MORPHOLOGICAL AND FUNCTIONAL ALTERATIONS IN PYRAMIDAL CELLS IN SCHIZOPHRENIC PREFRONTAL CORTEX.

By

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A Dissertation submitted to the Graduate Faculty in Biology in Partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

MORPHOLOGICAL AND FUNCTIONAL ALTERATIONS IN PYRAMIDAL CELLS IN SCHIZOPHRENIC PREFRONTAL CORTEX

By

Kevin Broadbelt

Advisor: Professor Liesl Jones

Schizophrenia, a disease of the brain, is one of the most debilitating mental illnesses known. Studies suggest morphological and functional alterations of pyramidal cells in the prefrontal cortex (PFC). Two regions of the PFC implicated in schizophrenia are Area's 9 and 32. Examination of Area 32 in 11 schizophrenics and 11 comparison subjects using Golgi-stained material showed a decrease in primary (33%) and secondary (29%) basilar dendrites on pyramidal neurons in layer V. Layer III showed a decrease in primary (17.4%) and secondary (15%) basilar dendrites also. Area 9 of the dorsolateral PFC exhibited a similar trend. A decrease in primary (23%) and secondary (23%) basilar dendrites in layer III were seen. In layer V a decrease in primary (29%) and (31%) secondary basilar dendrites was seen. A decrease in synaptic spine number on primary (18%) and secondary (19%) basilar dendrites in layer III were also seen. Layer V exhibited a decrease in synaptic spines on primary (17%) and secondary (18%) basilar dendrites. Using Scholl analysis, basilar dendritic trees in Area 32 were less extensive in the schizophrenia group.

Neurogranin immunostaining was reduced in layers III (70%) and V (50%) in area 9 using Area Fraction Analysis. Area 32 exhibited (36%) reduction in layer III and a (40%) reduction in layer V. There was no difference in either layer in density of positively stained pyramidal cells. A schizophrenia-associated decrease in Calmodulin area fraction in Areas 9 and 32 was seen. Area 9 (58 %) reduction in layer III and (44 %) reduction in layer V. Area 32 (51%) reduction in layer III and (32%) reduction in layer V.

A decrease in Neurogranin could allow more Calmodulin to interact with free calcium and activate the Calcium-calmodulin signal transduction pathway inappropriately. Activating this pathway incorrectly could, alter information processing of the pyramidal cell. Neurogranin and Calmodulin are greatly reduced in the same neuron population as the loss of dendrites. This data suggests that calcium signaling is altered in prefrontal cortical areas 9 and 32 in schizophrenia and these alterations could lead to loss of dendrites, altered signaling and aberrant information processing.

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<u>Chapter 1</u>: Introduction

GENERAL BACKGROUND INFORMATION

Schizophrenia is a chronic, severe, and disabling brain disorder that affects about one percent of people around the world (including 2.4 million Americans) and has been recognized throughout recorded history. It can trigger hallucinations, delusions, paranoia, and significant lack of motivation. Without treatment, schizophrenia affects the ability to think clearly, manage emotions, and interact appropriately with other people. The symptoms of schizophrenia can be categorized into three classes' negative, positive and cognitive symptoms. Positive symptoms are unusual thoughts or perceptions that include hallucinations, delusions and thought disorder. Positive symptoms are behaviors not seen in healthy people and usually involve a loss of contact with reality, which can come and go. Sometimes they are severe and at other times hardly noticeable, depending on whether or not the individual is receiving treatment. Negative symptoms relate to those abilities or personality traits that are "lost" with schizophrenia. Negative symptoms usually occur first and may still be present during periods of remission as the disease progresses. Negative symptoms represent a loss or a decrease in the ability to initiate plans, speak, express emotion, or find pleasure in everyday life. People with schizophrenia often neglect basic hygiene and need help with everyday living activities. Because it is not as obvious that negative symptoms are part of a psychiatric illness, people with schizophrenia are often perceived by others as lazy and not willing to better their lives. Cognitive symptoms (or cognitive deficits) are problems with attention, certain types of memory, and the executive functions (the ability to absorb and interpret information and make decisions based on that information), that allow us to plan and organize. Cognitive symptoms are subtle and are often detected only when neuropsychological tests are performed. Cognitive impairments often interfere with the patient's ability to live a normal life and can cause great emotional distress.

Scientists have long known that schizophrenia may have a genetic predisposition. As mentioned earlier it occurs in one percent of the general population, but is seen in ten percent of people with a first degree relative (a parent, brother, or sister) with the disorder. People who have second degree relatives with the disease also develop schizophrenia more often than the general population. For example, a monozygotic (identical) twin of a person with schizophrenia has the highest risk – 40 to 50 percent – of developing the illness (Reveley et al., 1982, Suddath et al., 1990). Several genes are thought to be associated with an increased risk of schizophrenia, but scientists currently believe that each gene has a very small effect and is not responsible for causing the disease by itself (Kendler et al., 2006; Derosse et al., 2006; Winantea et al., 2006). Candidate genes such as RAC-alpha serine/threonine-protein kinase (AKT1), brain-derived neurotrophic factor (BDNF), dopamine receptor-3 (DRD3), dystrobrevin binding protein-1 (DTNBP1), and neuregulin-1 (NRG1) have been identified as promising genes. (Kendler et al., 2006; Derosse et al., 2006; Winantea et al., 2006) It is not yet understood how the genetic predisposition is transmitted, and it cannot yet be accurately predicted whether a given person will or will not develop the disorder. Several regions of the human genome are being investigated to identify genes that may confer susceptibility for schizophrenia. Identification of specific genes involved in the development of schizophrenia will provide important clues into what occurs in the brain to produce and sustain the illness. Although there is a genetic risk for schizophrenia, genes alone are not likely to be sufficient to cause the disorder (Shelton et al., 1988, Egan et al., 2001).

A study done by Johnstone et al., (1976) was one of the first significant imaging findings related to schizophrenia. Johnstone et al (1976) carried out a landmark CT study that reported ventricular dilatation in a group of patients with chronic schizophrenia. There have now been a total of over 30 pneumoencephalography (PEG) studies over the past 40 years which in some cases has also shown gross ventricular enlargements. (Honer et al., 1994, Sharma et al., 1998, Silverman et al., 1998). Lateral ventricle studies showed a 20-75% increase in the ventricle to brain ratio (Daniel et al., 1991; Van Horn and McManus 1992) and a median (40%) increase in volume using Magnet Resonance Imaging (MRI) (Lawrie and Abukmeil, 1998). After thorough review Weinberger et al., (1983), identified six PEG studies showing gross abnormalities in the lateral and third ventricles that are accepted as credible (Brown et al., 1986, Pakkenberg et al., 1987, Crow et al., 1989. Problems in the selection of normal control scans may have had significant affect on the degree of dilation of the ventricles reported nevertheless, a significant ventricular enlargement remained as a constant in systematic reviews (Raz and Raz, 1990). A significant find using MRI is global reduction, about (4%) in the volume of cortical grey matter in schizophrenia, without concomitant white matter volume reductions (Woods et al., 1996, Cannon et al., 1998, Sharma et al., 1998, Marsh el al., 1999). Schaepfer et al., (1994) reported significant reductions in grey matter volume occurring primarily in areas of heteromodal association cortices (dorsolateral prefrontal cortex, inferior parietal lobule, and Wernicke's area) leading to the hypothesis that schizophrenia is a specific disorder of this kind of association cortex (Pearson et al., 1996). It has not yet been clarified whether the grey matter volume reduction is due to the reduction in thickness of the cortex (as suggested by some postmortem studies; e.g. Pakkenberg, 1987; Selemon et al., 1995), a reduction in cortical surface area, or both.

The frontal lobe is an area of interest in schizophrenia given the symptomatic, neuropsychological, and functional evidence that implicates this area (Morrison-Stewart et al., 1992; Andreason et al., 1986). Buchanan et al., 1998, identified new reductions in specific subregions of the frontal lobe; he found a 13% decrease in the inferior prefrontal grey matter compared with an average 5% decrease in other frontal regions. Hazlett et al., (2004, 2005), reported three findings on the thalamic measures: 1) a different pattern of glucose metabolism in the prefrontal lobe in response to cognitive activation for the patients with schizophrenia compared with the patients with schizotypal personality disorder and healthy subjects; 2) no volume differences between any of the groups; and 3) a smaller area of activation in the anterior left thalamus in the patients with schizophrenia and in the right mediodorsal region. The findings of functional but not volumetric differences may reflect a lack of normal frontothalamic afferent activity, which might result primarily from diminished frontal activity or possibly from disturbed connections between frontal-striatal-thalamic regions. Other studies using postmortem material have shown other regions of the brain to be affected. Some of the volumetric studies have also showed an (8%) decrease in the overall temporal lobe and 4-12% decrease in the volume of medial temporal structures, such as the hippocampus, parahippocampus and amygdale (Lawrie and Abukmeil, 1998).

Neurobiologists have hypothesized that schizophrenia may be a developmental disorder resulting when neurons form inappropriate connections during fetal development. These errors may lie dormant until puberty, when changes in the brain that occur normally during this critical stage of maturation interact adversely with the faulty connections. In a disorder notable for its neuropathological complexity, establishing the presence of gliosis in schizophrenia would equate a progressive, neurodegenerative process, as Kraeplin believed was the case. On the contrary, an absence of gliosis, in the presence of macroscopic and cytoarchitectural alterations, can be construed as supportive of a neurodevelopmental disease process (Bogerts et al., 1983; Benes et al., 1986; Falkai et al., 1986, Karson et al., 1993; Arnold et al., 1996). When defined neuropathologically, gliosis is often used as shorthand for, and is implicitly referring to astrocytic gliosis (also called reactive gliosis or astrogliosis). Since 1982 there has been a body of work investigating gliosis in schizophrenia (Roberts et al., 1987; Stevens et al., 1988; Crow et al., 1989; Bene et al., 1991; Selemon et al., 1995; Ongur et al., 1998; Rajkowska et al., 1998; Karson et al., 1999). Most of these studies investigating gliosis in schizophrenia have not confirmed the presence of and have largely ruled out the possibility of gliosis. Although each study has limitations, it is noteworthy that negative conclusions are seen in many brain areas, using different methods, and measuring different parameters of gliosis. Because gliosis is an invariable correlate of neural injury, its absence is taken to exclude any form of degenerative or progressive pathology, including classical neurodegenerative disorders, infective, post infective, or autoimmune processes, as occurring in schizophrenia. These data therefore support a hypothesis that schizophrenia is a neurodevelopmental neuropsychiatric disorder.

Schizophrenia, the Prefrontal Cortex and the Thalamus

In recent years a growing body of literature has indicated that the core features of the illness are represented by disturbances in certain cognitive functions, such as those mediated by neural circuits that include components of the dorsolateral prefrontal cortex (DLPFC) and the mediodorsal nucleus (MDN) of the thalamus (Elevag and Goldberg, 2000). The specific causal factors that give rise to these brain abnormalities remain unknown, with a number of genes and a range of environmental events identified as potential risk factors for schizophrenia (Lewis and Leiberman, 2000). Individuals with schizophrenia perform poorly on cognitive tasks that require the use of working memory: the ability to maintain information in order to guide behavior (Goldman-Rakic, 1994).

The DLPFC maintains reciprocal excitatory connections with specific subdivisions of the MDN, the principal source of thalamic inputs to the DLPFC (Figure. 1) (Goldman-Rakic and Porrino, 1985, Giguere and Goldman-Rakic, 1988, Barbas et al., 1991). Like the DLPFC, the MDN has been implicated as a site of dysfunction in schizophrenia (Lewis et al., 2000), it also plays a critical role in working memory functioning. In both rodent and primate brains, retrograde tracing studies have demonstrated that projection neurons in the MDN are organized such that different MDN subdivisions project to specific areas of the frontal lobe (Goldman-Rakic and Porrino, 1985, Barbas et al., 1991). In general the magnocellular MDN division is connected primarily with medial and orbital prefrontal regions, whereas the parvocellular subdivision projects to the DLPFC (Goldman-Rakic and Porrino, 1985, Giguere and Goldman-Rakic, 1988, Barbas et al., 1991). Within the DLPFC, MDN thalamic axons terminate primarily in deep layers 3 and 4 (Giguere and Goldman-Rakic, 1988), although other cortical layers do contain thalamic axons. The majority of axon terminals arising from the MDN form asymmetric synaptic contacts onto dendritic spines of the DLPFC pyramidal neurons. Although most axon terminals arising from the thalamic nuclei contact dendritic spines of pyramidal neurons, the dendrites of gamma-aminobutyric acid (GABA) –ergic cells are also targets of thalamic axons.

In the DLPFC, spines on the basilar dendrites of layer III pyramidal neurons are among the targets from the MDN. Layer 3 pyramidal neurons play a central role in the flow of information both between and within regions. The dendritic spines of other pyramidal neurons constitute the postsynaptic targets of almost all of both white matter and the long-range intrinsic projections of these neurons. The last link in MDN-DLPFC circuitry involves the reciprocal excitatory projections from the DLPFC back to the MDN. The majority of the MDN-projecting cells are small pyramidal neurons located in deep layer VI and large pyramidal cells in layer V. (Giguere and Goldman-Rakic, 1988, Erickson and Lewis, 2000). The corticothalamic axons target both the projection neurons of the MDN and the GABAergic neurons of the reticular thalamic nucleus, which in turn, provide inhibitory regulation of the MDN projection neurons. Studies done in monkeys have shown during postnatal development, substantial changes occur in the developmental trajectory for synaptic density in the DLPFC (Bourgeois et al., 1994). A similar process of synaptic overproduction and elimination also occurs in human DLPFC. Developmental changes in markers of synaptic inputs to the pyramidal neurons that participate in thalamocorticothalamic connections occur in similar fashion. Dendritic spines are the primary sites of excitatory synaptic inputs to pyramidal cells, and changes in spine number appear to reflect parallel alterations in the excitatory inputs to these neurons (Lund and Holbach, 1991).

Both imaging studies and postmortem studies have revealed abnormalities in the thalamus of subjects with schizophrenia (Pakkenberg et al., 1990; Goldman-Rakic, P.S., et al., 1995, Byne et al., 1999, 2001, 2002; Mitelman and Byne 2006). Recent analysis examining 15 magnetic resonance studies of thalamic volume found significant reductions in schizophrenia (Konick and Friedman, 2001). There has also been correlation studies between thalamic volumes and prefrontal white matter in individuals with schizophrenia but not in normal subjects (Portas et al., 1998), suggesting that reductions in thalamocortical projection neurons may contribute to the decrease in DLPFC volume. Two recent imaging studies that subdivided the thalamus support a selective reduction in MDN volume (Byne et al., 2001; Gilbert et al., 2001). Reductions in thalamic volume have also been reported in postmortem studies (Byne et al., 2002; Pakkenberg, 1990; Popken et al., 2000), and these changes may be localized to the MDN and related relay nuclei while not affecting the sensory and motor-related thalamic nuclei.

These reductions in MDN volume have been associated with a decreased number of MDN neurons in schizophrenia (Byne et al., 2002; Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000). Reduced neuronal numbers in the parvocellular and densocellular portions of the MDN, which furnishes projections to the DLPFC, have been reported, whereas neuronal number was unchanged in the magnocellular region, which is connected with the medial and orbital prefrontal regions (Popken et al., 2000, Byne et al., 2002). Some studies have shown negative results in that no change in thalamic volume, neuronal density and total neuronal number were detected (Danos et al., 2005; Cullen et al., 2003, Lewis et al., 2004). However, there were confounding variables that would suggest methodological problems with these studies. In the Danos study, tissue processing was unclear, tissue were sectioned at 18µm and mounted but after histological procedures the volume increased. Storage time and post mortem interval were also extremely high, when compared to other studies on the thalamus, referenced to the Cullen study.

Neuroimaging studies have revealed a 3-5% reduction in cerebral gray matter in subjects with schizophrenia (Pearlson and Marsh, 1999), with areas such as the DLPFC having the largest reductions. These reductions in gray matter may reflect decreases in synaptic connections in the DLPFC (Selemon and Goldman-Rakic, 1999). This abnormal synaptic connectivity may involve disturbances in afferent projections to the DLPFC as well as in excitatory and or inhibitory neurons within the DLPFC. Evidence for abnormalities in MDN to DLPFC projections includes reductions of both pre-and postsynaptic markers for these axons (Guidotti et al., 2000, Lewis et al., 2001, Cruz et al., 2003). Dendritic spine integrity depends on intact presynaptic inputs, reductions in the MDN axon projections may also cause reduced dendritic spine density in the layers that receive thalamic input in the DLPFC. Studies in schizophrenia have shown a reduced spine density on basilar dendrites of DLPFC layer III pyramidal cells (Glantz and Lewis, 2000). The largest reduction in spine density was for those pyramidal cells whose basilar dendrites extended through what is called the thalamic recipient zone. The findings mentioned above suggest that alterations in MDN-DLPFC circuitry play a critical role in the pathophysiology of cognitive dysfunction in schizophrenia.

More recent studies have shifted the focus to the examination of morphological alterations of cell populations in the PFC. Present studies of the neuropathology of schizophrenia suggest morphological and functional alterations of pyramidal cells in the PFC when comparing schizophrenics to control subjects. (Andreasen et al., 1994, Selemon et al., 1999, Rajkowska et al., 1998, Garey., et al 1998, Glantz et al., 2000, Kalus et al., 2000, Jones et al., 2001, Broadbelt et al., 2002, Jones et al., 2002, Pierri et al., 2002, Buxhoeveden et al., 2003, Jones et al., 2004).

Past analysis of the PFC cortex had shown a decrease in MAP2 staining in pyramidal cells (Jones et al., 2002); MAP2 is a protein that is found in dendrites and cell bodies. (Decamilli et al., 1984, Fischer et al., 1987, Li et al., 2000). Data from our lab had shown a loss of primary and secondary basilar dendrites on pyramidal cells in area 32 of the prefrontal cortex in schizophrenia (Broadbelt et al., 2002, chapter 2). A study by Pierri et al (Pierri et al., 2001) has shown a decrease in soma size of pyramidal cells in layer III in the PFC. Pyramidal cells are the primary output from the cortex (Jones et al., 2004, Shapiro et al., 1993, Frith et al., 1996) and their dendrites are the primary mechanism for receiving both excitatory and inhibitory contact (Glantz et al., 2000, Kalus et al., 2000). The pyramidal cells that make up layer III project to other ipsilateral cortical regions and to the same region contralaterally. The pyramidal cells of layer V project to sub-cortical regions. Studies have also found a decrease in spine density in layer III pyramidal neurons of the frontal association areas (Glantz et al., 1997, Garey., et al 1998, Glantz et al., 2000). Spine density is a marker of the number excitatory inputs to pyramidal neurons (Glantz et al., 1997, Garey., et al 1998, Glantz et al., 2000). Glutamate and dopamine afferents terminate on dendritic spines whereas GABA terminals are often found on dendritic shafts and cell bodies (Garey et al., 1998, Glantz et al., 2000). Several hypotheses implicate one or more of these neurotransmitters systems in the pathophysiology of schizophrenia (Benes et al., 1995, Blows et al., 2000). A loss of both spines and dendrites, which has been observed, could suggest a decrease in synaptic surface area for both excitatory and inhibitory inputs.

Morphological changes seen in pyramidal cells in the prefrontal cortex can be related to the disruption in neuronal circuitry in this region of the brain (Isseroff et al., 1982, Harrison et al., 1996, Selemon et al., 1999).

Changes such as a loss of dendrites and dendritic spines will reduce synaptic surface area for incoming afferents (Isseroff et al., 1982, Harrison et al., 1996, Selemon et al., 1999). This finding correlates with the reduction in neuropil; hypothesized by Selemon and Goldman-Rakic (Selemon et al., 1999). Studies showing cell loss in the MD nucleus may strengthen the belief of impaired activity in the dorsal DLPFC in schizophrenics to be associated with diminished thalamocortical connections. Based on the structural alterations seen on pyramidal cells, it is necessary now to look at the internal functional aspects. One link between structure and function in these cells is calcium signaling.

Role of calcium during development

During central nervous system development the thalamic afferents arrive at the developing cortex and enter the subplate (Benes et al., 1989, Van Peltet al., 1996, Sheu et al., 1996, Pakkenberg et al., 1997, Li et al., 1999, Thune et al., 2001, Lidow et al., 2001) prior to the birth of their primary target cell layer (Benes et al., 1989, Van Pelt et al., 1996, Sheu et al., 1996, Pakkenberg et al., 1997, Li et al., 1999, Thune et al., 2001, Lidow et al., 2001). For most cortical areas, layer IV is the primary target for thalamic afferents, however; in agranular cortices such as areas 9, 10 and 32 there is a very small layer IV, and the thalamic afferents have a dense input in layer III and also layer V (Honer et al., 1997, Chakravarthy et al., 1999, Novak et al., 2000, Jones et al., 2002). These excitatory projections from the thalamus to the cortex synapse primarily on layer III and layer V apical dendrites (Honer et al., 1997, Chakravarthy et al., 1999, Jones et al., 2002,). These thalamic afferents aid in the development of the cortex and more specifically in the activity-dependent process of dendritic pruning (Haroutunian et al., 1979, Goldman-Rakic et al., 1995, Holcomb et al., 1996, Goldman et al., 1997, Jones et al., 2001).

Neurons adapt their neuritic field to maintain a particular level of bioelectric activity (Haroutunian et al., 1979, Goldman-Rakic et al., 1995, Holcomb et al., 1996, Goldman et al., 1997). Electrical stimulation or depolarization has been shown to increase neurite outgrowth. These effects are dependent on the influx of extracellular calcium (Benes et al., 1995, Gerendsay et al., 1997, Blows et al., 2000). The rate of growth cone extension is dependent upon an optimal level of intracellular calcium, and if calcium levels fall below this level growth cones will become smaller and stop elongating or retract (Gerendsay et al., 1997, Blows et al., 2000). Lowering the level of intracellular calcium arrests axonal outgrowth, stops net addition of dendrites and dendritic branching at least in part by inhibiting polymerization of actin. Thus, lowered levels of intracellular calcium during development could lead to the changes in neuronal morphology that have been observed in schizophrenia.

One protein that plays a major role in calcium signaling in pyramidal cells is neurogranin. Neurogranin is a neural specific calmodulin binding protein that is postnatally expressed in cell bodies, dendrites and spines of the cerebral cortex (Alvarez-Bolado et al., 1996, Gerendsay et al., 1997, Li et al., 2000). Neurogranin contains a sequence homolog of 20 amino acids that has been designated as the IQ motif, where both the calmodulin binding domain and protein kinase C (PKC) phosphorylation site lies (Alvarez-Bolado et al., 1996; Prichard et al., 1999, Li et al., 2000). Neurogranin is an upstream regulator of both calcium and calmodulin and also functions to concentrate calmodulin in the cytosol of neurons (Alvarez-Bolado et al., 1996; Prichard et al., 1999, Li et al., 2000). Calmodulin has a high affinity for neurogranin in the absence of or low concentrations of calcium. If there is a large enough surge of calcium via the activation of the NMDA receptor, calmodulin is released and is available to bind free calcium. The resulting Ca-Calmodulin complexes can now move through out the cell to activate Ca/Calmodulin-dependent protein kinases (Sheu et al., 1996, Chakravarthy et al., 1999; Li et al., 1999, Prichard et al., 1999, Ho Pak et al., 2000; Novak et al., 2000).

Calcium reduces the affinity of neurogranin for calmodulin, neurogranin is also regulated by two molecules PKC and nitric oxide (NO) (Sheu et al., 1996, Gerendsay et al., 1997; Li et al., 1999, Li et al., 2000). PKC phosphorylates the Ser36 in the calmodulin binding domain of neurogranin therefore blocking calmodulin binding. Calcium-calmodulin complexes activate the nitric oxide synthase pathway (NOS) which produces nitric oxide (NO) (Chakravarthy et al., 1999). NO reconfigures the neurogranin calmodulin-binding domain on neurogranin that also inhibits calmodulin rebinding (Sheu et al., 1996, Gerendsay et al., 1997; Li et al., 1999, Li et al., 2000). Although the physiological functions of neurogranin have not been defined, its biochemical properties and postsynaptic localization have implicated it in several signal transduction pathways (Li et al., 1999, Prichard et al., 1999).

Changes in the expression or localization of neurogranin may add to the growing body of data which implicates dendritic alterations in schizophrenia. One reason any change in neurogranin expression can affect the cell is because of the role neurogranin plays in mature neurons. Neurogranin moves from the perikaryal region to the cell surface to participate in the formation and functioning of dendritic spines and synapses. Because of its role as a calmodulin-regulatory protein, alterations in neurogranin expression will have an adverse effect on calmodulin-stimulated enzymes and the calcium-calmodulin signal transduction pathway, which can affect the cell’s ability to process incoming information.

Recent studies in rats have shown that neurogranin is located in the soma of neurons in early development but in early postnatal development it is more concentrated in the neuropil, mainly in dendrites and spine (Alvarez-Bolado et al., 1996; Chakravarthy et al., 1999, Prichard et al., 1999). Quantitative results from neurogranin immunohistochemical experiments show a 50% decrease in staining in layer V and a 70% decrease in layer III of area 9 of the dorsolateral prefrontal cortex and a similar trend in area 32 of the medial prefrontal cortex showing a 40% decrease in staining in layer V and a 36% decrease in layer III of subjects with schizophrenia (chapter 4 {published}). A decrease or abnormal localization of neurogranin during development of the central nervous system may affect many early developmental processes (Ho Pak et al., 2000). Such processes include dendritic spine formation and synaptogenesis. Recent studies have shown a loss of dendritic spines and basilar dendrites on pyramidal cells in the prefrontal cortex of subjects with schizophrenia (Goldman et al., 1997, Garey., et al 1998, Selemon et al., 1999, Buxhoeveden et al., 2000; Glantz et al., 2000; Kalus et al., 2000, Jones et al., 2001, Broadbelt et al., 2002). A decrease in neurogranin can imply that while the cell is at rest free cytosolic unbounded calmodulin is capable of binding free calcium forming calcium-calmodulin complexes thus activating the pathway aberrantly (Chakravarthy et al., 1999; Li et al., 1999). The irregular activation of the calcium-calmodulin pathway may lead to a decrease in unwanted neuronal activity which can possibly disrupt the neuronal circuitry of these cells. Imaging studies have shown that there is a decrease in frontal lobe activity in subjects with schizophrenia (Levin et al., 1984, Weinberger et al., 1988; Seleman et al., 1992, Karson et al., 1996, Sharna et al., 1998; Levitt et al., 1993, Pakkenberg et al., 1997, Jaber et al., 1997, Rajkowska et al., 1998, Jones et al., 2001). The decrease in activity may be related to the cells compensating for the decrease in neurogranin by altering the levels of calmodulin to a lower level. This low level of calmodulin can have an effect on cell signaling and also be correlated with the decrease in neuronal activity in the frontal lobe of schizophrenics.

The NMDA receptor plays a major role in controlling calcium influx into the cell (see Figure 3). Once activated it allows influx of calcium which causes neurogranin to release bounded calmodulin (Alvarez-Bolado et al., 1996; Chakravarthy et al., 1999; Prichard et al., 1999). One of the enzymes activated down stream by the calcium-calmodulin complex is calmodulin kinase II (CaMKII) (Alvarez-Bolado et al., 1996; Chakravarthy et al., 1999; Prichard et al., 1999). Studies have shown that CaMKII is localized both on pre and postsynaptic cells (Liu et al., 1996; Shen et al., 1998; Zou D-Jet al., 1999, Novak et al., 2000, Borodinsky et al., 2002, McDonald et al., 2002). In the development of the nervous system CaMKII has an important role in controlling dendritic branch formation (Kater et al., 1988, Audesirk et al., 1997; Zou D-Jet al., 1999, Borodinsky et al., 2002). The size of dendritic branches is important for the proper functioning of the cell. If branches are too small or too large this will affect the way information is transmitted, interpreted and integrated. Changes therefore in CaMKII, as a result of the decrease of neurogranin and its action on calmodulin will have an adverse effect on cells causing structural anomalies and aberrant functioning.

This research aims to clarify and give a better understanding of how the morphological changes seen on pyramidal cells in layers III and V, in areas 9 and 32 of the prefrontal cortex may be related to changes or alterations in cell signaling in the prefrontal cortex in schizophrenia. This study puts us one step closer to our goal; to better decipher and understand the underlying mechanism in schizophrenia and to add to the growing body of data suggesting cytoarchitectural alterations in these regions of patients affected by the disease.

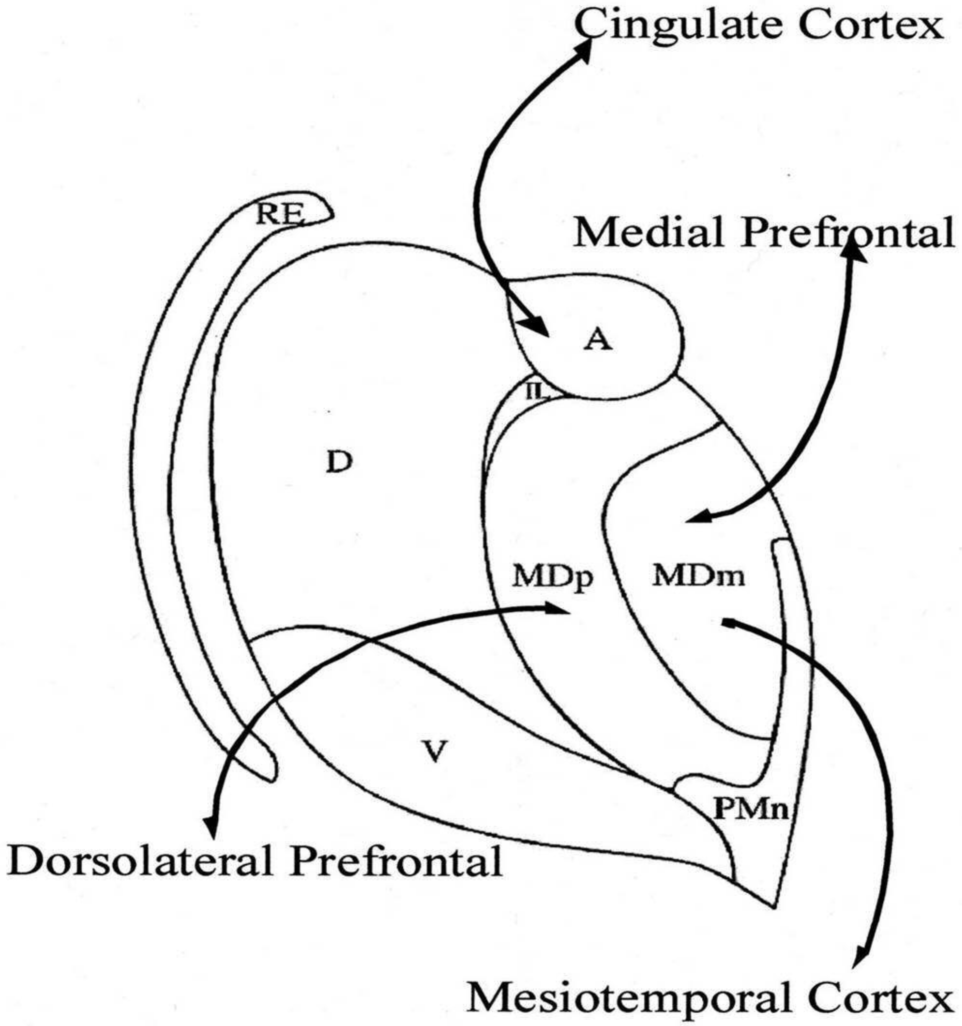


Figure 1. Connections of the Mediodorsal nuclei of the thalamus. (need reference)

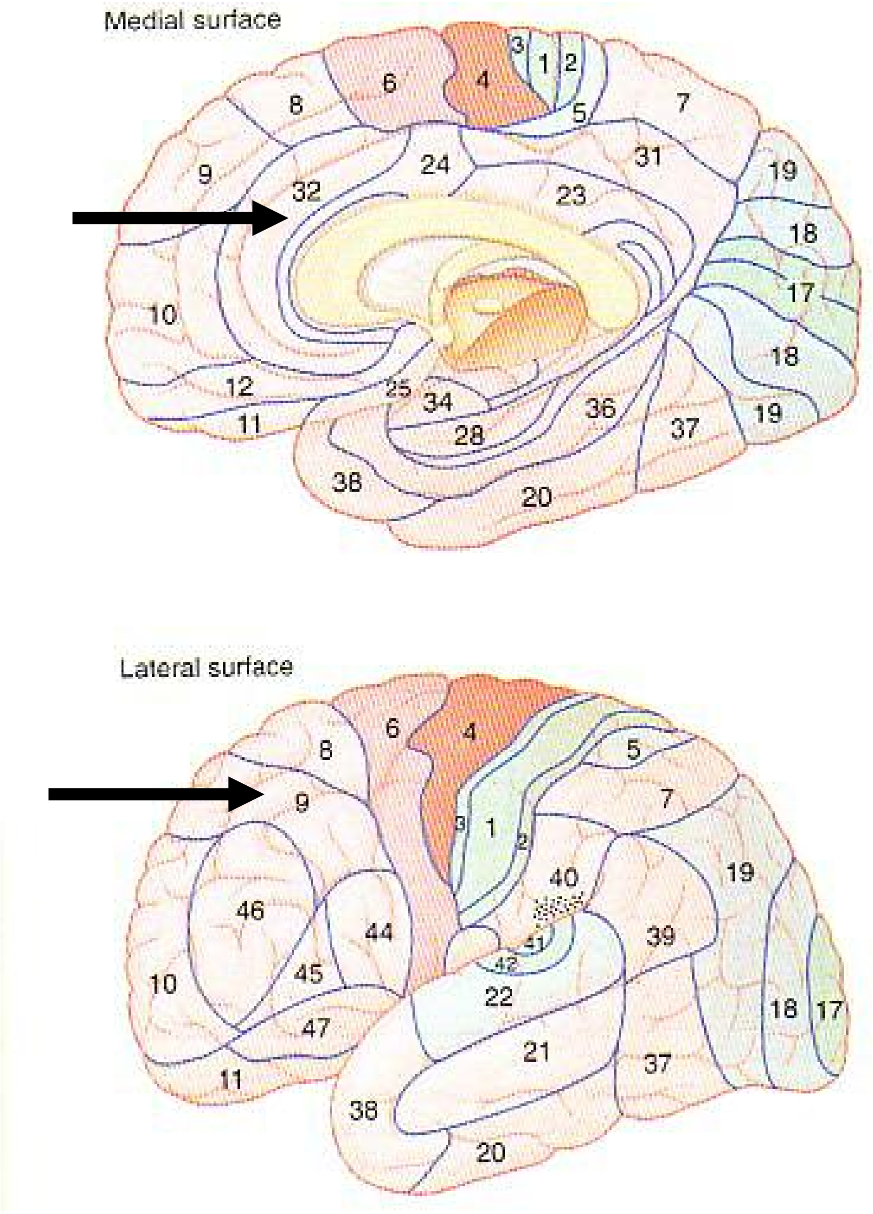


Figure 2. Broadman's map of the brain, A9 located on the dorsolateral region and A32 located in medial prefrontal. (Fitzgerald MJT, 2002. Clinical Neuroanatomy. P 244)

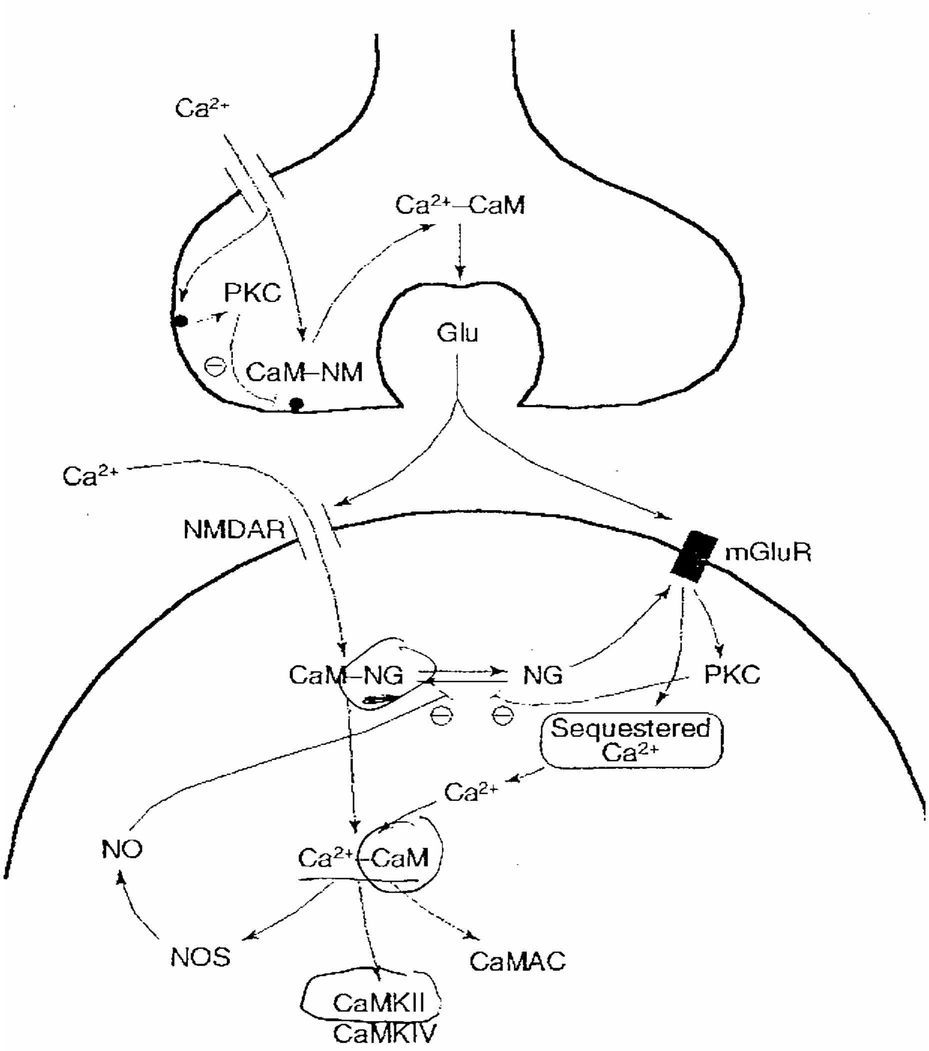


Figure 3. Schematic showing calcium dependent pathway involving neurogranin within the cell. (NG-neurogranin, NO-nitric oxide, NOS-nitric oxide synthase, Cam-Calmodulin, CaMAC-Calmodulin adenyl cyclase, PKC-protein kinase C)

CHAPTER 2: EVIDENCE FOR ALTERATIONS OF BASILAR DENDRITES ON PYRAMDAL CELLS IN A32 MEDIAL PREFRONTAL CORTEX AND A9 OF THE DORSOLATERAL PREFRONTAL CORTEX IN SCHIZOPHRENIA.

ABSTRACT

A variety of lines of converging evidence implicates the prefrontal cortex (PFC) in schizophrenia. Previous neuroanatomical studies have shown schizophrenia-associated changes in neuron density, soma size and spine number. We recently demonstrated a schizophrenia-associated decrease in microtubule-associated protein 2 (MAP2) immunostaining in laminae III and V of medial prefrontal area 32 and interpreted that finding as suggestive loss of dendritic material. We now present data from medial prefrontal area 32 and dorsolateral Area 9 of 11 schizophrenics and 11 comparison subjects. In Golgi-stained materials in Area 32, we describe a significant decrease in the number of both primary (29%) and secondary (46%) basilar dendrites on pyramidal neurons in layer V. Similarly, in layer III there was also a decrease in both primary (17%) and secondary (15%) basilar dendrites. We also found a significant schizophrenia associated decrease in the dendritic spines on primary and secondary basilar dendrites. In layer III a (18%) decrease was found on primary dendrites and in a (19%) decrease on the secondary dendrites. Similarly, in layer V there was also a decrease in synaptic spine density on both primary (17%) and secondary (18%). As measured by Sholl analysis, basilar dendritic trees of the pyramidal cells in Area 32 were less extensive in schizophrenia group than in the control group (Control= 2.96, Schizophrenia =1.38, p=0.007, t_{10}=2.57). Data from dorsolateral prefrontal area 9 of 11 schizophrenics and 11 comparison subjects in Golgi-stained materials, showed a significant decrease in the number of both primary (29%) and secondary (31%) basilar dendrites on pyramidal neurons in layer V. Similarly, in layer III there was also a decrease in both primary (23%) and secondary (31%) basilar dendrites. These finding

suggest a decrease in synaptic surface area which could lead to aberrant information processing.

INTRODUCTION

Postmortem studies have suggested that abnormalities in the PFC are associated with schizophrenia (Benes et al., 1991; Shapiro et al., 1993, Davis and Lewis, 1995, Selemon et al., 1995, Akbarain et al., 1996; Anderson et al., 1996; Glantz and Lewis, 1997; Honer et al., 1997; for review see Goldman-Rakic and Selemon, 1997; Garey et al., 1998, Rajkowska et al., 1998; Thompson et al., 1998; Harison, 1999; Dean et al., 1999). Studies of the PFC have found an increase in cell density in areas 9 and 10 (Benes et al., 1991; Selemon et al., 1995). The authors of those studies suggested that a schizophrenia-associated increase in neuronal density may reflect a decrease in the amount of neuropil. Research in the PFC has primarily focused on area 9, 10 and 46 while few studies have examined the role of Brodmann area 32 in schizophrenia. Some authors consider area 32 to be a part of the cingulofrontal transition cortex (Vogt et al., 1995; Conti et al., 1999); however; we and others (Brachevalier et al., 1997; Dombrowski et al., 2001) consider area 32 to be part of the medial prefrontal cortex. Like areas 9 and 10 and other regions of the PFC, area 32 is involved in higher cognitive function, working memory, mental imagery, willed action and active memory (Frith and Dolan, 1996).

Recently we described a decrease inmunostaining for microtubule-associated protein 2 (MAP2) in layers III and V of prefrontal areas 9 and 32 (Jones et al, 2002). MAP2 is a protein found in dendrites as well as cell bodies. These data are, therefore, consistent with the hypothesis (Selemon et al., 1995) of a schizophrenia-associated decrease of dendritic material. The primary cell type found in the affected laminae is the pyramidal cell, which is the main projection neuron from the cortex (Ghez, 1991). Pyramidal cells of area 32 receive thalamic input as well as GABAergic input. A loss of dendrites would therefore decrease the surface area from which the pyramidal cells could receive information and, therefore, transmit information (Table 1.).

Recent findings in the PFC suggest a schizophrenia-associated decrease of synapses formed by both local GABAergic neurons and thalamocortical neurons (Woo et al., 1998; Pierri et al., 2001; Lewis, 2000; Lewis et al., 2001). Pyramidal cells in layers III and V receive input from local GABAergic cells (Giguere and Goldman-Rakic, 1988). A study by Lewis (2000) has shown a deficit in GAT-1 cartridges of calbindin immunopositive terminals in the PFC suggesting that there may be a loss of GABAergic input onto the pyramidal cells in layers III and V. In addition to receiving local input, pyramidal cells in layers III also receive input from neurons of the medial dorsal nucleus of the thalamus (Bolz et al., 1996). At least four laboratories have describes a schizophrenia-associated decrease in cell number in the medial dorsal nucleus (Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000; Byne et al., 2002). A recent study by Lewis et al. (2001) has shown lamina specific deficits in parvalbumin immunoreactive varicosities. The varicosities are a marker for thalamic input into the cortex. Thus, data from local input and thalamic input suggest a decrease in synaptic contact onto the pyramidal cells have lost dendritic material, which may be suggestive of alterations in information processing.

In order to address the question of dendritic loss or any change in synaptic surface area, we employed Golgi-stained material to examine the number of primary and secondary basilar dendrites on pyramidal neurons, differences in synaptic spine density and Sholl analysis for changes in dendritic branching complexity in layers III and V of prefrontal area 32.

MATERIALS & METHODS

Analyses were conducted on postmortem brain tissues from 11 subjects diagnosed with schizophrenia and 11 non-schizophrenic comparison subjects matched for age, sex and postmortem interval (Table 1). All tissues were obtained from the Harvard Brain Tissue Resource Center (HBTRC) at McLean Hospital and were collected at the time of autopsy in accordance with an approval Institutional Review Board protocol. Diagnosis Conformed to DSM-4 criteria and were established retrospectively by reviewing medical records and interviewing knowledgeable informants. Tissues were not included in the study if there was evidence of neuropathology (e.g., Alzheimer's disease) or substance dependence as determined by the HBTRC. Formalin-Fixed specimens (1cm in thickness) from each cortical region of interest were dissected in the coronal plane by qualified neuropathologists at the midline rostral to the cingulate gyrus.

Modified Golgi Method for Silver Impregnation

A Golgi preparation described by Armstrong and Parker (1986) was used with some slight modifications as described below. Specimens were processed in pairs, control and schizophrenic. The tissue was cut into 2-mm slabs then wrapped in gauze. The tissue was first placed in a solution containing 100ml dH_2O**,** 3 g potassium dichromate, 12.5 g sucrose, 5ml 37% formalin, and 7.5 ml of 3% H_2O_2 and incubated in an oven at 55 °C for 6-7 hours. The tissue was removed from the oven and placed in fresh solution and left again at room temperature over night. The following day, the tissue was placed in a second solution containing 100 ml dH_2O, 3 g potassium dichromate and 12.5 g sucrose and incubated in an oven at 55 °C for 6-7 hours.

Following the second incubation, the tissue was placed in fresh solution and left again at room temperature over night. The following morning, the tissue was rinsed in 0.75% silver nitrate and placed in fresh 0.75% silver nitrate. Because silver nitrate is light sensitive, the jars were wrapped in aluminum foil and left at room temperature for 5 days. Finally, the tissue was serially sectioned on a vibratome into 150-µm thick sections and collected in 70% alcohol. The sections were dehydrated in graded alcohol starting at 70%, defatted in xylene, mounted onto slides and coverslipped.

Quantification of Dendritic Material

A Bioquant Image Analysis System interfaced with an Olympus AX70 microscope connected to a Ludl Motorized stage and a Sony 3-chip camera was used to count the number of primary and secondary basilar dendrites (Fig. 1). A contour was drawn to outline each lamina separately and a counting grid was placed inside the contour. The computer randomly marked half of the intersections to be measured and a counting box 200X200X50 µm (a buffer zone of 10 µm on either surface was employed so as not to include the cut surfaces) was placed inside the marked grids; and the dendrites of the cells within the grid, which met the criteria, were counted. Approximately 50 cells per layer per brain, randomly selected, as described below, were assessed for each specimen. The counting of spines along the primary and secondary basilar dendrites was done at 100X under oil. Ten cells per brain were chosen randomly. Cells were assessed only if the cell body was easily seen and had no secondary branches being obscured by background or by another cell. The counting of the primary basilar dendrites and secondary basilar dendrites was done using a 20X Plan Apo objective. Although a counting box was employed to select then neurons to be assessed, dendrites of selected neurons were counted even if they extended beyond the boundaries of the counting box. Tracing was performed using the program Neurolucida (MicroBrightfield). Dendritic tracing were quantified by Sholl Analysis, performed by the Neurolucida software (Neuro-Explorer). This procedure constructs a series of equally spaced, spherical shells around the center of the cell body and then determines the number of dendritic processes intersecting each successive shell (ie, at each radius). For anaylsis, each case was represented by an average value at a given shell radius for basilar dendrites of all traced neurons in the given region, a total of 10 neurons were counted for each brain.

To control for potential effects of age, postmortem interval and storage time, tissue was matched for those variables and processed in sets of matched pairs. Data were analyzed using paired t-tests (Microsoft Excel 2000). Since we were testing a directional hypothesis, all the tests employed used one-tailed probabilities.

RESULTS

Area 32

Compared to Non-psychiatric controls, schizophrenics exhibited a 29% decrease in the number of primary basilar dendrites (first branch from soma) in layer V (control=1.71±0.1, schizophrenic=1.11±0.06, t_{10}=-6.92, p=0.002). A 46% decrease in the number of secondary basilar dendrites (first branch coming off the primary dendrite) was observed in layer V (control=1.32±0.04, schizophrenic=0.95±0.03, t_{10}= 5.20, p=0.03) (Table 2.). Similarly, a 17.4% reduction was seen in primary basilar dendrites in layer III (control=2.13±0.35, schizophrenic=1.76±0.14, t_{10}= 2.34, p-0.02) (Table 2). In addition, a 15% decrease in secondary basilar dendrites were seen in layer III (control=1.44±0.1, schizophrenic=1.23±0.13, t_{10}=2.03, p=0.03) (Table 2). A decrease was also observed in synaptic spine density on primary and secondary dendrites. Compared to controls, schizophrenics exhibited a (18%) decrease in the number of spines on primary basilar dendrites in layer III (control=542±14.4, schizophrenic=442±29.8, t_{10}= 2.22, p=0.0004). A (19%) decrease was observed for synaptic spine density on secondary dendrites in layer III (control=426±10.17, schizophrenic=345±19, t_{10} =2.22, p=0.00001). Similarly, a (17%) reduction was seen on primary dendrites in layer V (control=603±14, schizophrenic= 502± 26, t_{10}= 2.23, p=0.00001). Also a decrease was observed on the secondary branches, (18%) in layer V (control=512± 9, schizophrenic=419±19, t_{10} = 2.23, p=0.00002). As measured by Sholl analysis, basilar dendritic trees of the pyramidal cells in Area 32 were less extensive in schizophrenia group than in the control group (Control= 2.96, Schizophrenia =1.38, p=0.007, t_{10}=3.61).

Area 9

Compared to Non-psychiatric controls, schizophrenics exhibited a 23% decrease in the number of primary basilar dendrites in layer V (control=1.97±0.3, schizophrenic=1.40±0.02, t_{10}=-6.80, $p=0.00004$). A 31% decrease in the number of secondary basilar dendrites was observed in layer V (control=1.66±0.04, schizophrenic=1.14±0.04, t_{10}= 5.83, $p=0.0001$) (Table 6, 7). Similarly, a 29% reduction was seen in primary basilar dendrites in layer V (control=1.97±0.3, schizophrenic=1.40±0.2, t_{10}= 5.83, $p=0.00004$) (Table 6, 7). In addition, a 23% decrease in secondary basilar dendrites were seen in layer III (control=3.34±0.50, schizophrenic=2.54±0.43, t_{10}=3.29, $p=0.0005$) (Table 5, 6).

DISCUSSION

The present data suggest a schizophrenia-associated decrease in the number of both primary and secondary dendrites and spines on the pyramidal neurons of layers III and V in both medial prefrontal area 32 and area 9 of the dorsolateral prefrontal cortex.

These differences are unlikely to be artifacts attributable to variation in age, sex or postmortem interval because specimens were matched for those variables; however, the present data cannot exclude an effect of neuroleptics. The matched-pair technique should also minimize any artifacts due to the capriciousness of Golgi-staining. While, in actuality, pyramidal neurons have more secondary than primary dendrites, in the material employed in the present study, more primary than secondary dendrites were stained. This may be because dendrites taper as they branch and the staining technique my favor impregnation of larger processes. In addition, we employed coronal sections so that the various cortical laminae could be precisely identified. While the dendrites of pyramidal neurons may be visualized more completely in sections cut parallel to the pial surface, the plane of sectioning employed should not influence the quantitative comparisons between control and schizophrenic subjects. Finally, we know of no evidence that schizophrenia alters the selectivity if the Golgi stain in a manner that would alter the interpretation of the present findings.

Selemon et al. (1995) hypothesized that the schizophrenia-associated increase in neural density they observed in PFC is due to a decrease of neuropil. Such a decrease could result from a loss of any of the components of neuropil including dendrites, synapses, axons or support cells. In addition to the present evidence, several previous studies are consistent with a schizophrenia-associated decrease in dendritic material. Specifically, MAP2 immunostaining has been found to be decreased in layers III and V in prefrontal areas 9 and 32 (Jones et al., 2002), and the density of dendritic spines has been found to be lower in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000). In addition, the size of a neuron’s soma is positively correlated with the amount of dendritic material it can support (van Pelt et al., 1996; van Ooyan et al., 1995). Thus, recent reports of a schizophrenia-associated decrease of neuronal somal size in the PFC (Rajkowska et al., 1998), and specifically the somal size of pyramidal neurons of layer III (Pierri et al., 2001) are also consistent with the hypothesis of a schizophrenia-associated decrease of dendritic material.

The decrease in the number of basilar dendrites and a change in synaptic spine density observed in the present study may reflect a loss of surface area for both excitatory and inhibitory inputs. Both cortical-cortical and cortical-subcortical communications have been found to be impaired in schizophrenia (Bertolino et al., 1999; Kalus et al., 1999; Lewis, 2000). While the loss of dendrites in layer III would primarily disrupt the transfer and integration of information between both ipsilateral and contralateral cortical regions (Ghez, 1991), the loss of layer V dendrites would affect primarily the exchange and integration of information between cortical and subcortical regions (Ghez, 1991), including the thalamus.

Two recent studies have reported a schizophrenia associated loss of neurons in the parvocellular division of the mediodorsal nucleus (Popken et al., 2000; Byne et al., 2002) which has extensive projections to the PFC (Goldman-Rakic and Porrino, 1985; Giguere and Goldman-Rakic, 1988), and markers for thalamic axons have been found to be decreased in the PFC of schizophrenics (Lewis et al., 2001). Thus, the schizophrenia-associated decrease of dendrites we observed in layer III may reflect, at least in part, a decrease of input from the thalamus, a nodal link in communication among multiple brain regions. The schizophrenia-associated decrease of dendrites in layer V may also reflect a loss of thalamic input as well, while both the decrease in layers III and V may reflect a loss of GABAergic input. The decrease in dendritic material and branching complexity is suggestive of a loss of synaptic surface area, which may alter information processing.

The present study represents our further examination of the PFC in schizophrenia to better understand how the disease alters neural circuitry. It remains to be shown whether the decrease of dendrites observed in the present study is restricted to the particular cortical area and the laminae we examined, or whether it more diffusely affects the PFC or other cortical regions. It also remains to be determined whether the present findings are specific to schizophrenia as opposed to other chronic mental illnesses including the schizophrenia spectrum and affective disorders.

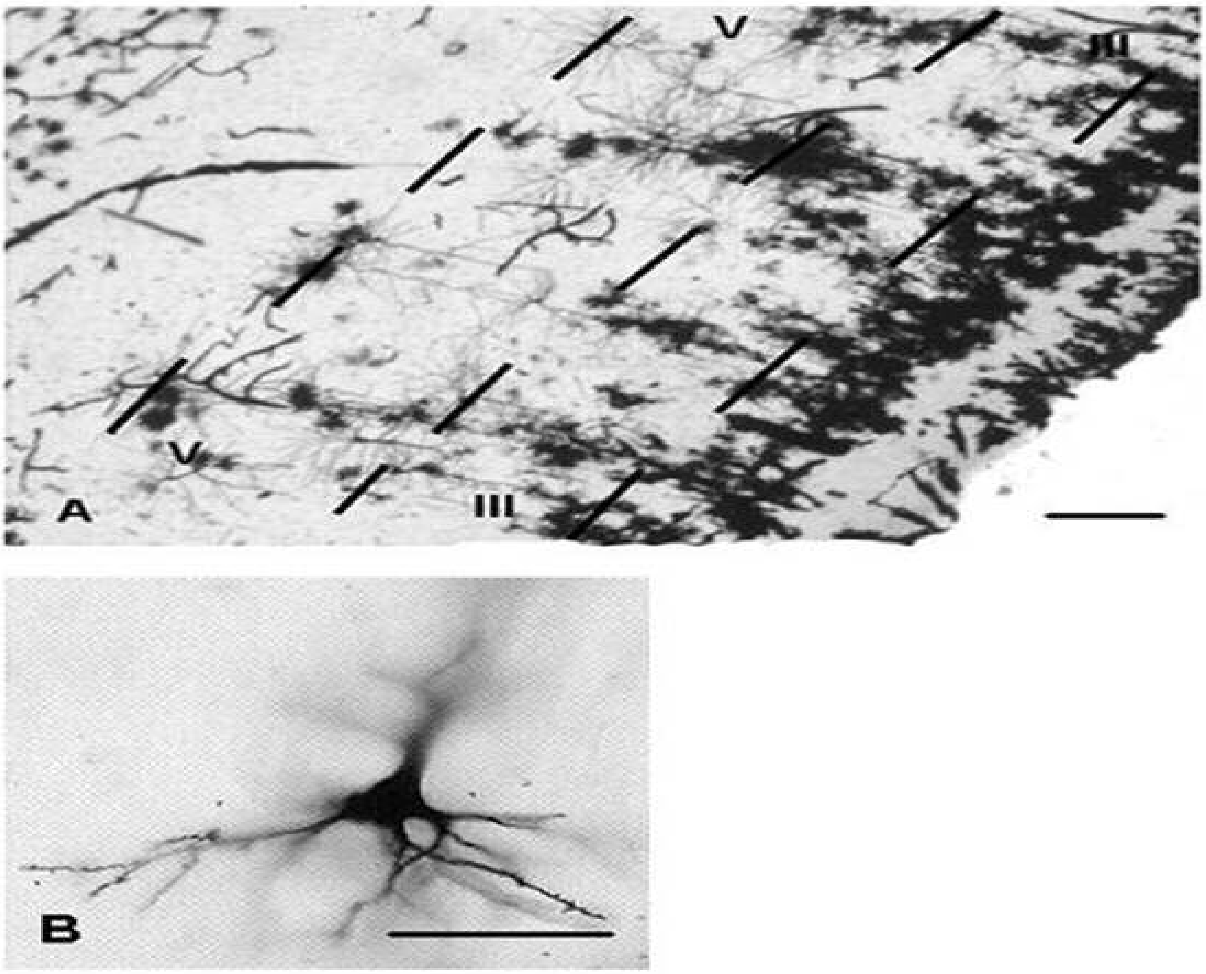


Figure 1. Photomicrograph of Golgi impregnated cells in Area 32 layers III and V, (B) golgi stained pyramidal cell. (Taken from Broadbelt et al, 2002)

Demographic data for the Golgi Study

Brain #	Distributive Dx	Age	Sex	PMI	Area	Hemisphere	Storage time
4158	Schizophrenia	42	M	18.1	32	Right	1 yr 4 mo
4150	Control	42	M	14.7	32	Right	1 yr 4 mo
4314	Schizophrenia	67	F	21.8	32	Left	10 mo
4072	Control	69	F	20.7	32	Right	1 yr 6 mo
4198	Schizophrenia	46	M	18.5	32	Right	1 yr 3 mo
4192	Control	46	M	25.9	32	Right	1 yr 3 mo
4539	Schizophrenia	62	M	25.7	32	Right	2 yrs
4635	Control	62	M	29.2	32	Right	1 yr 7 mo
4572	Schizophrenia	54	F	32.6	32	Right	1 yr 9 mo
4625	Control	53	F	24	32	Left	1 yr 7 mo
4707	Schizophrenia	60	F	19.6	32	Left	1 yr 1 mo
4810	Control	62	F	16.4	32	Right	No data
4375	Schizophrenia	31	M	-	32	Left	1 yr
4211	Control	30	M	23	32	Right	1 yr 2 mo
4256	Schizophrenia	47	M	19.3	32	Left	1 yr 1mo
4218	Control	48	M	12.8	32	Right	1 yr 2 mo
4496	Schizophrenia	49	M	19.8	32	Left	2 yrs
4596	Control	49	M	24.6	32	Right	1 yr 8 mo
4665	Schizophrenia	66	M	17.4	32	Right	1 yr 6 mo
4729	Control	66	M	17.8	32	Right	1 yr 3 mo
4984	Schizophrenia	58	F	33.5	32	Left	5 mo
4932	Control	67	M	22.3	32	Left	8 mo

Table 1. Demographic data collected from the Harvard Brain Tissue Resource Center for tissue used in the Golgi Study. (Taken from Broadbelt et al, 2002)

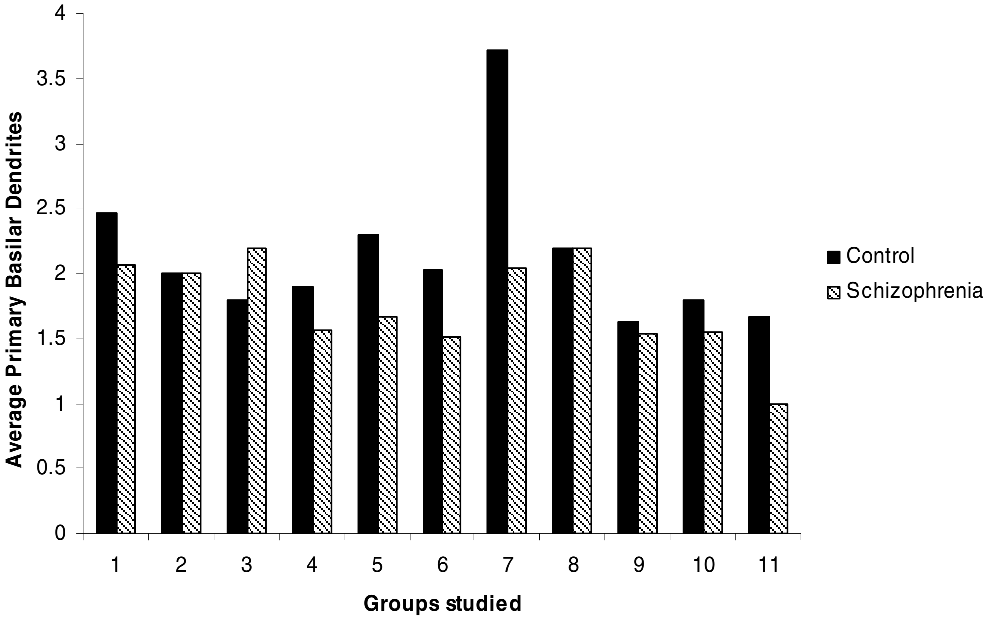


Figure 2. Raw data showing the number of primary basilar dendrites on pyramidal cells in layer III Area 32 for 11 pairs studied.

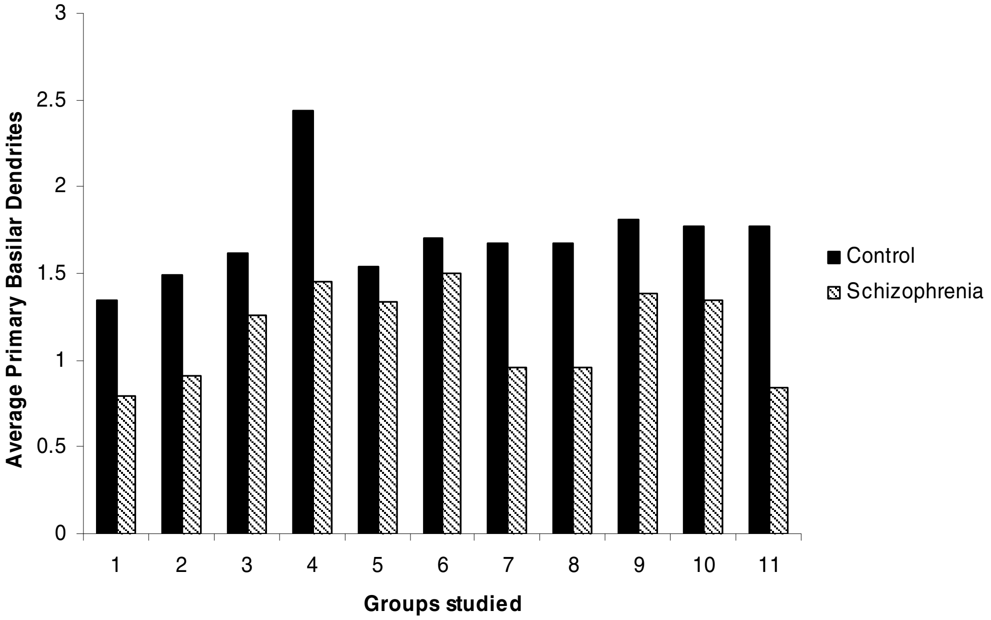


Figure 3. Raw data showing the number of primary basilar dendrites on pyramidal cells in layer V Area 32 for 11 pairs studied.

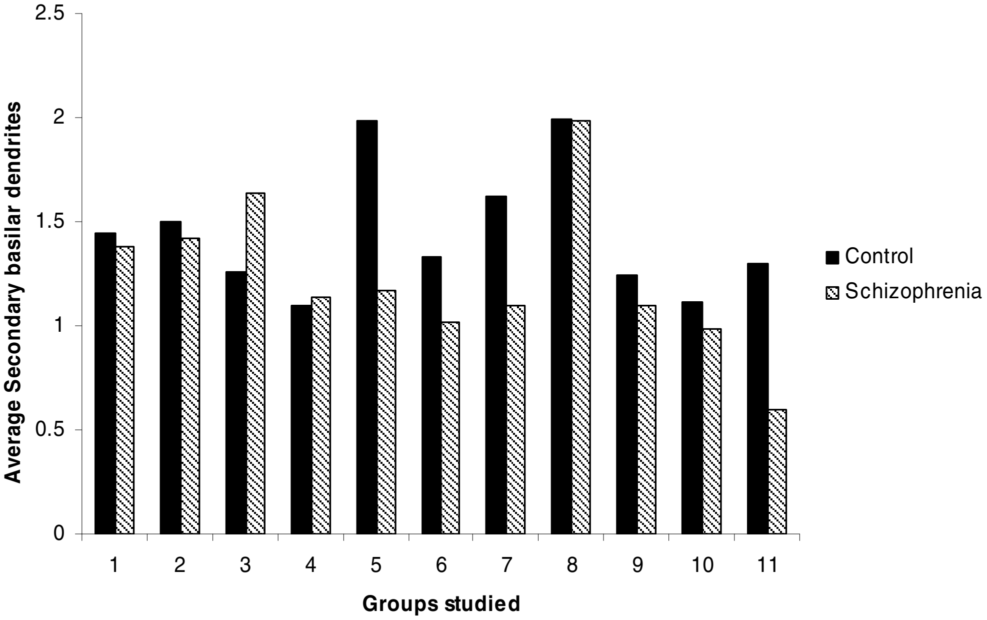


Figure 4. Raw data showing the number of secondary basilar dendrites on pyramidal cells in layer III Area 32 for 11 pairs studied.

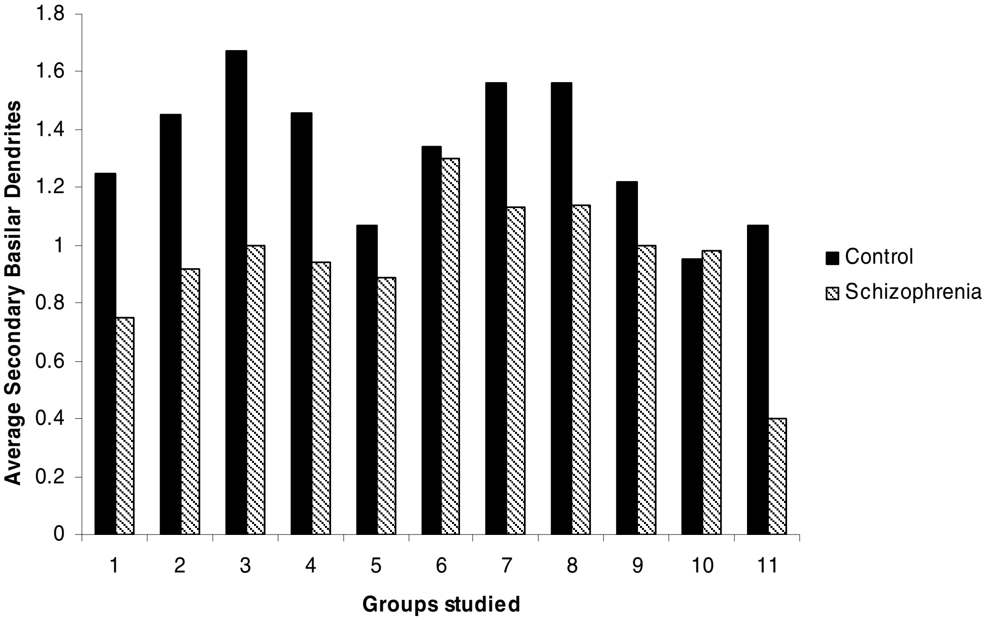


Figure 5. Raw data showing the number of secondary basilar dendrites on pyramidal cells in layer V Area 32 for 11 pairs studied.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A32	III	Primary	2.13±0.35	1.76±0.14	0.02	-2.34	17 %
A32	V	Primary	1.71±0.1	1.15±0.06	0.0002	-2.36	33%

Table 2. Table showing average primary dendrites of matched pairs control versus schizophrenics in Area 32, layers III and V with standard deviations and P-values.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A32	III	Secondary	1.44±0.1	1.23±0.13	0.03	-2.03	15%
A32	V	Secondary	1.32±0.04	0.95±0.03	0.0002	-2.36	28%

Table 3. Table showing average secondary dendrites of matched pairs, Control versus Schizophrenics in Area 32, layers III and V with standard deviations and P-values.

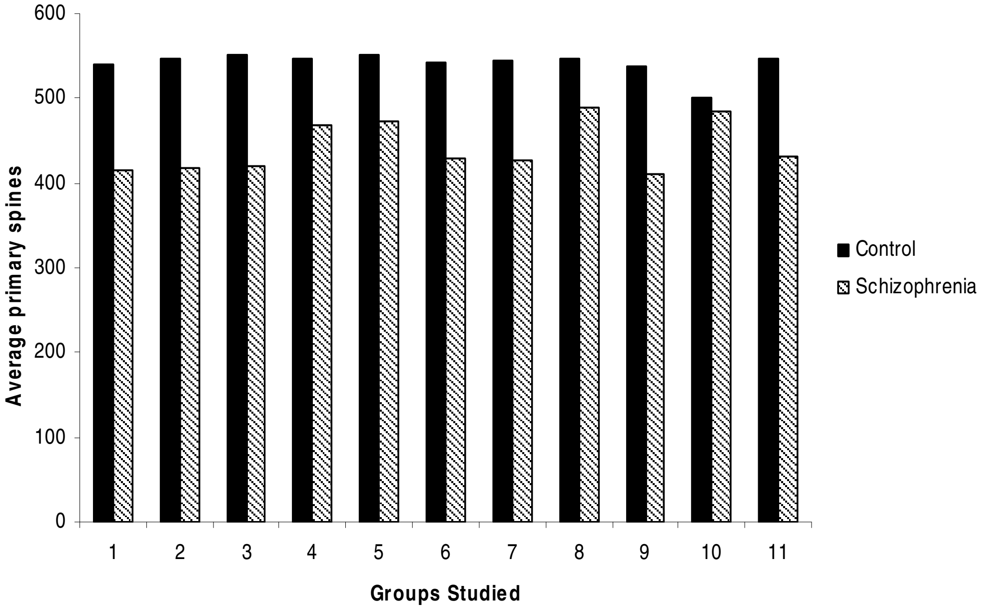


Figure 6. Raw data showing the mean synaptic spine distribution on primary dendrites on pyramidal cells in layer III Area 32 for 11 pairs studied.

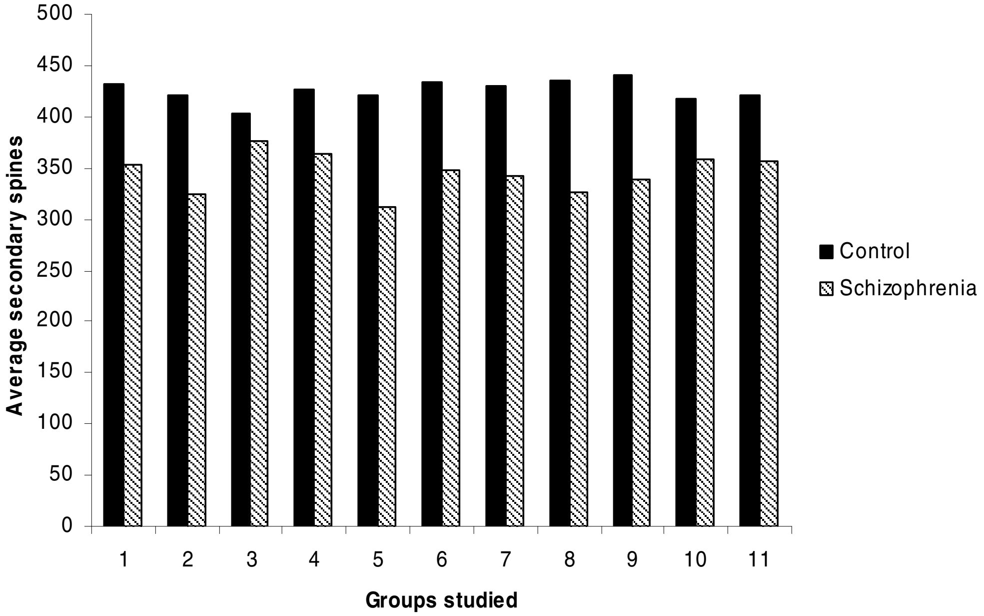


Figure 7. Raw data showing the mean synaptic spine distribution on secondary dendrites on pyramidal cells in layer III Area 32 for 11 pairs studied.

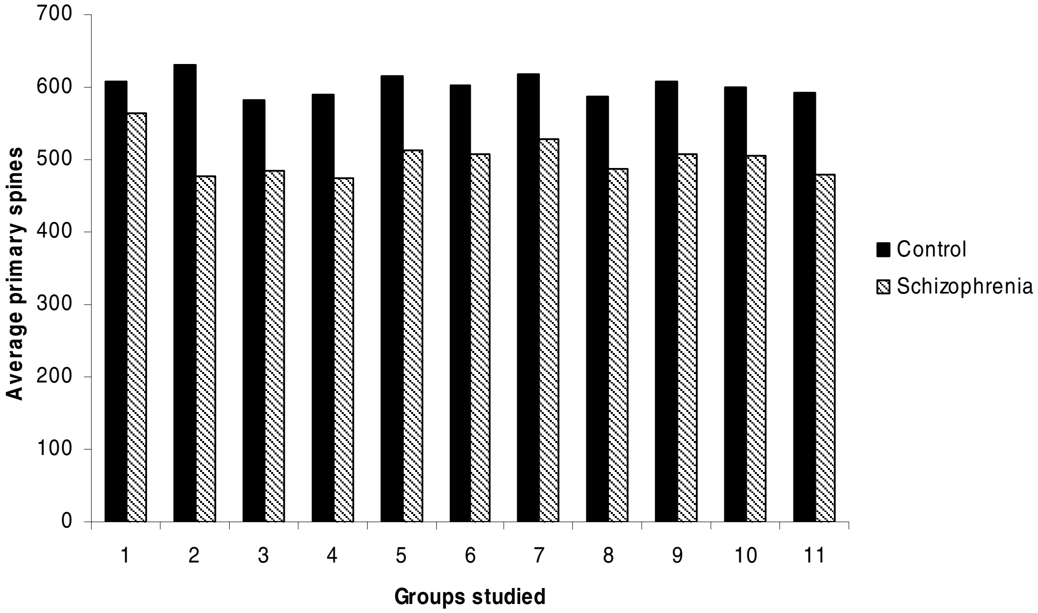


Figure 8. Raw data showing the average synaptic spine number on primary dendrites on pyramidal cells in layer V Area 32 for 11 pairs studied.

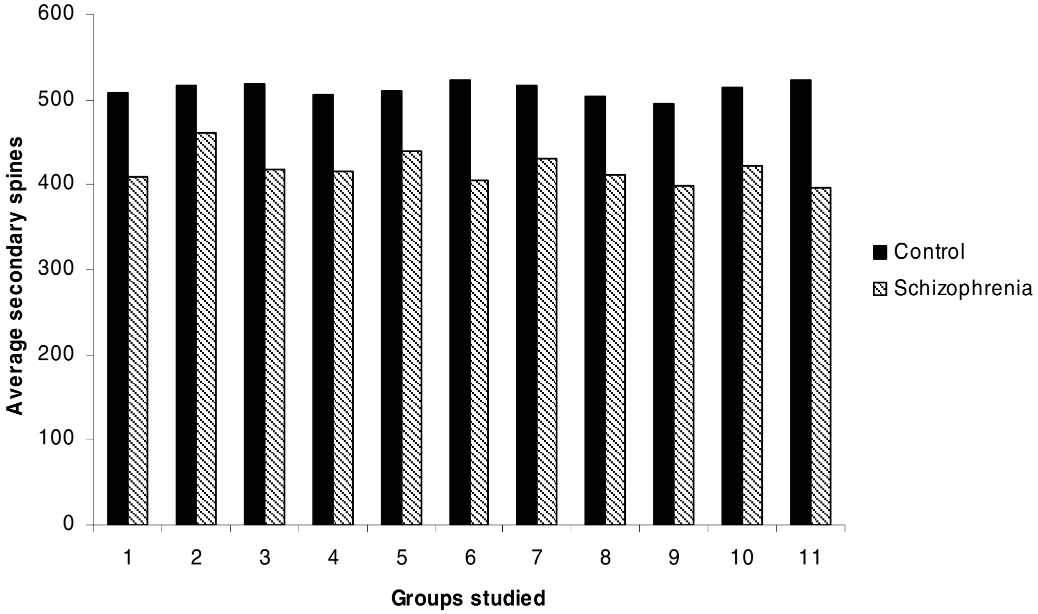


Figure 9. Raw data showing the average synaptic spine number on secondary dendrites on pyramidal cells in layer V Area 32 for 11 pairs studied.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A32	III	Primary	542± 14.4	442± 29.8	0.00004	2.22	18%
A32	V	Primary	603± 14	502± 26	0.00001	2.23	17%

Table 4 . Table showing average Spines on primary dendrites of matched pairs control versus schizophrenics in Area 32, layers III and V with standard deviations and P-values.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A32	III	Secondary	426± 10.17	345± 19	0.00001	2.22	19%
A32	V	Secondary	512± 9	419± 19	0.00002	2.23	18%

Table 5 . Table showing average Spines on secondary dendrites of matched pairs control versus schizophrenics in Area 32, layers III and V with standard deviations and P-values.

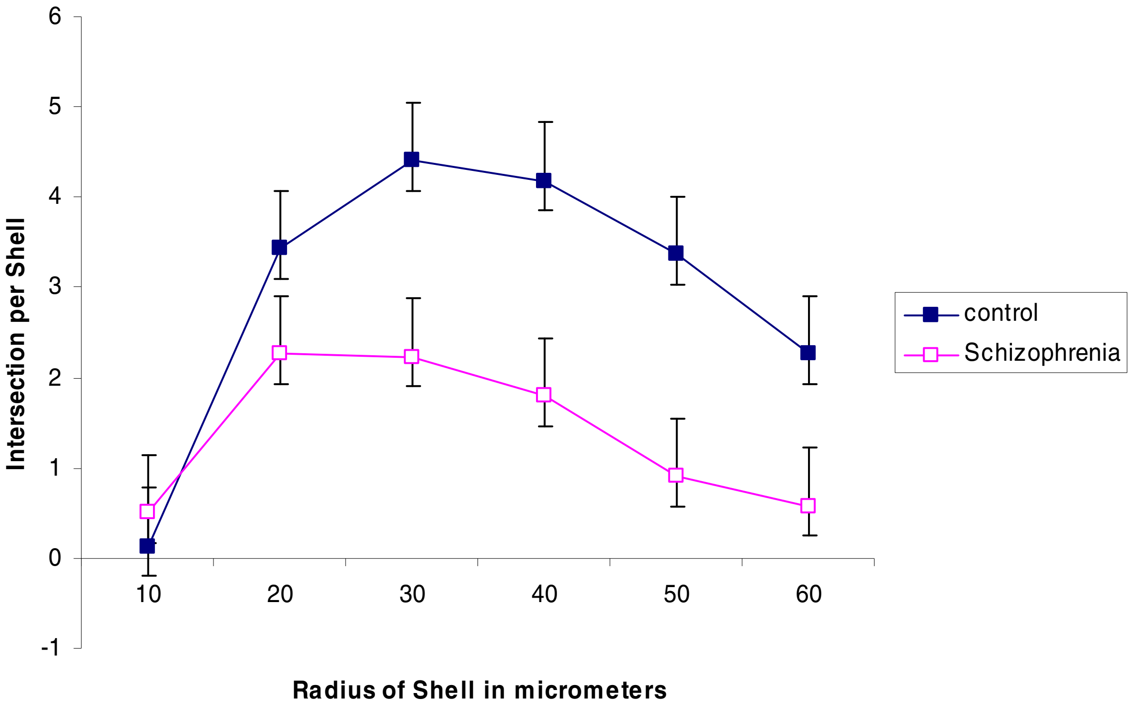


Figure 10. Sholl analysis of basilar dendrites of Area 32 pyramidal cells. Closed squares (blue) show control subjects (mean + SE) (n=6); open squares, indicates schizophrenia (pink) (mean + SE) (n=6).

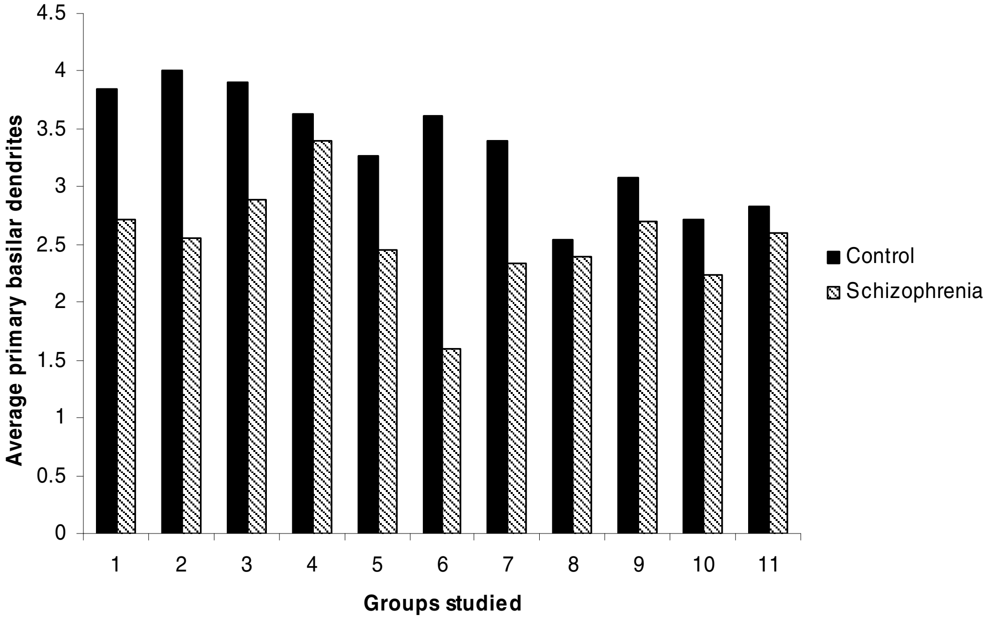


Figure 11. Raw data showing the number of primary basilar dendrites on pyramidal cells in layer III Area 9 for 11 pairs studied.

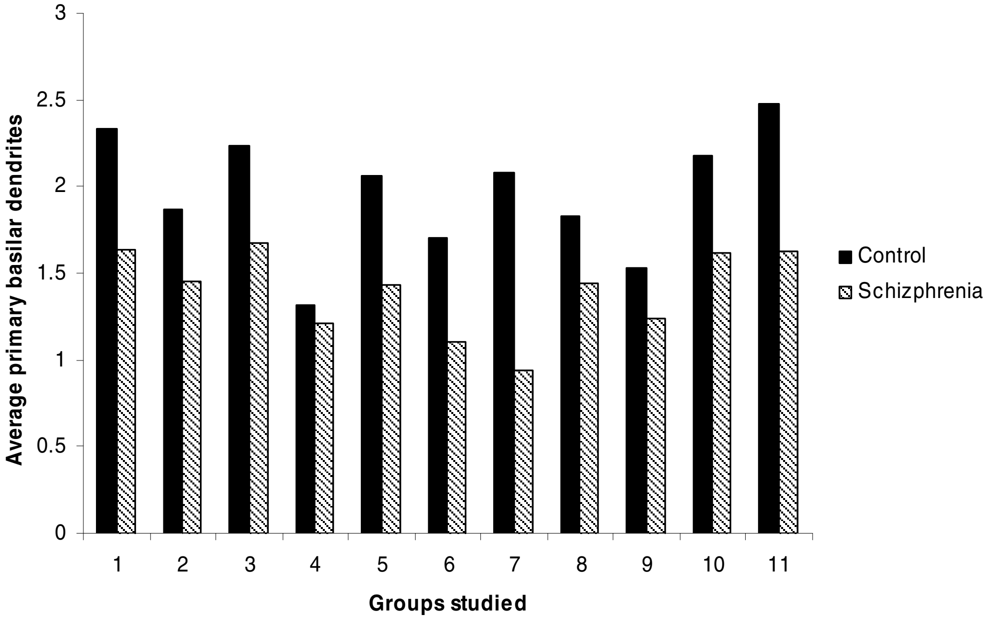


Figure 12. Raw data showing the number of primary basilar dendrites on pyramidal cells in layer V Area 9 for 11 pairs studied.

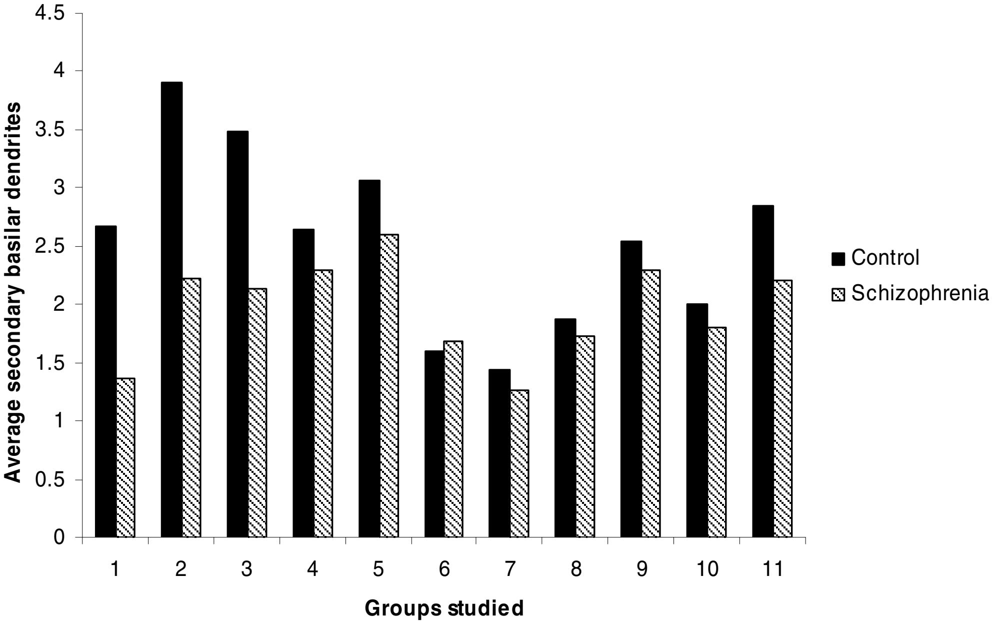


Figure 13. Raw data showing the number of secondary basilar dendrites on pyramidal cells in layer III Area 9 for 11 pairs studied.

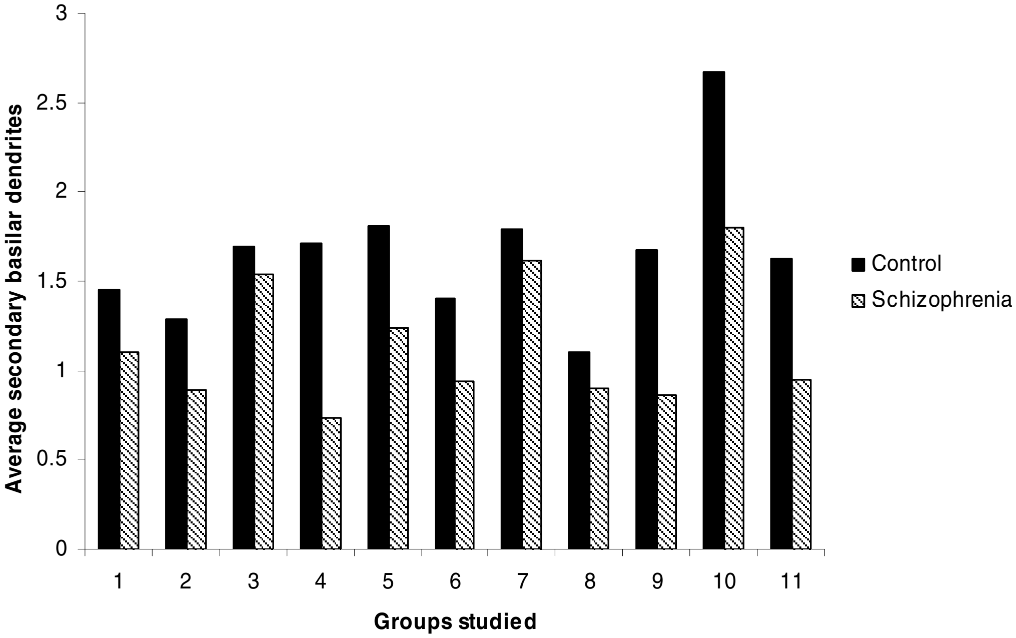


Figure 14. Raw data showing the number of secondary basilar dendrites on pyramidal cells in layer V Area 9 for 11 pairs studied.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A9	III	Primary	3.34+ 0.50	2.54+0.44	0.0005	4.57	23%
A9	V	Primary	1.97+ 0.3	1.40+ 0.2	0.00004	6.80	29%

Table 6. Table showing average primary dendrites of matched pairs, Control versus Schizophrenics in Area 9, layers III and V with standard deviations and P-values.

Brain Area	Layer	Type of Dendrite	Control mean/St.D	Schizophrenic Mean/St.D	p value	t value	% decrease
A9	III	secondary	2.55+ 0.6	1.96+ 0.4	0.004	3.29	23%
A9	V	secondary	1.66+ 0.4	1.14+ 0.4	0.0001	5.83	31%

Table 7. Table showing average secondary dendrites of matched pairs, Control versus Schizophrenics in Area 9, layers III and V with standard deviations and P-values.

CHAPTER 3: EVIDENCE OF ALTERED NEUROGRANIN IMMUNOREACTIVITY IN AREAS 9 AND 32 OF SCHIZOPHRENIC PREFRONTAL CORTEX.

Abstract:

Schizophrenia is a complex and poorly understood neuropsychiatric disorder. Much research has begun to implicate the PFC in the disease. Using immunocytochemistry we determined if neurogranin, a protein found in dendrites, spines and cell bodies and an upstream regulator of calcium was altered in areas 9 and 32 of schizophrenic prefrontal cortex. We examined its expression in pyramidal cells in layers III and V. Tissues from 7 controls and 7 schizophrenics (from our original MAP2 study Jones et al., 2002) matched for age, sex and postmortem interval were examined. Using area fraction analysis we quantified the immunostaining. Additionally, we counted the number of positively stained pyramidal cells in the same 7 pairs. Neurogranin immunostaining was dramatically reduced in both layers III (72%) and V (50%) in area 9. In area 32 there was a more modest reduction in both layers III (36%) and V (40%). There was no difference in either brain region or layer in the density of positively stained pyramidal cells. These data confirm mounting evidence suggesting dendritic loss in the prefrontal cortex and suggest that the loss of protein does not appear to be due to a change in the number of cells producing the protein but rather in the amount of protein being produced. Additionally, these data suggest that the loss of neurogranin may alter the calcium calmodulin dependent pathways due to its role as a regulator of calmodulin suggesting a link between structural and functional alterations of the pyramidal cells in the PFC.

Introduction

Schizophrenia is one of the most disabling of neuropsychiatric disorders whose pathogenic mechanisms are complex and poorly understood. A preponderance of evidence suggests that a combination of multigenic factors and early developmental insults could lead to a variety of brain abnormalities, which involve both cortical and subcortical structures. Mounting evidence implicates the prefrontal cortex (PFC) in schizophrenia (For review see Harrison 1999, Jones 2001, Shapiro 1993, Selemon and Goldman-Rakic 1999). The PFC, which includes areas 9 and 32, is an important region involved in higher cognitive functions, working memory, mental imagery, willed action and active memory (Frith and Dolan 1996) and, therefore, may play a role in the cognitive deficits observed in schizophrenia. Behavioral assessments of patients with schizophrenia show problems with spatial learning and verbal memory tasks, which are functions sub-served by the PFC (Peters et al., 2000, Weickert et al., 2000, Busch et al., 2002). Both PFC areas 9 and 32 receive inputs from the medial dorsal (MD) nucleus of the thalamus. Much research has shown a decrease in cell number and volume in the MD. Developmental studies of animals following lesions of either the PFC or its primary thalamic relay, the medial dorsal nucleus, have found alterations in a variety of behaviors similar to those exhibited by patients with schizophrenia (Aggleton and Mishkin 1983, Harrison and Mair 1996, Isseroff et al., 1982, Stokes and Best 1990). Therefore loss of input to these regions may affect pyramidal cell development.

Morphological studies in the prefrontal cortex have begun to show consistent changes that could alter information processing through a loss of synaptic surface area. Prefrontal cortical studies of pyramidal cells from our laboratory have shown a decrease in MAP2, a protein found in dendrites and cell bodies (Decamilli et al., 1984, Fischer et al., 1987), in layers III and V of areas 9 and 32 of the PFC (Jones et al., 2002). These data suggest a possible loss of dendritic material and or a change in soma size. A second study from our laboratory demonstrated a loss of primary and secondary basilar dendrites in area 32 of the prefrontal cortex (Broadbelt et al., 2002), consistent with the hypothesis that the decrease in MAP2 is due to a loss of dendritic material. Additional morphological studies have shown a decrease in dendritic spine density (Garey et al., 1998, Glantz and Lewis 2000). Finally, a study by Rajkowska et al. (1998) and confirmed by Pierri et al. (2001, 2003) and has shown a decrease in soma size of pyramidal cells in layer III in the prefrontal cortex. These data together suggest alterations in synaptic surface area on the pyramidal cells through a loss of dendrites and spines. One study found an increase in soma size of cells immunoreactive for Necab (Maldonado-Alviles et al 2006). While it is difficult to define the underlying mechanisms which cause changes in pyramidal cell morphology, such changes may be indicative of a greater problem in signal transduction.

Neurogranin is an important protein involved in signal transduction (Li et al., 2000). Neurogranin like MAP2 is located in cell bodies, dendrites and dendritic spines (Alvarez-Bolado et al., 1996) and a loss of neurogranin is a marker for dendritic lesions (Li et al., 2000). Functionally neurogranin is an upstream regulator of calcium and calmodulin (Ho Pak et al., 2000, Prichard et al., 1999). Neurogranin binds calmodulin and, therefore, renders it unable to interact with free calcium (Ho Pak et al 2000, Prichard et al., 1999). Knockout mice lacking neurogranin exhibit problems with spatial learning and long-term potentiation (Ho Pak et al., 2000) suggesting a role for neurogranin in processing and transmission of information and suggesting a possible role in schizophrenia. Therefore we chose to examine the expression of neurogranin in pyramidal cells in layers III and V in area 9 and 32 of prefrontal cortex. Our data suggest a dramatic decrease in neurogranin immunostaining in both layers III and V in areas 9 and 32, with no change in the number of cells expressing the protein confirming mounting evidence implicating morphological changes in the pyramidal cells and suggesting possible changes in signal transduction in the pyramidal cells.

Methods and Materials

Subjects and tissue

Postmortem brain tissues from 7 subjects diagnosed with schizophrenia and 7 subjects matched for age, sex and postmortem interval were obtained from the Harvard Brain Tissue Resource Center (HBTRC) at McLean Hospital. The tissues used were from the same brains that were used in our MAP2 study (Jones et al., 2000). Tissue was matched first for gender and then for age and PMI when we could not match for gender the tissue was matched for PMI and then for age (Table 1). In addition pairs were determined originally in the MAP2 study with in a given shipment and were kept the same for the current study. A power analysis was performed on the control data from the MAP2 study suggesting that an N of 4 was sufficient to obtain a significant result with an alpha of 0.05, and a power of 95. All tissues were collected at the time of autopsy in accordance with an approved Institutional Review Board protocol. Diagnoses conformed to Feigner criteria and were established retrospectively by a board certified psychiatrist (Dr. Francine Benes) through an examination of the patient files. Tissue was not included in the study if there was evidence of Alzheimer's disease, substance dependence or lesions with in the cortical area to be examined as determined by the HBTRC. Tissues were coded by the brain bank and the codes were kept by an individual not performing quantitative analysis until quantitative procedures were completed.

All specimens were formalin-fixed in a consistent manner. A longitudinal cut through the corpus callosum was made and one hemisphere was placed in 10% formalin, a commercial fixative from Fisher Scientific. One-centimeter thick blocks from areas 9 and 32 were dissected in the coronal plane by qualified neuropathologists at the HBTRC placed in 10% formalin and shipped to us. The blocks were never frozen prior to being cut on the sledge sliding microtome. The accuracy of the dissections was confirmed by the investigator by examining thionin-stained sections prepared from each block.

Specimens from area 9 were harvested from its dorsolateral extent, which is bounded by the superior frontal sulcus and contained with in the superior frontal gyrus. While area 9 does extend medially to the para-cingulate sulcus we did not harvest from this region. Compared with the medial region 9/46 and 46, the lateral region of area 9 does not have a well developed layer IV. Area 9 has a compact layer II and upper layer III. Deep layer III has large darkly stained pyramidal cells (Petrides and Pandya 1999; Rajkowska and Goldman-Rakic 1995).

Area 32 was taken in the midline in front of and dorsal to the cingulate gyrus. Area 32 resides rostral and dorsal to area 24 with its caudal extent residing between area 9 and area 24c. The main difference between areas 24 and 32 is the presence of large pyramidal cells in deep layer III that are not found in area 24. Area 32 also has a thin layer IV that becomes more attenuated as you move caudally through area 32. (Vogt et al 2003, Vogt et al 1995, Ongur et al 2003)

Immunocytochemistry

Coronal serial sections were cut at 50µm on a freezing sledge sliding microtome. Sections were collected sequentially into numbered wells and stored in cryoprotectant (see table 1 for length of storage in cryoprotectant) at –70°C until stained with thionin (all odd numbered sections) or were processed for neurogranin immunocytochemistry (every 10th section). Material was processed in pairs matched for age, sex and PMI (table 1) to control for variability on any given day.

A free-floating method of immunocytochemistry was employed to maximize impregnation and exposure to the antibody. The appropriate sections were removed from cryoprotectant, washed in phosphate buffered saline (PBS), and pretreated with 0.3% H_2O_2 for five minutes to remove any endogenous peroxidases. The sections were washed in PBS and incubated in 4% instant milk in PBS (BLOTTO) with 0.2% TritonX-100 for 1 hour. Sections were then incubated in neurogranin (Chemicon) diluted 1:500 in BLOTTO overnight at room temperature on an orbital shaker. The following day sections were washed in PBS 3 times for 10 minutes. The sections were then incubated in a biotinylated anti-mouse secondary (Vector Laboratories) at 1:100. Sections were incubated in an HRP-avidin/biotin complex according to the manufacturer's protocol followed by a standard diaminobenzadine reaction to view the immunocytochemical staining. All incubations were done in 12 well plates. Sections were then mounted, dehydrated and coverslipped.

Area Fraction Analysis:

Within a defined sampling box, area fraction refers to the ratio of the area occupied by immunopositive cells versus the total area of the box. The sampling box is a square with each side equal to the width of the lamina. The size of the sampling box remained constant through and was equal to 263687.2 μm^2. Bioquant software was employed to select pixels within the sampling box that match threshold criteria for the neurogranin positive profiles set by the investigator. The threshold was set at the level that selects the lightest stained cell bodies and dendrites without selecting background staining. However cells that had staining on the periphery were chosen as well because this staining tended to be darker than that of cells that had cytoplasmic staining. Once background staining was selected the threshold setting was set back to the previous level at which background was not selected. The selected levels for staining were on average 26% above threshold. The setting for illumination was kept constant throughout the analysis. The computer then outlined and summed the thresholded areas and determined the ratio of stained area to the total area of the sampling box. Sixteen sampling boxes were examined per section (8 within layer III and 8 within layer V). The sampling boxes were placed on the straight edges of the gyri. The 8 sampling boxes were taken consecutively as long as there were no histological artifacts in the tissue and the lamina could be determined. If there were artifacts in the tissue then the box was placed at the next straight edge after the artifact. All measurements were done using a Plan Apo 20x lens. Area fraction was averaged for all sampling boxes within a particular lamina for each specimen block. This method of analysis is routinely performed in the laboratory.

Cell Density Measurements

Following area fraction analysis all of the sections were counterstained with thionin for cell density measurements. Pyramidal cells were identified based on morphology and counted if they were immunostained. Pyramidal cells were counted in four evenly spaced sections per brain with a random start. Contours were traced for each layer and a counting grid was superimposed on the contour. Every third intersection with a random start was marked for counting so that 30% of the intersections were marked. A counting box of 200um/200um/17um (a buffer zone of 5um on either surface was employed so as not to include the cut surfaces) was placed inside the marked intersections. Counting was done using a 40x Plan Apo objective under oil. Every pyramidal cell inside the box with a visible nucleolus that did not touch exclusion lines on the left and bottom of the counting box was counted. Neuron density was determined by dividing the average number of cells per box by the volume of the counting box.

Statistical Analysis

Because specimens were processed in matched pairs the data were analyzed by paired t-tests. Two-tailed probabilities were employed. Data are reported as mean ± standard deviation (SD). Intraclass Correlation (ICC) was performed on the means from the control population. Data from every control brain (N = 7) was included in the calculation. Our ICC was .90 which suggests a high correlation and reliability of the methods.

Results:

Area Fraction Results

Area 9

Area Fraction analysis was completed on seven non-psychiatric controls and seven schizophrenics. Compared to normal controls, schizophrenics exhibited a 50% reduction in Neurogranin area fraction in layer V (Control = 0.12 ± 0.018, Schizophrenic = 0.06 ± 0.018, t =-7.32, P =0.0003) (Table 2, Fig. 1, 2). Similarly, there was a 72% reduction in layer III (Control = 0.11 ± 0.021, Schizophrenic = 0.03 ± 0.010, t =-13.691, P =0.000009) (Table 2, Fig. 1, 2).

Area 32

Area fraction analysis was completed on seven non-psychiatric controls and seven schizophrenics. Compared to controls, schizophrenics exhibited a 40% reduction in

Neurogranin area fraction in layer V (Control=0.045±0.03, Schizophrenic=0.027±0.02 t=2.44, P= 0.0005) (Fig.3). Similarly, there was a 36% reduction in layer III (Control= 0.047±0.003, Schizophrenic=0.03±0.002, t=2.44, P=0.003) (Fig. 3).

Cell Density Results

Cell density measurements were conducted on five non-psychiatric controls and five schizophrenics. Two pairs 3546/3118 and 3915/3932 were removed from the study because one of the brains in the pair did not counter-stain with thionin and therefore, could not be accurately analyzed. Previous power analysis showed that and n of 4 was sufficient to reach significance with an alpha of 0.05 and a confidence level of 95. Examining the current data with that previously published pyramidal cell density data from the same cohort of brains (Jones et al., 2000) on average 76% of the pyramidal cells were stained with neurogranin.

Area 9

Compared to controls there was no significant difference in either layer III (Control = $12.3\text{x}10^6 \pm 1.9\text{x}10^5$, Schizophrenic = $12.3\text{x}10^6 \pm 3.8\text{x}10^6$ t= 0.023 p= 0.98) or V (Control = $13.5\ \text{x}10^6 \pm 2.5\text{x}10^5$, Schizophrenic =$13.5\text{x}10^6 \pm 1.6\text{x}10^6$ t= 0.017 p= 0.98) in stained-pyramidal cell density (Table 3).

Area 32

Compared to controls there was no significant difference in either layer III (Control = $15.3\text{x}10^6 \pm 1.6\text{x}10^6$, Schizophrenic $15\text{x}10^6 \pm 2.3\text{x}10^6$ t=0.385 p = 0.41) or V (Control = $15\text{x}10^6 \pm 1.7\text{x}10^6$, Schizophrenic $15\text{x}10^6 \pm 3.1\text{x}10^6$ t=0.222 p = 0.42) in stained-pyramidal cell density (Table 3).

Discussion

Area fraction analysis shows a marked decrease in neurogranin immunostaining in both area 9 and 32 of the prefrontal cortex with no difference in the number of pyramidal cells positively stained with neurogranin. We believe that these results are due to schizophrenia-associated alterations and not due to an artifact of sampling or tissue processing for the following reasons. First, because tissues were matched for age, sex and postmortem interval it is unlikely that the observed differences are due to any of those factors. Moreover, the tissues were processed in matched pairs to control for variation due to tissue processing or staining. The potential role of neuroleptics must also be considered because all of the schizophrenic subjects had been exposed to neuroleptics. A recent study by Lidow et al., (2001) examined the possible role of neuroleptics and their effects on dendrite and spine associated proteins. The study showed a neuroleptic induced increase in phosphorylation of MAP2 but no differences in the level of MAP2 or in spinophylin in the PFC. In addition long postmortem intervals and poor fixation could affect the present data. The average PMI was less than 24 hours for both the control material and the schizophrenic material. A recent study by Viel et al., (2001) shows that neurons stain positively for MAP2 following a 24-hour PMI. Suggesting that immunostaining is possible with specimens whose PMI's were equal to or less than 24 hours. All of our tissues had PMI's less than 24 hours (Table 1). All specimens in the study were fixed according to a standard protocol so that all tissues received the same treatment and therefore an effect of fixation should affect the material consistently. Studies have shown that using antigen retrieval methods can enhance immunocytochemistry (Shi et al., 2001, Van den Broek and Van de Vijvier 2000, MacIntyre 2001) however, other studies have shown that use of antigen retrieval methods on tissue stored in formalin for a year or more has no affect on immunohistochemical staining of human material (Van den Broek and Van de Vijvier 2000). The average storage time for our material was over a year and therefore we felt the use of antigen retrieval would not have an effect on our staining (Table 1). The variability inherent in immunocytochemical staining has to be considered. Impregnation of the primary antibody in our material appeared to be consistent throughout the thickness of the section and our statistics showed very little within group variability. In addition Hoff et al., (2000) have shown using comparable section thickness, that staining is consistent throughout the section. While area fraction analysis does not directly measure changes in expression of the protein it does measure differences in staining patterns and amount of stain in a given cell population which suggests loss of protein or alterations in rate of expression of the protein. A more direct method would be to perform western blots to confirm the loss of the protein. Finally, we preformed an interclass correlation on the control raw data to examine variation among the brains as well as reliability of our analytic method. For these reasons we believe that the effects we have observed are associated with schizophrenia and not due to confounding variables.

Neurogranin and Calcium Signaling

Neurogranin is located in cell bodies, dendrites and dendritic spines (Alvarez-Bolado et al., 1996). Neurogranin is an important protein involved in signal transduction (Prichard et al., 1999) and a loss of neurogranin is suggestive of dendritic lesions as well as alterations in cellular signaling. Studies using knockout mice lacking neurogranin exhibited problems with spatial learning and long-term potentiation (Ho Pak et al., 2000), suggesting a role for neurogranin in processing and transmission of information and in schizophrenia. Our data suggest a schizophrenia-associated 50% decrease in staining in layer V and a 72% decrease in staining in layer III of area 9. We saw a similar reduction in area 32 for layers III (40%) and V (36%). The decrease in immunostaining is quite dramatic and may appear surprising. However, at least one other study has shown such dramatic results in the PFC in schizophrenia (Novak et al., 2000). This study examined mRNA levels of CAMKinase II beta and found an 85% increase in the message. CAMKinase II beta is involved in the signaling pathway regulated by neurogranin lending further evidence that this pathway is dramatically altered in patients with schizophrenia. Additionally, we examined the density of stained pyramidal cells in controls and schizophrenics and found no difference in either layer or region suggesting that the cells are producing the protein but at a much decreased rate.

The binding of calmodulin by neurogranin regulates the Ca-calmodulin pathway, controlling activation while the cell is at rest (Chakravarthy et al., 1997). Binding of glutamate to the NMDA receptor allows large amounts of calcium to enter the cell and signals the release of calcium from internal cellular stores. These two physiological changes cause neurogranin to be rendered inactive and thereby permit the release calmodulin to interact with calcium (Chakravarthy et al., 1997). A decrease in neurogranin levels could allow more calmodulin to interact with free calcium and to activate the Ca-calmodulin signal transduction pathway inappropriately.

The calcium-calmodulin signal pathway activates CaMKinase II and nitric oxide, both of which are involved in neuromodulation, synaptic plasticity, and long-term potentiation (Chakravarthy et al., 1997). Activating this pathway incorrectly could, therefore, alter information processing of the pyramidal cell. As mentioned above research examining mRNA levels of CaM kinase II beta show a dramatic 85% increase in mRNA levels (Novak et al 2000). Elevated levels of message are suggestive of an increase in protein levels; however message levels do not directly correlate with protein expression. A second study (Molnar et al., 2002), examining protein levels of CaM kinase II alpha found no change. The lack of a finding may be due to sampling technique. Studies of neurogranin knock out mice have shown a decrease in activated Cam Kinase II levels in the cortex (Ho Pak et al., 2000). These data argue that similar alterations may be observed in patients with schizophrenia due to a marked decrease of neurogranin and that these proteins may play a role in cognitive deficits observed in schizophrenia. A loss of neurogranin during development could have adverse affects on dendritic spine formation and synaptogenesis. Research has suggested a loss of dendritic spines as well as a loss of basilar dendrites in the prefrontal cortex of subjects with schizophrenia (Broadbelt et al., 2002, Garey et al., 1998, Glantz and Lewis 2000). Studies using neurogranin knock out mice have not found any gross anatomical alterations; however, no cellular studies have been performed.

A loss of neurogranin is suggestive of both morphological and functional alterations in the prefrontal cortical areas 9 and 32. Much evidence has pointed to morphological changes in the pyramidal cells in the prefrontal cortex. Recent data have shown functional alterations in these cells as well. Our data represent the first link between morphological alterations and functional alterations in pyramidal cells in the prefrontal cortex. Future work needs clarify the significance of these alterations and how they contribute to the behavioral and cognitive problems observed in patients with schizophrenia.

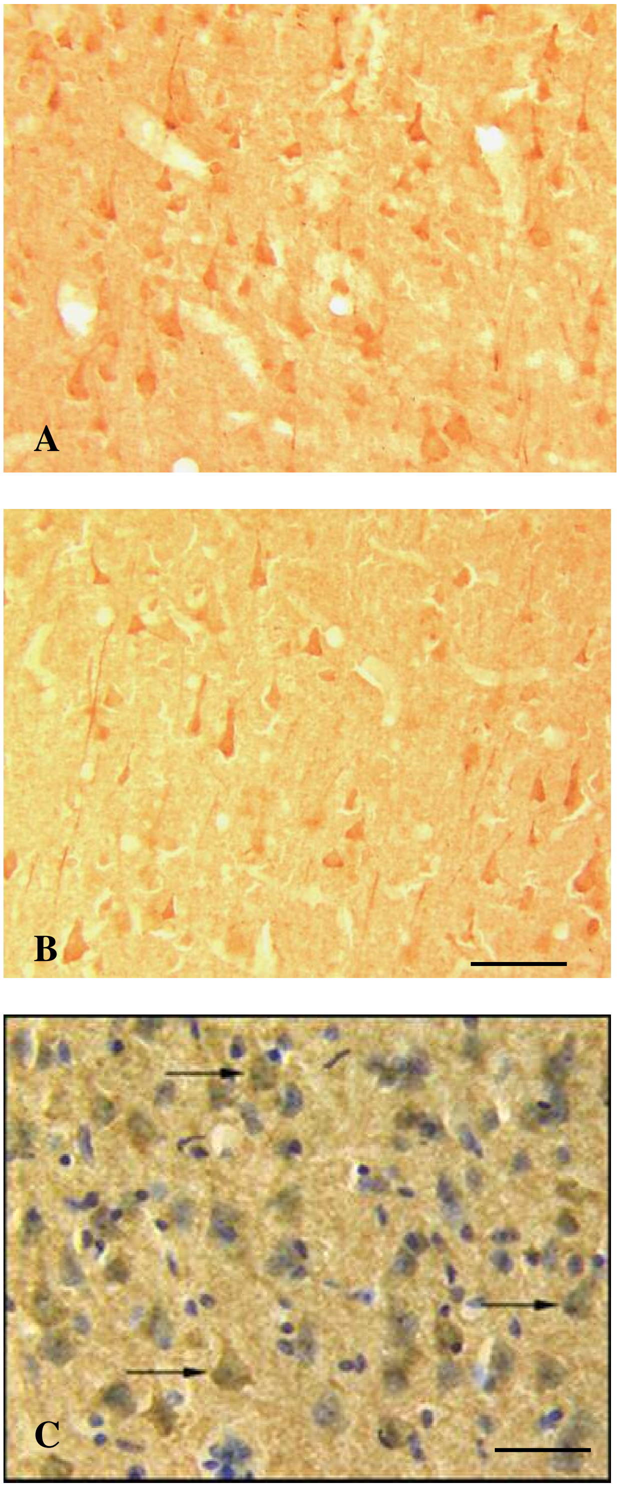
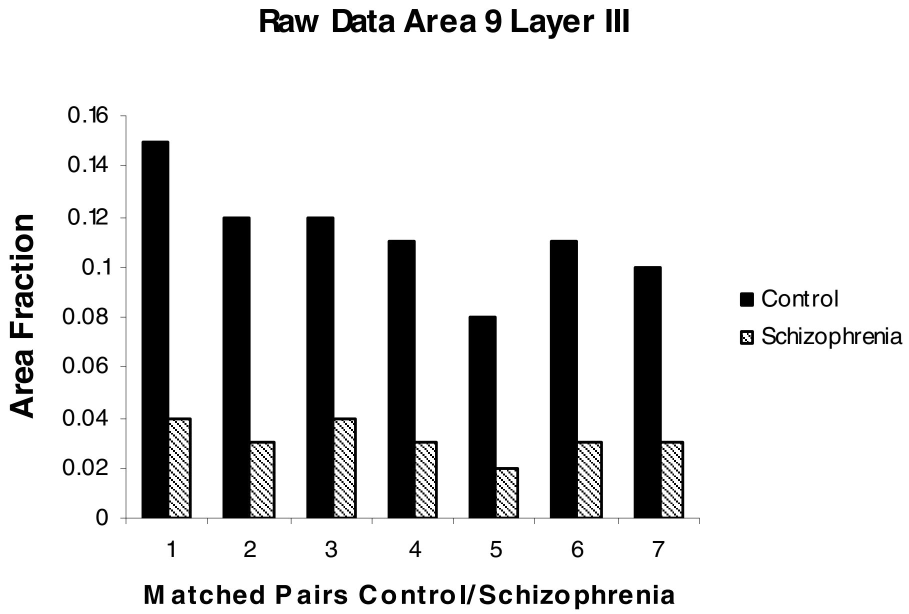


Figure. 1: Photomicrograph of area 9 layer III neurogranin immunostaining A) Control, B) Schizophrenic taken with a 20x lens final magnification of 200 C) Control counterstained with thionin taken with a 40x lens under oil final magnification of 400. Arrows point to pyramidal cells that are immunopositive. Scale bar A,B = 50µm C= 30µm.

Brain #	Distributive Dx	Age	Sex	PMI	Layer	Raw Data	Storage time in months *
3673	schizophrenia	43	m	20.8	III, V	0.04, 0.04	10
3748	control	44	m	23	III, V	0.15, 0.11	8
3833	schizophrenia	67	m	21.3	III, V	0.03, 0.04	13
3878	control	65	m	13	III, V	0.12, 0.12	11
3742	schizophrenia	71	m	19	III, V	0.04, 0.05	7
3875	control	68	f	14	III, V	0.12, 0.11	12
3915	schizophrenia	34	m	17.4	III, V	0.03, 0.06	10
3932	control	38	m	21.9	III, V	0.11, 0.09	10
3546	schizophrenia	69	m	17.83	III, V	0.02, 0.06	13
3619	control	74	f	19.8	III, V	0.08, 0.14	11
3557	schizophrenia	66	f	16.75	III, V	0.03, 0.09	13
3625	control	70	f	18.25	III, V	0.11, 0.12	11
3634	schizophrenia	71	m	18	III, V	0.03, 0.07	15
3626	control	65	f	16.6	III, V	0.1, 0.13	11

Table 1. Demographic data for the matched pairs (*= storage times are from the time the brains were removed at HBRC to the time they were processed for MAP2. An additional 36 months of storage in cyroprotectant needs to be added to all of the brains for neurog. processing.



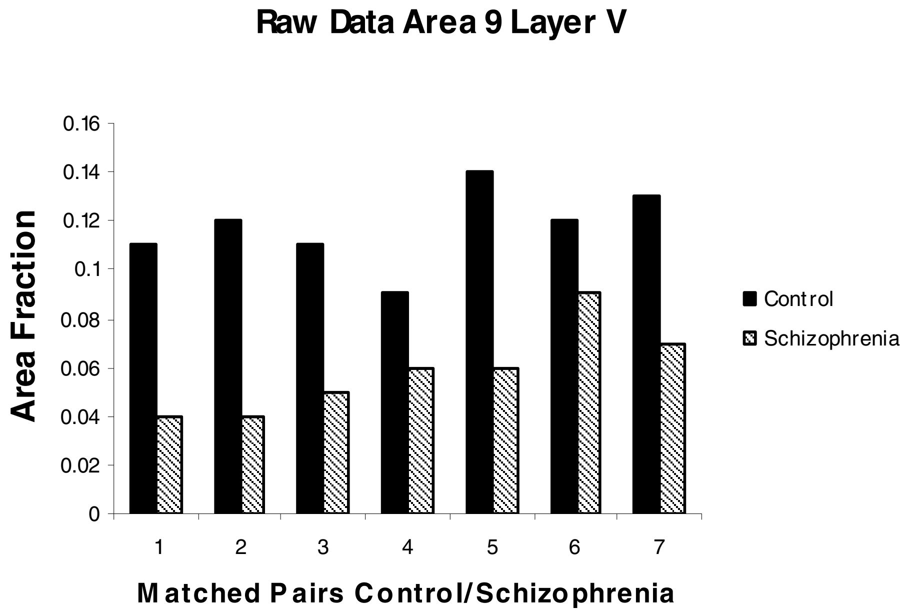
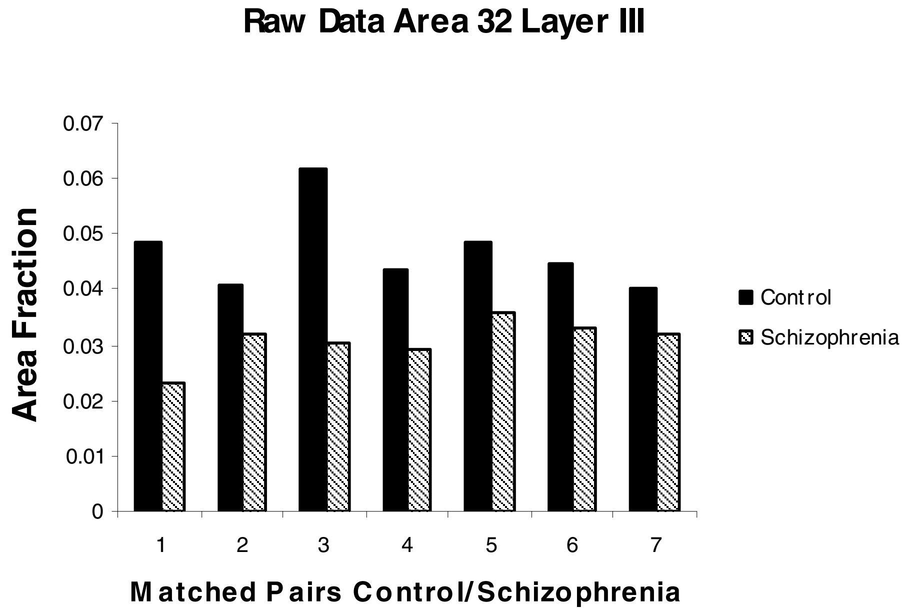


Figure 2. Raw data showing neurogranin area fraction for area 9 layers III and V.


Raw Data Area 32 Layer III

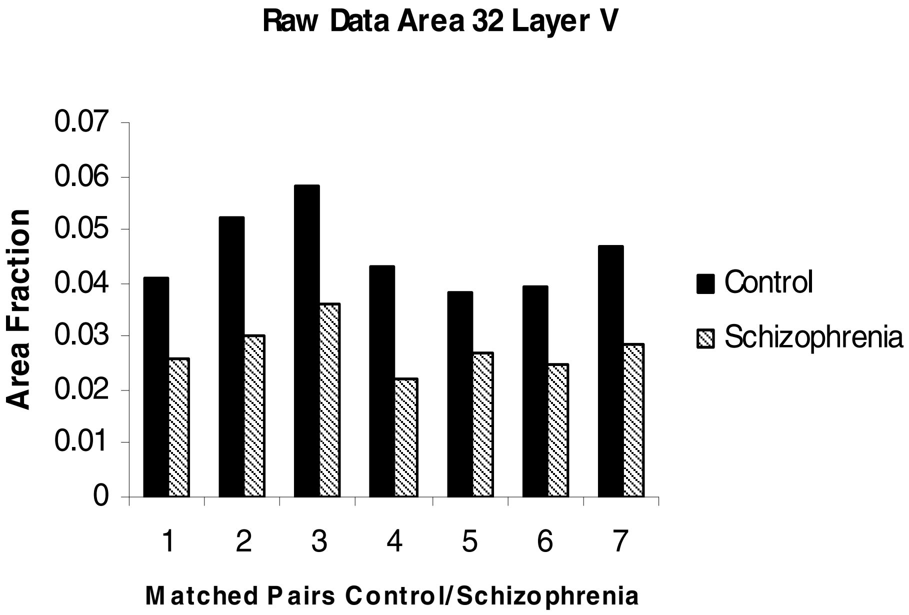

Raw Data Area 32 Layer V

Figure 3. Raw data showing Neurogranin area fraction for area 32 layers III and V.

Brain Area	Layer	Control mean	Control SD	Schizophrenic Mean	Schizophrenic SD	t value	p value
9	III	0.11	± 0.021	0.03	± 0.010	13.691	0.000009
9	V	0.12	± 0.018	0.06	± 0.018	7.32	0.0003
32	III	0.047	±0.003	0.031	±0.002	4.846	0.003
32	V	0.045	±0.003	0.027	±0.002	10.88	0.0005

Table 2. Area fraction data for layers III and V Brain Area's 9 and 32.

Brain Area	Layer	Control mean $x10^6$	Control SD $x10^6$	Schizophrenic Mean $x10^6$	Schizophrenic SD $x10^6$	t value	p value
9	**III**	**12.3**	**±1.9**	**12.3**	**±3.8**	**0.023**	**0.98**
9	**V**	**13.5**	**±2.5**	**13.5**	**±1.6**	**0.017**	**0.98**
32	**III**	**15.3**	**±1.6**	**15**	**±2.3**	**0.385**	**0.41**
32	**V**	**15**	**±1.7**	**15**	**±3.1**	**0.222**	**0.42**

Table 3. Positive stained profile densities for layers III and V of BA 9 and 32.

CHAPTER 4: EVIDENCE OF ALTERED CALMODULIN IMMUNOREACTIVITY IN AREAS 9 AND 32 OF SCHIZOPHRENIC PREFRONTAL CORTEX.

Abstract

Schizophrenia one of the most severe and disabling neuropsychiatric disorders of which little is known about the causality and etiology if the disease. One region that has been of interest is the prefrontal cortex. Previous anatomical and imaging studies have implicated this region (Haroutunian et al., 1979; Levin et al., 1984; Weinberger et al., 1988; Andreasen et al., 1990; Andreasen et al., 1994; Sharma et al., 1998). Immunocytochemical studies show a decrease in neurogranin (chapter 4), a protein found in dendrites, spines and cell bodies (Alvarez-Bolado et al., 1996; Gerendsay et al., 1997; Li et al., 2000). Neurogranin is an upstream regulator of both calcium and calmodulin and functions to concentrate calmodulin in the cytosol of neurons (Alvarez-Bolado et al., 1996; Prichard et al., 1999; Li et al., 2000). Calmodulin has a high affinity for neurogranin in the absence of or low concentrations of calcium (Sheu et al., 1996; Chakravarthy et al., 1999; Prichard et al., 1999; Li et al., 1999; Novak et al., 2000; Ho Pak et al., 2000). We performed a free floating immunocytochemical technique to exam calmodulin expression in pyramidal cells in layers III and V. Tissues from 6 controls and 6 schizophrenics (from our original MAP2 study Jones et al., 2002) matched for age; sex and postmortem interval were examined. Using area fraction analysis we quantified the immunostaining. Additionally, we counted the number of immunopositive pyramidal, immunonegative pyramidal and immunopositive interneurons for the same 6 pairs. Area Fraction analysis showed reductions in calmodulin in both layers III (58%) and V (44%) in area 9. Similarly, in area 32 there was reduction in both layers III (51%) and V (32%). Pyramidal cell density measurements of positively stained profiles showed a reduction in area 9 (11%) layer III, (20%) layer V, area 32 (16%) layer III and (17%) layer V and no difference observed in unstained pyramidal cells. Positively stained interneuron profiles

also showed no significant difference in both layers III and V in areas 32 and 9. These data confirm mounting evidence of calcium signaling in the prefrontal cortex and suggest that the loss of protein does appear to be due to a change in the number of cells producing the protein. Additionally, these data suggest that the loss of neurogranin and calmodulin may alter the calcium calmodulin dependent pathways suggesting a link between structural and functional alterations of the pyramidal cells in the prefrontal cortex.

Introduction

Schizophrenia is a chronic, severe, and disabling brain disease whose pathogenic mechanisms are complex and poorly understood. Evidence suggests that combination of multigenic factors and early developmental insults could lead to a variety of brain abnormalities, which involve both cortical and subcortical structures. Mounting evidence implicates the PFC in schizophrenia (For review see Harrison 1999, Jones 2001, Shapiro 1993, Selemon and Goldman-Rakic 1999). The PFC, which includes areas 9 and 32, is an important region involved in higher cognitive functions, working memory, mental imagery, willed action and active memory (Frith and Dolan 1996) and, therefore, may play a role in the cognitive deficits observed in schizophrenia. Behavioral assessments of patients with schizophrenia show problems with spatial learning and verbal memory tasks, which are functions sub-served by the PFC (Peters et al., 2000, Weickert et al., 2000, Busch et al., 2002). Areas 9 and 32 receive inputs from the medial dorsal (MD) nucleus of the thalamus. Developmental studies of animals following lesions of either the PFC or its primary thalamic relay, the medial dorsal nucleus, have found alterations in a variety of behaviors similar to those exhibited by patients with schizophrenia (Aggleton and Mishkin 1983, Harrison and Mair 1996, Isseroff et al., 1982, Stokes and Best 1990). Therefore loss of input to these regions may affect pyramidal cell development.

Morphological studies performed on the PFC have shown consistently changes that could alter information processing through a loss of synaptic surface area. Prefrontal cortical studies of pyramidal cells from our laboratory have shown a decrease in MAP2, a protein found in dendrites and cell bodies (Decamilli et al., 1984, Fischer et al., 1987), in layers III and V of areas 9 and 32 of the prefrontal cortex (Jones et al., 2002). These data suggest a possible loss of dendritic material and or a change in soma size. A second study from our laboratory has shown a loss of primary and secondary basilar dendrites in area 32 of the prefrontal cortex (Broadbelt et al., 2002), consistent with the hypothesis that the decrease in MAP2 is due to a loss of dendritic material. Additional morphological studies have shown a decrease in dendritic spine density (Garey et al., 1998, Glantz and Lewis 2000). Data from our lab has shown a significant decrease in basilar dendrites and synaptic spine density in both layers III and V in area 9. Compared to Non-psychiatric controls, schizophrenics exhibited a (23%) decrease in the number of primary basilar dendrites in layer V. A (31%) decrease in the number of secondary basilar dendrites was observed in layer V. Similarly, a (29%) reduction was seen in primary basilar dendrites in layer V. In addition, a (23%) decrease in secondary basilar dendrites was seen in layer III. These data together suggest alterations in synaptic surface area on the pyramidal cells through a loss of dendrites and spines. While it is difficult to define the underlying mechanisms which cause changes in pyramidal cell morphology, such changes may be indicative of a greater problem in signal transduction.

Altered levels of many proteins involved in Ca^{++} signaling have been reported in schizophrenia (reviewed by Lidow MS.), and we have now reported a loss of immunoreactivity for protein, Neurogranin a regulator of Calmodulin (Broadbelt et al., 2006 [article in press]). Many postsynaptic processes, including the determination of the size and number of spines, are mediated by calcium-dependent mechanisms that are initiated by glutamate receptor-mediated influx of Ca^{++} ions. Calmodulin is a ubiquitous calcium-binding protein of eukaryotic cells that mediates a variety of cellular responses to calcium. The calcium-calmodulin complex can acts as a messenger, affecting the activity of many enzymes and nonenzyme proteins, including the calcium pump, numerous specific protein kinases and cyclic nucleotide phosphodiesterases. We chose to examine calmodulin expression in light of the previous results that show a decrease in neurogranin expression, and both proteins are dependant on another's expression. As a result of the decrease seen in neurogranin an upstream regulator of calmodulin, it is likely that a decrease in calmodulin would be observed to compensate for the decrease in neurogranin.

Methods and Materials

Subjects and tissue

Postmortem brain tissues from 6 subjects diagnosed with schizophrenia and 6 subjects matched for age, sex and postmortem interval were obtained from the Harvard Brain Tissue Resource Center (HBTRC) at McLean Hospital. The tissues used were from the same brains that were used in our MAP2 study (Jones et al., 2000). Tissue was matched first for gender and then for age and PMI when we could not match for gender the tissue was matched for PMI and then for age (Table 1). In addition pairs were determined originally in the MAP2 study with in a given shipment and were kept the same for the current study. A power analysis was performed on the control data from the MAP2 study suggesting that an N of 4 was sufficient to obtain a significant result with an alpha of 0.05, and a power of 95. All tissues were collected at the time of autopsy in accordance with an approved Institutional Review Board protocol. Diagnoses conformed to Feigner criteria and were established retrospectively by a board certified psychiatrist (Dr. Francine Benes) through an examination of the patient files. Tissue was not included in the study if there was evidence of Alzheimer's disease, substance dependence or lesions with in the cortical area to be examined as determined by the HBTRC. Tissues were coded by the brain bank and the codes were kept by an individual not performing quantitative analysis until quantitative procedures were completed.

All specimens were formalin-fixed in a consistent manner. A longitudinal cut through the corpus callosum was made and one hemisphere was placed in 10% formalin, a commercial fixative from Fisher Scientific. One-centimeter thick blocks from areas 9 and 32 were dissected in the coronal plane by qualified neuropathologists at the HBTRC placed in 10% formalin and shipped to us. The blocks were never frozen prior to being cut on the sledge sliding microtome. The accuracy of the dissections was confirmed by the investigator by examining thionin-stained sections prepared from each block.

Specimens from area 9 were harvested from its dorsolateral extent, which is bounded by the superior frontal sulcus and contained with in the superior frontal gyrus. While area 9 does extend medially to the para-cingulate sulcus we did not harvest from this region. Compared with the medial region 9/46 and 46, the lateral region of area 9 does not have a well developed layer IV. Area 9 has a compact layer II and upper layer III. Deep layer III has large darkly stained pyramidal cells (Petrides and Pandya 1999 Rajkowska and Goldman-Rakic 1995a,b).

Area 32 was taken in the midline in front of and dorsal to the cingulate gyrus. Area 32 resides rostral and dorsal to area 24 with its caudal extent residing between area 9 and area 24c. The main difference between areas 24 and 32 is the presence of large pyramidal cells in deep layer III that are not found in area 24. Area 32 also has a thin layer IV that becomes more attenuated as you move caudally through area 32. (Vogt et al 2003, Vogt et al 1995, Ongur et al 2003)

Immunocytochemistry

Coronal serial sections were cut at 50µm on a freezing sledge sliding microtome. Sections were collected sequentially into numbered wells and stored in cryoprotectant (see table 1 for length of storage in cryoprotectant) at –70°C until stained with thionin (all odd numbered sections) or were processed for calmodulin immunocytochemistry (every 10th section). Material was processed in pairs matched for age, sex and PMI (table 1) to control for variability on any given day.

A free-floating method of immunohistochemistry will be employed to maximize impregnation and exposure to the antibody. The appropriate sections will be removed from cryoprotectant, washed in phosphate buffered saline (PBS) for 15 minutes, and pretreated with 0.3% H_2O_2 for five minutes to remove any endogenous peroxidases. The sections will be washed in PBS 3 times for 10 minutes and incubated in 0.4g instant milk in 10mM $CaCl_2$ ($CaCl_2$-BLOTTO) with 0.2% Triton X-100 for 1 hour. Sections will be incubated in Calmodulin (Chemicon) diluted 1:100 in $CaCl_2$-BLOTTO overnight at room temperature on an orbital shaker. A positive control will not be necessary because the material will be processed as matched pairs (control versus schizophrenic). Twenty fours later, the sections will be washed in PBS 3 times for 10 minutes. The sections will be incubated in a biotinylated anti-mouse secondary (Vector Laboratories) at a dilution of 1:100 for 1hour. The sections will be washed in PBS 3 times for 10 minutes and then incubated in HRP-avidin/biotin complex according to the manufacturer's protocol followed by a standard diaminobenzadine reaction to view the immunohistochemical staining.

Area Fraction Analysis:

Within a defined sampling box, area fraction refers to the ratio of the area occupied by immunopositive cells versus the total area of the box. The sampling box is a square with each side equal to the width of the lamina. The size of the sampling box remained constant through and was equal to 263687.2 μm^2. Bioquant software was employed to select pixels within the sampling box that match threshold criteria for the neurogranin positive profiles set by the investigator. The threshold was set at the level that selects the lightest stained cell bodies and dendrites without selecting background staining. However cells that had staining on the periphery were chosen as well because this staining tended to be darker than that of cells who had cytoplasmic staining. Once background staining was selected the threshold setting was set back to the previous level at which background was not selected. The selected levels for staining were on average 26% above threshold. The setting for illumination was kept constant throughout the analysis. The computer then outlined and summed the thresholded areas and determined the ratio of stained area to the total area of the sampling box. Sixteen sampling boxes were examined per section (8 within layer III and 8 within layer V). The sampling boxes were placed on the straight edges of the gyri. The 8 sampling boxes were taken consecutively as long as there were no histological artifacts in the tissue and the lamina could be determined. If there were artifacts in the tissue then the box was placed at the next straight edge after the artifact. All measurements were done using a Plan Apo 20x lens. Area fraction was averaged for all sampling boxes within a particular lamina for each specimen block. This method of analysis is routinely performed in the laboratory.

Cell Density Measurements

Following area fraction analysis all of the sections were counterstained with thionin for cell density measurements. Pyramidal cells were identified based on morphology and counted if they were immunostained. Pyramidal cells were counted in four evenly spaced sections per brain with a random start. Contours were traced for each layer and a counting grid was superimposed on the contour. Every third intersection with a random start was marked for counting so that 30% of the intersections were marked. A counting box of 200um/200um/17um (a buffer zone of 5um on either surface was employed so as not to include the cut surfaces) was placed inside the marked intersections. Counting was done using a 40x Plan Apo objective under oil. Every pyramidal cell inside the box with a visible nucleolus that did not touch exclusion lines on the left and bottom of the counting box was counted. Neuron density was determined by dividing the average number of cells per box by the volume of the counting box.

Statistical Analysis

Because specimens were processed in matched pairs the data were analyzed by paired t-tests. Two-tailed probabilities were employed. Data are reported as mean ± standard deviation (SD). Intraclass Correlation (ICC) was performed on the means from the control population. Data from every control brain (N = 6) was included in the calculation. Our ICC was .90 which suggests a high correlation and reliability of the methods.

Results:

Area Fraction Results

Area 9

An ICC of 0.90 was obtained for the control tissue. Area Fraction analysis was completed on seven non-psychiatric controls and seven schizophrenics. Compared to normal controls, schizophrenics exhibited a 44% reduction in Calmodulin area fraction in layer V (Control = 0.11 ± 0.02, Schizophrenic = 0.065 ± 0.013, t =-13.7, P =0.002) (Table 2, Fig. 1, 2). Similarly, there was a 58% reduction in layer III (Control = 0.13 ± 0.01, Schizophrenic = 0.055 ± 0.013, t =5.22, P =0.001) (Table 2, Fig. 1, 2).

Area 32

Area fraction analysis was completed on seven non-psychiatric controls and seven schizophrenics. Compared to controls, schizophrenics exhibited a 32% reduction in Neurogranin area fraction in layer V (Control=0.075±0.008, Schizophrenic=0.051±0.007 t=7.62, P= 0.006). Similarly, there was a 51% reduction in layer III (Control= 0.083±0.014, Schizophrenic=0.04±0.014, t=7.31, P=0.0007).

Cell Density Results

Cell density measurements were conducted on six non-psychiatric controls and six schizophrenics. Previous power analysis showed that and n of 4 was sufficient to reach significance with an alpha of 0.05 and a confidence level of 95.

Area 9

Stained Pyramidal cells

Compared to controls there was a significant difference in both layer III (Control = $9.0x10^6$ ±$0.81x10^6$, Schizophrenic = $8.0x10^6$ ±$1.1x10^6$ t= 3.59, p= 0.02) and V (Control = $9.3x10^6$ ±$2.5x10^6$, Schizophrenic =$8.0x10^6$ ±$1.1x10^6$ t= 6.65, p= 0.01) in stained-pyramidal cell density. In layer V a 20% decrease was noted and an 11% decrease in layer III.

Non-Stained Pyramidal cells

Compared to controls there was no significant difference in either layer III (Control = $6.8x10^6$ ±$9.0x10^6$, Schizophrenic $6.5x10^6$ ± $9.53x10^6$ t=0.514, p = 0.60) or V (Control = $5x10^6$ ±$9.5x10^6$, Schizophrenic $5.4x10^6$ ±$1.5x10^6$ t=-0.896, p = 0.40) in Non-stained-pyramidal cell density.

Total Pyramidal cells

Compared to controls there was no significant difference in either layer III (Control = $15.7x10^6$ ±$9.2x10^6$, Schizophrenic $15x10^6$ ± $9.0x10^6$ t=1.63 p = 0.2) or V (Control = $14.1x10^6$ ±$9.7x10^6$, Schizophrenic $13.1x10^6$ ±$3.0x10^6$ t=1.19 p = 0.40) in Total pyramidal cell density.

Area 32

Stained Pyramidal cells

Compared to controls there was a significant difference in both layer III (Control = $8.8x10^6$ ±$3.3x10^6$, Schizophrenic = $7.4x10^6$ ±$4.4x10^6$ t= 11.77 p= 0.0001) and V (Control = $9.0x10^6$ ±$1.4x10^6$, Schizophrenic =$7.4x10^6$ ±$0.7x10^6$ t= 4.54 p= 0.006) in stained-pyramidal cell density. In layer V a 17% decrease was noted and a 16% decrease in layer III.

Non-Stained Pyramidal cells

Compared to controls there was no significant difference in either layer III (Control = $4.7 \times 10^6 \pm 1.5 \times 10^6$, Schizophrenic $4.7 \times 10^6 \pm 1.5 \times 10^6$ $t=0.128$ $p = 0.90$) or V (Control = $5.2 \times 10^6 \pm 1 \times 10^6$, Schizophrenic $5 \times 10^6 \pm 1.5 \times 10^6$ $t=0.377$ $p = 0.71$) in Non-stained-pyramidal cell density.

Total Pyramidal cells

Compared to controls there was no significant difference in either layer III (Control = $15.5 \times 10^6 \pm 4.9 \times 10^6$, Schizophrenic= $12.1 \times 10^6 \pm 1.2 \times 10^6$ $t=1.60$ $p = 0.2$) or V (Control = $14 \times 10^6 \pm 1.6 \times 10^6$, Schizophrenic= $12 \times 10^6 \pm 1.7 \times 10^6$ $t=2.18$ $p = 0.08$) in Total pyramidal cell density.

Area 9

Stained Interneurons

Compared to controls there was no significant difference in either layer III (Control = $11.4 \times 10^6 \pm 9.6 \times 10^5$, Schizophrenic= $11.11 \times 10^6 \pm 8.17 \times 10^5$ $t=1.83$ $p = 0.12$) or V (Control = $11.2 \times 10^6 \pm 7.9 \times 10^5$, Schizophrenic=$11 \times 10^6 \pm 7.0 \times 10^5$ $t=1.18$ $p = 0.29$) in positively stained interneuron profiles.

Area 32

Stained Interneurons

Compared to controls there was no significant difference in either layer III (Control = $9.8 \times 10^6 \pm 1.4 \times 10^5$, Schizophrenic= $9.9 \times 10^6 \pm 1.0 \times 10^5$ $t=-0.416$ $p = 0.69$) or V (Control =

$9.0 \times 10^6 \pm 1.1 \times 10^5$, Schizophrenic=$9.1 \times 10^6 \pm 1.7 \times 10^5$ $t=-1.88$ $p = 0.12$) in positively stained interneuron profiles.

Discussion

The present data shows a significant decrease in Calmodulin immunostaining in both layers III and V in areas 9 and 32 in schizophrenia as compared to controls with a decrease in the number of pyramidal cells positively stained for calmodulin. We believe that these results are due to schizophrenia-associated alterations and not due to an artifact of sampling or tissue processing for the following reasons. First, because tissues were matched for age, sex and postmortem interval it is unlikely that the observed differences are due to any of those factors. Moreover, the tissues were processed in matched pairs to control for variation due to tissue processing or staining. All specimens in the study were fixed according to a standard protocol so that all tissues received the same treatment and therefore an effect of fixation should affect the material consistently. The variability inherent in immunocytochemical staining has to be considered. Impregnation of the primary antibody in our material appeared to be consistent throughout the thickness of the section and our statistics showed very little within group variability. Area fraction analysis does not directly measure changes in expression of the protein it does measure differences in staining patterns and amount of stain in a given cell population which suggests loss of protein or alterations in rate of expression of the protein. A more direct method would be to perform western blots to confirm the loss of the protein. An interclass correlation on the control raw data to examine variation among the brains as well as reliability of our analytic method. An ICC of 0.90 was obtained which is highly suggestive of reliability of the method and low variation among brains and within a given brain. For these reasons we believe that the effects we have observed are associated with schizophrenia and not due to confounding variables.

The binding of calmodulin by neurogranin regulates the Ca-calmodulin pathway, controlling activation while the cell is at rest (Chakravarthy et al., 1997). Calcium influx is controlled by activation of the NMDA receptor. Binding of glutamate to the NMDA receptor causes an influx of calcium, which triggers the release of calcium from internal stores and the release of calmodulin from neurogranin. These two physiological changes cause neurogranin to be rendered inactive and thereby permit the release calmodulin to interact with calcium (Chakravarthy et al., 1997). A decrease in calmodulin levels could lead to a decrease in the amount of calcium that could bind to calmodulin which would activate the Ca-calmodulin signal transduction pathway and therefore decrease signaling within the cell. Imaging studies of patients with schizophrenia have suggested a decrease in frontal lobe activity (Hill et al., 2004; Holms et al., 2005, Hoshi et al., 2006). The calcium-calmodulin signal pathway activates CaMKinase II and nitric oxide, both of which are involved in neuromodulation, synaptic plasticity, and long-term potentiation. Activating this pathway incorrectly or at low levels could, therefore, alter information processing within the cell. These changes could lead to aberrant information processing in patients diagnosed with schizophrenia.

A loss of calmodulin and neurogranin is suggestive of both morphological and functional alterations in the prefrontal cortical areas 9 and 32. Binding of glutamate to the NMDA receptor allows large amounts of calcium to enter the cell and signals the release of calcium from internal cellular stores. This physiological changes cause neurogranin to be rendered inactive and thereby permit the release calmodulin to interact with calcium (Chakravarthy et al., 1997). The calcium-calmodulin signal pathway activates CaMKinase II and nitric oxide, both of which are involved in neuromodulation, synaptic plasticity, and long-term potentiation (Chakravarthy et al., 1997). Much evidence has pointed to morphological changes in the pyramidal cells in the prefrontal cortex. Recent data have shown functional alterations in these cells as well. Our data represent the first link between morphological alterations and functional alterations in pyramidal cells in the prefrontal cortex.

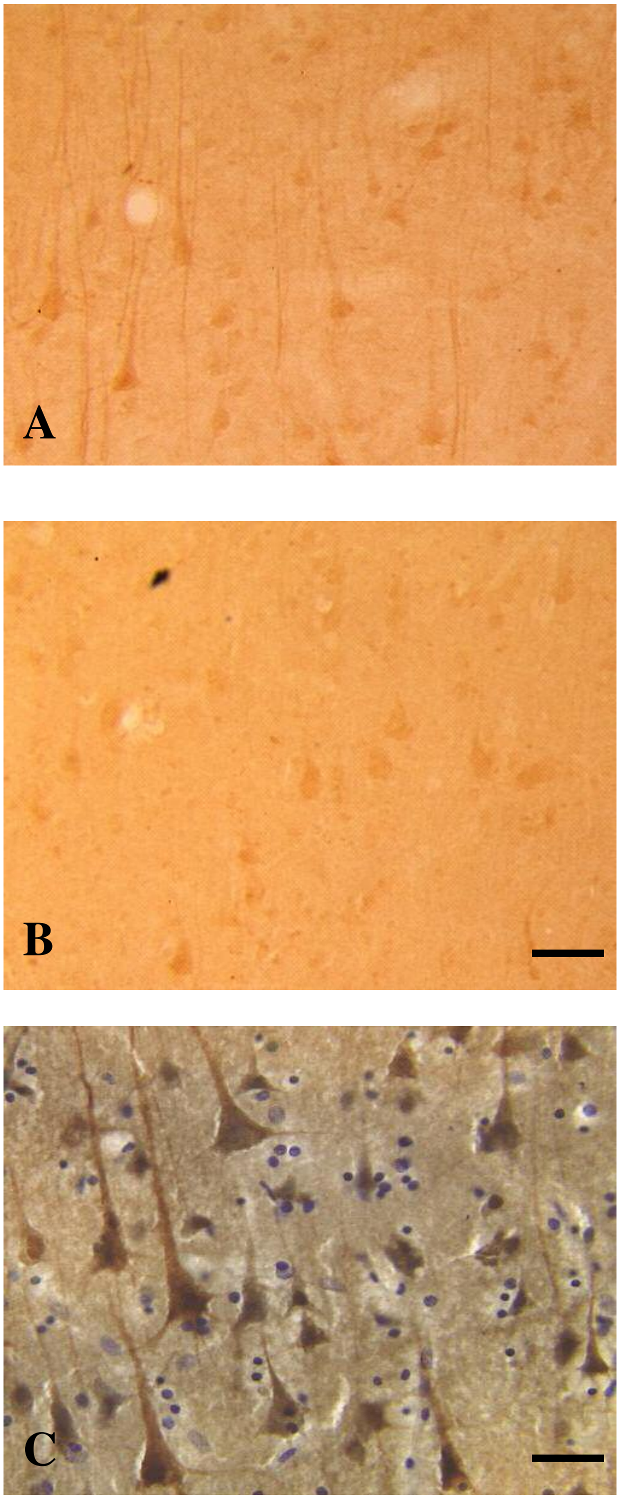


Figure. 1: Photomicrograph of area 9 layer III Calmodulin immunostaining A) Control, B) Schizophrenic taken with a 20x lens final magnification of 200 C) Control counterstained with thionin taken with a 40x lens under oil final magnification of 400. Scale bar A, B = 50µm C= 30µm.

Brain #	Distributive Dx	Age	Sex	PMI	Layer	Storage time in months *
3833	schizophrenia	67	M	21.3	III, V	13
3878	control	65	M	13	III, V	11
3742	schizophrenia	71	M	19	III, V	7
3875	control	68	F	14	III, V	12
3915	schizophrenia	34	M	17.4	III, V	10
3932	control	38	M	21.9	III, V	10
3546	schizophrenia	69	M	17.83	III, V	13
3619	control	74	F	19.8	III, V	11
3557	schizophrenia	66	F	16.75	III, V	13
3625	control	70	F	18.25	III, V	11
3634	schizophrenia	71	M	18	III, V	15
3626	control	65	F	16.6	III, V	11

Table 1. Demographic data for the matched pairs (*= storage times are from the time the brains were removed at HBRC to the time they were processed fro MAP2. An additional 36 months of storage in cyroprotectant needs to be added to all of the brains for neurogranin processing.)

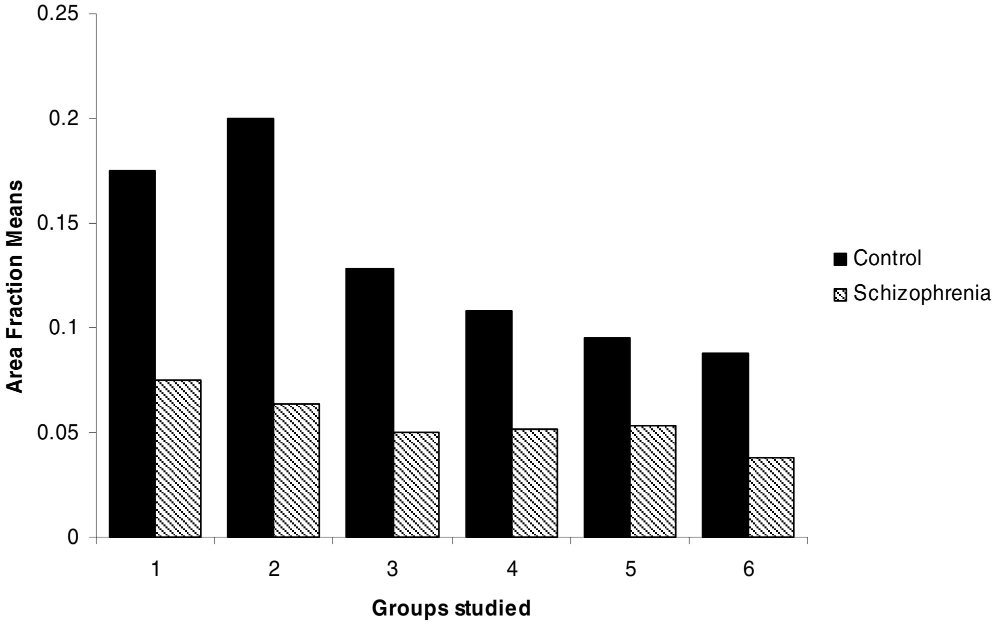


Figure 2. Raw data showing the distribution of means for Calmodulin Area Fraction in pyramidal cells in layer III Area 9.

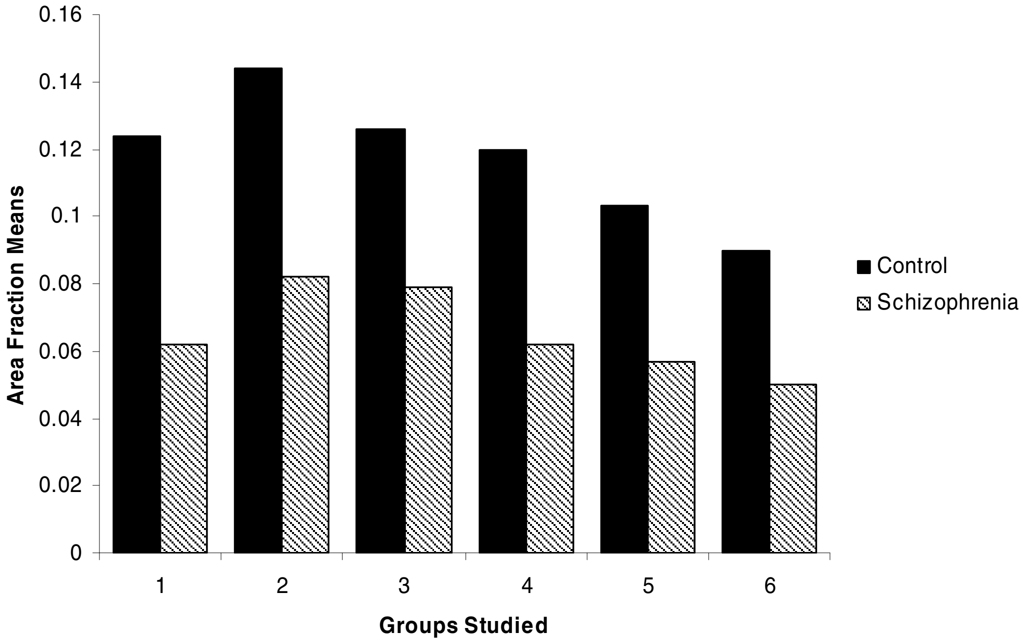


Figure 3. Raw data showing the distribution of means for Calmodulin Area Fraction in pyramidal cells in layer V Area 9.

Brain Area	Layer	Control mean	Control St.d	Schizo. Mean	Schizo. St.d	T value	P value	% decrease
A9	III	0.133	0.01	0.055	0.013	5.22	0.001	58%
A9	V	0.117	0.02	0.065	0.013	13.7	0.002	44%

Table 2. Table showing Area fraction data for layers III and V in Brain Area 9.

Brain Area	Lay	Control mean x10^6	Control St.d x10^6	Schizo Mean x10^6	Schizo St.d x10^6	T value	P value	% decrease
A9	III	9.0	0.81	8.0	1.1	3.59	0.02	11%
A9	V	9.3	0.74	8.0	1.1	6.65	0.01	20%

Table 3. Calmodulin positive stained profile densities for layers III and V in Area 9.

Brain Area	Layer	Control mean x10^6	Control St.D x10^6	Schizo. Mean x10^6	Schizo. St.D x10^6	t value	p value
A9	III	6.8	9.0	6.5	9.5	0.514	0.62
A9	V	5.0	9.5	5.4	1.5	-0.896	0.40

Table 4. Table showing Non-stained pyramidal cell densities in Area 9 layers III and V.

Brain Area	Layer	Control mean x106	Control St.D x106	Schizo. Mean x106	Schizo. St.D x106	t value	p value
A9	III	15.7	9.2	15.0	9.0	1.63	0.2
A9	V	14.1	9.7	13.1	3.0	1.19	0.3

Table 5. Table showing Total pyramidal cell densities in Area 9 layers III and V.

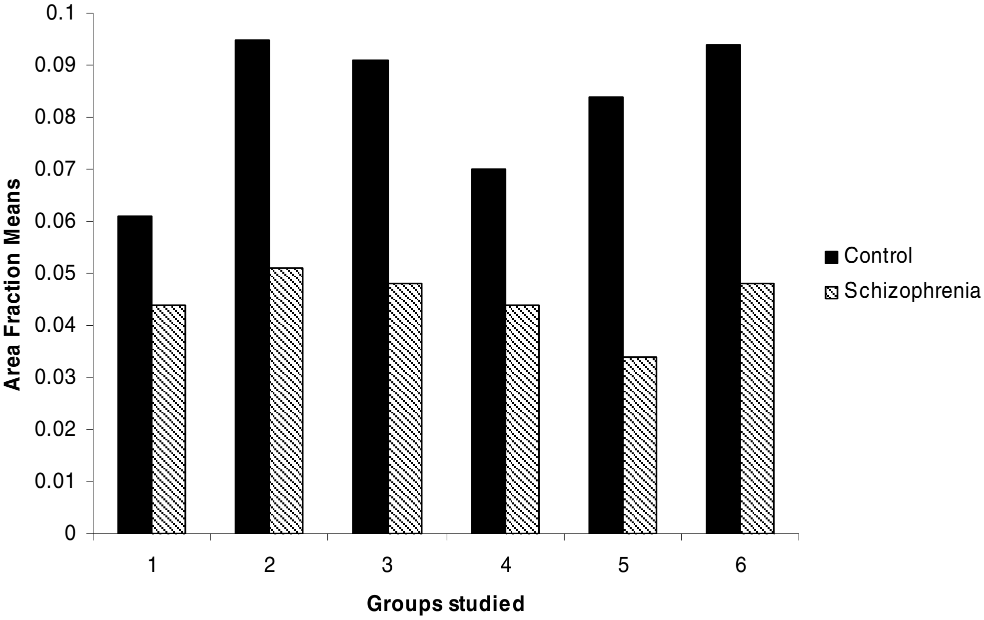


Figure 4. Raw data showing the distribution of means for Calmodulin Area Fraction in pyramidal cells in layer III Area 32.

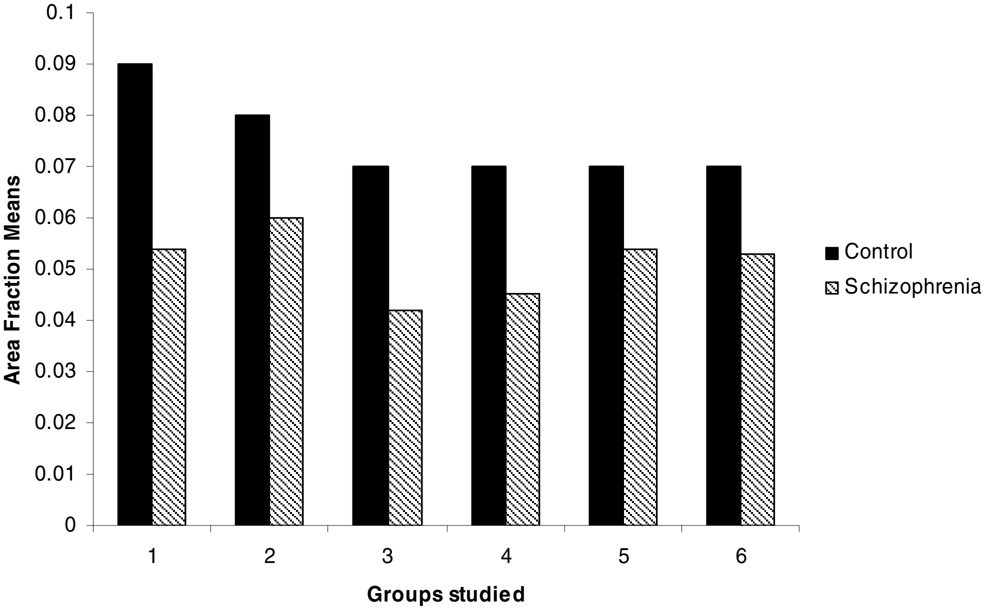


Figure 5. Raw data showing the distribution of means for Calmodulin Area Fraction in pyramidal cells in layer V Area 32.

Brain Area	Layer	Control mean	Control St.d	Schizo. Mean	Schizo. St.d	T value	P value	% decrease
32	III	0.083	0.014	0.04	0.014	7.31	0.0007	51%
32	V	0.075	0.008	0.051	0.007	7.62	0.006	32%

Table 6. Table showing Area fraction data for layers III and V in Brain Area 32.

Brain Area	Lay	Control mean x10^6	Control St.d x10^6	Schizo Mean x10^6	Schizo St.d x10^6	T value	P value	% decrease
A32	III	8.8	3.3	7.4	4.4	11.77	0.0001	16%
A32	V	9.0	1.4	7.4	0.7	4.54	0.006	17%

Table 7. Calmodulin positive stained profile densities for layers III and V in area 32.

Brain Area	Layer	Control mean x10^6	Control St.D x10^6	Schizo. Mean x10^6	Schizo. St.D x10^6	t value	p value
A32	III	4.7	1.5	4.7	1.1	0.128	0.90
A32	V	5.2	1.0	5.0	1.5	0.377	0.71

Table 8. Table showing Cell density for Non-stained pyramidal cells in Area 32, layer III and layer V.

Brain Area	Layer	Control mean x10^6	Control St.D x10^6	Schizo. Mean x10^6	Schizo. St.D x10^6	t value	p value
A32	III	15.5	4.9	12.1	1.2	1.60	0.2
A32	V	14.0	1.6	12.0	1.7	2.18	0.08

Table 9. Table showing total pyramidal cell density in Area 32, in layers III and V.

Brain Area	Layer	Control mean x10^6	Control St.D x10^5	Schizo. Mean x10^6	Schizo. St.D x10^5	t value	p value
A9	III	11.4	9.6	11.11	8.17	1.83	0.13
A9	V	11.2	7.9	11.0	7.0	1.18	0.29

Table 10. Table showing cell density for positive stained interneurons in area 9, layers III and V.

Brain Area	Layer	Control mean x10^6	Control St.D x10^5	Schizo. Mean x10^6	Schizo. St.D x10^5	t value	p value
A32	III	9.8	1.43	9.9	1.0	-0.416	0.69
A32	V	9.0	1.1	9.1	1.7	-1.88	0.12

Table 11. Table showing cell density for positive stained interneurons in area 32, layers III and V.

<u>Chapter 5:</u> CONCLUSION

There is suggestive evidence that the PFC cortex is a major site affected in schizophrenia. Support has implicated area 9 of the PFC and now findings are also suggesting involvement of Brodman's area 32. Both imaging and morphological studies suggest deficits in the ultrastructure of the PFC (Weinberger et al., 1986, Buchsbaum et al., 1990; Benes et al., 1991; Goldman-Rakic et al., 1995; Beasely et al., 1997; Glantz and Lewis et al., 1997; Garey et al., 1998; Harrison et al., 1999; Andreason et al., 2000; Thune et al., 2001; Jones et al., 2002; Broadbelt et al., 2002). The PFC maintains reciprocal excitatory connections with specific subdivisions of the MD, the principal source of thalamic inputs to the PFC (Goldman-Rakic and Porrino, 1985, Giguere and Goldman-Rakic, 1988, Barbas et al., 1991). Like the PFC, the MD has been implicated as a site of dysfunction in schizophrenia (Lewis et al., 2000). In the PFC, spines on the basilar dendrites of layer III pyramidal neurons are among the targets from the MD (Giguere and Goldman-Rakic, 1988). Layer III pyramidal neurons play a central role in the flow of information both between and within regions. The dendritic spines of other pyramidal neurons constitute the postsynaptic targets of almost all both white matter and the long-range intrinsic projections of these neurons. More recent studies have began to examine morphological alterations of cell populations in the prefrontal cortex. Present studies of the neuropathology of schizophrenia suggest morphological and functional alterations of pyramidal cells in the prefrontal cortex (Rajkowska et al., 1998, Andreasen et al., 1994, Garey., et al 1998, Selemon et al., 1999, Glantz et al., 2000, Kalus et al., 2000, Pierri et al., 2001, Jones et al., 2001; Jones et al., 2001, Broadbelt et al., 2002, Jones et al., 2002; Buxhoeveden et al., 2003)

Past analysis of the prefrontal cortex had shown a decrease in MAP2 staining in pyramidal cells (Jones et al., 2001); MAP2 is a protein that is found in dendrites and cell bodies. (Decamilli et al., 1984, Fischer et al., 1987, Li et al., 2000). Pyramidal cells are the primary output from the cortex (Shapiro et al., 1993, Frith et al., 1996, Jones et al., 2001,) and their dendrites are the primary mechanism for receiving both excitatory and inhibitory contact (Glantz et al., 2000, Kalus et al., 2000). Studies have also found a decrease in spine density in layer III pyramidal neurons of the frontal association areas (Garey., et al 1998, Glantz et al., 1997, Glantz et al., 2000). Spines primarily receive excitatory synapses and the dendritic shafts primarily receive inhibitory synapses (Garey et al., 1998, Glantz et al., 2000). Morphological changes seen in pyramidal cells in the prefrontal cortex can be related to the disruption in neuronal circuitry in this region of the brain (Isseroff et al., 1982, Harrison et al., 1996; Selemon et al., 1999, Staal et al., 2000). Changes, such as a loss of dendrites and dendritic spines will reduce synaptic surface area for incoming afferents (Isseroff et al., 1982, Harrison et al., 1996, Selemon et al., 1999, Staal et al., 2000).

The aim of the first part of this study was to determine whether morphological changes seen on the pyramidal cells were schizophrenia associated. The dendritic branches of a neuron are essential for receiving and transmittance of information. If the arbors are too expansive or too small, that can alter the transfer of information and in turn alter integration of information. The data presented from the Golgi study (chapter 2) showed a significant decrease in the primary and secondary basilar dendrites in both areas 9 and 32 in both layers III and V. In addition, we believe that there is a relationship between the loss of dendrites and spines, and the loss of MAP2. These differences are unlikely to be artifacts attributable to variation in age, sex or postmortem interval because specimens were matched for those variables; however, the present data cannot exclude an effect of neuroleptics. The matched-pair technique should also minimize any artifacts due to the capriciousness of Golgi-staining. Selemon et al. (1995) hypothesized that the schizophrenia-associated increase in neural density they observed in PFC is due to a decrease of neuropil. Such a decrease could result from a loss of any of the components of neuropil including dendrites, synapses, axons or support cells. In addition to the present evidence, several previous studies are consistent with a schizophrenia-associated decrease in dendritic material. Specifically, MAP2 immunostaining has been found to be decreased in layers III and V in prefrontal areas 9 and 32 (Jones et al., 2002), and the density of dendritic spines has been found to be lower in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000). In addition, the size of a neuron's soma is positively correlated with the amount of dendritic material it can support (van Pelt et al., 1996; van Ooyan et al., 1995). Thus, recent reports of a schizophrenia-associated decrease of neuronal somal size in the PFC (Rajkowska et al., 1998), and specifically the somal size of pyramidal neurons of layer III (Pierri et al., 2001) are also consistent with the hypothesis of a schizophrenia-associated decrease of dendritic material.

The decrease in the number of basilar dendrites and a change in synaptic spine density observed in the present study may reflect a loss of surface area for both excitatory and inhibitory inputs. Both cortical-cortical and cortical-subcortical communications have been found to be impaired in schizophrenia (Bertolino et al., 1999; Kalus et al., 1999; Lewis, 2000). While the loss of dendrites in layer III would primarily disrupt the transfer and integration of information between both ipsilateral and contralateral cortical regions (Ghez, 1991), the loss of layer V dendrites would affect primarily the exchange and integration of information between cortical and subcortical regions (Ghez, 1991), including the thalamus.

Two recent studies have reported a schizophrenia associated loss of neurons in the parvocellular division of the mediodorsal nucleus (Popken et al., 2000; Byne et al., 2002) which has extensive projections to the PFC (Goldman-Rakic and Porrino, 1985; Giguere and Goldman-Rakic, 1988), and markers for thalamic axons have been found to be decreased in the PFC of schizophrenics (Lewis et al., 2001). Thus, the schizophrenia-associated decrease of dendrites we observed in layer III may reflect, at least in part, a decrease of input from the thalamus, a nodal link in communication among multiple brain regions. The schizophrenia-associated decrease of dendrites in layer V may also reflect a loss of thalamic input as well, while both the decrease in layers III and V may reflect a loss of GABAergic input. The decrease in dendritic material is suggestive of a loss of synaptic surface area, which may alter information processing.

The present study represents our further examination of the PFC in schizophrenia to better understand how the disease alters neural circuitry. It remains to be shown whether the decrease of dendrites observed in the present study is restricted to the particular cortical area and the laminae we examined, or whether it more diffusely affects the PFC or other cortical regions. It also remains to be determined whether the present findings are specific to schizophrenia as opposed to other chronic mental illnesses including the schizophrenia spectrum and affective disorders.

Based on the structural alterations observed on pyramidal cells, it was necessary now to examine the internal functional aspects. One link between structure and function in these cells is calcium signaling. Neurogranin is an important protein involved in signal transduction (Li et al., 2000). Neurogranin like MAP2 is located in cell bodies, dendrites and dendritic spines (Alvarez-Bolado et al., 1996) and a loss of neurogranin is a marker for dendritic lesions (Li et al., 2000). Functionally neurogranin is an upstream regulator of calcium and calmodulin (Ho Pak et al., 2000, Prichard et al., 1999). Neurogranin binds calmodulin and, therefore, renders it unable to interact with free calcium (Ho Pak et al 2000, Prichard et al., 1999). Knockout mice lacking neurogranin exhibit problems with spatial learning and long-term potentiation (Ho Pak et al., 2000) suggesting a role for neurogranin in processing and transmission of information and suggesting a possible role in schizophrenia. We therefore chose to examine the expression of neurogranin in pyramidal cells in layers III and V in area 9 and 32 of prefrontal cortex. Our data suggest a dramatic decrease in neurogranin immunostaining in both layers III (72%) and V (50%) in areas 9 and in area 32, (30%) in layer III and (40%) in layer V with no change in the number of cells expressing the protein confirming mounting evidence implicating morphological changes in the pyramidal cells and suggesting possible changes in signal transduction in the pyramidal cells. Neuron density measurements of immunopositive pyramidal cells showed no difference in the number of pyramidal cells positively stained with neurogranin. We believe that these results are due to schizophrenia-associated alterations and not due to an artifact of sampling or tissue processing for the following reasons. The potential role of neuroleptics must also be considered because all of the schizophrenic subjects had been exposed to neuroleptics. A recent study by Lidow et al., (2001) examined the possible role of neuroleptics and their effects on dendrite and spine associated proteins. The study showed a neuroleptic induced increase in phosphorylation of MAP2 but no differences in the level of MAP2 or in spinophylin in the PFC. In addition long postmortem intervals and poor fixation could affect the present data. The average PMI was less than 24 hours for both the control material and the schizophrenic material. A recent study by Viel et al., (2001) shows that neurons stain positively for MAP2 following a 24-hour PMI. Suggesting that immunostaining is possible with specimens whose PMI's were equal to or less than 24 hours. While area fraction analysis does not directly measure changes in expression of the protein it does measure differences in staining patterns and amount of stain in a given cell population which suggests loss of protein or alterations in rate of expression of the protein. A more direct method would be to perform western blots to confirm the loss of the protein. For these reasons we believe that the effects we have observed are associated with schizophrenia and not due to confounding variables.

The decrease in neurogranin immunostaining is quite dramatic and may appear surprising. However, at least one other study has shown such dramatic results in the PFC in schizophrenia (Novak et al., 2000). We examined the density of stained pyramidal cells in controls and schizophrenics and found no difference in either layer or region suggesting that the cells are producing the protein but at a much decreased rate. Examination of Calmodulin, a second protein in the pathway is a protein directly regulated by neurogranin is decreased in both layers III and V in both areas 9 and 32. However there was a significant trend towards a decrease in the number of positive stained pyramidal cells, which is suggestive of fewer cells making the protein. Calmodulin is also present in interneurons after careful analysis of and using a three dimensional counting technique there was no significant difference between groups in both layers in both areas. The binding of calmodulin by neurogranin regulates the Ca-calmodulin pathway, controlling activation while the cell is at rest (Chakravarthy et al., 1997). Binding of glutamate to the NMDA receptor allows large amounts of calcium to enter the cell and signals the release of calcium from internal cellular stores. These two physiological changes cause neurogranin to be rendered inactive and thereby permit the release of calmodulin to interact with calcium (Chakravarthy et al., 1997). A decrease in neurogranin levels could allow more calmodulin to interact with free calcium and to activate the Ca-calmodulin signal transduction pathway inappropriately. A loss of both neurogranin and calmodulin during development could have adverse affects on dendritic spine formation and synaptogenesis. Studies using neurogranin knock out mice have not found any gross anatomical alterations; however, no cellular studies have been performed. A loss of neurogranin is suggestive of both morphological and functional alterations in the prefrontal cortical areas 9 and 32. Much evidence has pointed to morphological changes in the pyramidal cells in the prefrontal cortex. Recent data have shown functional alterations in these cells as well. This data represent the first link between morphological alterations and functional alterations in pyramidal cells in the prefrontal cortex. There are future studies that can be done to continue to elaborate on this work. Using a controlled specimen one can try to replicates the morphological finding and relate through correlations between both studies. A more quantitative experimental design to expand on the protein can be done by the use of applied molecular techniques. The antibody that was used detected total neurogranin while there are two forms of the proteins in this pathway, phosphorylated and non-phosphorylated. Antibodies have now been found that detects the active or phosphorylated form of the protein, it essential to find out which form of this protein is significantly affected. Using the same cohort of brains as well as an animal model currently being worked on and modifying the immunohistochemical method, applied florescent confocal microscopy can be performed to triple label for neurogranin, MAP2, and calmodulin, where all proteins can be analyzed for localization, cell density, and intensity with in a given population. This work has been insightful and has the possibility of opening or exploring many ideas which can be used as part of the much larger task at and to try and understand this disease.

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