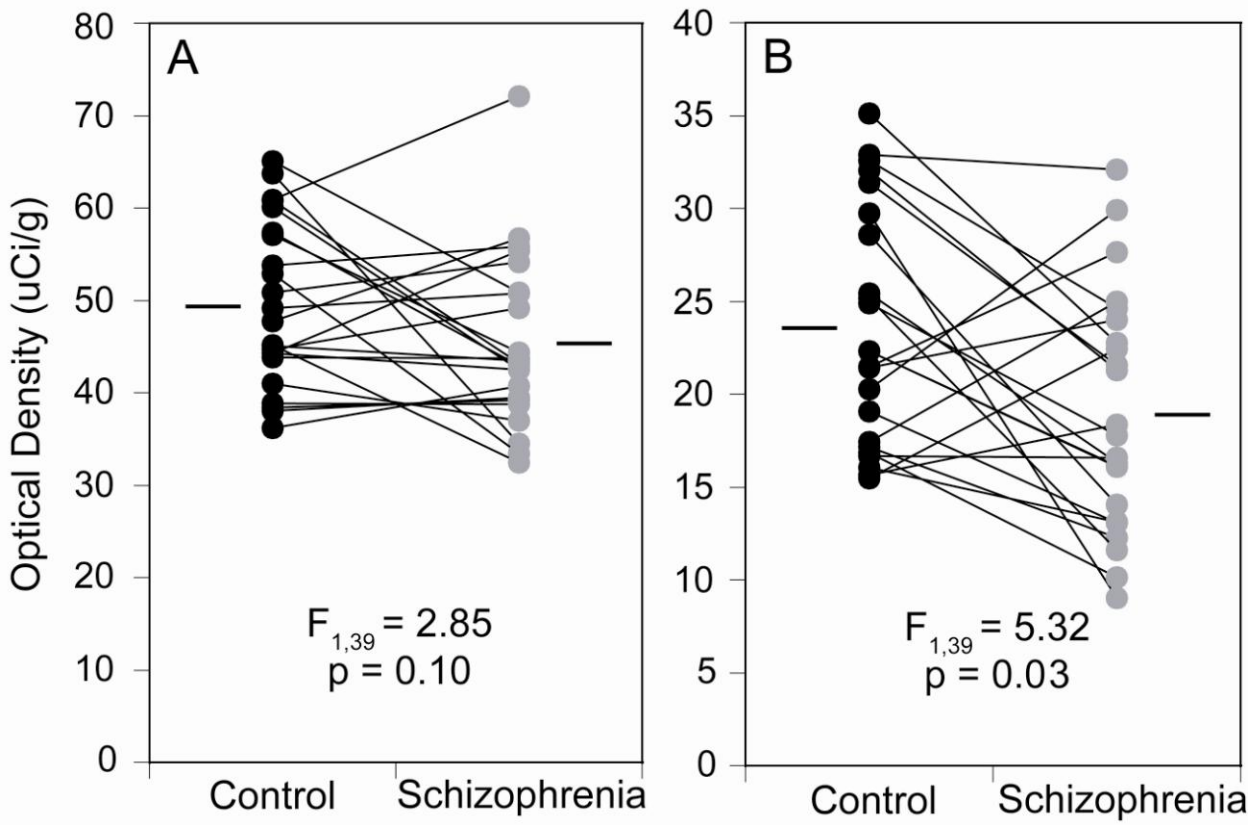

Figure 19 SSTR1 and SSTR2 mRNA expression levels assessed by autoradiographic film optical density (OD) measures in DLPFC area 9 of subjects with schizophrenia (solid gray circles) and control subjects (solid black circles).

Pairs are connected by a solid black line and the mean expression levels for each subject group are represented by a horizontal line. The expression of SSTR1 mRNA is not significantly altered (A), but expression of SSTR2 mRNA is significantly reduced (B).

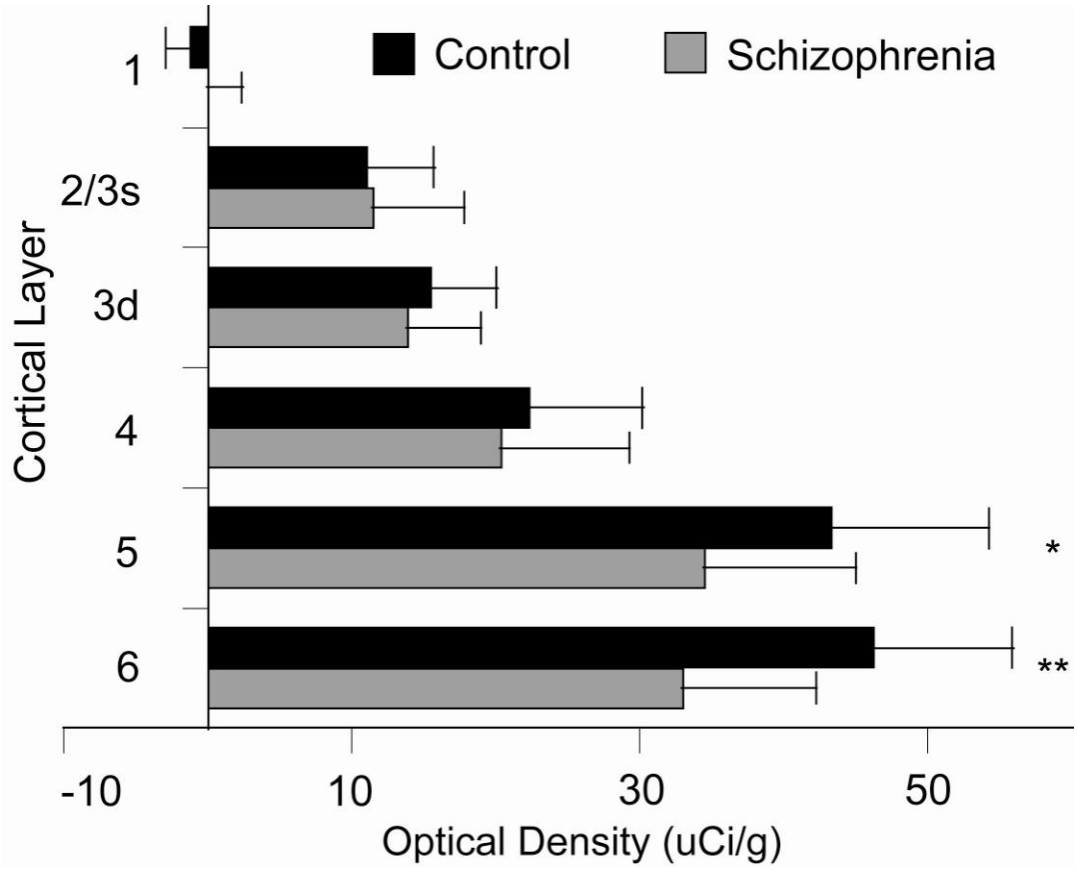

Figure 20 Mean (SD) expression levels of SSTR2 mRNA by layer as assessed by film autoradiography optical density (OD) measures in each cortical layer.

* Adjusted $p < 0.05$. ** Adjusted $p < 0.001$.

use of benzodiazepines/valproate ATOD, suicide, antipsychotic medication use ATOD, or use of haloperidol ATOD (all $t_{(21)} < 1.74$, all $p > 0.10$) **(Figure 21)**.

To evaluate the potential effect of long-term exposure to typical and atypical antipsychotic medications, we examined the expression of SSTR2 mRNA in the PFC of monkeys chronically exposed to placebo, haloperidol, or olanzapine (n = 6 per group). Utilizing a ^{35}S-labeled riboprobe, the laminar distribution of SSTR2 mRNA in the 3 groups matched the pattern observed in the human DLPFC **(Figure 22)**. There was a significant effect of treatment group ($F_{2,15} = 7.29$; $p = 0.006$) on the expression of SSTR2 mRNA. Post-hoc analysis revealed a significant reduction in the mean (±SD) OD in the haloperidol group (26.4 ± 3.3 nCi/g) as compared to the placebo group (42.3 ± 9.3 nCi/g) or the olanzapine group (37.6 ± 8.2 nCi/g) ($p = 0.005$ and $p = 0.048$, respectively); there was no significant difference ($p = 0.53$) between the placebo and olanzapine groups. In order to confirm these results, we also utilized ^{33}P-CTP, a relatively high emitter, to radiolabel a separate SSTR2 riboprobe and repeated the experiment in the same monkey cohort. The ^{33}P-labeled riboprobe revealed a laminar distribution of SSTR2 mRNA that matched the pattern seen with the ^{35}S-labeled riboprobe in humans and monkeys **(Figure 22)**. Similar to our previous experiment, there was a significant effect ($F_{2,15} = 5.18$; $p = 0.02$) of treatment group of which there was a significant reduction ($p = 0.02$) in the mean (±SD) OD in the haloperidol group (20.4 ± 5.1 nCi/g) as compared to the placebo group (31.6 ± 6.4 nCi/g); however, the mean (±SD) OD of the olanzapine group (27.7 ± 6.8 nCi/g) was not significantly different than the haloperidol group ($p = 0.13$) or the placebo group ($p = 0.54$).

These results suggested that chronic exposure to haloperidol affects the expression of SSTR2 mRNA in the PFC which we did not observe in the subjects with schizophrenia **(Figure 21)**; therefore, we wanted to confirm these results in a separate monkey cohort. Thus, we examined the expression of SSTR2 mRNA in the PFC of monkeys that were chronically exposed to higher levels of haloperidol decanoate (mean trough plasma levels >4 ng/mL) as compared to the haloperidol-exposed group in the previous monkey cohort (steady-state trough plasma levels of ~1.5 ng/mL). Mean (±SD) OD did not differ ($t_{(3)} = -1.78$; $p = 0.17$) between the haloperidol-exposed monkeys (38.8 ± 5.7 nCi/g) and their sex-, age-, and weight-matched controls (46.8 ± 9.0 nCi/g).

Since SSTR2 mRNA expression was significantly affected by chronic exposure to haloperidol in at least one of the monkey cohorts, we wanted to further examine the effect of haloperidol or,

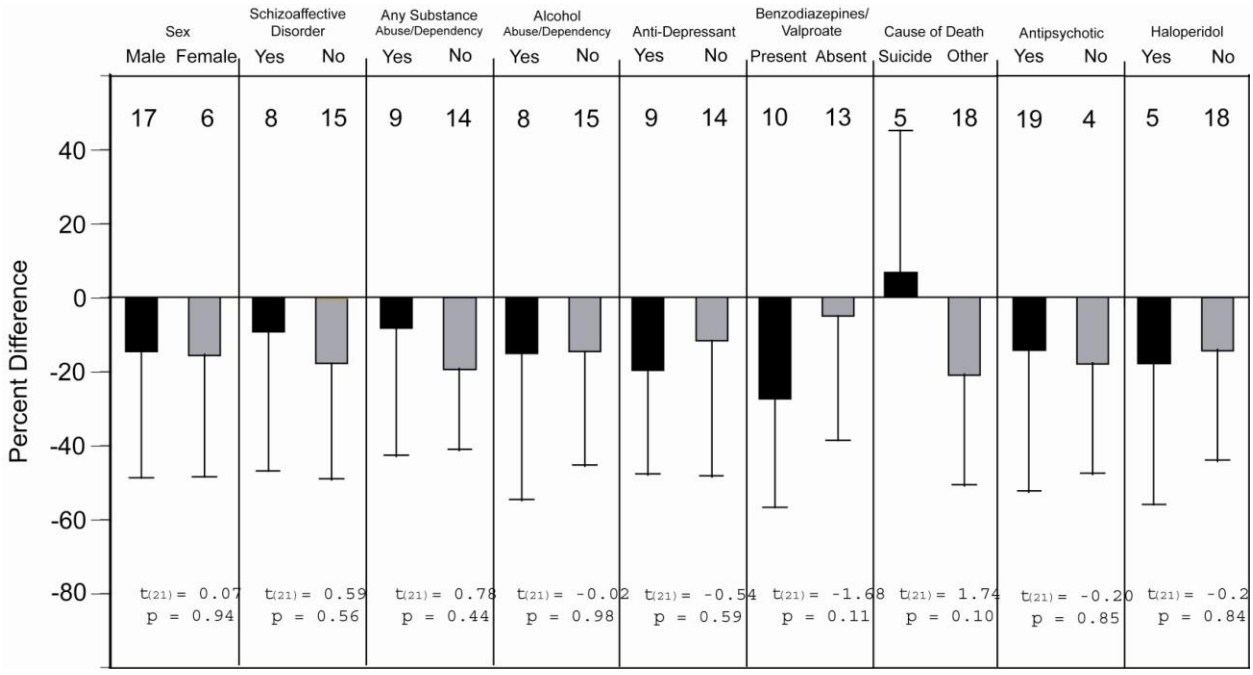

Figure 21 The effects of potential confounding factors on the expression differences in SSTR2 mRNA in subjects with schizophrenia.

The bars represent the mean (SD) percent differences from control subjects for SSTR2 mRNA within subject pairs; numbers above each bar indicate the number of subject pairs. Sex, diagnosis of schizoaffective disorder, diagnosis of substance or alcohol abuse or dependency ATOD, use of antidepressant medications ATOD, use of benzodiazepines/valproate ATOD, death by suicide, use of antipsychotic medication ATOD, or use of haloperidol ATOD did not significantly affect the expression changes in SSTR2 mRNA.

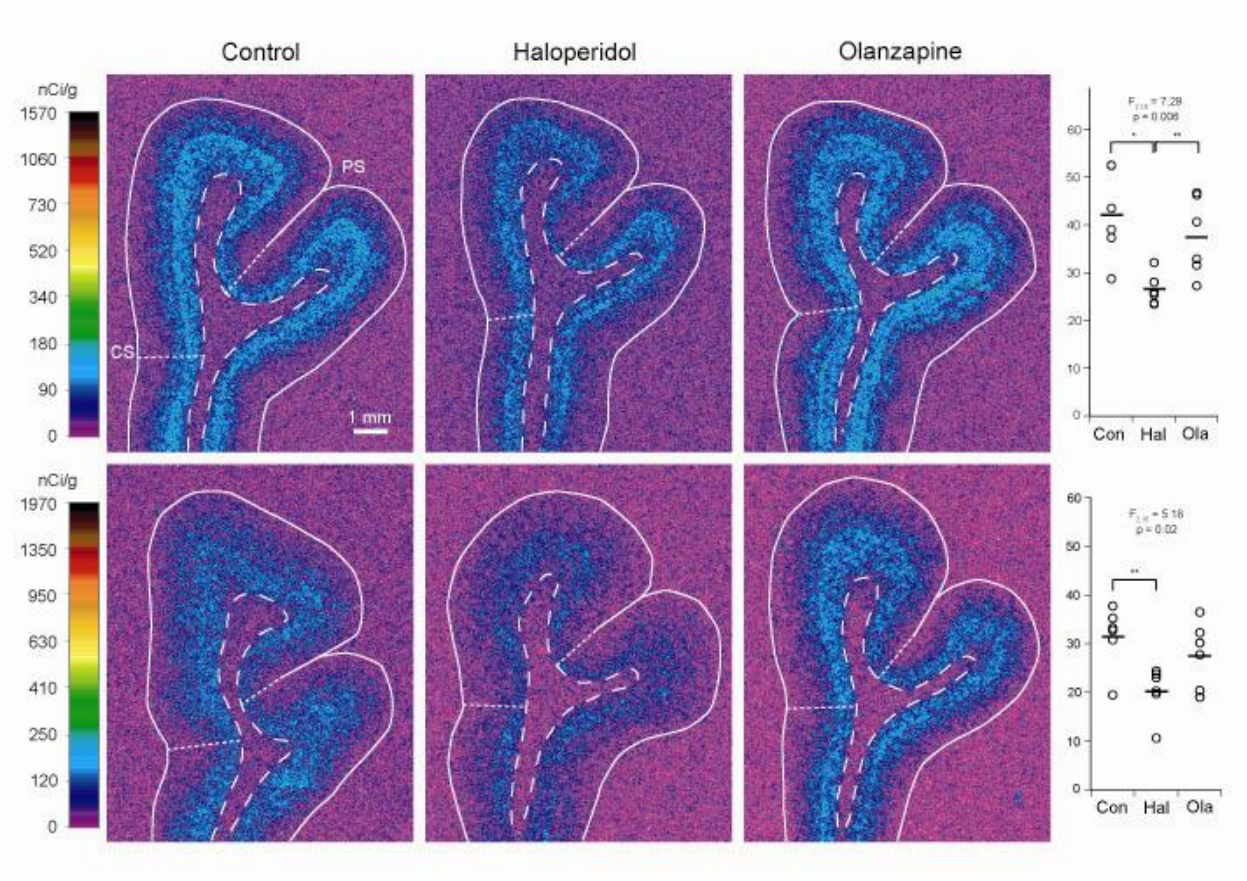

Figure 22 The effect of chronic exposure to antipsychotic medications on the expression of SSTR2 mRNA in the monkey PFC.

Representative film autoradiograms demonstrate the expression of SSTR2 mRNA utilizing a ^{35}S-labeled riboprobe (upper panels) and a ^{33}P-labeled riboprobe (lower panels) in the PFC of a control (left panels), a haloperidol-exposed (center panels) and an olanzapine-exposed (right panels) monkey. The densities of hybridization signals are presented in a pseudocolor manner according to the calibration bars (left; applies to all panels in a row). Solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. Expression of SSTR2 mRNA was assessed between the cingulate sulcus (CS) and the principal sulcus (PS). Graphs (right) show the mean (±SD) expression levels of SSTR2 mRNA utilizing a ^{35}S-labeled riboprobe (upper) and ^{33}P-labeled riboprobe (lower) in the placebo-, haloperidol-, and olanzapine-exposed monkey groups.

more generally, typical antipsychotic medication usage in the subjects with schizophrenia. The within-subject pair percent differences in SSTR2 mRNA expression did not differ as a function of use of haloperidol ATOD **(Figure 21)**. Furthermore, when we removed all subject pairs in which the subject with schizophrenia was taking a typical antipsychotic medication ATOD and performed an ANCOVA on the remaining pairs (n = 11 pairs) there was a significant reduction ($F_{(1,15)} = 4.81$; $p = 0.04$) of 19.9% in mean (±SD) OD in subjects with schizophrenia (20.1 ± 4.5 nCi/g) as compared to control subjects (25.1 ± 7.1 nCi/g)

5.5 DISCUSSION

The expression level of SSTR2 mRNA was significantly decreased in deep cortical layers 5 and 6 of the DLPFC area 9 in subjects with schizophrenia compared to matched controls; in contrast, SSTR1 mRNA expression did not differ between subjects groups. Furthermore, the observed differences in SSTR2 mRNA, but not SSTR1 mRNA, expression were significantly correlated with those in SST mRNA in the same subject pairs suggesting that the down regulation of SSTR2 is related to the alteration found in the SST interneuron population (Morris et al., 2008) although a cause and effect relationship cannot be clearly established. Furthermore, we found a significant effect of chronic haloperidol exposure on SSTR2 mRNA expression in the PFC of one of the monkey cohorts; however, haloperidol or typical antipsychotic usage ATOD was not the cause of the altered SSTR2 mRNA expression in subjects with schizophrenia.

Several lines of evidence suggest that the SSTR2 mRNA expression deficit is not a result of factors frequently associated with illness. First, the within-subject pair percent differences in SSTR2 mRNA expression did not differ as a function of the use of antipsychotic medications ATOD **(Figure 21)**. Second, SSTR2 mRNA expression was not altered in the PFC of monkeys with chronic long-term exposure to atypical (olanzapine) antipsychotics **(Figure 22)**. Third, within-subject pair percent differences in SSTR2 mRNA expression did not differ between subject pairs in which the subject with schizophrenia was receiving haloperidol ATOD and pairs in which the subject with schizophrenia was not taking haloperidol ATOD **(Figure 21)**.

Consistent with this observation, when all subject pairs in which the subject with schizophrenia was taking typical antipsychotics ATOD were removed from the analyses SSTR2 mRNA expression remained significantly reduced. Furthermore, long-term haloperidol exposure in rats demonstrated normal levels of SST binding in the frontal cortex after one week of withdrawal suggesting that previous haloperidol exposure does not permanently affect SSTR expression (Perez-Oso et al., 1990). Finally, neither sex, diagnosis of schizoaffective disorder, diagnosis of substance or alcohol abuse ATOD, use of antidepressant or benzodiazepines/valproate ATOD, nor death by suicide accounted for the decreased expression of SSTR2 mRNA in the subjects with schizophrenia.

In the neocortex of rats, SSTR2s are primarily concentrated in the ascending dendrites of pyramidal neurons located in the deep layers (Schindler et al., 1997). Furthermore, in the rat and monkey neocortex SST-containing interneurons predominately innervate the dendritic shafts of pyramidal neurons (Hendry et al., 1984; DeLima and Morrison, 1989; Kawaguchi and Kubota, 1996; Melchitzky and Lewis, 2008). Combined with our study and others (Thoss et al., 1996; Schindler et al., 1997) demonstrating a primary localization of SSTR2 mRNA or immunoreactive-cell bodies in the deep layers of the neocortex of the human, these data suggest that SSTR2 is primarily expressed by layer 5 and 6 pyramidal cells on their ascending dendritic shaft which receive input from SST-containing inhibitory interneurons.

SSTRs have a putative inhibitory effect on neuronal excitability (Vezzani and Hoyer, 1999; Baraban and Tallent, 2004). In the presence of SST, SSTRs hyperpolarize rodent hippocampal pyramidal neurons *in vitro* (Pittman and Siggins, 1981) by augmenting M-currents (Moore et al., 1988) and voltage-insensitive K^+ leak current (Schweitzer et al., 1998). These data suggest that in the DLPFC of subjects with schizophrenia there is not only a reduction in presynaptic inhibitory drive onto pyramidal dedritic domains as evidenced by a robust reduction in SST mRNA expression (Morris et al., 2008), but there is also a reduction in postsynaptic inhibitory drive as evidenced by the reduction in SSTR2 mRNA expression. Reductions in both presynaptic and postsynaptic inhibitory drive suggest that the correlated changes in SST and SSTR2 mRNA expression are a result of an "upstream" event that effects inhibitory drive in pyramidal neurons. For example, a leading hypothesis concerning the pathophysiology underlying schizophrenia is that there is a disruption in the glutamatergic system which eventually leads to disruptions in other neurotransmission systems such as disturbances seen in

the GABA system (Lewis and Moghaddam, 2006). Several converging lines of evidence support this hypothesis. First, many of the putative schizophrenia susceptibility genes are associated with excitatory neurotransmission (Harrison and Owen, 2003; Moghaddam, 2003) suggesting that genetic risks involving this type of neurotransmission may be a key component in the etiology of schizophrenia. Second, administration of antagonists of the NMDA subtype of glutamate receptors impair cognitive functions in control subjects similar to those found in subjects with schizophrenia (Krystal et al., 1994; Adler et al., 1999; Newcomer et al., 1999; Krystal et al., 2000). For example, control subjects receiving subanesthetic doses of ketamine demonstrated reductions in working memory performance and increases in thought disorder similar to those seen in subjects with schizophrenia (Adler et al., 1999). Third, many post-mortem studies in individuals with schizophrenia have demonstrated significant alterations in glutamate receptor efficacy and subunit transcript and protein expression (Konradi and Heckers, 2003). Therefore, the coordinated reduction in the expression of SST and SSTR2 mRNA could be a compensatory mechanism which reduces inhibitory drive in order to increase the efficacy of excitatory drive on the dendritic domain of pyramidal neurons in the DLPFC of subjects with schizophrenia.

6.0 GENERAL DISCUSSION

Previous studies raised the possibility that the SST-containing interneurons in the DLPFC of indivuals with schizophrenia are altered. Therefore, in order to better understand the pathophysiology of SST interneurons and how they could contribute to cognitive deficits in schizophrenia, this thesis outlines the alterations in and possible upstream mechanisms of alterations in SST interneurons in the DLPFC of individuals with schizophrenia. We concluded that the correlated alterations in SST and GAD_{67} mRNAs indicate that GABA neurotransmission from SST interneurons is dysfunctional in schizophrenia and these changes are likely downstream from alterations in neurotrophin signaling via the trkB receptor. Furthermore, the correlated reductions in SST and SSTR2 mRNAs are indicative of reduced inhibitory modulation at the dendritic domain of pyramidal neurons. We interpret these correlated changes to represent, in schizophrenia, a downstream compensatory response to reduced excitatory drive at the dendritic domain of pyramidal cells in order to increase the efficacy of excitatory inputs to pyramidal cells in the DLPFC of subjects with schizophrenia.

Since SST interneurons seem to play a critical role in the coordinated firing of pyramidal cells during working memory function, these results indicate that alterations in SST-containing interneurons in the DLPFC of subjects with schizophrenia represent a plausible neuropathological entity that contributes to altered theta oscillations and, therefore, to the manifestation of the clinical manifestation of altered working memory performance. In addition, previous postmortem studies have suggested that in the DLPFC of subjects with schizophrenia parvalbumin-containing interneurons such as the chandelier class of GABA neurons have reduced GABA synthesis (Lewis et al., 2005) and lead to altered coordination of pyramidal cell firing in the gamma range. As will be explained later in this discussion, synchrony of pyramidal cell firing at the gamma range is also important for working memory function; therefore, these data combined with the data from this dissertation suggest that the coordinated network activity

necessary for normal working memory function is severely altered in individuals with schizophrenia.

In the following sections, I will integrate the data from this dissertation into the existing literature to propose a working model of schizophrenia that outlines the disease process leading to working memory dysfunction, discuss some limitations of the studies in the thesis, and propose future directions.

6.1 PROPOSED DISEASE PROCESS OF SCHIZOPHRENIA

It is paramount that the results from the human postmortem studies from this dissertation be integrated into a plausible disease process that can explain the etiology that leads to the observed alterations in the neural substrate, where the observed alterations fall within a cascade of events (i.e. Is it a causal factor, a downstream consequence, or a compensatory response?), and how the observed alterations in the neural substrate lead to the neuropathophysiology that underlies the clinical symptoms. Such a disease model is important for the development of novel treatments because it would allow for the identification of alterations that are most proximal to the neuropathophysiology (i.e. altered network activity) underlying a core feature of the disease (i.e. working memory dysfunction).

Chapter 1.2.2 outlined previous studies demonstrating selective reductions of GAD_{67} mRNA in PV-containing interneurons representing an alteration in the neural substrate of the DLPFC of subjects with schizophrenia. This dissertation demonstrated alterations in mRNAs involved in the functionality of SST-containing interneurons which represent a novel neuropathological entity in the DLPFC of subjects with schizophrenia and a plausible upstream mechanism involving alterations in trkB signaling. The following sections will propose a model of the disease process incorporating the observations of alterations in PV-containing chandelier and SST-containing neurons which converge on a common neuropathophysiology underlying a core feature of schizophrenia (Figure 23).

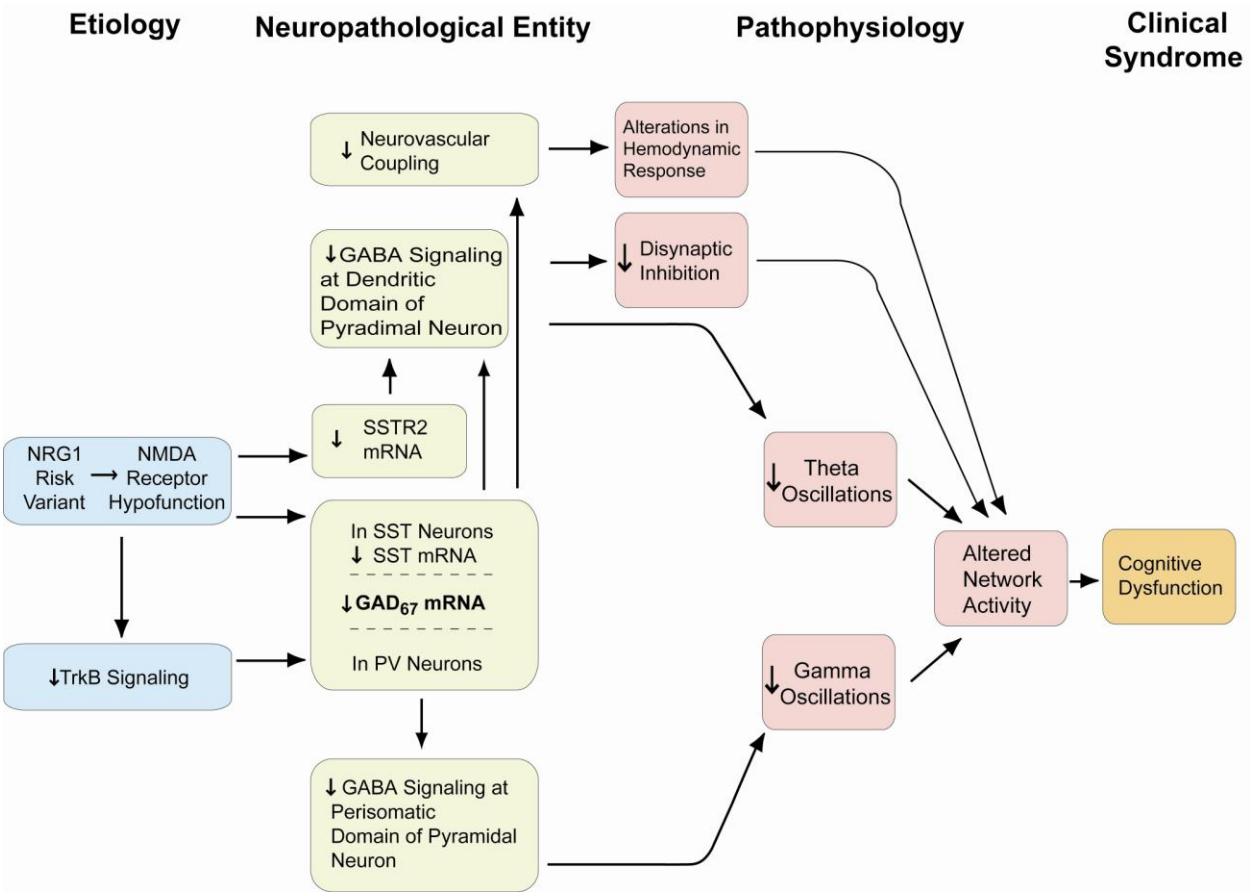

Figure 23 Proposed disease model illustrating potential cascade of events of the disease process in schizophrenia.

A set of genetic liabilities and environmental risk factors produce reduced GABA signaling from two distinct subclasses of GABA neurons. Despite apparent compensatory responses, several downsteam mechanisms from alterations in these two neuron types converge to produce altered network activity, which gives rise to impairments in working memory.

6.1.1 Etiological mechanism of reduced GAD_{67}

This section will review an upstream causal mechanism that can account for the observed alteraltions seen in PV- and SST-containing neurons. This plausible mechanism was chosen because it can account for the data presented in this dissersation; however, many other possibilities exist.

6.1.1.1 Alterations in excitatory neurotransmission in schizophrenia

As mentioned in Chapter 5, several lines of evidence suggest that disruptions in the glutamatergic system in subjects with schizophrenia are a pathophysiology. Besides, the mentioned studies other factors that affect the efficacy or function of modulatory sites on the NMDA receptor or proteins involved in the NMDA downstream intracellular pathways have been implicated in schizophrenia. For example, variants in the gene for neuregulin 1 (*NRG1*) have been shown to increase risk for schizophrenia (Stefansson et al., 2002) and alterations in this protein can reduce NMDA receptor activation through interactions with its erbB4 receptors in the DLPFC of subjects with schizophrenia (Hahn et al., 2006). Thus, these data suggest that NDMA hypofunction perhaps through occurrence of variants in *NRG1* could contribute to the neuropathophysiology of schizophrenia (Figure 23).

Reduced excitatory transmission can be an independent neuropathology in schizophrenia that underlies cognitive impairments in the illness (Moghaddam, 2003). However, because GABA interneuronal activity is affected by glutamatergic inputs, these disturbances in excitatory neurotransmission found in schizophrenia could be affecting GABA neurotransmission and, thus, indirectly affecting working memory function. For example, blockade of NMDA glutamate receptors induces a robust deficit in GAD_{67} levels in many areas of the forebrain (Qin et al., 1994; Bacci et al., 2002; Paulson et al., 2003). Furthermore, GABA neurons are more sensitive to the antagonistic effects of drugs blocking NMDA receptors, as compared to surrounding pyramidal neurons (Li et al., 2002) leading others to suggest that NMDA antagonists that reproduce cognitive deficits seen in schizophrenia primarily affect GABA neurons (Coyle,

2004). Therefore, these data suggest that NMDA hypofunction may be an upstream causal factor contributing to the reductions of GAD_{67} mRNA expression observed in schizophrenia (Figure 23).

Several lines of evidence suggest that excitatory neurotransmission may prefentially affect certain subtypes of interneurons, thus, explaining the selective alterations seen in PV-, but not CR-containing neurons in the DLFPC of subjects with schizophrenia (Chapter 1.2.2). First, glutamatergic drive is stronger to PV-containing than to CR-containing neurons as seen by a much larger total number of excitatory synapses onto PV-containing neurons than on to CR-containing neurons in the rodent hippocampus and monkey DLPFC (Gulyas et al., 1999; Melchitzky and Lewis, 2003). Second, PV- and CR-containing neurons preferentially express different NMDA receptor subunits. In the primate cortex, approximately 50-90% of PV-containing, but only 10% of CR-containing neurons were immunoreactive for the NR1 subunit of the NMDA receptor (Huntley et al., 1994; Huntley et al., 1997). Third, PV-containing interneurons seem to be particularly sensitive to the effects of NMDA receptor antagonists as demonstrated by long-term phencyclidine (PCP) exposure in the rodent. The effects of chronic PCP exposure were a reduction in the expression level of PV mRNA per neuron by 25% without any change in the density of PV mRNA-positive neurons in the rat frontal cortex (Cochran et al., 2003). This pattern of change mimics the alterations seen in PV mRNA expression in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2003). Together, these findings suggest that the selective reduction of GAD_{67} mRNA expression in the PV-containing neurons could be downstream of consequence of NMDA hypofunction (Figure 23).

It is unclear whether alterations in SST-containing neurons are a direct consequence of NMDA hypofunction. However, at least one study suggests that SST-containing neurons are sensitive to reductions in excitatory neurotransmission. In rat somatosensory cortex, both PV- and SST-containing interneurons were robustly and persistently activated by thalamocortical stimulation *in vitro* (Tan et al., 2008) demonstrating SST-containing interneurons also have extensive excitatory inputs. This study suggests that the alterations in GAD_{67} and SST mRNA expression in the DLPFC of subjects with schizophrenia could be another downstream consequence of reduced excitatory drive.

In this dissertation, we demonstrated correlated reductions in SST and its receptor, SSTR2, mRNA. This correlated reduction would have the effect of reducing inhibitory drive at

the dendritic domain of pyramidal cells. We speculated that such a reduction could be a compensatory mechanism in order to help increase excitatory input to the pyramidal neurons (Figure 23). Unfortunately, the effects of reduced exctitatory drive on the expression of SSTR2 mRNA have not been investigated.

Together, these data suggest that in schizophrenia there are reductions in excitatory drive perhaps through NMDA hypofunction mediated by alterations in NRG1 signalling. This reduction in excitatory drive could be an upstream causal factor of reductions in GAD_{67} specifically in PV- and perhaps SST-containing neurons although a direct causal mechanism is unclear for SST-containing neurons. Furthermore, this reduction in excitatory drive may also drive compensatory reductions in SSTR2 mRNA in order to increase the efficacy of excitatory drive at the dendritic domain of pyramidal cells (Figure 23).

6.1.1.2 Altered neurotrophin signaling via trkB receptors in schizophrenia

Another possible upstream causal mechanism that could account for the reductions in GABA-related markers is reduced signaling through the trkB receptor. As previously mentioned in 1.2.2, 1.3.5, and demonstrated in this thesis, it seems that the alterations in GABA-related markers are specific to the SST subpopulations of GABA neurons, but not the CR interneuron subpopulation. Furthermore, the PV-containing neurons may also be selectively affected by reductions in trkB signaling. Therefore, the reductions in BDNF and trkB mRNA expression seen the DLPFC of subjects with schizophrenia (chapter 1.3.5) expression could be an upstream mechanism responsible for reductions in the affected interneuron subpopulations in schizophrenia.

In the cortex, pyramidal neurons synthesize and secrete BDNF in an activity-dependent manner; and, BDNF acts as a growth factor for many GABA neurons that express trkB (Cellerino et al., 1996; Marty et al., 1997). Furthermore, activity in pyramidal neurons enhances dendritic growth and branching in nearby inhibitory interneurons through a BDNF-dependent mechanism (Jin et al., 2003) and BDNF/trkB signaling could mediate levels of GABA throughout the cortex (Rutherford et al., 1997). These alterations could be selective for PV and SST interneurons because most PV interneurons (Cellerino et al., 1996; Gorba and Wahle, 1999) and ~50% of SST interneurons express trkB (Gorba and Wahle, 1999), but CR interneurons do not (Gorba and Wahle, 1999). Furthermore, trkB hypomorphic mice demonstrate selective

reductions in PV mRNA and SST mRNA, but not CR mRNA expression (Hashimoto et al., 2005). Therefore, the reduction in excitatory inputs could lead to the reductions in BDNF and trkB expression found in the DLPFC of subjects with schizophrenia and indirectly alter GAD_{67} expression in and function of PV interneurons and at least a subset of SST interneurons (Figure 23).

6.1.2 Alterations in network activity of the DLPFC of subjects with schizophrenia converge upon a common pathophysiology

Figure 23 summarizes the proposed disease process in which various upstream causal mechanisms such as NMDA hypofunction and BDNF/trkB signaling alterations can converge downstream to account for the alterations seen in GAD_{67} mRNA in PV- and SST-containing neurons. The reduction seen in SSTR2 mRNA was interpreted to be a compensatory mechanism utilized to increase excitatory drive at the dendritic domain of pyramidal neurons. However, such a compensatory mechanism may not be adequate to alleviate a core feature of schizophrenia, working memory deficits. The following sections will propose that alterations in PV- and SST-containing neurons result in several downstream consequences resulting in alterations to coordinated network activity needed for the normal function of working memory is altered in the DLPFC of subjects with schizophrenia and can not be compensated for by a simple increase in efficacy in excitatory drive at the dendritic domain of pyramidal cells.

In chapter 2 we hypothesized that alterations in SST-containing neurons affect disynaptic inhibition which is involved in filtering distracting stimuli and, thus, may be involved in the correlated reductions in sensory gating and working memory performance (Figure 23). We also hypothesized that the alterations in SST-containing neurons affect frontal theta EEG oscillations and, therefore, are involved in working memory dysfunction (Figure 23). Thus, the observed reductions in SSTR2 mRNA may be a compensatory response to reductions in excitatory drive; however, the reduction in inhibitory tone may also have deleterious effects on the coordinated network activity needed for working memory function.

Furthermore, other downstream consequences from reductions in SST mRNA expression could be affecting the proper function of network activity. For example, somatostatin has been

implicated in the proper function of neurovascular coupling. Neurovascular coupling refers to the tightly controlled system of alterations in local blood perfusion to neurons with altered activity. Therefore, this coupling is thought to supply blood to cortical areas which have higher metabolic demands and is the basis of the signals utilized in many functional neuroimaging techniques (Logothetis et al., 2001). In the cerebral cortex, local GABA neurons have been implicated as the mediator between changes in the local activation of neurons and subsequent changes in local vasculature needed for changes in blood perfusion. (Kawaguchi and Kubota, 1997). This is supported by a study demonstrating that GABA neurons provide rich innervation onto local microvessels (small blood vessels within the brain) (Vaucher et al., 2000). Several lines of evidence suggest that SST-containing interneurons are at least one cell type involved in neurovascular coupling. First, evoked cell firing of single SST-containing interneurons in acute frontoparietal rat sections elicited contractions in neighboring microvessels (Cauli et al., 2004). Second, direct perfusion of somatostatin to these acute slices produced constrictions in microvessels (Cauli et al., 2004). Third, RT-PCR analyses demonstrated that smooth muscle cells from these microvessels expressed mRNAs that encode for several SSTR subtypes including SSTR2 mRNA (Cauli et al., 2004). Fourth, *in vivo* activation of SST-containing neurons (indicated by c-Fos-immunoreactivity) with monitoring of cerebral blood flow in rat frontal cortex indicates that these neurons are involved in neurovascular coupling in an intact system (Kocharyan et al., 2008). Furthermore, humans with small-vessel disease, or cerebral microangiopathy (CMA), display degeneration of the cerebral microcirculation (Roman et al., 2002) which seems to affect the cerebral blood flow primarily in the frontal lobes (Hund-Georgiadis et al., 2002) and is accompanied by cognitive dysfunction including reductions in memory task performance (Prins et al., 2005). Since occlusion of blood flow in rats has been shown to reduce neuronal cell firing at least in the brain stem (Sakata et al., 2000), together these data suggest that reduction in somatostatin expression in the DLPFC of subjects with schizophrenia is affecting neurovascular coupling which in turn has deleterious effects on neuronal cell firing and leads to dysfunction of memory tasks. Thus, although SSTR2 mRNA reductions may be a mechanism in which to increase excitatory drive to pyramidal cells it also may have deleterious affects on neurovascular coupling providing a separate mechanism in which to reduce working memory performance in schizophrenia (Figure 23).

Finally, as mentioned in chapter 6.1.2 the PV-contining subclass of GABA-containing interneurons are affected in the DLPFC of subjects with schizophrenia. In contrast to SST-containing neurons, PV-containing neurons tend to innervate the perisomatic region of pyramidal cell suggesting that these neurons are involved in regulating the output of pyramidal cells (Lewis et al., 2005). Networks of chemically and electrically-coupled PV-containing neurons are essential for the synchronization of large cell ensembles of neurons and give rise to oscillatory activity in the gamma-band range (20-80 Hz) (Whittington and Traub, 2003). In the human DLPFC, gamma oscillations appear at the onset of, and are maintained during, the delay period of working memory tasks (Tallon-Baudry et al., 1998) and the power of gamma band oscillations increases in proportion to working memory load (Howard et al., 2003). In individuals with schizophrenia, deficits in working memory are associated with reductions in DLPFC gamma-band oscillations (Cho et al., 2006). Therefore, the alterations seen in PV-containing neurons may impair inhibition at the perisomatic domain of pyramidal cells and consequently, contribute to reduced gamma-band oscillations associated with working memory dysfunction in individuals withs schizophrenia (Figure 23).

These data suggest that within the DLPFC of individuals with schizophrenia there are selective alterations in PV- and SST-containing interneurons. Through several downstream mechanisms these alterations can affect neuronal network activation which ultimately contribute to the working memory dysfunction in schizophrenia. These mechanisms have been attributed to the DLPFC; however, other studies have demonstrated alterations in PV- and SST-containing interneurons in other brain regions of subjects with schizopohrenia (Hashimoto et al., 2008d) suggesting that these mechanisms are not specific to this brain region and may affect other types of cognitive dysfunction in subjects with schizophrenia.

6.1.3 Diagnostic specificity of the profile of GABA-related alterations: implications for the etiology of schziohrenia

Since Kraepelin, a dichotomy between schizophrenia (dementia praecox) and BPD (manic-depressive insanity) has been classically viewed, with specific diagnostic criteria defining schizophrenia and BPD as separate diseases (Kempf et al., 2005). However, based on genetic and

neurochemical findings, many researchers are challenging this position suggesting that instead these disorders are different manifestations of the same disease along a "continuum" of symptom severity (Moller, 2003). As mentioned in 1.2.3, BPD, but not MDD, shares many susceptibility genes and GABA-related pathophysiology with schizophrenia. However, in chapter 3, when we examined the expression profile GABA-related mRNA in the DLPFC of subjects with BPD or MDD, to that seen in schizophrenia each disorder demonstrated unique profiles of alterations. As seen in Table 5, subjects with schizophrenia demonstrated selective reduction in GAD_{67}, PV, and SST whereas subjects with BPD or MDD had selective reductions in PV or SST, respectively. No one transcript demonstrated reductions in all three disorders, suggesting that the the profile of alterations found in each disorder is unique and not due to some common environmental factor that is associated with a chronic debilitating psychiatric illness (e.g. hospitalizations or social isolation).

Instead, these unique profiles suggest each disorder has separate etiological factors contributing to the neuropathophysiology of the DLPFC within these individuals. Although the following conclusions must be confirmed by exprimentation, the selective alterations in certain subpopulations of GABA neurons in BPD (PV interneurons), MDD (SST interneurons), and schizophrenia (both PV and SST interneurons) suggests that each disorder has specific etiologies that uniquely affect these specific subpopulations. These could include, but are not limited to, alterations to the instrinsic efferents to or targets of these subpopulations of GABA neurons. Alternatively, place of origin of PV and SST interneurons, could be selectively affected during development in each of these disorders. Culture experiments and experiments involving the homotopic transplant of progenitors demonstrated that CR-containing interneurons originate from the dorsal region of the caudal ganglionic eminence (CGE) whereas PV- and SST-containing interneurons origate from the medial ganglionic eminence (MGE) in the mouse (Xu et al., 2004; Butt et al., 2005). Furthermore, within the MGE of the mouse, SST interneurons originate from the dorsal region and PV interneurons from the caudal region (Wonders et al., 2008). These data suggest in individuals in schizophrenia the trajectory of interneuron development is affected in the MGE, but not the CGE; futhermore, in individuals with BPD or MDD the developmental trajectories of interneurons from the dorsal or caudal regions, respectively, are altered. The etiologic mechanisms that would confer risk for selective alterations in the respective interneuron developmental trajectories remains unknown; however,

each of these interneuron subpopulations at least in the mouse express specific transcription factors important for normal development and differentiation (Butt et al., 2007) which could be differentially affected within these disorders.

Table 5 Profiles of GABA-related mRNA alterations

Psychiatric Group	Calretinin	GAD_{65}	GAD_{67}	Parvalbumin	Somatostatin
Schizophrenia	↔	↓(?)	↓	↓	↓
BPD	↔	↔	↔	↓(?)	↓
MDD	↔	↔	↔	↔	↓

6.2 LIMITATIONS AND FUTURE DIRECTIONS

The studies within this thesis only examined mRNA expression of GABA-related markers. The functional implications of reductions in mRNA expression can only be true if there is a concurrent reduction in the cognate protein. Unfortunately, small peptides in the brain such as SST are rapidly degraded post-mortem making accurate quatification of the SST protein difficult (Hayes et al., 1991). However, both in the rat and monkey brain SST mRNA and protein are highly correlated (Hayashi et al., 1997; Vela et al., 2003) suggesting the reductions that we observed in SST mRNA expression reflects a concurrent reduction in the cognate protein. The correlation between SSTR2 mRNA and its protein is unknown, thus, confirmation of reductions in the protein concurrent with the reduction in mRNA must be confirmed.

6.2.1 Confirmation that alterations in GABA neurotransmission from SST interneurons affect working memory

We have argued that the correlated reductions in SST and GAD_{67} mRNAs in the same subjects suggest that GABA neurotransmission is altered in SST interneurons. However, these correlations are not direct evidence for a reduction in GABA neurotransmission in this subpopulation.

6.2.1.1 Identification of unique markers for SST interneurons

We did not pursue a dual-label experiment utilizing SST and GAD_{67} mRNAs in order to determine if SST interneurons express less GAD_{67} in schizophrenia because of a potential confound that we identified in Chapter 2. In the DLPFC of subjects with schizophrenia, the density of SST mRNA+ interneurons was significantly reduced. Because in the DLPFC of subjects with schizophrenia there is no reduction in neuronal density, we interpreted this finding

to mean that there is a normal density of SST interneurons in the DLPFC of subjects with schizophrenia; however, some of these interneurons do not express detectable levels of SST mRNA. This suggests that any co-labeling study that is performed with SST mRNA in schizophrenia would be inaccurate since the marker (SST mRNA) is not detectable in some neurons. Therefore, we propose a microarray study in which SST interneurons are selectively isolated via laser microdissection in order to determine potential alternative markers for SST interneurons that are not altered in schizophrenia.

6.2.1.2 Proof of principle experiment: Selective reductions in GABA neurotransmission in SST interneurons

In Chapter 2 and in 2.1.2, we predicted that the functional consequences of alterations in SST interneurons would include reductions in disynaptic inhibition and theta oscillations leading to working memory deficits. Therefore, we propose a series of experiments utilizing mouse models in which SST interneurons express reduced levels of GABA in order to determine if our predictions have merit. These could include either mice with genetic manipulations in which GAD_{67} is selective knocked out or *in vivo* knock-down of GAD_{67} in SST interneurons utilizing viral-mediated RNA-interference.

7.0 CONCLUSION

In the DLPFC of individuals with schizophrenia, GAD_{67} mRNA levels were reduced in layers 2-5 with reduced PV mRNA expression in layers 3 and 4 which suggested that another subpopulation of GABA neurons were affected in layers 2 and 5. The high density of SST interneurons in prefrontal laminar layers 2/superficial 3 and 5 suggested that this subpopulation of GABA neurons were the affected neurons; consistent with this line of reasoning, of several GABA-related mRNA expression alterations, reductions in the level of SST mRNA were the most robust in the DLFPC of subjects with schizophrenia. This thesis confirmed and elucidated the alterations in the SST-containing subpopulation of the DLPFC of individuals with schizophrenia.

In Chapter 2, we demonstrated selective reductions in the expression of SST mRNA in layers 2-superificial 6 and that the changes in SST mRNA expression correlated with GAD_{67} mRNA expression changes in the same subject pairs. Furthermore, this reduction is likely mediated by impaired neurotrophin signaling through the trkB receptor. In Chapter 3, we illustrated that the profile of GABA-related mRNA expression changes were specific to schizophrenia suggesting that other major psychiatric disorders have differing etiologic factors contributing to their respective disease processes. Chapter 4 demonstrated that the affected SST neurons do not include the subset that colocalizes NPY. And, finally, Chapter 5 illustrated that the reductions in SST mRNA expression parallels reductions the expression of mRNA encoding for its post-synaptic receptor, SSTR2.

We concluded that the correlated changes in SST and GAD_{67} mRNAs represent alterations in GABA neurotransmission from the subpopulation of SST-, but not NPY-, containing interneurons; furthermore, the correlated changes in SST and SSTR2 mRNAs reflect a dysfunction of inhibition at the dendritic domain of pyramidal neurons. We interpret these findings to indicate that, in the DLFPC of inviduals with schizophrenia, the pre- and post-

synaptic reductions in inhibition from SST interneurons is an attempt to compensate for a general reduction of excitatory inputs to the dendritic domain of pyramidal neurons. In addition, because SST-containing interneurons play a role in disynaptic inhibition and the synchronization of pyramidal neuron firing in the theta range, both of which are likely important for working memory, alterations in this subpopulation of GABA neurons contribute to the cognitive dysfunction in schizophrenia.

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