Early Mediodorsal Thalamic Damage Induces Alterations in Prefrontal Cortex: Potential Model for Schizophrenia

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ABSTRACT

Early Mediodorsal Thalamic Damage Induces Alterations in Prefrontal Cortex: Potential Model for Schizophrenia

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One of the most consistent findings in schizophrenia is a decrease in volume and neuronal number in the medial dorsal nucleus of the thalamus (MD) (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002). The MD is reciprocally connected to the prefrontal cortex (PFC), another region implicated in schizophrenia. Focusing on the interplay between the MD and the PFC, this study examined the hypothesis that early damage to the MD may lead to alterations in morphology of pyramidal cells in the PFC, similar to that observed in schizophrenics. Unilateral electrolytic lesions of the MD in Long-Evans rat pups were made on postnatal day 4 (P4) and animals developed to P60. We examined morphological profiles for pyramidal cells in three subregions of the PFC: prelimbic (PL), anterior cingulate 1(CG-1), and Dorsolateral anterior cingulate (DL) cortices, which receive afferents from the MD. Structural alterations were assessed by three meausures: immunostaining levels for microtubule-associated protein 2, an indicator of dendritic integrity (Caceres et al., 1992), number of basilar dendrites, as well as spine density. Lesions causing mean MD volume decreases of 12.30% led to significant decreases in MAP2 immunostaining. No difference was observed in pyramidal cell density in any of the regions in or layers, so the reduction in MAP2 staining likely occurred as a function of reduced protein levels and not due to lower cell densities in these regions. Early postnatal thalamic lesions led to significant reductions in the number of primary and secondary dendrites for pyramidal cells in the PFC, suggesting early MD damage

affected the dendritic arbors. Spines on pyramidal dendrites are the predominant targets of the MD (Kuroda et al., 1995), and are induced by afferent input activity (Kossel et al., 1997). Mean nuclear volume decreases of 14.82% in the MD led to decreases in the density of spines along basilar dendrites. The data showed that early loss of cells in the MD could affect the morphology of pyramidal neurons in the PFC, and support the hypothesis that the alterations in PFC observed in schizophrenic subjects could arise as a consequence of early MD damage.

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Chapter 1. Introduction

Schizophrenia is a debilitating psychiatric illness, affecting approximately two million people in the United States, and over fifty million people worldwide (Buchanan and Carpenter 2000). Individuals suffering from this disorder suffer a constellation of clinical features divided into positive, negative, and cognitive symptoms. Positive symptoms are defined by the presence of abnormal behaviors. These symptoms include dramatic hallucinations, most commonly of the auditory type, and paranoid delusions. The patients suffer from abnormal perception, and aberrant inferential thinking, rendering them unable to process reality accurately (Buchanan and Carpenter 2000). Disorganized speech, a positive symptom, is characterized by derailment and incoherence (Keef and Harvey 1994). Negative symptoms refer to the absence or diminution of normal behavior. These symptoms include decreased spontaneous communication, movement, eye contact, and facial expression. Flat affect, anhedonia, avolition and withdrawal may also be present (Keef and Harvey 1994). Cognitive dysfunction may be the most persistent and disabling clinical feature. It has been suggested that the severity of the cognitive decline may predict the long term outcome of the disorder (Lewis et al., 2001). These dysfunctions include impairments in executive functioning and certain memory modalities (Stone et al., 1998; Conklin et al., 2000; Perry et al., 2001). Executive functions involve conscious control of thought such as inhibition, consequential thinking, planning, and goal directed behavior (Frith and Dolan 1996), and patients with schizophrenia show loss of these executive functions (Stone et al., 1998; Conklin et al., 2000; Perry et al., 2001), as well as deficits in orienting tasks and conflict tasks (Romer and Walker 2007). An individual may express any combination of the different symptom categories: positive, negative, and cognitive. Different individuals may also be afflicted to varying degrees.

Schizophrenia is a complex neuropathology characterized by a wide spectrum symptomatology, impacting subjects differentially.

Some of the schizophrenic symptoms are diminished or abolished through the use of antipsychotic drugs. The mechanism of action of these drugs was found to be related to the action of dopamine (for review see Carlsson 1978), which provided insight into a potential pathogenesis for schizophrenia. Antipsychotic drugs are dopamine receptor antagonists, suggesting the disorder is characterized by hyperactivity of dopamine. The earlier, typical antipsychotic drugs have high affinity for D2 receptors, which are expressed abundantly in forebrain subcortical areas (Khan et al., 1998). The atypical neuroleptics have higher affinity for the D4 receptor subtype (Seeman et al., 1997), preferentially expressed in the hippocampus and in the cortex (Khan et al., 1998). Further evidence showed that the increase in dopamine was not global; many subjects with schizophrenia showed no signs of increased dopamine activity, and patients with predominantly negative symptoms improved after dopamine activity was increased; furthermore, 20% of patients do not respond to dopamine-blocking medication (Keef and Harvey 1994). It has been postulated that excessive dopamine transmission, primarily within the forebrain regions, is responsible for the psychotic symptoms of schizophrenia (Seeman 1987). Yet, there is no direct evidence linking aberrant dopamine neurotransmission as a causal factor in schizophrenia pathogenesis. Pharmacological evidence has also linked other neurotransmitter systems: glutamate has been suspected to play a role in this disorder because PCP, an NMDA receptor antagonist, was shown to give rise to symptoms and cognitive dysfunction similar to those seen in schizophrenia, such as social withdrawal, apathy and lack of executive functions (Javitt and Zukin 1991). Furthermore, Kynurenic acid, an endogenous glutamate antagonist, is reported to be elevated in the cerebral spinal fluid of schizophrenic patients as compared to

healthy controls (Ehrhardt et al., 2003); however, average brain concentrations of kynurenic acid are far below those required to affect NMDA-receptors as revealed from in vitro studies (Scharfman et al., 2000). Much debate continues between the dopamine and glutamate hypothesis. Recent arguments incorporate both theories and state that NMDA dysfunction may lead to secondary dopaminergic dysregulation in striatal and prefrontal brain regions (For review see Javitt 2007). They speculate that the imbalance in dopamine signaling is a consequence of an original glutamate dysfunction. However, it is unlikely that simple hyperactivity or hypoactivity of a neurotransmitter system can explain all the complex aspects of this disorder. Dysregulation of the transmitter systems best explains the psychosis of schizophrenia (Seeman and Kapur 2000) in part, because treatment with pharmacological agents can mimic or antagonize the symptoms; however, the pathophysiology of cognitive abnormalities remains unclear. Theories on neurotransmitter dysfunction also cannot explain the timing of onset of symptoms and the lifetime of waxing and waning symptoms (McCullumsmith et al., 2004). Schizophrenia is a very complex neuropathology which cannot be explained by the disruption of neurotransmitter homeostasis alone.

The onset of schizophrenic symptoms occurs during late adolescence or early adulthood (Alda et al., 1996). The “sudden” presence of positive symptoms in an individual, accompanied by a decline in social and cognitive functioning may suggest a chronic, progressive, neurodegenerative model for schizophrenia. In such model, excitotoxicity, dysregulated apoptosis, and oxidative stress, among other processes, lead to pathological changes in brain circuitry which then manifest as symptoms of schizophrenia (Deutsch et al., 2001; Weinberger and McClure 2002). However, such degeneration would be accompanied by an increase in glial density, and a decrease in the neuron-glial ratio (Benes 1988), and postmortem assessments of

schizophrenic brains have found no evidence of gliosis (Benes et al., 1986; Pakkenberg 1990; Falkai et al., 1999; Popken et al., 2000; Young et al., 2000). The lack of gliosis suggested schizophrenia may be a developmental disorder. Early epidemiological studies reported that the incidence of schizophrenia among first degree relatives was ten times higher than for the general population (Kety and Rosenthal 1968). Moreover, in monozygotic twins the concordance rate was 40-50%, while in dizygotic twins it was only 10% (For review see Gottesman 1991), suggesting that schizophrenia might involve a genetic factor. Evidence for the involvement of specific genes in schizophrenia has been extensive (for review see Harrison and Weinberger 2005), ranging from immediate early genes fos and jun (Kyosseva 2004) to neuroregulin 1 (Stefansson et al., 2002), and dysbindin (Straub et al., 2002), and Disc-1 (Mackie et al., 2007), among several others (Harrison and Weinberger 2005). The concordance among twins and the disruption of so many genes in schizophrenia further supports the notion that it may not be a degenerating pathology, but one that arises in early development. Furthermore, recent research reveals the presence of behavioral deficits, particularly social and cognitive, prior to the onset of full symptomatology (Cannon et al., 2000; Niemi et al., 2003; Rapoport et al., 2005), as well as some structural abnormalities including enlargement of the ventricles (Benes 1989); postmortem examinations indicate that the enlargement is unaccompanied by changes in glial population (for review see Benes 1989). Moreover, studies in young schizophrenic patients, with symptoms less than six months duration, showed a similar degree of ventricular enlargement as their chronic counterparts (Weinberger et al., 1982; Schulz et al., 1983; Landrich et al., 1986). Other structural anomalies include volume deficits in temporal lobe structures, such as in the entorhinal cortex (Bogerts et al., 1993) with no increased gliosis (Falkai et al., 1988) and volume reductions in the hippocampus (Suddath et al., 1989; Bogerts et al., 1990; Shenton et al., 1992) with evidence of

neuronal cells loss in the area (Falkai and Bogerts 1986). There are reports of volume reductions in the parahippocampal gyrus (Brown et al., 1986) and the amygdala (Bogerts et al., 1985). Moreover, thalamic abnormalities associated with schizophrenia are regularly reported, including volume and neuronal number reductions (Pakkenberg 1990; Andreasen et al., 1994; Byne et al., 2001; Byne et al., 2002; Mitelman et al., 2006), as well as decreased number of oligodendrocytes (Byne et al., 2006). Alterations associated with schizophrenia are reported in the cerebral cortex as well. Imaging studies report global reduction of nearly 4% in the volume of cortical gray matter in schizophrenia (Cannon et al., 1998; Sharma et al., 1998; Marsh et al., 1999), while other investigators show a significant gray matter volume decline in association cortices: PFC, inferior parietal lobule, and Wernicke's area (Shaepfer et al., 1994), as well as alterations in white matter volume and number of oligodendrocytes in the frontal cortex (Hof et al., 2003). The variations in volume and neuronal numbers have been extensively replicated in schizophrenia, and these reductions are unaccompanied by changes in glial population (Benes et al., 1986; Falkai and Bogerts 1986; Falkai et al., 1988; Benes et al., 1991; Rajkowska et al., 1998; Selemon et al., 2003). In the absence of gliosis, it can be inferred that these volume and neuronal population abnormalities associated with schizophrenia appear early in development and are not the result of a neurodegenerative process. Schizophrenia may be masked during early childhood by the presence of excess synaptic connections, so that aberrant circuitry may be compensated by other connections. The wave of synaptic pruning occurring during late adolescence (for review see Arnold 1999) minimizes the excess connectivity and exposes the defective circuitry. Furthermore, associative cortical areas show increased myelin staining during the second decade of life (Benes 1989), thus the late myelination of key circuits allows for the expression of a previously concealed defect. Schizophrenia is associated with genetic,

behavioral, and physical abnormalities which predate the onset of symptoms; there are no physical remnants of degeneration, and the late onset of symptoms coincinding with a wave of synaptic pruning and myelination of key circuits may suggest that schizophrenia follows a developmental pathogenesis.

A complex neurodevelopmental disorder characterized by a large diversity of symptoms with multiplicity of brain regions affected may be partially explained as resulting from a defect in an area where multiple functional systems converge. This may be a region receiving and projecting information to the many areas affected, such region may be the thalamus, which mediates communication and distributes information between the periphery and central circuits. Of the approximately fifty thalamic nuclei identified, the association nuclei are of interest in schizophrenia research; these nuclei have widespread but topographically defined projections to the cortex, and they include the pulvinar and the mediodorsal nucleus (MD).

Mediodorsal Thalamic Nucleus:

The MD is a principal source of subcortical input to the frontal cortex, as well as a major relay nucleus of the limbic system (Paxinos 1985; Pirot et al., 1994; Kuroda et al., 1995). In the nonhuman primate, the MD receives projections from the amygdala, ventral pallidum, substantia nigra, superior colliculus, entorhinal and olfactory cortex (Goldman-Rakic et al., 1985; Barbas et al., 1991; Ray and Price 1993; Kuroda et al., 1998). MD efferent fibers ascend ipsilaterally and terminate on multiple cortical regions, dorsolateral frontal cortex, medial prefrontal cortex, orbital cortex, premotor cortical areas and striatum (Goldman-Rakic et al., 1985; Barbas et al., 1991; Ray and Price 1993). (See figure 1).

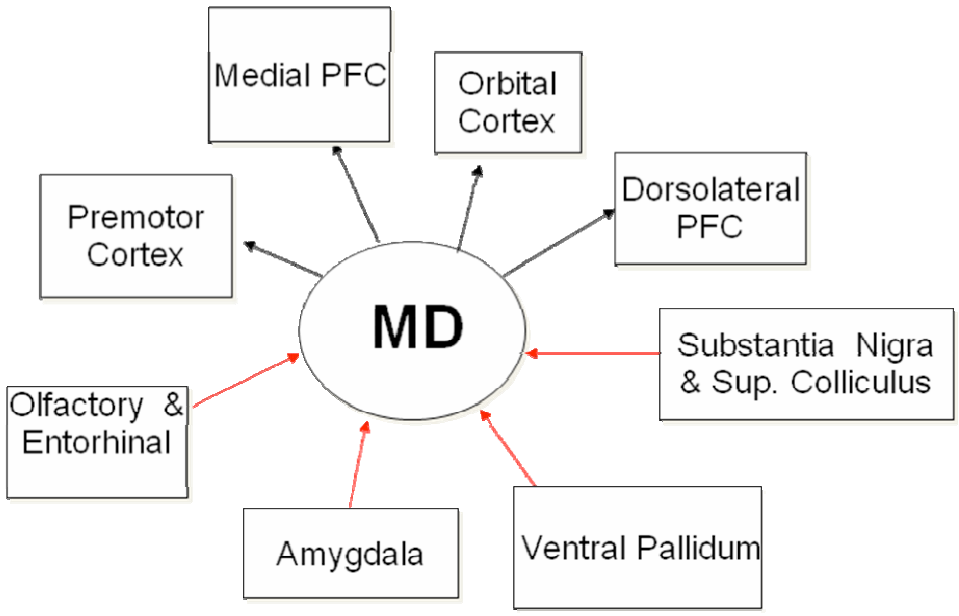


Figure 1: Schematic of nonhuman primate MD thalamus afferents and cortical projections. Referenced from Ray and Price 1993, and Barbas and colleagues, 1991.

The MD nucleus is a homogeneous nucleus where relay cells are the predominant population, namely Stellate and Fusiform cells (Kuroda et al., 1998; Negyessy et al., 1998). Stellate cells are medium to large sized relay cells with spherical dendritic fields, while fusiform cells are smaller cells with distorted dendritic fields (Kuroda et al., 1998). Some MD cells cannot be easily categorized into either group because their morphology shares characteristics from both types, and are thus called transitional cells (Kuroda et al., 1998). The MD is divided into three subdivisions, each with specific projection profiles. The anteromedial magnocellular component receives afferents from the olfactory cortex and amygdala, and projects reciprocally to orbitomedial prefrontal cortex (PFC) and hypothalamus. The parvocellular subnucleus, on the dorsolateral aspect, reciprocally connects to the dorsolateral PFC, while the caudodorsal densocellular subnucleus projects to premotor cortical areas and striatum (Goldman-Rakic et al., 1985; Barbas et al., 1991; Ray and Price 1993). The abundant interconnections of the MD to the

cortex and other subcortical structures indicate the MD as a site where multiple systems converge.

The MD afferents terminate heavily on prefrontal cortical regions (Herkerham 1980; Goldman-Rakic et al., 1985; Barbas et al., 1991; Ray and Price 1993; Pirot S et al., 1994; Kuroda et al., 1995; Kuroda et al., 1998; Negyessy et al., 1998; Wang and Shyu 2004). The dendrites on pyramidal cells in the PFC represent the principal targets for incoming afferents (Schade and Baxter 1960). Thalamocortical afferents innervating these cells terminate differentially across the cortical layers (for review see Kuroda et al., 1998): Pyramidal cells in layer III of the PFC possess a highly branched apical dendrite and a rich arborization of the basilar dendritic tree, and are thus optimum targets for the MD fibers. These layer III cells send ipsilateral projections to other cortical regions, and some of the PFC cells receiving MD input are callosal cells, projecting contralaterally to corresponding regions of the opposite hemisphere (Kuroda et al., 1998). Apical dendrites of pyramidal cells in layer V receive thalamocortical afferents to a lesser extent (Kuroda et al., 1998); these cells give rise to subcortical fibers innervating subcortical structures and the MD. Excitatory neurotransmission in the central nervous system takes place mainly at dendritic spines (for review see Harris 1999) and thalamocortical terminations on PFC form asymmetric synapses with dendritic spines (Kuroda et al., 1998), suggesting their excitatory nature. Moreover, PFC efferents arising from layers V and VI make similar contacts with their thalamic targets (Kuroda et al., 1998; Negyessy et al., 1998), suggesting an excitatory circuitry between the PFC and the MD (Kuroda et al., 1995; Kuroda et al., 1998).

In humans, the close association between the MD and the PFC may initiate early in development. Prenatally, between the seventh and tenth weeks of gestation, migration and accumulation of postmitotic cells at the intermediate zone form the cortical plate in human

neocortex (for review see Holmes 1986); this is followed by increased thickness of the plate and subsequent divisions into inner and outer zones. During the thirteenth and fifteenth weeks of gestation neurons migrate into the external zones of the cortical plate and begin differentiation (Holmes 1986), which appears completed before 28 weeks' gestational age (Sidman and Rakic 1973). Pyramidal neurons in the PFC appear to develop later than in more posterior neocortical areas (for review see Conel 1969), at birth, appearing as smaller pyramidal cells with few short basal dendritic branches and no branches arising from apical dendrites. Axons from pyramidal neurons reach their targets well before the parent neurons have developed a substantial dendritic tree and before afferent fibers to layers III and V have been completed (Holmes 1986), suggesting the close developmental interactions between the cortex and subcortical regions. Dendritic development advances between 1 and 3 months postpartum, at this age there appears to be no difference between cortical regions (Conel 1969). Dendritic growth continues beyond this age with considerable elongation of branches after 2 years of age (Mrzljak et al., 1990), as well as maturational changes into puberty including growth of dendrites, especially on layer III cells, and regressive changes, with loss of spines especially on interneurons (Mrzljak et al., 1990). Excess synaptic connections develop between age 1 to 7, when synaptic density is about 40% above the adult value, followed by synaptic pruning and elimination occurring during late childhood and adolescence (Huttenlocher and Dabholkar 1997). The development of functionally significant neural circuits likely depends on input to the system (Huttenlocher and Dabholkar 1997), such as input arising from the MD thalamocortical afferents.

The intricate interdependency between the cortex and thalamus is evident in rat neurodevelopment as well. In the rat, developing thalamocortical axons first proceed ventrally from the dorsal thalamus and then turn dorsolaterally at the diencephalon-telencephalon junction,

where they join the internal capsule (Molnar et al., 2003). At the striatocortical junction, these thalamic afferents interact with descending early corticofugal fibers before further advancing to their targets (Molnar et al., 2003), and these corticofugal fibers may even assist thalamocortical afferents thru the distal part of their journey (Molnar 2000). Significant brain development occurs perinatally. A large number of MD afferents reach the upper cortical plate on postanatal day 1 or 2, reaching its maximum level between postnatal day 4 and 5, and that level remains high to postnatal day 10 (Van Eden 1986). Simultaneously, the prefrontal cortex is undergoing structural changes and development, the cortical plate is differentiating from a densely packed zone of immature cells into laminae resembling future cortical layers (Van Eden 1986); by postnatal day 4 and 5, layer V can be distinguished from the upper cortical plate containing the elements to become layers II and III. The first evidence of retrograde labeling from PFC to MD becomes apparent at this time (Van Eden 1986). It seems that the arrival of the MD afferent fibers in the upper cortical plate precedes the completion of layer III differentiation, occurring on postnatal days 9 to 10 (Van Eden 1986). It is unknown whether these fibers make synaptic contacts within the developing layers, but it is likely they exert great influence on the development of the cortical plate.

When the thalamic afferents arrive in the cortex, the dendritic tree is premature (Wise et al., 1979). Because formation of dendritic branches and spines is induced by afferent innervation and influenced by activity (Kossel et al., 1997; Wedzony et al., 2005), it is probable that the thalamocortical fibers affect the modeling of the pyramidal arbor and formation of spines in PFC cells. Considerable dendritic growth and spine formation occur after the afferent fibers establish an adult pattern of distribution, with spines appearing around postnatal day four (Wise et al., 1979), and reaching mature configuration after three to four weeks postnatally. However, the

dendritic arbor remains plastic, with further development being marked by extension and branching of existing dendrites. Dendritic structure is dynamically shaped by synaptic activity, so that the size of the dendritic arbor is proportional to the level of excitatory innervation (Bouwmeester et al., 2002). Therefore, because the MD afferents are excitatory fibers (Kuroda et al., 1998), their synaptic activity could cause depolarization on PFC postsynaptic elements, subsequently opening voltage-gated and receptor-linked calcium channels, altering calcium influx which would lead to changes in dendritic structure and spine density.

Calcium signaling plays a crucial part in the formation and regulation of neuronal processes and their migration (Van Pelt et al., 1996; Novak et al., 2000; Ramakers et al., 2001; Sola et al., 2001; Lidow 2003; Aizawa et al., 2004). The elongation and branching of neurites is modulated by the concentration of calcium in the growth cones, which is dictated by membrane depolarization and excitability (Van Pelt et al., 1996). As calcium enters the cell, there is further release of the cation from internal stores; calmodulin, usually obstructed by neurogranin, is released and allowed to interact with calcium. The calcium-calmodulin complex activates CaMKII, which in turn phosphorylates MAP2 (See figure 2). This phosphorylation increases the space between microtubules, destabilizing the microtubule bundle, which leads to dendritic sprouting (Hely 2001). There is an optimal range of calcium concentration for neurite growth; if calcium levels are above or below this range, elongation and outgrowth stops (Van Pelt et al., 1996), thus calcium homeostatic levels must be maintained to ensure dendritic integrity.

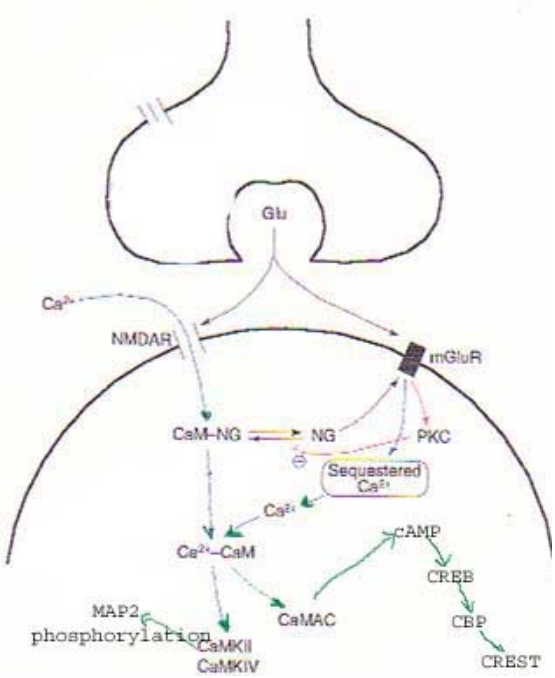


Fig.2. Schematic showing calcium dependent pathway in the formation and regulation of neuritis. (Chakravarthy et al., 1999).

The MD and the PFC are regions of interest in schizophrenia research because both areas are reported to be affected by this disorder (Pakkenberg 1990; Pakkenberg 1992; Rajowska et al., 1998; Stone et al., 1998; Bunney and Bunney 2000; Conklin et al., 2000; Kalus et al., 2000; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Perry et al., 2001; Thune et al., 2001; Byne et al., 2002; Broadbelt et al., 2002; Jones et al., 2002; Lipska et al., 2002; Selemon et al., 2003; Black et al., 2004; Broadbelt et al., 2006). Several postmortem studies showed a decrease in the number of neurons and volume in the MD (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002). Pakkenberg (1990) reported a 40% decrease in the total number of neurons in the MD of postmortem schizophrenic brains, as well as a significant decrease in volume. This finding has been replicated (Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Byne et al., 2002; Danos et al., 2005; Mitelman et al., 2006). The reported MD cell loss was shown to be

unrelated to drug treatment (Pakkenberg 1992). Structural imaging studies have further supported the postmortem reports of MD volume reductions (Staal et al., 2000; Byne et al., 2001; Sim et al., 2006). Aside from the extensive morphometric findings, imaging studies have reported deficiencies in glucose metabolism in the MD in schizophrenia (Hazlett et al., 2004), as well as a metabolic disconnection between the MD and associative cortical networks (Mitelman 2005). These imaging studies correlate the degree of MD dysfunction with more severe negative symptoms. Some studies did not find a change in volume or neuronal density in the MD associated with schizophrenia (Lesch and Bogerts 1984; Cullen et al., 2003; Dorph et al., 2004; Danos et al., 2005). There were methodological issues in these studies including the lack of stereological methodology (Lesch and Bogerts 1983), long postmortem interval of 47 hours (Cullen et al., 2003), and long storage time of over one year in formalin (Dorph et al., 2004). One study measured section thickness increasing with histological procedures instead of shrinking (Danos et al., 2005). Extensive evidence points to the MD as a structure affected in schizophrenia; it is consistently reported to have decreased volume and neuronal number; this loss of cells in the MD may in turn affect other regions.

The PFC is reported to be affected by schizophrenia as well. The PFC mediates higher cognitive functions such as working memory, active memory, mental imagery, planning, emotional processing of sensory information and willed action (Frith and Dolan 1996) and in schizophrenia, the most prevalent symptoms include loss of these executive functions (Stone et al., 1998; Conklin et al., 2000; Perry et al., 2001). Morphological alterations in the PFC have been reported as well (Garey et al., 1998; Rajkowska et al., 1998; Kalus et al., 2000; Broadbelt et al., 2002; Jones et al., 2002; Selemon et al., 2003; Black et al., 2004; Kolluri et al., 2005; Broadbelt et al., 2006), including a significant decrease in microtubule-associated protein 2

(MAP2) area fraction in layers III and V of the PFC (Jones et al., 2002). MAP2 is a marker for dendrites (Crandel et al., 1989), that is used to indicate dendritic integrity (Caceras et al., 1992) and its decrease may signify aberrant dendritic morphology of pyramidal cells in the PFC. This was further evidenced by reports of an affected basilar dendritic system (Kalus et al., 2000), followed by accounts of a pronounced decrease in primary and secondary basilar dendrites in pyramidal cells of layers III and V of area 32 of the PFC (Broadbelt et al., 2002). Descriptions of a 40% decrease in the basilar dendritic field of layer V pyramidal neurons, as well as shorter dendritic length and decreased spine density of layer III pyramidal neurons (Black et al., 2004) further support this notion. Decreased soma size of pyramidal cells in layer III was also reported (Rajkowska et al., 1998; Pierri et al., 2001); soma size correlates with the extent of the dendritic arbor (Van Oooyen et al., 1995), and its decrease implies a compromised dendritic field. Further, a significant reduction of pyramidal dendritic spines in schizophrenic brains has been described (Garey et al., 1998; Glantz and Lewis 2000). Spines are the major sites for excitatory neurotransmission on pyramidal cells (Pirot et al., 1994; Drakew et al., 1995; Van Pelt 1996); spines disappear after prolonged sensory deprivation (Berry 1974) and inactivity, and their decrease, as seen in schizophrenic brains, would reflect a loss of input from the thalamus. Altered dendritic integrity as evidenced by decreased levels of MAP2, basilar dendrites, and spine density would hinder the cells from proper transmission and may suggest that pyramidal cells in the PFC have compromised communication abilities.

It is speculated that the MD plays a role in the etiology of schizophrenia for several reasons: first, the MD has many reciprocal connections to the PFC (Herkerham1980; Barbas et al., 1991; Pirot et al., 1994; Kuroda et al., 1995; Kuroda et al.,1998; Negyessy et al., 1998; Wang and Shyu 2004); second, thalamic axons aid in the development of cortex in an activity

dependent manner (Kuroda et al., 1995; Van Ooyan et al., 1995; Molnar et al., 1998; Molnar et al., 2000), and third, the afferents from the MD to the PFC are myelinated by the second decade of life, coinciding with the onset of schizophrenic symptoms (Benes 1989). It is speculated that the late insulation of these fibers may enhance the defective circuitry between these two regions, triggering the onset of symptoms. The reported cell loss of the MD (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002), if occurring early in development, would have prominent consequences on the pyramidal cells of the PFC. Deficit of these MD afferents can translate into decreased synaptic activity, and due to their excitatory nature, decreased depolarization of the dendritic postsynaptic elements in the PFC. Dendritic trees are stimulated to grow and are maintained by afferent input (Kossel et al., 1997), and such loss of activity could result in alterations in the dendritic arbor, such as a loss of dendrites and spines, both of which have been reported in schizophrenia (Garey et al., 1998; Glantz and Lewis 2000; Broadbelt et al., 2002). The observed decrease in primary and secondary basilar dendrites in pyramidal cells of the PFC, together with reductions in spine density, may indicate a loss of surface area for excitatory and inhibitory inputs, which could affect the ability of the prefrontal pyramidal cells to process information. The evidence suggests that loss of MD neurons may affect the morphology of pyramidal neurons in the PFC in a developmental manner.

Determining Developmental Factors: Lesion Studies.

The problem arises of how to investigate the developmental components of this disorder. It is possible to investigate functional and behavioral alterations in live subjects using imaging techniques, but by the time individuals are diagnosed, the disorder is fully expressed, and any developmental milestones of this detrimental disorder are past the reach of scientific

investigation. The use of postmortem material offers the benefit of ample anatomical, morphological and cellular investigations, nonetheless, the developmental components of the disease cannot be investigated. A potential method to study development is through the use of animal models. It is possible to examine this issue by focusing on the interaction between structures that are relevant to schizophrenia research. Manipulations of one area in early development, followed by schizophrenia-like effects in connecting areas, can broaden our understanding of connectivity and the role development plays in the final outcome.

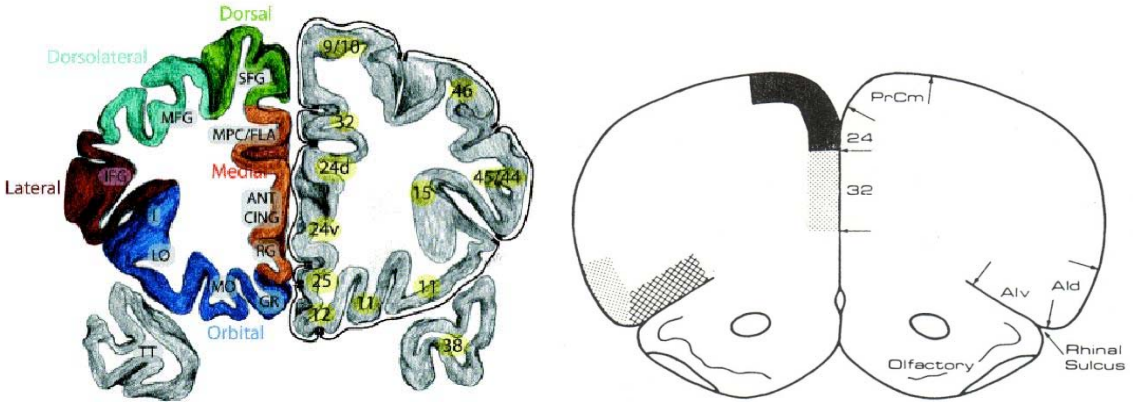
One method used to examine connected areas is to lesion one region and to study the effects on the other region. Previous lesion manipulations have predominantly focused on mesiotemporal limbic areas and their effect on behavior. One such model involves the neonatal damage of the rat ventral hippocampus; typically, ibotenic acid, causing an excitatoxic lesion, is applied to the ventral hippocampus at postnatal day 7 (Sams-Dodd et al., 1997; Becker et al., 1999; Lipska et al., 2002); then, the animals are allowed to mature and are tested for social interactions (Sams-Dodd et al., 1997), aggression (Becker et al., 1999), and performance in working memory tasks (Lipska et al., 2002). This model mimics a spectrum of behavioral features of schizophrenia; it produces functional pathology in other brain regions also implicated in schizophrenia, such as the striatum, the nucleus accumbens, and the PFC; furthermore, the social and functional effects are not evident until the rat subjects reach adolescence, thus mimicking the timing of schizophrenia onset of symptoms (for review see Lipska 2004). Although this model is consistent with some of the behavioral aspects of schizophrenia, it is inconsistent with the anatomical research. Although studies have reported morphometric abnormalities in the hippocampal formation, such as decreased volumes (Falkai and Bogerts 1986; Heckers et al., 1991) to decreased number of neurons and smaller pyramidal cells in

schizophrenia (Falkai and Bogerts 1986; Jonsson et al., 1999), other studies have not been able to replicate such findings (Heckers et al., 1991; Walker et al., 2002). Further discrepancy is seen in the morphological evaluation of this model which reports an increase in synaptic density, number of branches, and dendritic length in the pyramidal cells of the PFC (Robinson and Kolb 1997), which contradicts the compromised morphology evidence in schizophrenia (Garey et al., 1998; Bunney and Bunney 2000; Glantz and Lewis 2000; Kalus 2000; Byne et al., 2001; Pierri et al., 2001; Broadbelt et al., 2002; Black et al., 2004). Although the discrepancy in anatomical findings between this model and schizophrenia are great, it prevails to be an attractive model because of its implications in the dopaminergic system, a neurotransmitter system known to be affected in this disorder and a major target for therapeutic agents (Carlsson 1978). Recent investigations using this model examined cell excitability in PFC neurons, and it was concluded that the PFC dopamine-glutamate interactions were altered after puberty in lesioned rats (Tseng et al., 2007). Specifically, the PFC neurons showed enhanced excitability in lesioned animals, which contradicts the common concept of hypofrontality, characteristic of schizophrenic subjects.

Excitatory lesions of the entorhinal cortex (EC) have been used to further investigate the effects on the dopaminergic system. It was observed that an EC lesion resulted in enhancement of methamphetamine-induced dopamine release in the nucleus accumbens and basolateral amygdala (Uehara et al., 2007), implying dysregulation in the dopaminergic neurotransmission in the limbic regions. Although these models offer great insight into circuitry of the dopaminergic system, and potential for development of therapeutic agents, it is evident that models based on manipulations of the dopamine system have limited promise. They can imitate a

spectrum of schizophrenic behaviors, but they fall short on morphological and physiological findings.

Another mesiotemporal limbic area used for lesion studies is the amygdala. Lesions of the amygdala have been carried out testing for locomotor stereotypy (Daenen et al., 2002) and play and exploratory activities (Woletrink et al., 2001). These lesion studies tend to find deficits in social behavior (Sams-Dodd et al., 1997; Becker et al., 1999), working memory (Lipska et al., 2002), and abnormalities in locomotor stereotypy (Daenen et al., 2002). One study also looked at the effect of a mesiotemporal limbic lesion on signals from N-acetyl-aspartate (NAA), a neuronal viability marker in the PFC, and they found a developmental effect in the early-lesioned animals, which was absent from the animals lesioned as adults (Bertolino et al., 1997). In humans, these temporal association areas have widespread connections with the pulvinar nucleus of the thalamus (Byne et al., 2001), which has also been implicated in schizophrenia (Dom et al., 1981; Hazlett et al., 1999; Byne et al., 2001; Byne et al., 2002; Kemether et al., 2003; Byne et al., 2007). This nucleus, although a promising site of research, is difficult to study in a rat animal model since this nucleus is absent in rodents (Paxinos 1985; Byne et al., 2001). Because rats do possess the other thalamic association nucleus, the MD (Paxinos 1985), with reciprocal connections to the PFC as seen in human (Paxinos 1985; Kuroda et al., 1998) (See figure 3), the MD represents an important relevant target for lesion studies.



A. B.

Figure 3. Prefrontal cortices in human A. (Fallon et al., 2003), and rats B. (Paxinos 1985). The MD nuclei of both species have similar projections to the PFC. In humans, MD projects to medial, dorsolateral and orbital cortices (Ray and Price 1993). In rats, the MD projects to medial prelimbic (32), CG-1 (24), and dorsolateral anterior cingulated or medial precentral cortex (PrCm) (Paxinos 1985).

Previous MD lesion studies have primarily looked at the effect of the lesions on behavior (Isseroff et al., 1982; Stokes and Best 1990; Van Eden et al., 1994; Loredana and Mair 1996; Parker et al., 1997; Hunt and Aggleton 1998). Studies have shown that a lesion of the MD leads to impairments in spatial memory tasks in rats (Isseroff et al., 1982; Loredana and Mair 1996), as well as in monkeys (Parker et al., 1997). This spatial memory loss is qualitatively similar to that seen after damage of the prefrontal cortex (Isseroff et al., 1982; Loredana and Mair 1996). MD lesions can also affect working memory as assessed by radial maze tests (Stokes and Best 1990; Hunt and Aggleton 1998). These findings are consistent with reports of working and spatial memory deficits in schizophrenic patients (Stone et al., 1998; Conklin et al., 2000; Perry et al., 2001). Another animal lesion model for schizophrenia includes intrauterine radiation of rhesus monkeys during thalamic neurogenesis, which results in a 25% loss of thalamic volume, neuron loss, and nonuniform damage to the thalamic complex (Schindler et al., 2002). Although this

model is simulating the consistent finding of neuron loss and decreased volume in the thalamus (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002), it offers too many variables as the entire fetus is subjected to radiation and thus a spectrum of possible side effects. Additional research has focused on the relevant circuitry between the MD and the PFC. One study examined the structural and functional effects of a MD lesion on the PFC in the rat (Van Eden et al., 1994). They performed an electrothermal lesion on the MD on the day of birth and analyzed prefrontal architecture on day 35, as well as performance on a delayed alternation task. They found no significant gross changes in the PFC, except for local decreases in cortical width. The behavioral ability in spatial task was also unaffected (Van Eden et al., 1994). These negative results may be explained by the insensitivity of tests looking at the morphology in the PFC; they only examined gross morphology and not specific cellular morphology, which is known to be affected in schizophrenic brains (Garey et al., 1998; Broadbelt et al., 2002; Glantz and Lewis 2000). In rats, thalamic fibers grow into the cortex between postnatal day 0 and 7 (Wise et al., 1979; Van Eden 1986), and a lesion done too early on, may reflect the plasticity ability of the brain. Another MD lesion study examined whether an acute excitatoxic lesion of the MD on periadolescent monkeys could produce decreased PFC glutamate decarboxylase mRNA expression (Volk and Lewis 2003). They found that a substantial lesion did not reduce levels of this GABA-synthesizing enzyme in the PFC four weeks after lesions were performed (Volk and Lewis 2003). Their inability to see a change in PFC enzymatic levels may have occurred as a result of the acute lesion in prepubescent animals; the connections between the MD and the PFC may have been established by the time the lesions were done. Therefore, this model may not accurately reflect the developmental timecourse for the disorder.

Hypothesis:

There are consistent reports of schizophrenia-associated decrease in MD volume and neuronal number (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002). The MD is intricately connected to the PFC (Herkerham 1980; Barbas et al., 1991; Pirot et al., 1994; Kuroda et al., 1995; Kuroda et al., 1998; Negyessy et al., 1998; Wang and Shyu 2004) and aids in its development in an activity dependent manner (Kuroda et al., 1995; Van Ooyan et al., 1995; Kossel et al., 1997; Molnar et al., 1998; Molnar et al., 2000). It is therefore hypothesized that a loss of MD thalamic afferents in early development can cause morphological alterations in the pyramidal cells of the PFC. The hypothesis is tested by mimicking the MD loss observed in schizophrenia in an animal model through electrolytic lesions on the MD of rat pups. A postanatal day 4 lesion will translate into a loss of MD afferents innervating the PFC during development, and we hypothesize that such loss of synaptic input activity onto the nascent pyramidal cells would cause aberrant dendritic morphology. Evidence for such morphological alterations may include reductions in MAP2 immunostaining, less complex basilar dendritic branches and decreased spine densities. The objective of this project is to clarify the causality of the changes seen in the circuit between the PFC and the MD in schizophrenia, and to provide an insight into the role of development and the etiology of this devastating disorder.

Chapter 2. Early Postnatal Lesion of the Medial Dorsal Nucleus Leads to Loss of MAP2 Immunostaining in Adult Prefrontal Cortical Regions

Abstract:

Background: Schizophrenia is a devastating psychiatric illness. One of the most consistent findings in this disorder is a decrease in volume and neuronal number in the medial dorsal nucleus of the thalamus (MD) (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002). The MD is reciprocally connected to the prefrontal cortex (PFC), another region implicated in schizophrenia. Focusing on the interplay between the MD and the PFC, this study examined the hypothesis that early damage to the MD may lead to alterations in microtubule-associated protein-2 (MAP2) immunostaining in the PFC, similar to that observed in schizophrenics (Jones et al., 2002). **Methods: U**nilateral electrolytic lesions of the MD in Long-Evans rat pups were made on postnatal day 4 (P4) and animals developed to P60. We examined MAP2 immunostaining and pyramidal cell density in prelimbic (PL), anterior cingulate 1(Cg1), and Dorsolateral anterior cingulate (DL) cortices, which receive afferents from the MD. **Results:** Lesions causing mean MD volume decreases of 12.30% led to a 26.09% decrease in MAP2 staining in the superficial layers and a 34.78% decrease in deep layers of PL. A similar 28.00% decrease was observed in the superficial layers in the DL and a 26.09% decrease in the deep layers. In Cg1 we observed a 28.57% decrease in MAP2 in the superficial layers and a 27.27% decrease in the deep layers. No difference was observed in pyramidal cell density in any of the regions or layers. **Conclusions:** Our data are thus consistent with the hypothesis that the alterations in PFC observed in schizophrenic subjects could arise as a consequence of early MD damage.

Introduction

A preponderance of evidence suggests that schizophrenia is the result of a combination of genetic factors and early developmental insults that lead to a variety of brain abnormalities, involving cortical and subcortical structures. Mounting evidence suggests functional and structural involvement of the prefrontal cortex (PFC) (Benes et al., 1986; Benes et al., 1991; Davis and Lewis 1995; Perone et al., 1996; Beasley and Reynolds 1997; Glantz and Lewis 1997; Honer et al., 1997; Thompson et al., 1998; Bertolino et al., 1999; Dean et al., 1999; Buxhoeveden et al., 2000; Kalus et al., 2000; Peters et al., 2000; Lewis et al., 2001; Pierri et al., 2001; Reynolds and Beasley 2001; Broadbelt et al., 2002; Jones et al., 2002) and for review see (Shapiro 1993; Hirsch et al., 1997; Harrison 1999; Selemon and Goldman 1999), which plays a crucial role in executive function, working memory, mental imagery, willed action and active memory (Frith and Dolan 1996). Morphological studies of the PFC in schizophrenia have demonstrated pronounced decreases in number of basilar dendrites and spines (Garey et al., 1998; Glantz and Lewis 2000; Broadbelt et al., 2002), size of pyramidal cell bodies and immunoreactivity for MAP2 (Jones et al., 2002), neurogranin (Broadbelt et al., 2006) and calmodulin (submitted). Less dramatic decreases in presynaptic proteins have also been described in many studies (for review see Honer and Young 2004, Eastwood 2004).

Normal development of the cerebral cortex is dependent upon reciprocal connections with the thalamus. The predominant thalamic input to the PFC is from the medial dorsal nucleus (MD: also known as medial thalamus). Medial prefrontal area 32 also makes extensive connections with the medial pulvinar (Faull and Mehler 1985; Byne et al., 2001). Most postmortem studies report a loss of neurons and decrease in volume of the MD nucleus (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001;

Lewis et al., 2001; Byne et al., 2002) in schizophrenia. Recently two studies using the brains of schizophrenic subjects have reported no change in volume or cell density within the MD (Cullen et al., 2003; Anton et al., 2004), while another study found no change in cell density but a decrease in volume on the left hemisphere (Danos et al., 2005). However, these studies all had methodological shortcomings such as postmortem intervals of 47 hours (Cullen et al., 2003), storage time of over one year in formalin (Dorph et al., 2004), section thickness increasing with histological procedures as opposed to shrinking (Danos et al., 2005), and 3D measurements done on flat sections (Young et al., 2004). Most postmortem and *in vivo* studies including these suggest a disconnection, a change in communication, between the MD and the PFC, which may account for the observed alterations in the PFC both functional and morphological. For example Lewis and colleagues (2001) showed a PFC loss of parvalbumin-containing vertical processes, the presumed thalamocortical afferents. Myelination of the axons from the MD to the PFC occurs approximately at the age of onset of schizophrenia (Benes 1989), suggesting that the onset of the illness may be linked to the failure to fulfill an important role for these connections that normally occurs at this time. Developmental maturation of prefrontal cortical cells is dependent upon the activity from the MD (Van Pelt et al., 1996); an early lesion of the MD could result in abnormal development of the PFC. In particular, loss of thalamic input to PFC may result in loss of calcium-mediated stimulation of dendritic remodeling. Altered levels of several proteins involved in this process have been shown in schizophrenia (for review see Lidow 2003), and we have recently demonstrated a dramatic loss of neurogranin (Broadbelt and Jones 2006), a crucial regulator of neuronal responsiveness to calcium (Li et al., 1999). We suggest that the decrease in cell number and volume observed in the MD may result in a loss of innervation to the PFC, and this deficit could be directly responsible for dysfunction of the PFC in schizophrenia.

Developmental studies of animals following lesions of either the MD or the PFC have found cognitive deficits similar to those exhibited by patients with schizophrenia. Lesions of the MD produce a variety of deficits of working memory in rats (Stokes and Best 1990; Harrison and Mair 1996) and deficits of spatial working memory (Isseroff et al., 1982) and visual recognition (Aggleton and Mishkin 1983) in monkeys similar to that observed in schizophrenia. We examined three rat prefrontal cortical regions that receive projections from the MD: prelimbic (Pr) (homologous to human area 32), Dorsolateral Anterior cingulate cortex (DL) (homologous to Brodmann 24b (Gabbott et al., 2003) and anterior cingulate cortex (Cg1) following a lesion at P4 to determine if an early developmental insult in the MD leads to altered dendritic development of cortical pyramidal cells as seen in schizophrenia. Our data suggest that the morphological alterations observed in the PFC in schizophrenia may indeed be related to altered levels of excitatory input due to loss of projections from the MD.

Materials and Methods

Lesion Protocol:

Time pregnant Long Evans rats were obtained from Jackson Laboratories and the day of birth was designated as P0. Surgery was performed on P4. Subjects were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (6-8 mg/kg).The pups were lesioned on the right hemisphere using stereotactic methods. The scalp was incised and reflected to expose the skull. Preliminary lesions showed the MD at P4 to be located 4.8mm caudal to Bregma and 0.75mm lateral to midline. An electrode was inserted 6mm deep, at which point a current of 5*u*A was passed for 3 seconds; three consecutive lesions were performed at 0.2 mm intervals to ensure that the lesions would meet criteria. The incisions were sutured and the pups were returned to the mother and allowed to survive to P60. At this time, the pups received an intraperitoneal overdose of sodium pentobarbital, followed by a transcardial perfusion with 0.9%

saline followed by 4% Peases fixative. The brains postfixed overnight *in-situ* for 24 hours, then removed and placed in increasing gradients of sucrose solution for cryoprotection.

Areas of Interest

Medial Dorsal Nucleus

The medial dorsal nucleus is easily distinguished in thionin stained sections. The intermedullary lamina borders approximately 80% of the MD. The remaining medial boundary is the paraventricular nucleus. Superficially it is bounded by the third ventricle.

Prefrontal Cortex

The prefrontal cortex in the rat extends from the frontal poles to the rostral end of the corpus callosum. The three regions investigated in this study were the prelimbic cortex (PL), anterior cingulate cortex (CG-1) and dorsolateral anterior cingulate cortex (DL); these prefrontal cortical subregions were chosen because they have reciprocal connections to the MD (Paxinos 1985; Hoover and Vertes 2007), and perform cognitive functions analogous to the PFC in humans (Seamans et al., 1995; Heidbreder and Groenewegen 2003). To distinguish the cytoarchitectural borders between the PFC subregions we used criteria from Donoghue and Wise (1982), Paxinos and colleagues (1999), as well as Gabott and colleagues (2003). PL is on the midline just dorsal to infralimbic cortex (Paxinos 1985; Gabbot et al., 2003), and has a very thin layer IV with a homogeneous layer V (Radley et al., 2006). CG-1 is dorsal to PL; it is completely dysgranular and characterized by a sparse layer III and loosely packed broad layer V. The DL is dorsolateral to CG-1, Dysgranular, marked by a pale staining layer III and compact layer II (Donoghue and Wise 1982). All of the sections that fall within the prefrontal regions were used for immunohistochemistry.

Histological procedures

Sections were stained using thionin to better differentiate areal and laminar boundaries to determine MD volumes, and to perform cell counts both in the MD and the PFC. The slides were mounted and defatted by increasing gradients of ethyl alcohol, exposed to xylene for ten minutes, and then rehydrated and stained with thionin. Sections were then dehydrated, cleared and coverslipped

MAP2 Immunohistochemistry

An antibody from Chemicon international was used specific for $MAP2_a$ and $MAP2_b$, which correspond to the two high molecular weight forms found in the adult brain. The corresponding sections were removed from cryoprotectant and washed in phosphate buffered saline (PBS) three times, then pretreated with 3% hydrogen peroxide for ten minutes to remove any endogenous peroxides. Sections were again washed three times with PBS, followed by one hour incubation in a blocking solution of 4% instant milk in PBS (BLOTTO) with 0.2% Triton-X. Following the blocking procedure, sections were incubated in primary antibody in a 1:500 dilution overnight on an orbital shaker at room temperature. The next day the sections were washed with PBS and treated with secondary antibody (Vector Laboratories) solution diluted 1:100 for one hour. After three PBS washes, the sections were treated with tertiary solution (ABC kit: Vector Laboratories) to allow for greater sensitivity and decreased background staining, followed by a standard diaminobenzadine (DAB) reaction to visualize label. The sections were then mounted on slides, dehydrated, defatted, and coverslipped. Following area fraction analysis the coverslips were removed and the sections were counterstained with thionin for neuron density measurements.

Quantification of the MD volume

The material was quantified using a Bioquant Image Analysis system interfaced with an Olympus AX70 microscope and a Sony 3-chip color camera. Area of the MD was determined for each slide by drawing a contour around the nucleus. The specimen thickness (z) was calculated as an average of three randomly selected slides. MD volume was then obtained by multiplying the nuclear area by section thickness and the number of sections through each nucleus. Volumes of lesioned nuclei were compared to controls to calculate percent variation. Because we lesioned only one hemisphere (the right) we used the left as a control; thus each animal acted as its own control. Only brains that met the criterion of an MD lesion greater than or equal to 9% were included in the MAP2 analysis.

Quantification of cell density in MD and PFC

Cells were counted in four randomly selected thionin sections. Contours were traced around the selected region, and a counting grid was superimposed on the contour. Every third intersection was marked, with a random start, so that 30% of the intersections were marked. A counting box of 100x100 μm was placed inside the marked intersections. Counting was done using a 40X objective under oil. Cells were counted if they had a distinguishable nucleolus, except for those touching the exclusion lines. Neuron density was determined by dividing the average number of cells per box (Q) by the volume of the counting box. The volume of the dissector was calculated as the product of the area of the frame and the average thickness of the section corrected for shrinkage. The estimate of the total number of neurons in the MD was determined as the average neuron density of four randomly selected sections multiplied by the total nucleus volume.

Area Fraction of MAP2

We measured area fraction in both superficial and deep layers in the regions of interest in the PFC. We measured every section, thus removing bias from picking the sections that stained best with MAP2, and allowing for a more accurate assessment of the brain region. Within a defined sampling box, area fraction refers to the ratio of the area occupied by MAP2-immunostaining to the total area of the box. The sampling box is a square with each side equal to the width of the lamina. Bioquant software was employed to select pixels within the sampling box that matched threshold criteria for the MAP2 positive immunostaining. Threshold was set at the level that selected the lightest stained cell bodies and dendrites without selecting background staining. The setting for illumination was kept constant throughout the analysis. The computer outlined and added the thresholded areas, and determined the ratio of stained area to the total area of the sampling box. Eight sampling boxes were examined per section (4 within deep layers and 4 within superficial layers). The 4 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, to avoid distortion due to round pial edges, after the artifact. Area fraction was averaged for all sampling boxes within a particular lamina for each brain.

Statistics

Specimens were processed in pairs with unilateral lesions to the right hemispheres; the left hemispheres served as controls and data were analyzed by paired t-tests. One tailed probability was used to test the directionality of the hypothesis. ($P < 0.05$ considered significant).

Results

MD Volumes and Cell counts

Five animals received unilateral lesions. The lesions ranged in size from 4.1% to 23.4% difference in nuclear volume as compared to the opposite hemisphere (Figures 1, 2). Paired t-tests were used to determine significance ($p< 0.05$) because each animal was used as its own control comparing left and right thalami. Brains with lesions smaller than 9% were not used in the study; therefore one animal whose lesion was 4.1% was excluded from further study. The remaining brains had lesions greater than 9% with a significant average decrease of 12.30% (Control=0.000366, Lesion=0.000321, p= 0.00009, t= 21.7). The same four brains were used to determine cell loss in the MD due to the lesion. There was a significant 10.85% decrease in total cell number (Control= 45889.35, Lesion=40912.6, p= 0.04, t= 2.6) (Figure 3). These data suggest that we accurately lesioned the MD.

MAP2 Area Fraction

Three prefrontal cortical regions were examined in P60 rats for changes in MAP2 expression following an MD lesion at P4 (Figures 4, 5, 6). Paired t-tests were used to determine significance ($p<0.05$). We examined superficial layers (deep layer II and layer III) and deep layers (layer V). The cortical areas chosen have a minimal if any layer IV; the thalamic afferents synapse primarily on layer III cells. In the PL there was a significant 26.09% decrease in MAP2 immunostaining in the superficial layers (Control=0.023, Lesion=0.017, p=0.015, t=3.27). In the deep layers there was a significant 34.78% decrease in MAP2 immunostaining (Control=0.023, Lesion=0.015, p=0.0014, t=6.59). In the DL there was a significant 28.00% reduction in MAP2 immunostaining in the superficial layers (Control= 0.025, Lesion=0.018, p=0.02, t=2.96). There was a similar 26.09% reduction in the deep layers (Control=0.023 Lesion=0.017, p= 0.003, t= 6.22). In CG-1 there was a significant 28.57% reduction in immunostaining in the superficial layers (Control=0.021 Lesion=0.015, p= 0.0017, t= 6.22).

There was a similar 27.27% significant reduction in immunostaining in the deep layers (C=0.022 L=0.016, p=0.004, t= 4.898). There appears to be no correlation between the size of the MD lesion and the percent reduction in MAP2 immunostaining for either superficial or deep layers in any of the prefrontal subregions studied (Figures 7, 8).

Prefrontal Cortex Cell Density

To determine if the MD lesion caused retrograde cell loss we counted the number of thionin positive pyramidal cells in each of the three regions (Figures 9, 10). In the prelimbic region there was no significant change in the superficial layers (Control=1406.06, Lesion= 1461.82, p=0.23, t=-1.41) or in the deep layers (Control= 1511.4, Lesion=1538.8, p=0.53, t= -0.69). In the dorsolateral anterior cingulate region we found no significant change in pyramidal cell density in the superficial layers (Control=2295.7, Lesion=2291.9, p=0.98, t= 0.033). In the deep layers we found a non-significant change in cell density (Control=2632.18, Lesion=2817.58, p=0.58, t=-0.60). In the third region Cg1 we found no difference in cell density in the superficial layers (Control=612.18, Lesion=609.46, p=0.76, t=0.33). In the deep layers we found a non-significant change in cell density (Control=664.08, Lesion=718.44, p=0.66, t=-0.47).

Discussion

Our results suggest that an early postnatal lesion of the MD leads to disruption of dendritic development of the pyramidal cells in the prefrontal cortical regions connected to the MD. Following a lesion at P4 we found significant decreases in MAP2 immunoreactivity in the three PFC regions innervated by the MD. The loss of MAP2 appears to be unrelated to a loss of cells in the region as seen in the cell density measurements. We feel that our results are due to the lesion and not artifact for the following reasons. The lesions observed were large enough to

cause loss of input as seen in the volume decrease and in the number of cells lost. Our decision to perform the lesion at P4 is based on the study by Kolbe and Cioe (2000) showing that early cortical lesions caused morphological and behavioral changes but that lesions after P7 caused no long term effects in either morphology or behavior. Rats have a prolonged postnatal cortical development with thalamocortical projections reaching maximum levels between P4 and P6 (Van Eden et al., 1986); this time window also coincides with the first indication of reciprocal projections from the PFC to the MD (Van Eden et al., 1986). Manipulations prior to P7 have permanent lasting results that persist in adulthood; later manipulations do not cause similar problems suggesting that the manipulations have little or no effect on the development of that cortical region (Kolb and Cioe 2000). Dendritic maturation appears to be dependent upon synaptic activity. Deafferentation causes many changes on the target regions leading to loss of dendrites and reduction in neuropil (Berry 1974). Moreover, Rajakumer and Williamson (1997) lesioned P4 pups and showed significant behavioral abnormalities with potential analogies to schizophrenia. Additionally much of dendritic development occurs postnatally in rodents and the lesion occurred at a time prior to myelination of the axons from the MD to the PFC and prior to the final development of the region. While the MD does have connections to the contralateral PFC those connections are minimal (Minciacchi and Granato 1989), and we do not believe that they would have a significant effect on the opposing cortex. Our data suggest that loss of cells in the MD very early can affect dendritic development in the PFC.

Much data suggest that schizophrenia is a developmental disorder and that loss of innervation of the MD to the PFC may account for the morphological changes seen in schizophrenia. During development, the arrival of MD fibers in the upper cortical plate precedes laminar differentiation, and although it is unknown whether these fibers make synaptic contacts

with the developing layers, it is likely they exert an influence on the nascent PFC (Van Eden 1986). 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 (Kuroda et al., 1995; Kuroda et al., 1995; Kuroda et al., 1998). These excitatory projections from the thalamus to the cortex synapse primarily on layer III and layer V apical dendrites (Kuroda et al., 1995; Kuroda et al., 1995; Kuroda et al., 1998). These thalamic afferents aid in the development of the cortex and more specifically in the activity-dependent process of dendritic pruning (Wise et al., 1979; Van Ooyen et al., 1995; Van Pelt et al., 1996; Baker and van Pelt 1997; Kossel et al., 1997). Electrical stimulation or depolarization has been shown to increase neurite outgrowth; therefore, changes in the perceived activity of a cell may be reflected in the number and length of dendritic branches. These effects are dependent on the influx of extracellular calcium (Kater et al., 1988; Petit et al., 1988; Ramakers et al., 2001). 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 stop elongating or retract (Kater et al., 1988; Ramakers et al., 2001). Lowering the level of intracellular calcium arrests axonal outgrowth and stops net addition of dendrites and dendritic branching; this is in part due to 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. Data from our laboratory have shown a decrease in MAP2, a decrease in the number basilar dendrites, loss of spines, decreased expression of neurogranin and calmodulin (Jones et al., 2002; Broadbelt et al., 2002; Broadbelt et al., 2006), suggesting alterations in morphology and calcium signaling, all of which may be due to a loss of afferents from the MD. Current data suggest that a MD volume loss exceeding 8% and a cell loss

averaging 10.85% lead to a decrease in MAP2 expression in the PFC in rats similar to the loss of MAP2 seen in schizophrenics. Our data suggests that an early developmental insult of the MD may be responsible for the morphological alterations observed in the PFC of schizophrenics. Further studies need to be performed to determine if the loss of MAP2 results from a decrease in the number of basilar dendrites and spines.

Figures and Tables.

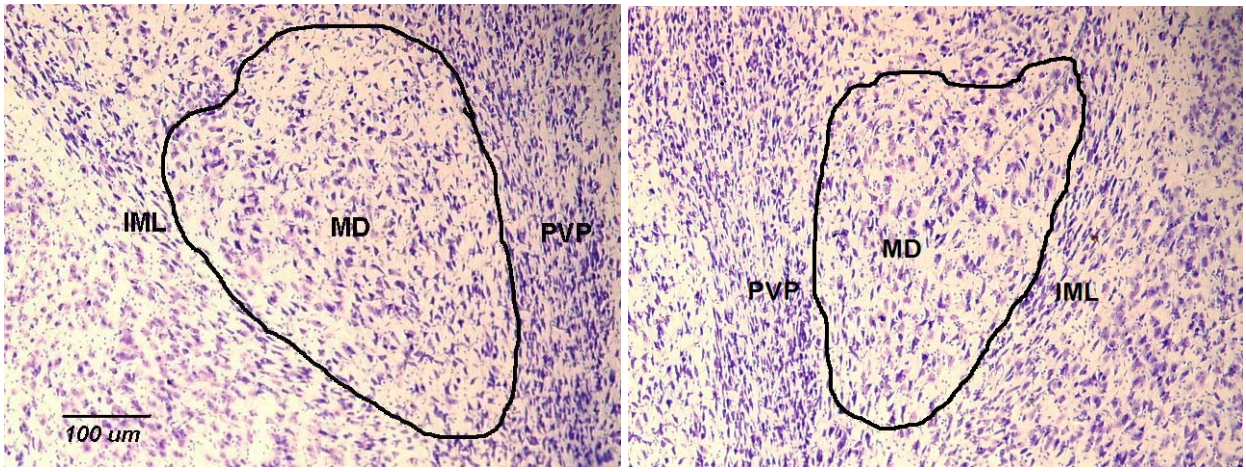


Figure 1. Photomicrograph of P60 MD nuclei sections: control (left) versus lesion (right). The MD in the rat is bounded by the intermedullary lamina (IML), and medially by the Paraventricular Nucleus (PVP). Scale bar =100µm.

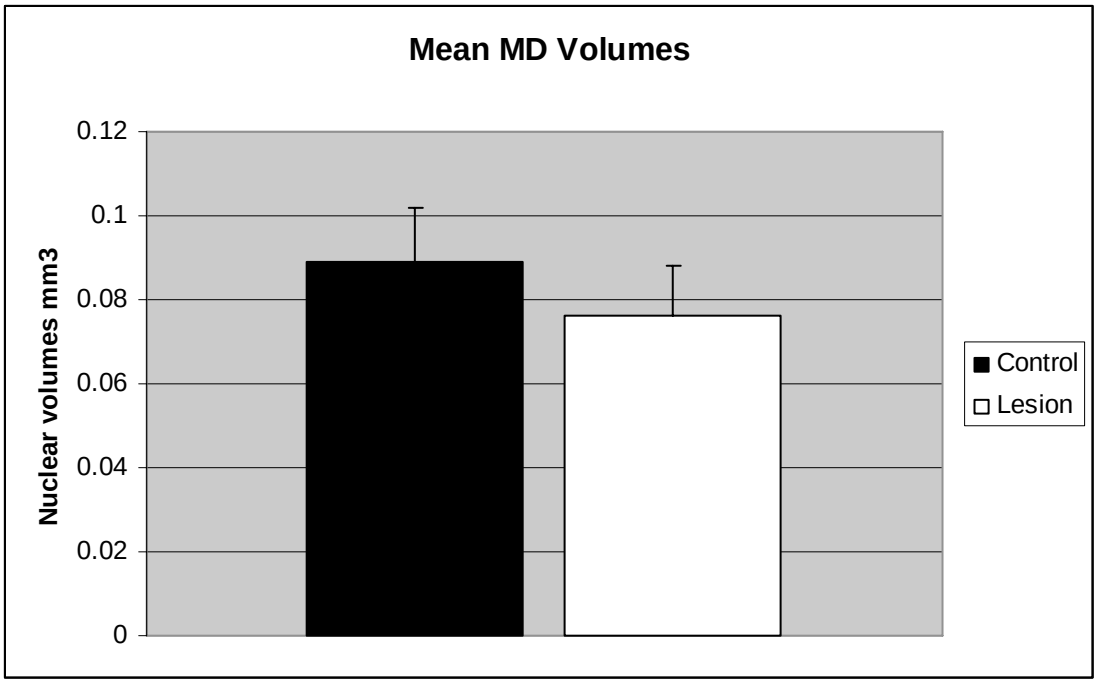


Figure 2. Histogram showing the hemispheric difference in average MD nuclear volumes at postnatal day 60 after a unilateral lesion performed on postnatal day 4. There was a significant average decrease of 12.30% (Control=0.000366, Lesion=0.000321, p= 0.00009, t= 21.7) in MD volume in the hemisphere receiving the lesion when compared to the intact side.

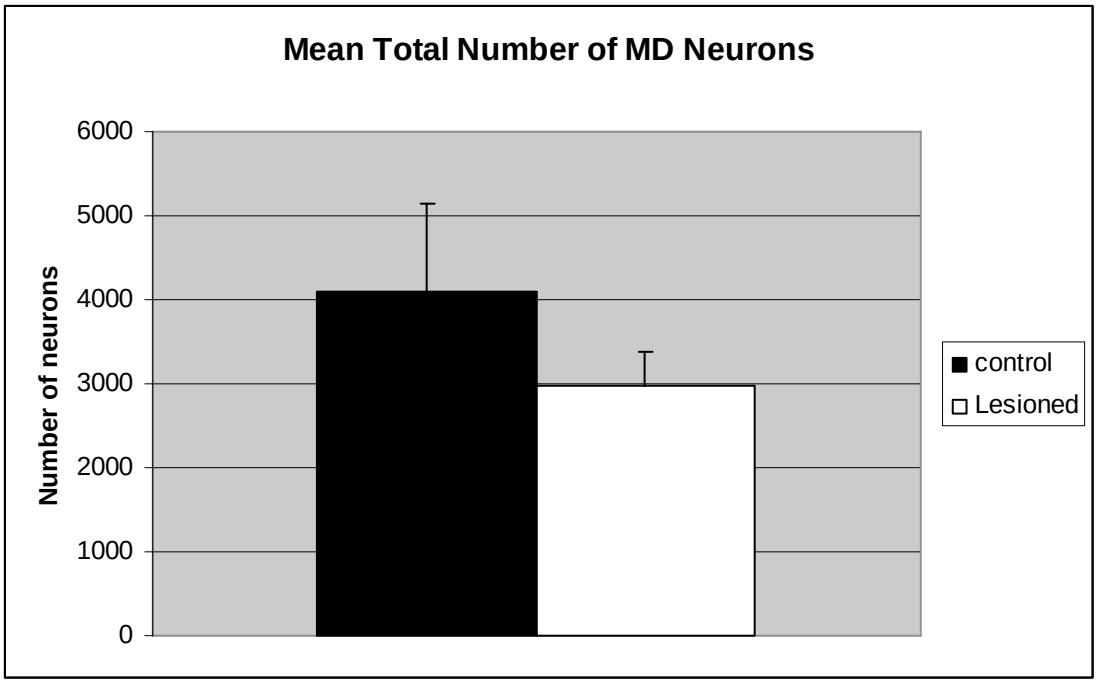


Figure 3. Histogram showing the average decrease in cell population in the MD at P60 following a lesion of the MD at P4. The loss in MD volume was accompanied by a significant 10.85% decrease in total cell number (Control= 45889.35, Lesion=40912.6, p= 0.04, t= 2.6)

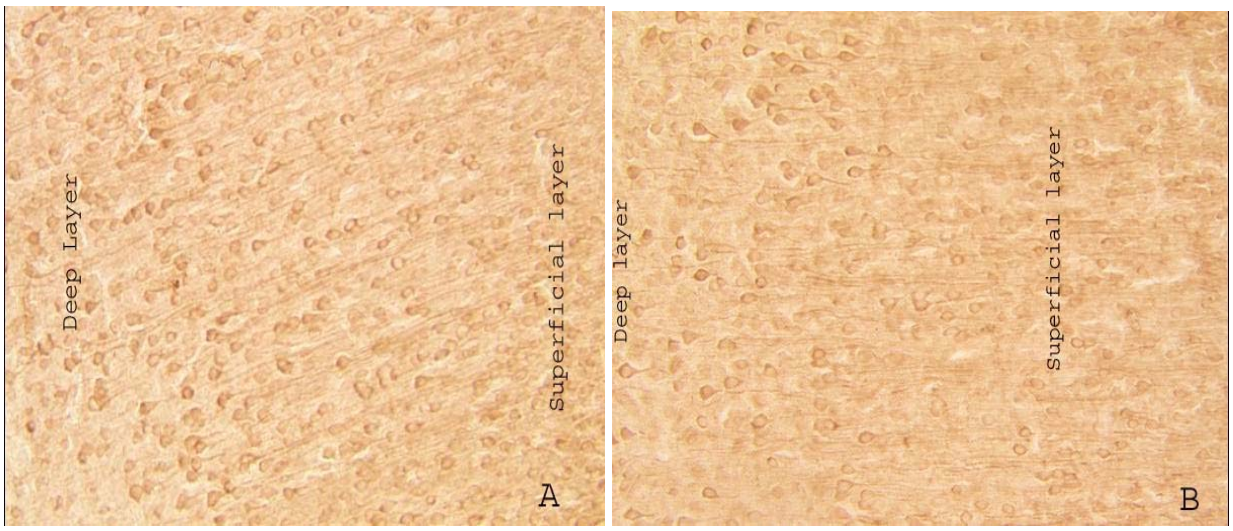


Figure 4. Example of MAP2 immunostaining in P60 prelimbic cortex (A) intact hemisphere (B) treated hemisphere following a unilateral electrolytic lesion on postnatal day 4.

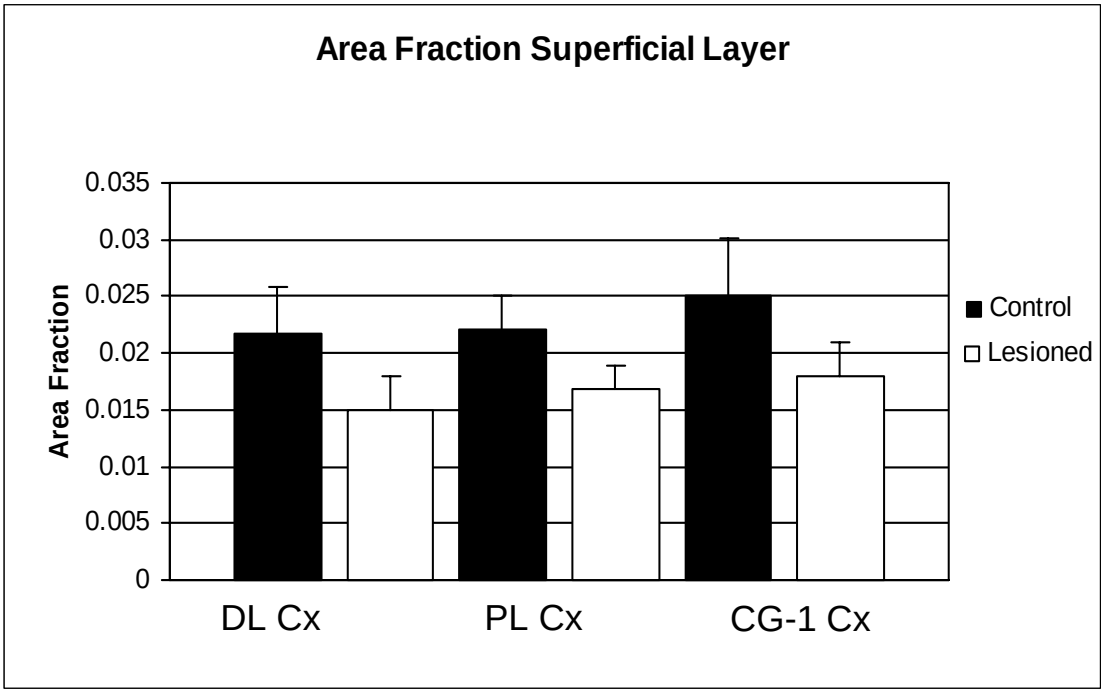


Figure 5. Histogram showing the average difference in immunostaining area fraction analysis for the three prefrontal cortical regions examined. In all regions, in the superficial layers there was a significant decrease in MAP2 staining on the lesioned side as compared to the intact side. In the dorsolateral area there was a significant 28.00% reduction in MAP2 immunostaining (Control= 0.025, Lesion=0.018, p=0.02, t=2.96). In the prelimbic cortex, the mean reduction in stain was 26.09% (Control=0.023, Lesion=0.017, p=0.015, t=3.27). In Cg1 cortex, there was a significant 28.57% decrease in MAP2 immunostaining (Control=0.021 Lesion=0.015, p= 0.0017, t= 6.22).

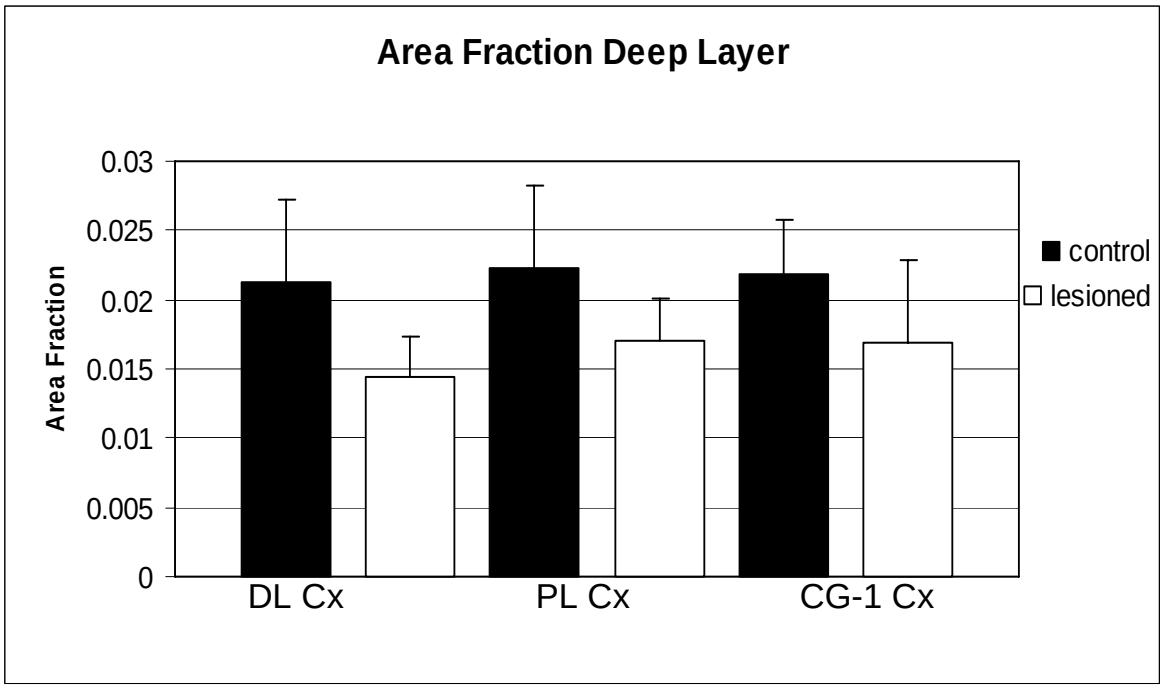


Figure 6. Histogram showing the average difference in area fraction analysis for MAP2 staining for the three PFC regions examined. In all regions in the deep layers, there was a significant decrease in MAP2 staining on the lesioned side as compared to the control or unlesioned side. There was a 26.09% reduction in MAP2 area fraction in the dorsolateral cortex (Control=0.023 Lesion=0.017, p= 0.003, t= 6.22). In the prelimbic deep cortex, there was a significant 34.78% decrease in MAP2 immunostaining (Control=0.023, Lesion=0.015, p=0.0014, t=6.59). Similarly, the CG-1 deep area showed a 27.27% decrease in MAP2 immunostaining (C=0.022 L=0.016, p=0.004, t= 4.898) in the lesioned side versus control.

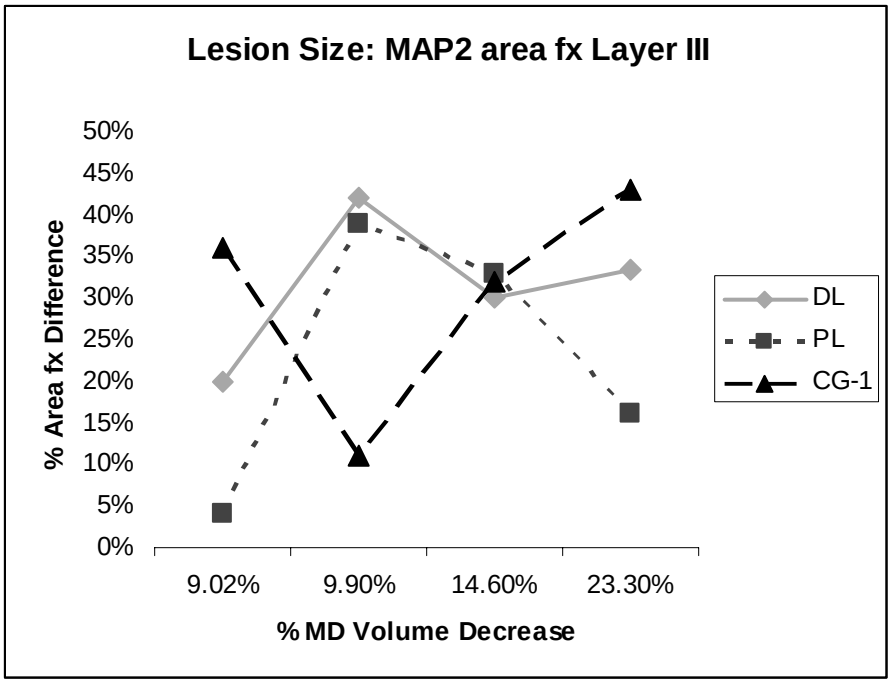


Figure 7. Comparison between the size of the thalamic lesion versus the effect on MAP2 immunostaining for the superficial layers across all three PFC subregions. There is no correlation between the size of the lesion and the extent of the effect on MAP2.

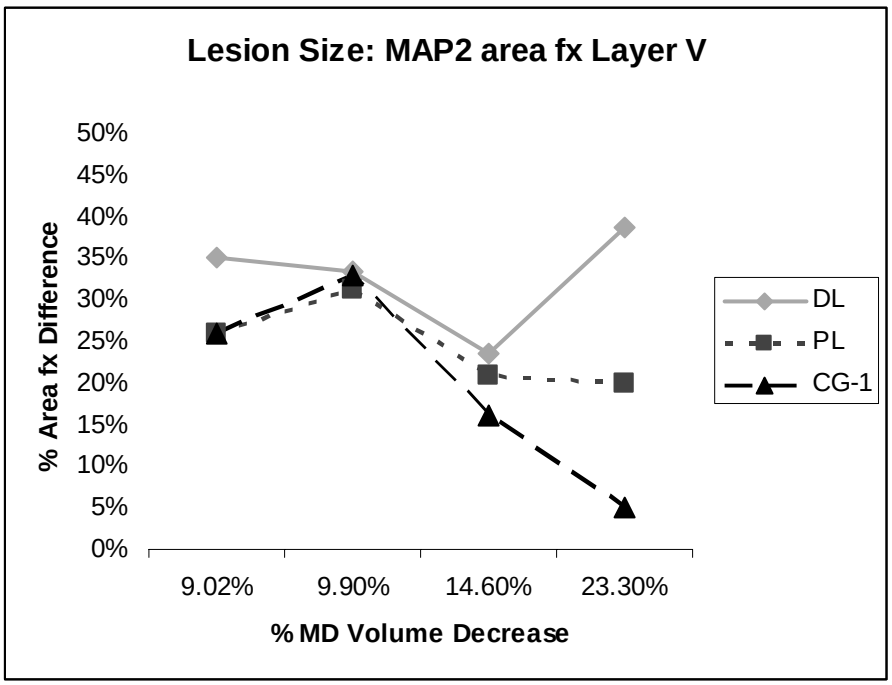


Figure 8. Graph depicting the correlation between the size of the MD lesion and the percent difference in MAP2 immunostaining in the deep layers for all three PFC subregions. No significant correlation is evident.

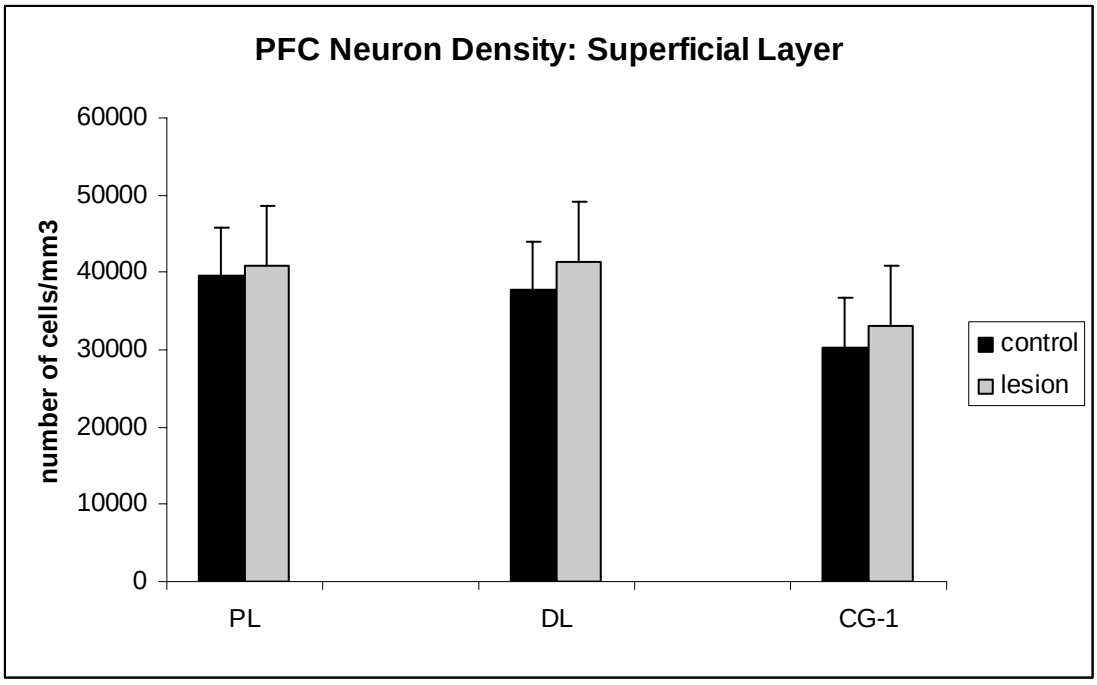


Figure 9. Histogram showing the average pyramidal cell density in the superficial layers for all three prefrontal cortical regions examined. There was no difference in density in any of the regions control versus lesion. Prelimbic cortex: Control=39503.62, Lesion= 40744.57, p=0.23, t=-1.4. DL average pyramidal cell density: Control=37645.19, Lesion=41365.05, p=0.98, t= 0.033. For CG-1, there was also no significant difference in pyramidal cell density between the two hemispheres (Control=30382.63, Lesion=33009.31, p=0.76, t=0.33).

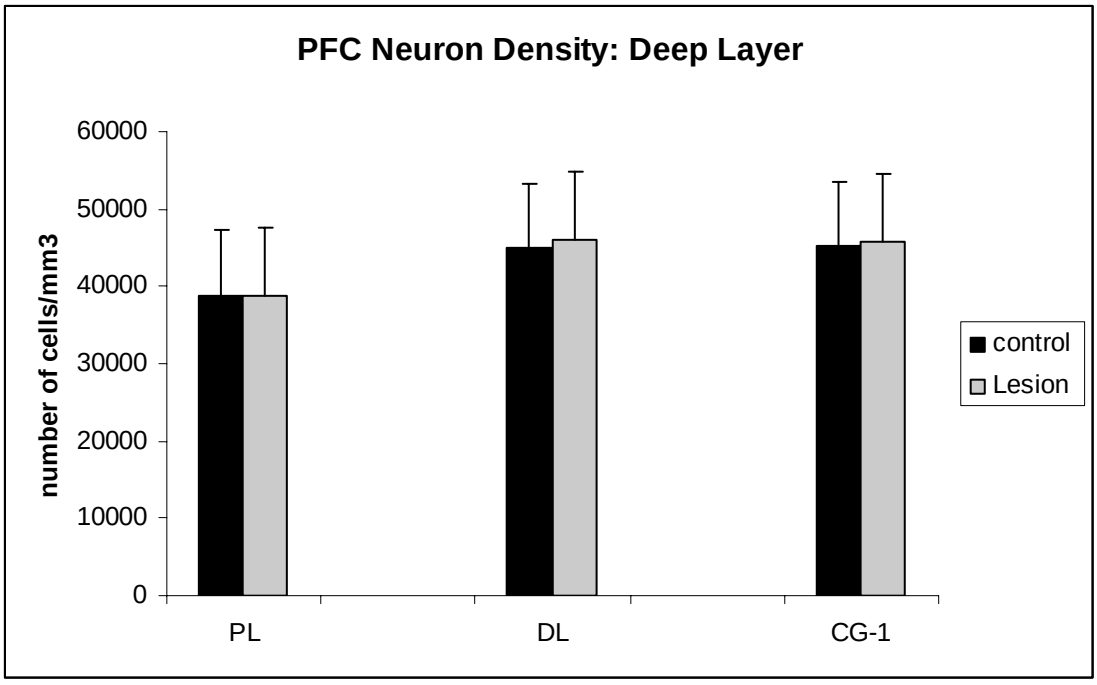


Figure 10. Histogram showing the estimated average density of pyramidal neurons in the deep layers of the three PFC regions examined. There was no difference in pyramidal cell density in any of the cortical regions, when comparing the control versus lesioned hemispheres. DL average pyramidal density: Control=44881.08, Lesion=45915.2, p=0.58, t=-0.60. PL: Control= 38883.14, Lesion=38676.32, p=0.53, t= -0.69). The deep layer of CG-1 exhibited a non-significant change in pyramidal cell density (Control=45149.95, Lesion=45667.01, p=0.66, t=-0.47).

Chapter 3. Early Thalamic Lesions Alter Basilar Dendritic Development in Pyramidal Cells of the PFC

Abstract

Background: Decreased volume and cell number in the mediodorsal nucleus of the thalamus (MD) has consistently been reported in schizophrenia (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al 2002). The MD is closely associated with the PFC, a region also implicated in schizophrenia (Garey et al., 1998; Rajkowska et al., 1998; Stone et al., 1998; Conklin et al., 2000; Kalus et al., 2000; Perry et al., 2001; Pierri et al., 2001; Broadbelt et al., 2002; Jones et al., 2002; Selemon et al., 2003; Black et al., 2004; Kolluri et al., 2005). The MD-PFC circuit is suspected to play a role in the etiology of this disorder for several reasons: both, the MD and PFC are intricately connected (Kuroda et al., 1995; Kuroda et al., 1998; Wang and Shyu 2004); MD afferents aid in the development of the PFC in an activity dependent manner (Kuroda et al., 1995), and this circuit gets myelinated during the period of early adulthood (Benes 1989), which coincides with the onset of symptoms. We hypothesize that early damage to the MD may lead to morphological alterations in the PFC. We have previously reported that an electrolytic lesion of the rat MD performed during the first postnatal week resulted in alterations of microtubule associated protein-2 (MAP2) immunostaining profiles. MAP2 is an indicator of dendritic integrity (Caceres et al., 1992), and therefore it is believed that such MD lesion may affect the development of basilar dendrites on pyramidal neurons in the PFC as seen in schizophrenic brains. **Methods:** Unilateral electrolytic lesions of the MD in Long-Evans rat pups were made on postnatal day 4 (P4), and sacrificed on P60, when the basilardendrites of pyramidal cells in prefrontal cortical regions, the dorsolateral anterior cingulate cortex (DL), medial prelimbic (Pr), and anterior

cingulate cortex (CG-1), were analyzed. **Results:** Electrolytic lesions of the MD nucleus resulted mean volume decreases of 14.82%, which led to a 25% decrease in primary basilar dendrites in the superficial layers, and a 24.84% decrease in deep layers of Pr cortex. Secondary dendrites showed a more prominent difference across both layers: 39.7% in superficial cortex and 31.84% in the deep cortex. There were 24.24 % fewer primary dendrites in lesion DL in the superficial layer. The difference in the deep layer was 14.55%. Secondary dendrites in this region had a reduction of 40.46% in the superficial aspect and 30.04% in the deep layer. CG-1 cortex showed a 25.31% reduction in primary basilar dendrites superficially and 23.06% in the deep layers. Secondary dendrites decreased 39.74% in the superficial layer of the lesioned hemisphere and 34.41% in the deep layer. **Conclusions:** Decreased dendritic arbors in PFC observed in schizophrenic subjects could arise as a consequence of early MD damage.

Introduction:

Schizophrenia is a debilitating disorder, the etiology of which remains a mystery. Much evidence suggests that the disease is developmental in origin. Two brain regions implicated in schizophrenia, the mediodorsal nucleus of the thalamus (MD) and the prefrontal cortex (PFC) have extensive reciprocal connections. Postmortem research has shown a decrease in the number of neurons and volume in the MD in schizophrenic brains (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002; Danos et al., 2005; Mitelman et al., 2006), which has been supported by structural imaging studies (Staal et al., 2000; Byne et al., 2001; Sim et al., 2006). In addition to the extensive morphometric findings, imaging studies have reported deficiencies in glucose metabolism in MD in schizophrenia (Hazlett et al., 2004), as well as a metabolic disconnection between the MD and associative cortical networks (Mitelman 2005).

The PFC is also affected by schizophrenia; the most prevalent symptoms include loss of executive functions sub-served by the PFC (Stone et al., 1998; Conklin et al., 2000; Perry et al., 2001). Postmortem investigations have revealed morphological alterations in the PFC (Garey et al., 1998; Rajkowska et al., 1998; Stone et al., 1998; Conklin et al., 2000; Kalus et al., 2000; Perry et al., 2001; Pierri et al., 2001; Broadbelt et al., 2002; Jones et al., 2002; Selemon et al., 2003; Black et al., 2004; Kolluri et al., 2005; Broadbelt et al., 2006), including a significant decrease in microtubule-associated protein 2 (MAP2) area fraction in layers III and V of the PFC (Jones et al., 2002). MAP2 is a protein found in the dendrites and cell bodies of pyramidal cells (Fischer et al., 1987), and the loss of this protein may indicate changes in dendritic integrity (Caceras et al., 1992). Moreover, schizophrenia is associated with a less complex dendritic tree (Kalus et al., 2000), which includes a pronounced decrease in the number of primary and

secondary basilar dendrites in pyramidal cells of layers III and V of area 32 of the PFC (Broadbelt et al., 2002), in addition to a significant reduction of pyramidal dendritic spines (Garey et al., 1998; Glantz et al., 2000; Kalus et al., 2000). Hence, these cells have compromised dendritic arbors.

We have previously shown that loss of thalamic input following early MD damage in neonatal rats leads to a decrease in MAP2 immunostaining in the adult PFC, indicating a loss of dendritic material. This suggests that a reduction in input activity from the MD during development results in a less complex dendritic arborization in the PFC. Therefore, in the present study we used the same model to examine whether early electrolytic lesions of the MD caused a change in the number of basilar dendrites on pyramidal cells in rat PFC.

Materials and Methods:

Lesion Protocol:

Time pregnant Long Evans rats were obtained from Jackson Laboratories and the day of birth was designated as P0. Surgery was performed on P4. Subjects were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (6-8 mg/kg).The pups were lesioned on the right hemisphere using stereotactic methods. The scalp was incised and reflected to expose the skull. Preliminary lesions showed the MD at P4 to be located 4.8mm caudal to Bregma and 0.75mm lateral to midline. An electrode was inserted 6mm deep, at which point a current of 5*u*A was passed for 3 seconds; three consecutive lesions were performed at 0.2 mm intervals to ensure that the entire nucleus was lesioned. The incisions were sutured and the pups were returned to the mother and allowed to survive to P60. At this time, the pups received an intraperitoneal overdose of sodium pentobarbital, followed by a transcardial perfusion with 0.9% saline followed by 4% Peases fixative. The brains were removed and postfixed overnight in the

same solution. The frontal cortex anterior to Bregma was severed from the rest of brain to be processed for Golgi, while the posterior portion of the brain was exposed to increasing gradients of sucrose solution for cryoprotection.

Areas of Interest

Medial Dorsal Nucleus

The medial dorsal nucleus is easily distinguished in thionin stained sections. (See figure 1). The intermedullary lamina borders approximately 80% of the MD. The remaining medial boundary is the paraventricular nucleus. Superficially it is bounded by the third ventricle.

Prefrontal Cortex

The prefrontal cortex in the rat extends from the frontal poles to the rostral end of the corpus callosum. The three regions investigated in this study were the prelimbic cortex (Pr), anterior cingulate cortex (CG-1) and dorsolateral anterior cingulate cortex (DL); these prefrontal cortical subregions were chosen because they have reciprocal connections to the MD (Paxinos 1985; Hoover and Vertes 2007), and perform cognitive functions analogous to the PFC in humans (Seamans et al., 1995; Heidbreder and Groenewegen 2003). To distinguish the cytoarchitectural borders between the PFC subregions we used criteria from Paxinos and colleagues (1999), Donoghue and Wise (1982), as well as Gabott and colleagues (2003), Pr is on the midline just dorsal to infralimbic cortex (Paxinos 1985; Gabbot et al., 2003), and has a very thin layer IV with a homogeneous layer V (Radley et al., 2006). CG-1 is dorsal to Pr; it is completely agranular and characterized by a sparse layer III and loosely packed broad layer V. The DL is dorsolateral to CG-1, agranular, marked by a pale staining layer III and compact layer

II (Donoghue and Wise 1982). All of the sections that fall within the prefrontal regions were impregnated with golgi.

Histological Procedures:

The posterior portions of the brains containing the mediodorsal nucleus of the thalamus were cut on a freezing sledge sliding microtome. Serial coronal sections 50μm in width were collected to be processed for histological staining. Sections were stained using thionin to better differentiate boundaries to determine MD volumes, and perform cell counts in the MD. The slides were mounted, defatted by increasing gradients of ethyl alcohol, exposed to xylene for ten minutes, and then rehydrated and stained with thionin. Sections were then dehydrated, defatted and coverslipped.

Golgi Method:

The frontal cortex anterior to Bregma was severed from the more caudal portion of the brain. Frontal cortices were wrapped in clean gauze and immersed in 100 mL of Golgi solution. Both Golgi solution and gauze were changed after 24 hours, followed by extended 12 week incubation in the dark. After impregnation the tissue was dehydrated thru increasing gradients of alcohol followed by three day incubations in serial celloidin. The tissue was put in paper boats and allowed to harden in chloroform. 12% parlodion was added to the paper boats and stored for 3 to 24 hours until hardened. Finally the tissue was serially sectioned on a vibratome into 200-*u*m thick sections and collected in 70% alcohol. The sections were washed in distilled water, incubated in 19% NH_4OH for thirty minutes, and rinsed again in water. The sections were then fixed in Kodak Rapid Fix, followed by a final wash in distilled water. The sections were

dehydrated in graded ethanol starting at 50%, defatted in toluene, mounted onto slides and coverslipped.

Quantification of MD volume:

The material was quantified using a Bioquant Image Analysis system interfaced with an Olympus AX70 microscope and a Sony 3-chip color camera. Area of the MD was determined for each slide by drawing a contour around the nucleus. The specimen thickness (z) was calculated as an average of three randomly selected slides. MD volume was then obtained by multiplying the averaged nuclear area by section thickness and the number of sections thru each nucleus. Volumes of lesioned nuclei were compared to controls to calculate the extent of the lesion.

Quantification of MD cell density:

Cells were counted in four randomly selected thionin sections. Contours were traced around the selected region, and a counting grid was superimposed on the contour. Every third intersection was marked, with a random start, so that 30% of the intersections were marked. A counting box of 100x100 μm was centered at the marked intersections. Counting was done using a 40X objective under oil. Cells were counted if they had a distinguishable nucleolus, rejecting those touching the exclusion lines. Neuron density was determined by dividing the average number of cells per box (Q) by the volume of the counting box. The volume of the dissector was calculated as the product of the area of the frame and the average thickness of the section corrected for shrinkage. The estimate of the total number of neurons in the MD was determined as the average neuron density of four randomly selected sections multiplied by the total nucleus volume.

Quantification of dendritic material:

A contour was drawn to outline each lamina separately in each of the regions of interest in the PFC. A counting grid was placed inside the contour, and the computer randomly selected half of the intersections to be measured with a counting box of 100 X 100um placed inside the marked grids. The dendrites of the cells within the grid, whose cell bodies did not touch the exclusion lines, were counted at 40X Plan Apo objective under oil. Although a counting box was used to select neurons randomly, basilar dendrites of the selected neurons were counted even if they extended beyond the boundaries of the counting box. Primary dendrites (first branches off the soma) and secondary dendrites (bifurcations of primary dendrites) were counted. (See figure 2). 20 cells per layer per region were assessed for each brain.

Statistics:

Specimens were processed in pairs with unilateral lesions to the right hemispheres; the left hemispheres served as controls and data were analyzed by paired t-tests. One tailed probability was used to test the directionality of the hypothesis. ($P < 0.05$ considered significant).

Results:

MD Volumes

We analyzed the brains of nine animals that had received an electrolytic lesion to the MD in the right hemisphere. The lesions ranged in size from 0.89% to 17.64%. Brains whose lesions were below 9% were not used in the study, therefore R28, R31, and R35 with corresponding lesions of 8.13%, 5.26%, and 0.89% were excluded from further study. The mean MD volume decrease as a result of the lesion ranged from 0.0984±0.014 mm3. to 0.0836± 0.0044 mm3. ($p=0.00033$, $t=8.72$) (Figure 3).

MD Cell Counts

Six brains were examined for cell loss in the MD due to the lesion. There was a significant decrease in cell density of 10.84% Control: 9.68± 2.1. Lesion: 8.63±1.7 (p=0.016 t=3.56). There was an average decrease in total cell number of 23.43% Control: 4947.5± 1539.7. Lesion: 3719.4±976.4 (p=0.0044 , t=4.92) (Figure 4).

Basilar Dendrites

Three prefrontal cortical regions were examined in P60 rats for changes in basilar dendrites due to a MD lesion at P4. In the medial prelimbic area there was a significant 25% (Control: 4.72±0.81, Lesion: 3.54±0.37. p=0.0031, t=5.35) and 24.84% (Control: 4.71±0.72, lesion: 3.54±0.33. p=0.002, t=5.790) decrease in the average number of primary dendrites in the superficial (Figure 5) and deep layers respectively (Figure 6) . The average number of secondary dendrites showed a more prominent difference across both layers: 39.70% for superficial cells (Control: 7.28±1.24, Lesion: 4.39±0.61. p=0.0016, t=6.19) (Figure 5) and 31.84% for cells found in the deep cortex (Control: 7.13±0.81, Lesion: 4.86±0.76. p=0.0015, t=6.3) (Figure 6).

In the DL where there were 24.24 % fewer primary dendrites in lesioned hemispheres versus controls in the superficial layer (Control: 4.62±0.73, Lesion:3.5±0.36, p=0.0059, t=4.59) (Figure 7). The difference in the deep layer was not as pronounced, 14.55%, but was still significant (Control: 4.4±0.73, Lesion: 3.76±0.85, p=0.016, t=3.57) (Figure 8). There was an increased averaged number of secondary dendrites in the control hemispheres in this region of cortex 40.46% in the superficial aspect (Control:7.76±0.53, Lesion: 4.62±0.36, p=0.000043, t=13.2) (Figure 7), and 30.04% more secondary basilar dendrites in the deep layer (Control: 7.19±0.61, Lesion: 5.03±0.5, p=0.00021, t=9.54) (Figure 8).

This pattern of decline was also seen CG-1 showed similar reductions in basilar dendrites: primary dendrites decreased 25.31% (Control: 4.82±0.79, Lesion: 3.6±0.37, p= 0.008,

t=4.89), in superficial and 23.06% (Control: 4.77±0.998, Lesion: 3.67±0.4, p=0.022, t=3.66) (Figure 9) deep layers respectively, while there were substantially fewer secondary dendrites in lesioned hemispheres: 39.74% (Control: 7.7±0.48, Lesion: 4.64±0.40, p=0.00016, t=13.74) superficially and 34.41% deep (Control: 7.73±1.07, Lesion: 5.07±0.44, p=0.0012, t=8.16) (Figure 10), mirroring the effects found in the previous cortical region.

Discussion:

An electrolytic lesion was performed on the MD of rat pups on postnatal day 4. The objective was to cause a loss of volume and cells in this nucleus similar to that reported in schizophrenia research. It was hypothesized that such a lesion would cause morphological alterations in the pyramidal cells in the PFC regions, closely associated with the MD, and these alterations would be evidenced by a reduced dendritic arborization. A MD volume loss higher than 9% was sufficient to cause changes in the dendritic trees of PFC pyramidal neurons. Average numbers of primary and secondary basilar dendrites were significantly decreased in the cells of the lesion hemisphere as compared to the intact hemisphere for all three cortical regions investigated, across both layers. It was evident that the effect on the secondary dendrites was more pronounced.

The MD is of particular importance because it is a principal source of subcortical input to the PFC, as well as a major relay nucleus of the limbic system (Paxinos 1985; Pirot et al., 1994; Kuroda M et al., 1995); moreover, it has been consistently reported as an affected structure in this disorder (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Staal et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002; Hazlett et al., 2004; Danos et al., 2005; Mitelman et al., 2005; Sim et al., 2006). For these reasons, the MD is an efficient target for a lesion animal model. In the rat, there is reciprocal connectivity between the MD and

the PFC, with distinct cytoarchitectonic areas in the two regions being topographically related (Kuroda et al., 1995; Kuroda et al., 1998; Negyessy et al., 1998; Wang and Shyu 2004). The medial MD sends projections to medial cortex area 32, prelimbic and dorsal insular cortices, while the lateral MD sends afferents to anterior cingulate and medial precentral (dorsolateral anterior cingulate) cortices (Paxinos 1985; Pirot et al., 1994). Because of this intricate interconnectivity with the MD, the three cortical regions: dorsolateral anterior cingulate (DL), prelimbic (Pr) and anterior cingulate (CG-1) were chosen as study targets to assess the effect of the lesions. The MD afferents terminate mainly on layer III of the PFC (Herkenham 1990; Kuroda et al., 1995); in turn, each subregion of the PFC projects to the MD region from which it receives information (Pirot et al., 1994), and these corticothalamic fibers arise predominantly from layers V and VI (Kuroda et al., 1995). Hence, we focused on pyramidal cells in layer III (superficial) and layer V (deep) for each cortical region. The reciprocity between the MD and PFC in the rat is similar to human connectivity; the MD reciprocally projects to orbital, medial, and dorsolateral PFC (Ray and Price 1993). This similarity in connections between human and rat makes this animal a viable vehicle for this lesion investigation.

In the rat, significant brain development and synaptogenesis occurs postnatally. Thalamic fibers grow into the cortex during the first seven days post-birth (Wise SP et al., 1979; Van Eden 1986), so that organization and innervation of the cortex appears virtually mature by postnatal day eight (Molnar et al., 1998). No new primary dendrites are formed after postnatal day seven, but the dendritic arbor remains plastic with further development being marked by extension and branching of existing dendrites (Petit et al., 1988). The more pronounced reduction in secondary dendrites may be explained by the timing and the sequence of their development. Primary dendrites are formed during the first week (Petit et al., 1988), so that by the time the lesion was

performed on postnatal day 4, some primary dendrites had been already formed. Secondary dendrites continue to proliferate to postnatal day 15-20 (Petit et al., 1988); hence by the time they developed, there was a significant reduction in afferent activity, leading to their more pronounced retraction.

It can be argued that the decreased number of cells in the MD, seen in postmortem schizophrenic brains, does not result from neurodegenerative effects (Benes 1988; Pakkenberg 1990; Popken et al., 2000; Young et al., 2000). Fewer cells and hence fewer afferents during early development can impact the anatomy and functionality of the circuitry between the MD and the PFC. The arrival of MD fibers into the cortical plate precedes the termination of cortical lamination (Van Eden 1986), thus, it is likely that these fibers influence the development of cortical pyramidal neurons. MD afferents are excitatory (Kuroda et al., 1998) causing depolarization in the postsynaptic PFC cells. Consequently, N-methyl-D-aspartate receptors (NMDAR) and voltage-sensitive calcium channels open leading to an influx of calcium into the postsynaptic PFC elements.

Calcium signaling plays a crucial role in the formation and regulation of neuronal processes (Van Pelt et al., 1996; Ramakers et al., 2001; Sola et al., 2001; Lidow 2003). Elevated calcium in the cytosol participates in the activation of calcium-dependent protein kinases (Bito et al., 1997) including calmodulin protein and CamKII, which in turn phosphorylates microtubule-associated-protein-2 (MAP2) (Hely et al., 2001). This phosphorylation increases the space between microtubules, destabilizing the microtubule bundle, which leads to dendritic sprouting (Bouwmeester et al., 2002; Hely et al., 2001). Thus, thalamic afferent input activates calcium cascades on the cortical postsynaptic cells resulting in increased branching of the dendritic arbor. In schizophrenia, fewer MD cells and fewer afferents would translate into decreased synaptic

input to the PFC cells; several studies indicate that blocking synaptic activity during development can prevent the normal elaboration of dendritic arbors (Kalb 1994; Vogel and Prittie 1995), which may explain the dendritic alterations reported for pyramidal cells in the PFC (Kalus et al., 2000; Broadbelt et al., 2002; Black et al., 2004).

An electrolytic lesion of the MD thalamus during this first week of life causes significant effects in the anatomy of the circuitry between the MD and the PFC. Deficit of MD afferents can translate into decreased synaptic activity, decreased depolarization and decreased influx of calcium into the pyramidal cells of the PFC. Since the concentration of calcium modulates the elongation and branching of neurites (Van Pelt et al., 1996), an imbalance in calcium homeostasis would disrupt the activity of the different calcium-dependent proteins, resulting in alterations of the dendritic arbor. The observed decrease in primary and secondary basilar dendrites in pyramidal cells of the PFC may indicate a loss of surface area for excitatory and inhibitory inputs, which would hinder the cells from proper transmission and may suggest that pyramidal cells in the PFC have compromised communication abilities.

Figures.

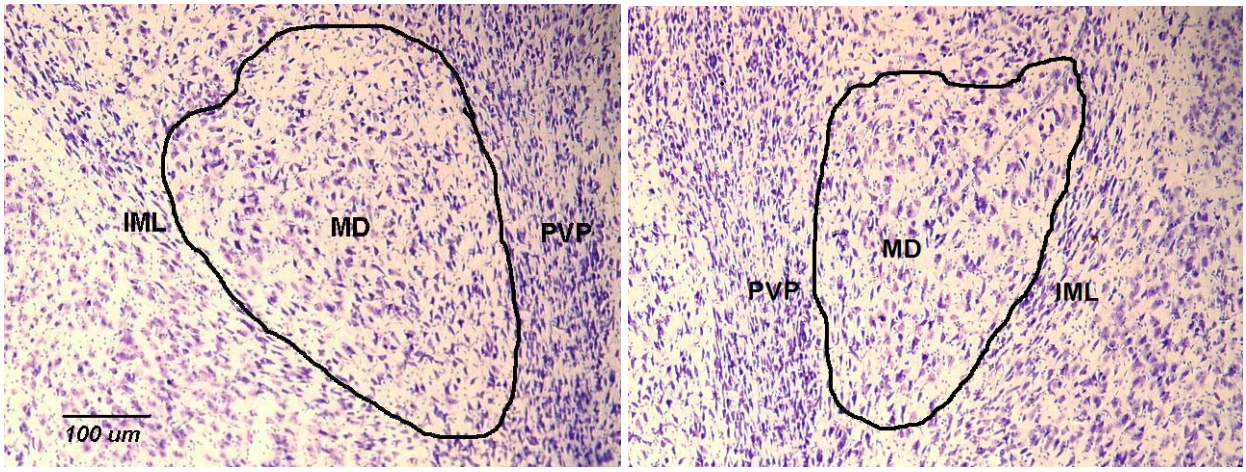


Figure 1. Photomicrograph of P60 MD nuclei sections: control (left) versus lesion (right). The MD in the rat is bounded by the intermedullary lamina (IML), and medially by the Paraventricular Nucleus (PVP). Scale bar =100µm.

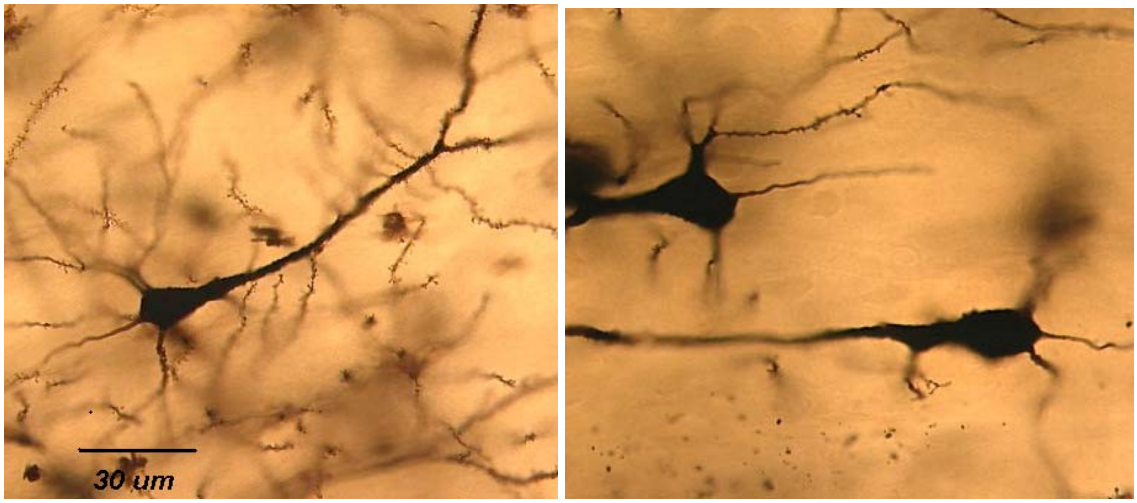


Figure 2. Photomicrograph (60X) with immersion oil of pyramidal neurons in the superficial layer of PFC. Left is cell in the control hemisphere vs. right image showing cells in the lesioned hemisphere. Scale bar: 30*u*m.

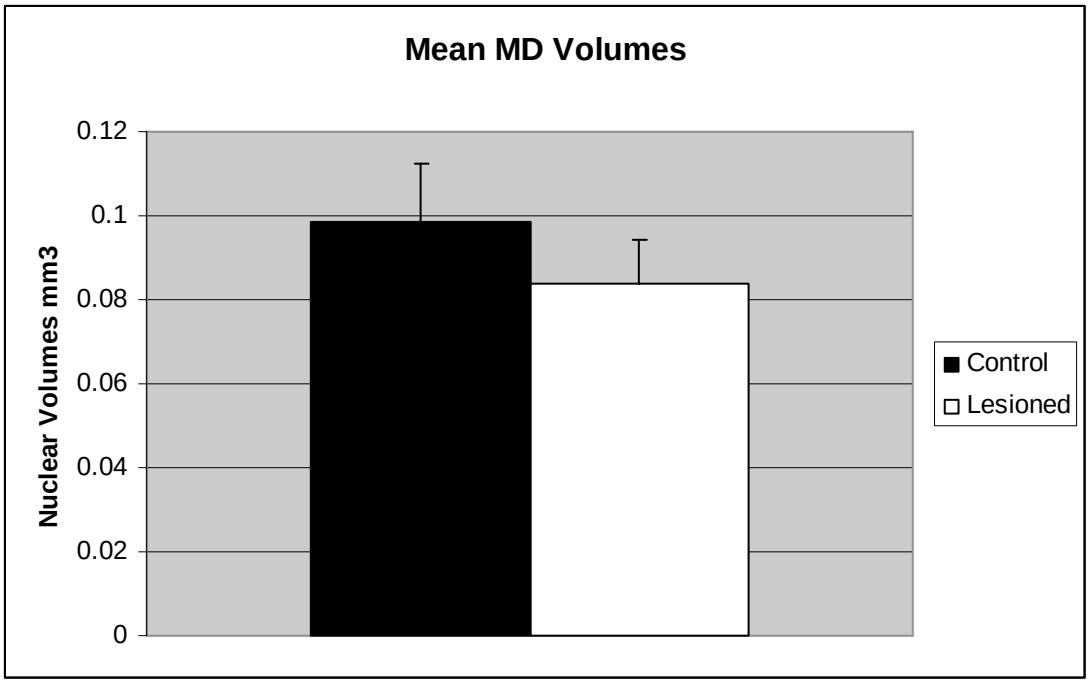


Figure 3. Histogram showing the average decrease in volume in the MD at P60 following a lesion of the MD at P4. The six brains used for this study had a lesion above 9% decrease. Average decrease in MD nuclear volume in lesioned hemisphere versus intact hemisphere was 14.82%. Mean Control: 0.0984±0.014 mm3. Lesion: 0.0836± 0.0044 mm3. ($p=0.00033$, $t=8.72$).

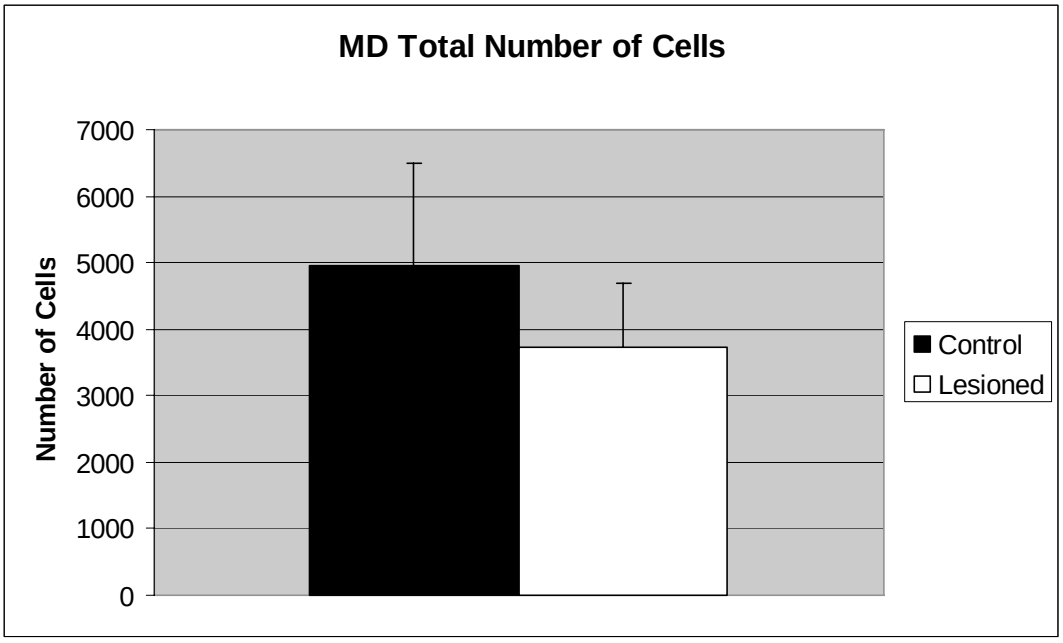


Figure 4. Histogram showing the average decrease in cell number in the MD at P60 following a lesion of the MD at P4. There was an averaged decrease in total cell number of 23.43%. Control: 4947.5±1539.7. Lesion: 3719.4±976.4 (p=0.0044 , t=4.92).

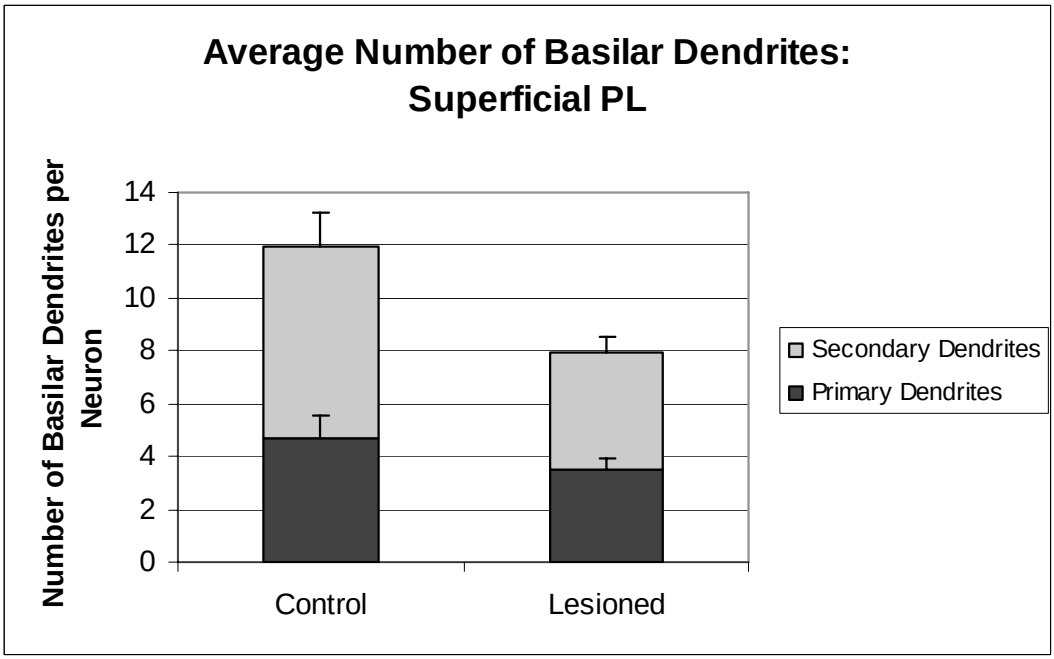


Figure 5. In the superficial Prelimbic cortex, there was a significant 25.00% (Control: 4.72±0.81, Lesion: 3.54±0.37. p=0.0031, t=5.35) decrease in primary dendrites of pyramidal cells in the

lesioned hemispheres, while secondary dendrites showed a more prominent difference 39.70% (Control: 7.28±1.24, Lesion: 4.39±0.61. p=0.0016, t=6.19).

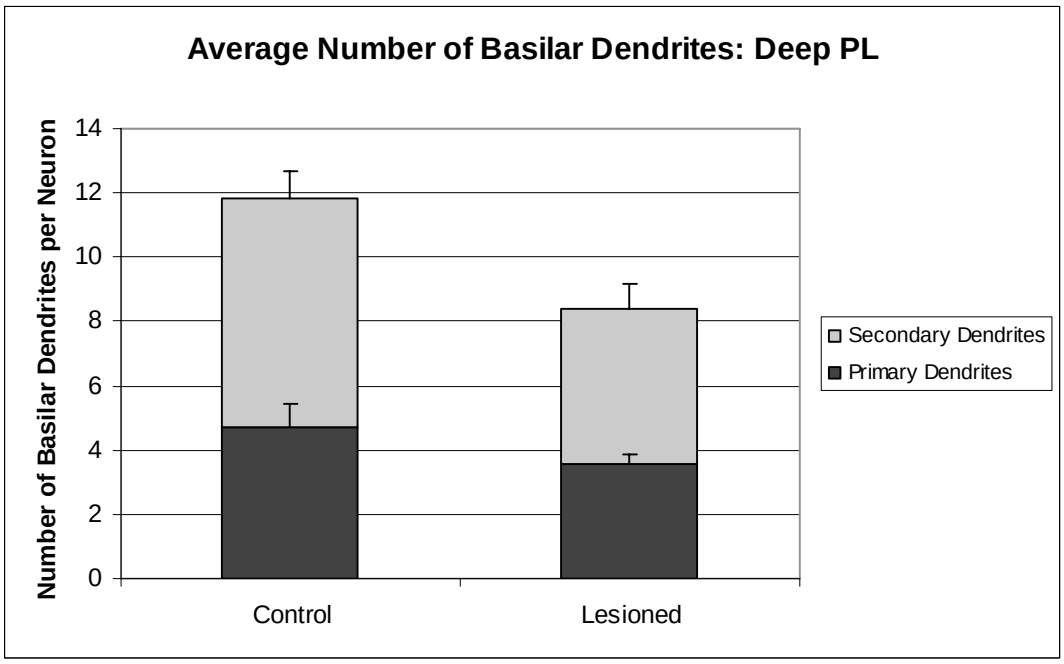


Figure 6. Pyramidal cells in the deep prelimbic cortex corresponding to the lesioned side exhibited a 24.84% decrease in averaged primary basilar dendrites (Control: 4.71±0.72, lesion: 3.54±0.33. p=0.002, t=5.790) and a 31.84% decrease in averaged secondary dendrites. (Control: 7.13±0.81, Lesion: 4.86±0.76. p=0.0015, t=6.3)

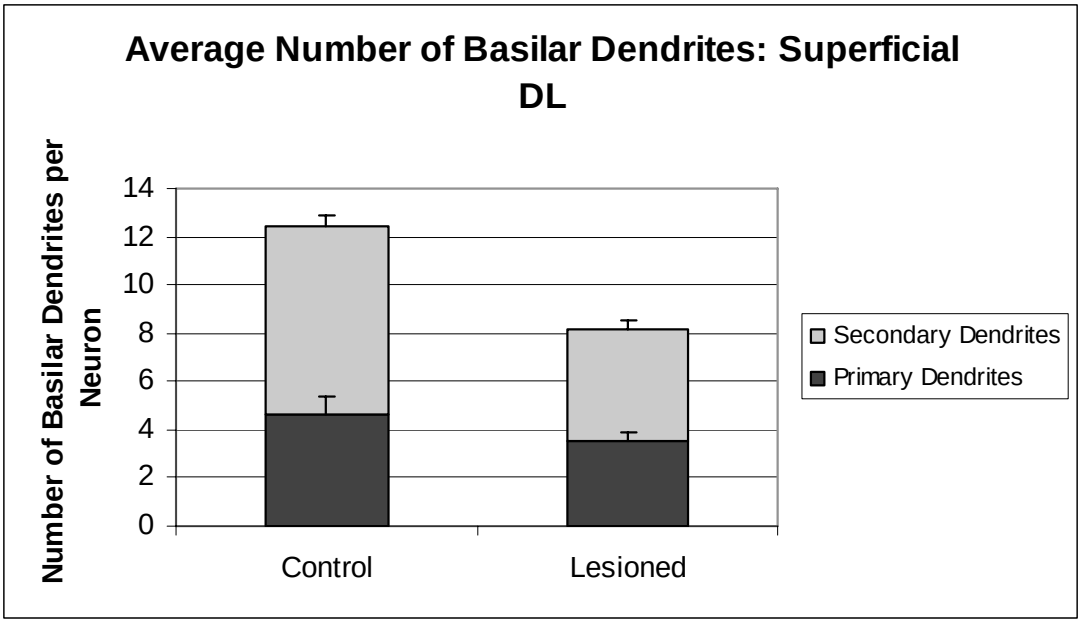


Figure 7. In the superficial DL, there was a 24.24 %decrease in the average of primary basilar dendrites for pyramidal neurons of the lesioned hemispheres as compared to controls (Control: 4.62±0.73, Lesion: 3.5±0.36, p=0.0059, t=4.59). The reduction was pronounced for the secondary dendrites 40.46% (Control: 7.76±0.53, Lesion: 4.62±0.36, p=0.000043, t=13.2).

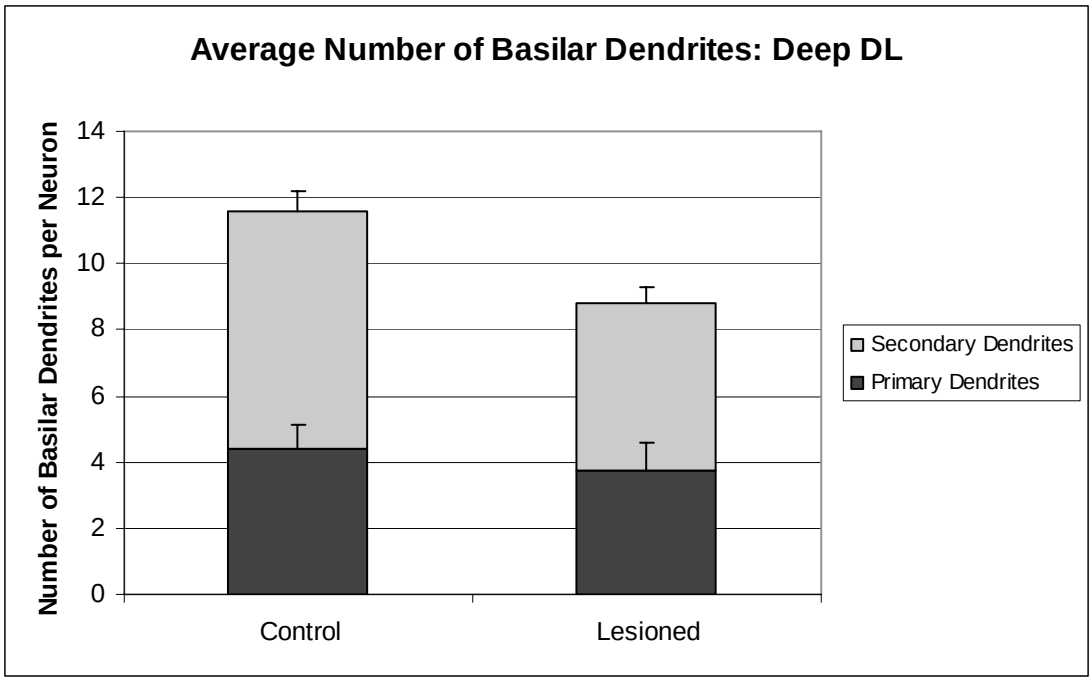


Figure 8. There was a 14.55% decrease in primary dendrites in the deep DL of the lesioned side (Control: 4.4±0.73, Lesion: 3.76±0.85, p=0.016, t=3.57). In this region, the difference in averaged pyramidal secondary basilar dendrites was more evident 30.04% (Control: 7.19±0.61, Lesion: 5.03±0.5, p=0.00021, t=9.54).

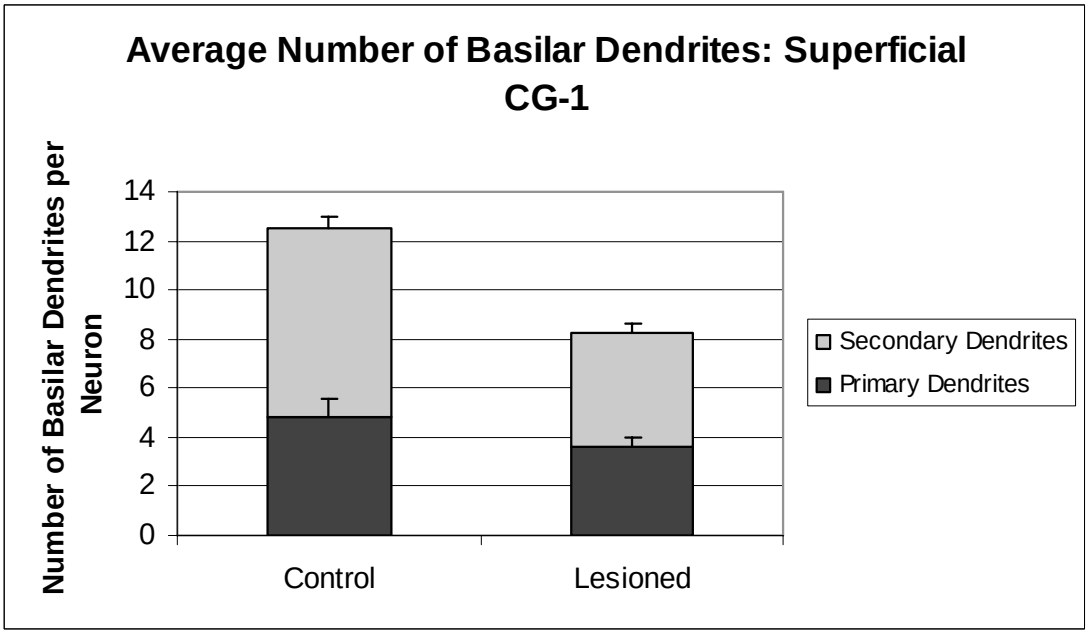


Figure 9. Pyramidal cells in Superficial CG-1 had fewer averaged primary basilar dendrites: 25.31% (Control: 4.82±0.79, Lesion: 3.6±0.37, p=0.008, t=4.89), accompanied by a 39.74%

decrease in secondary basilar dendrites for the same population (Control: 7.7±0.48, Lesion: 4.64±0.40, p=0.00016, t=13.74).

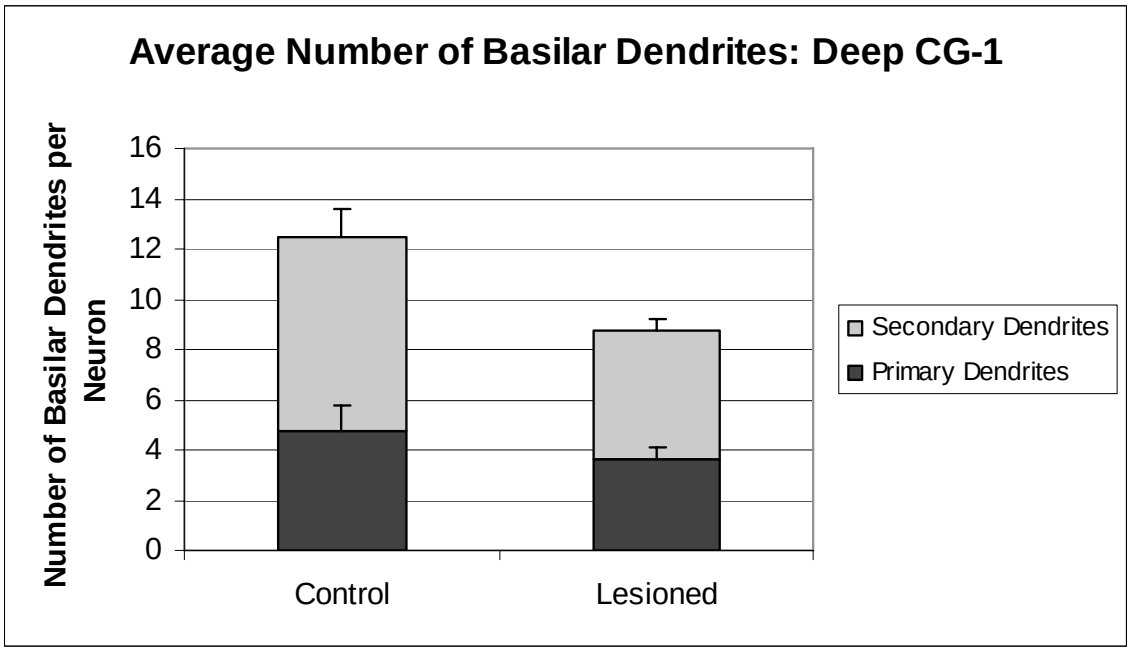


Figure 10. Pyramidal cells in the deep CG-1 showed similar effects to the early MD lesions as the previous regions: 23.06% fewer primary basilar dendrites (Control: 4.77±0.998, Lesion: 3.67±0.4, p=0.022, t=3.66), and 34.41% fewer secondary basilar dendrites in the hemispheres corresponding to the lesion (Control: 7.73±1.07, Lesion: 5.07±0.44, p=0.0012, t=8.16).

Chapter 4. Neonatal Mediodorsal Thalamic Lesion affects Spine Levels in Pyramidal Cells of the PFC in Adult Rat.

Abstract

Background: Schizophrenia is associated with reports of decreased spine populations in pyramidal cells of the prefrontal cortex (PFC) (Garey et al., 1998; Glantz et al., 2000; Kalus et al., 2000). Spines are the main targets for excitatory inputs (Kuroda et al., 1995) and their decrease may affect the ability of cells for synaptic transmission. Spines are the predominant targets of the mediodorsal nucleus of the thalamus (MD) (Berry M 1974; Kuroda et al., 1995), a region consistently showing a schizophrenia associated reduction in volume and cell number. Spines are induced by input activity (Kossel et al., 1997), and it is speculated that a loss of such input resulting from the schizophrenia associated MD loss may result in a reduced number of spines. It has been reported that lesioning the MD of rat pups during the first postnatal week may affect dendritic development as evidenced by decreased levels of MAP2 immunostaining and decreased numbers of basilar dendrites. It is hypothesized that an early lesion of the MD may lead to decreased number of spines and decreased spine densities on basilar dendrites of pyramidal cells in the PFC. **Methods:** Unilateral electrolytic lesions of the MD in Long-Evans rat pups were made on postnatal day 4 (P4) and animals developed to P60. Dendritic spines along basilar dendrites were counted and density was assessed. **Results:** Spine density measurements revealed a pronounced reduction in spine density for distal segments of pyramidal cells in prefrontal cortical regions in the lesioned hemisphere as compared to the intact hemisphere. Spine counts along primary and secondary basilar dendrites of pyramidal cells in the PFC of the lesioned hemisphere showed reductions in the number of spines along secondary dendrites in Prelimbic cortex (Pr) across both layers: 32.4% for superficial cells and 46.2% for deep cells. Dorsolateral anterior cingulate cortex (DL) showed reductions in secondary dendritic

spines of 37.2% in the superficial aspect and 41.6% in the deep layer. In the anterior cingulate cortex (CG-1) of the lesioned side, there were fewer spines along both primary basilar dendrites (53.4% in superficial layer and 28.4% in deep layer) and secondary basilar dendrites (46.7% superficially and 47.6% deep). **Conclusion:** Early MD thalamic damage may contribute to the reductions in the number of spines reported in schizophrenia.

Introduction:

The circuitry between the mediodorsal nucleus (MD) and the prefrontal cortex (PFC) is of particular importance in schizophrenia research. Evidence from postmortem material consistently shows there is a loss of cells in the MD associated with schizophrenia (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002; Danos et al., 2005; Mitelman et al., 2006) with no evidence of gliosis (Pakkenberg 1990; Falk et al., 2000; Popken et al., 2000; Young et al., 2000); suggesting therefore, that the loss of cells may occur early in development. The MD is intricately connected to the PFC (Herkerham 1980; Barbas et al., 1991; Pirot et al., 1994; Kuroda et al., 1995; Kuroda et al., 1998; Negyessy et al., 1998; Wang and Shyu 2004), a region affected by schizophrenia as well (Garey et al., 1998; Rajkowska et al., 1998; Stone et al., 1998; Conklin et al., 2000; Kalus et al., 2000; Perry et al., 2001; Broadbelt et al., 2002; Jones et al., 2002; Selemon et al., 2003; Black et al., 2004; Kolluri et al., 2005; Broadbelt et al., 2006). Research suggests that schizophrenia affects dendritic architecture of pyramidal neurons in the prefrontal cortex (Garey et al., 1998; Glantz et al., 2000; Kalus et al., 2000; Broadbelt et al., 2002; Kolluri et al., 2005). Postmortem schizophrenic brains exhibit a decrease in the number of basilar dendrites (Broadbelt et al., 2002), in addition to significant reductions in the density of spines (Garey et al., 1998; Glantz et al., 2000; Kalus et al., 2000)(for review see Jones 2004). Pyramidal cells in the PFC of schizophrenics exhibit a compromised morphology that may affect the ability of these cells for synaptic communication.

Spines are attached to the dendritic shaft by a thin neck, which increases resistance, and can cause large voltage changes despite the size of the synaptic input (Nikonenko et al., 2002); this structure makes spines efficient postsynaptic elements. Thalamic afferents, among other

afferents, synapse on pyramidal cell spines (Berry 1974; Kuroda et al., 1995). These synapses are asymmetrical and assumed to be excitatory and glutamatergic (Kuroda et al., 1995). Excitation causes depolarization of postsynaptic elements, which in turn opens L-type voltage-gated calcium channels (Ramakers et al, 2001) and receptor-linked calcium channels (Van pelt et al, 1996). The subsequent influx of calcium elevates the concentration of this cation in the cytosol. Calcium may then activate calcium-dependent protein kinases (Bito et al., 1997) that eventually affect transcription of CREST genes (for review see Chakravarty et al., 1999). Calcium activation of CREST mediated transcription induces de novo protein synthesis (West et al., 2001) and helps to regulate neuronal morphogenesis (for review see Aisawa 2004). Thus, input activity from thalamocortical afferents, via excitation, may influence spine formation and stability thru activation of calcium cascades.

Evidence suggests that some proteins relevant in the calcium cascade are affected in schizophrenia. Phosphorylation of Microtubule-associated protein-2 (MAP2), by calcium-calmodulin complex, leads to neurite branching (Hely et al., 2001). MAP2 is significantly decreased in areas 9 and 32 of the PFC (Jones et al., 2002). There is also a schizophrenia-associated decrease in Neurogranin in area 32 (Broadbelt et al., 2006); this protein binds calmodulin and inhibits its interaction with calcium (Chakravarty et al., 1999). Calmodulin is affected in schizophrenia as well, with significant reductions in area 9 and 32 (In press). Dysregulation of the calcium associated proteins may imply that the calcium cascade is compromised in schizophrenia. Alterations in the optimum level for calcium causes neurites to stop growing or retract (Ramakers et al., 2001). MD afferents directly affect calcium influx into the PFC spines through their release of glutamate (Kuroda et al., 1995). The loss of MD cells reported in schizophrenia (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et

al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002; Danos et al., 2005; Mitelman et al., 2006) implies a decrease in thalamic input. The deprivation in input activity may translate into the loss of target dendritic spines by affecting postsynaptic calcium cascade events.

It is possible to investigate the effects caused by cell loss of the MD on the dendritic spines of pyramidal neurons. Because input from the MD afferents is suspected to affect spine morphology, it is speculated that the MD loss reported in schizophrenia may lead to reductions in spine population in the pyramidal cells of the PFC. The schizophrenia-associated effect on this circuit can be mimicked by lesioning the MD of neonatal rat pups. It was hypothesized that such lesion could alter the dendritic spine population in the PFC. This was assessed by calculating the total number of spines along primary and secondary basilar dendrites, as well as the spine densities (number of spines/length of dendrite) along these dendrites.

Methods and Materials:

Lesion Protocol:

Time pregnant Long Evans rats were obtained from Jackson Laboratories and the day of birth was designated as P0. Surgery was performed on P4. Subjects were anesthetized with intraperitoneal injections of ketamine (75 mg/kg) and xylazine (6-8 mg/kg).The pups were lesioned on the right hemisphere using stereotactic methods. The scalp was incised and reflected to expose the skull. Preliminary lesions showed the MD at P4 to be located 4.8mm caudal to Bregma and 0.75mm lateral to midline. An electrode was inserted 6mm deep, at which point a current of 5*u*A was passed for 3 seconds; three consecutive lesions were performed at 0.2 mm intervals to ensure that the entire nucleus was lesioned. The incisions were sutured and the pups were returned to the mother and allowed to survive to P60. At this time, the pups received an

intraperitoneal overdose of sodium pentobarbital, followed by a transcardial perfusion with 0.9% saline followed by 4% Peases fixative. The brains were removed and postfixed overnight in the same solution. The frontal cortex anterior to Bregma was severed from the rest of brain to be processed for Golgi, while the posterior portion of the brain was exposed to increasing gradients of sucrose solution for cryoprotection.

Areas of Interest

Medial Dorsal Nucleus

The medial dorsal nucleus is easily distinguished in thionin stained sections. The intermedullary lamina borders approximately 80% of the MD. The remaining medial boundary is the paraventricular nucleus. Superficially it is bounded by the third ventricle.

Prefrontal Cortex

The prefrontal cortex in the rat extends from the frontal poles to the rostral end of the corpus callosum. The three regions investigated in this study were the prelimbic cortex (Pr), anterior cingulate cortex (CG-1) and dorsolateral anterior cingulate cortex (DL); these prefrontal cortical subregions were chosen because they have reciprocal connections to the MD (Paxinos 1985; Hoover and Vertes 2007), and perform cognitive functions analogous to the PFC in humans (Seamans et al., 1995; Heidbreder and Groenewegen 2003). To distinguish the cytoarchitectural borders between the PFC subregions we used criteria from Paxinos and colleagues (1999), Donoghue and Wise (1982), as well as Gabott and colleagues (2003). Pr is on the midline just dorsal to infralimbic cortex (Paxinos 1985; Gabbot et al., 2003), and has a very thin layer IV with a homogeneous layer V (Radley et al., 2006). CG-1 is dorsal to Pr; it is completely agranular and characterized by a sparse layer III and loosely packed broad layer V.

The DL is dorsolateral to CG-1, agranular, marked by a pale staining layer III and compact layer II (Donoghue and Wise 1982). All of the sections that fall within the prefrontal regions were used for immunohistochemistry.

Histological Procedures:

The posterior portions of the brains containing the mediodorsal nucleus of the thalamus were cut on a freezing sledge sliding microtome. Serial coronal sections 50μm in width were collected to be processed for histological staining. Sections were stained using thionin to better differentiate boundaries to determine MD volumes, and perform cell counts in the MD. The slides were mounted, defatted by increasing gradients of ethyl alcohol, exposed to xylene for ten minutes, and then rehydrated and stained with thionin. Sections were then dehydrated, defatted and coverslipped.

Golgi Method:

The frontal cortex anterior to Bregma was severed from the more caudal portion of the brain. Frontal cortices were wrapped in clean gauze and immersed in 100 mL of Golgi solution. Both Golgi solution and gauze were changed after 24 hours, followed by extended 12 week incubation in the dark. After impregnation the tissue was dehydrated thru increasing gradients of alcohol followed by three day incubations in serial celloidin. The tissue was put in paper boats and allowed to harden in chloroform. 12% parlodion was added to the paper boats and stored for 3 to 24 hours until hardened. Finally the tissue was serially coronally sectioned on a vibratome into 200-*u*m thick sections and collected in 70% alcohol. The sections were washed in distilled water, incubated in 19% NH_4OH for thirty minutes, and rinsed again in water. The sections were then fixed in Kodak Rapid Fix, followed by a final wash in distilled water. The sections were

dehydrated in graded ethanol starting at 50%, defatted in toluene, mounted onto slides and coverslipped.

Quantification of MD volume:

The material was quantified using a Bioquant Image Analysis system interfaced with an Olympus AX70 microscope and a Sony 3-chip color camera. Area of the MD was determined for each slide by drawing a contour around the nucleus. The specimen thickness (z) was calculated as an average of three randomly selected slides. MD volume was then obtained by multiplying the averaged nuclear area by section thickness and the number of sections thru each nucleus. Volumes of lesioned nuclei were compared to controls to calculate the extent of the lesion.

Quantification of MD Cell Density

Cells were counted in four randomly selected thionin sections. Contours were traced around the selected region, and a counting grid was superimposed on the contour. Every third intersection was marked, with a random start, so that 30% of the intersections were marked. A counting box of 100x100 μm was centered at the marked intersections. Counting was done using a 40X objective under oil. Cells were counted if they had a distinguishable nucleolus, rejecting those touching the exclusion lines. Neuron density was determined by dividing the average number of cells per box (Q) by the volume of the counting box. The volume of the dissector was calculated as the product of the area of the frame and the average thickness of the section corrected for shrinkage. The estimate of the total number of neurons in the MD was determined as the average neuron density of four randomly selected sections multiplied by the total nucleus volume.

Quantification of dendritic spines:

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 dendritic spines. A contour was drawn to outline each lamina separately in each of the regions of interest in the PFC. A counting grid was placed inside the contour, and the computer randomly selected half of the intersections to be measured with a counting box of 50 X 50um placed inside the marked grids. The spines on the primary and secondary branches off the somae within the grid, which met the criteria, were counted at 100X Plan Apo objective under oil. Although a counting box was used to select neurons randomly, dendritic spines of the selected neurons were counted even if the basilar dendrites extended beyond the boundaries of the counting box. 10 cells per layer per region were assessed for each brain. One tailed probability was used to test the directionality of the hypothesis. ($P < 0.05$ considered significant).

Quantification of spine density:

Three prefrontal cortical subregions were analyzed. Five neurons from the superficial and deep layers were traced for each brain. The soma was traced first, followed by every single basilar dendrite with its primary (branch off the soma) and secondary (bifurcation of the primary) segments (See Figure 1). Every spine emanating from the primary and secondary segments was traced. While the dendritic processes were traced on a two dimensional model, the software registered the three dimensional coordinates for every point allowing an exact virtual reconstruction of the dendritic tree. Neurolucida and Neuroexplorer softwares were used to measure the length of the dendrites. Spine density was calculated as the number of spines over the length of the given dendrite. The software generated concentric shells at equidistant spaces

from the center of the soma. Spine densities were compared as the radii of such circles increased, moving distally from the cell body. Univariate analysis of variance was performed for spine densities in the control and lesioned groups at each radius.

Results:

MD Volumes

We analyzed the brains of nine animals that had received an electrolytic lesion to the MD in the right hemisphere. The lesions ranged in size from 0.89% to 17.64%. Brains whose lesions were below 9% were not used in the study. Paired t-tests were performed between the means of control MD nuclei and lesioned MD nuclei to assess significant differences. The mean MD volume decreased as a result of the lesion from 0.0984±0.014 mm3. to 0.0836± 0.0044 mm3. ($p=0.00033$, $t=8.72$) (Figure 2).

MD Cell Counts

The six brains were examined for cell loss in the MD due to the lesion. There was a significant decrease in cell density of 10.84% Control: 9.68± 2.1. Lesion: 8.63+/1.7 ($p=0.016$ $t=3.56$). There was an average decrease in total cell number of 23.43% Control: 4947.5± 1539.7. Lesion: 3719.4±976.4 ($p=0.0044$, $t=4.92$) (Figure 3).

Number of Basilar Dendritic Spines

A general view of the spine dynamics following a thalamic lesion was surveyed by counting the total number of spines along primary and secondary basilar dendrites. Ten random neurons in each of the three prefrontal subregions in both superficial and deep layers were assessed. The total number of spines on primary dendrites was only significantly reduced for the CG-1 subregion, superficial: 53.4% (control: 26.1±9.4, lesioned: 12.2±2.7, $p= 0.03$) and deep: 28.4% (control: 22.3±7.8, lesioned: 15.9±5.2, $p= 0.048$). The other two PFC subregions, Pr and

DL, displayed tendencies towards decreased primary dendritic spines but did not reach statistical significance. Pr III (control: 21.8±7.4, lesioned: 15±5.2, p= 0.12), Pr V(control: 21.4±8.4, lesioned: 12.5±4.1, p=0.07), DL III(control: 15.4±5.9, lesioned: 10.9±1.3, p=0.059), DL V (control: 12.9±2.9, lesioned: 11.4±2.1, p=0.21). (See figures 4-9).

Spines along secondary dendrites were significantly reduced in the lesion hemisphere versus the intact side for all three cortical subregions for superficial and deep layers. In PL, secondary dendritic spines showed a difference across both layers: 32.4% for superficial cells (control: 90.5±11, lesioned: 61.2±5.7, p=0.0003) and 46.2% for cells found in the deep cortex (control: 97±17.1, lesioned: 52.2±9.9, p=0.0006) (Figures4, 5). This pattern of decline was also seen in the DL, where there were considerably fewer secondary dendritic spines in the lesion hemispheres, 37.2% in the superficial aspect (control: 118.8±5.7, lesioned: 74.1±4.7, p=0.0007), and 41.6% decrease in the deep layer (control: 101.1±6.8, lesioned: 59.3±4, p=0.00016) (Figures 6, 7). The number of secondary spines in lesioned hemispheres in CG-1 were substantially decreased: 46.7% superficially (control: 100.3±27.9, lesioned: 53.6±4.8, p=0.01) and 47.6% deep (control: 101.4±34.7, lesioned: 53.8±8.4, p=0.021) (Figures 8, 9).

Spine Density

Somae with radiating primary and secondary basilar dendrites were traced for neurons in superficial and deep layers of all three prefrontal cortical subregions. Spine density was calculated as the total number of spines over the length of the dendrite. The statistical significance of the reduction in spine density was assessed along the dendritic segments beginning at proximal segments (when the radius of the concentric shell intersecting the dendritic tree was 20 um) and moving distally from the soma. In the superficial prelimbic subregion, there was a significant difference in mean spine density between the control and

lesion hemispheres when the shell intersecting the pyramidal tree was 20µm from the cell body (p=0.001) and at increasing distances as well. At radius 30 µm, p=0.03; 40 µm, p=0.02: 50 and 60 µm, p=0.001; at distance 70 µm, p=0.03, and at 80 µm p=0.02 (see figure 10). There was also an evident reduction in spine density for cells in the deep aspect of prelimbic cortex that only became significant when the distance from the soma was at or above 40 µm, p=0.005; 50 µm, p=0.004. At 60 and 70µm, p=0.000, and at a distance of 80 µm, p=0.006 (see figure 11). Spine density in the superficial DL was reduced for cells in the lesioned hemisphere even at distances proximal to the somae: at 20 µm, p=0.019; 30 µm, p=0.000; 40 µm, p=0.001; 50 and 60 µm, p=0.004; 70 µm, p=0.037, 80 µm, p=0.003, and at a distal 90 µm, p=0.001 (see figure 12). In the deep layer of DL the difference in spine density was evident from a distance of 30 µm, p=0.042 from the cell body to 90 µm, p=0.027 (see figure 13). CG-1 subregion showed a more variable change in spine density for the lesioned hemispheres, and although it showed a decreasing trend at every distance from the somae, this reduction only reached significance at 30 µm, p=0.03; 70 µm, p=0.01 and 80 µm, p=0.001 (see figure 14). Finally, in the deep layer of CG-1, the decrease in spine density was substantial, even close to the somae: 20 µm, p=0.001 and extending outward to more distal segments 90 µm, p=0.000 (see figure 15).

Discussion:

We assessed total spine counts along primary and secondary dendrites, as well as spine density in the control and lesion hemispheres. Spine counts on primary dendrites only showed a significant decrease for the CG-1 subregion, while the other two subregions only showed a tendency towards a decrease in the number of spines associated with the lesion. The lesion effect on the mean total number of spines was more prominent in secondary dendrites where the reduction was statistically significant in all three cortical regions and across superficial and deep

layers. The reduction in the mean total number of spines on secondary dendrites between the lesion and control hemispheres was further corroborated by spine density measurements, decreased along dendritic segments for all cortical subregions examined. These data are consistent with schizophrenia postmortem investigations which observed a decrease in dendritic spines (Garey et al., 1998; Glantz et al., 2000; Kalus et al., 2000). Further, the spine density difference between schizophrenic and control subjects, although obvious in proximal segments, reaches significance in more distal segments of the dendritic tree (Kalus et al., 2000). Spine density analysis of proximal segments showed a significant reduction in spine density, whereas spine counts reported only a trend decrease. This may be due to the different techniques: for the latter, total number of spines were counted along the entire dendrite, not considering the length of the dendrite, while density measurements considered the number of spines over the length of the dendrite. The spine counts offered a global view of the difference in the spine population, while the density measurements offered a more detailed picture of the changes along the dendrites associated with an early MD lesion.

The three prefrontal cortical regions investigated: anterior cingulate, medial prelimbic, and dorsolateral anterior cingulate cortices were chosen because they are innervated by the MD: The medial MD sends projections to medial cortex area 32, prelimbic and dorsal insular cortices, while the lateral MD sends afferents to anterior cingulate and medial precentral (dorsolateral anterior) cortices (Paxinos 1985; Pirot et al., 1994). The MD afferents terminate mainly on layer III of the PFC (Herkenham 1990; Kuroda et al., 1995); in turn, each subregion of the PFC projects to the MD region from which it receives information (Pirot et al., 1994), and these corticothalamic fibers arise predominantly from layers V and VI (Kuroda et al., 1995). Hence,

we focused on pyramidal cells in layer III (superficial) and layer V (deep) for each cortical region.

The lesion was performed during the first postnatal week for several reasons: Previous studies have shown that early cortical lesions caused morphological and behavioral changes, but lesions after P7 caused no long term effects in either morphology or behavior (Kolbe and Cioe 2000). Furthermore, it is during this time that the rat MD afferents arrive in the cortex (Kuroda et al., 1995; Molnar et al., 2000); therefore, a lesion during this week has the potential to affect the connections between the MD and the PFC. This time period is especially sensitive for the formation of spines, which begin to appear on postnatal day 4 (Wise et al., 1979), therefore lesions were performed on this day. A lesion of the MD at this time translates into loss of afferents and decreased input activity, affecting the activity dependent formation of spines (Berry 1974; Drakew et al., 1995; Kossel et al., 1997; Kirov and Harris 1999). Loss of excitatory afferents implies less glutamate release at the synaptic contact. Because glutamate activates calcium influx into the postsynaptic spine (Chakravarty et al., 1999), the lower release of such neurotransmitter may impact the calcium current into the postsynaptic spine. There is an optimal range of calcium concentration for neurite growth (Van Pelt et al., 1996; Ramakers et al., 2001), which may be breached by loss of input activity. The dysregulation of calcium cascade homeostasis as evidenced by disruption in several calcium associated proteins (Jones et al., 2002; Broadbelt et al., 2006) may play a role in the reduction of spines. Spines are postsynaptic targets of input by afferents, and their decrease, as reported in postmortem schizophrenia investigations and in the current lesion study, suggests the pyramidal cells in the PFC experience reduced synaptic transmissions. This hypoactivity may translate into altered processing capabilities for pyramidal cells in the PFC.

Figures.

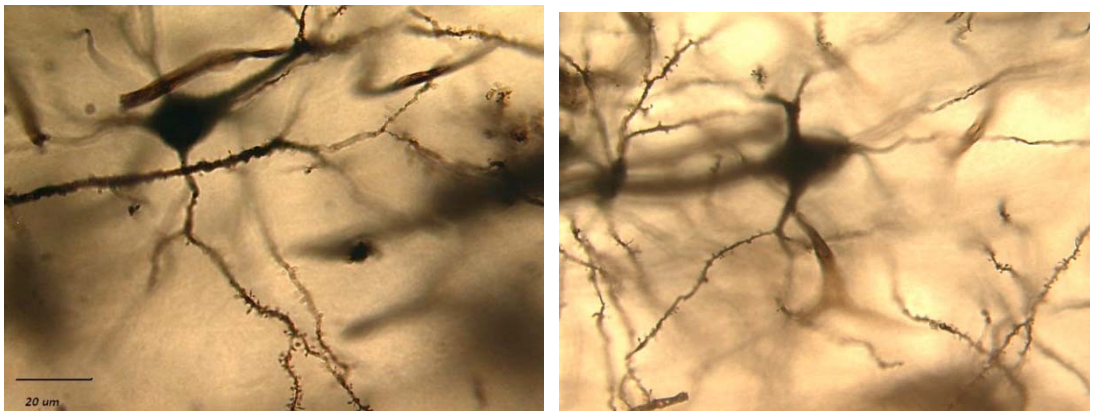


Figure 1. Photomicrograph of distal spines on pyramidal cells in medial prelimbic cortex control (left) versus lesion hemisphere (right). Magnification 100X under oil. Scale Bar: 20*u*m.

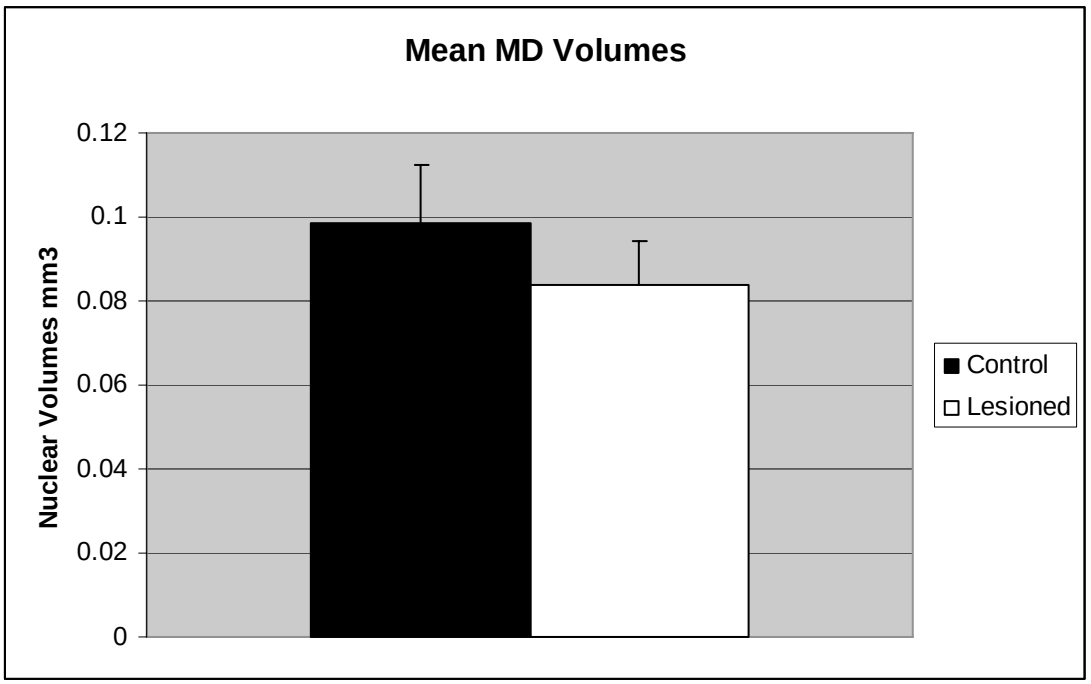


Figure 2. Histogram showing the average decrease in volume in the MD at P60 following a lesion of the MD at P4. The six brains used for this study had a lesion above 9% decrease.

Average decrease in MD nuclear volume in lesioned hemisphere versus intact hemisphere was 14.82%. Mean Control: 0.0984±0.014 mm3. Lesion: 0.0836± 0.0044 mm3. (p=0.00033, t=8.72).

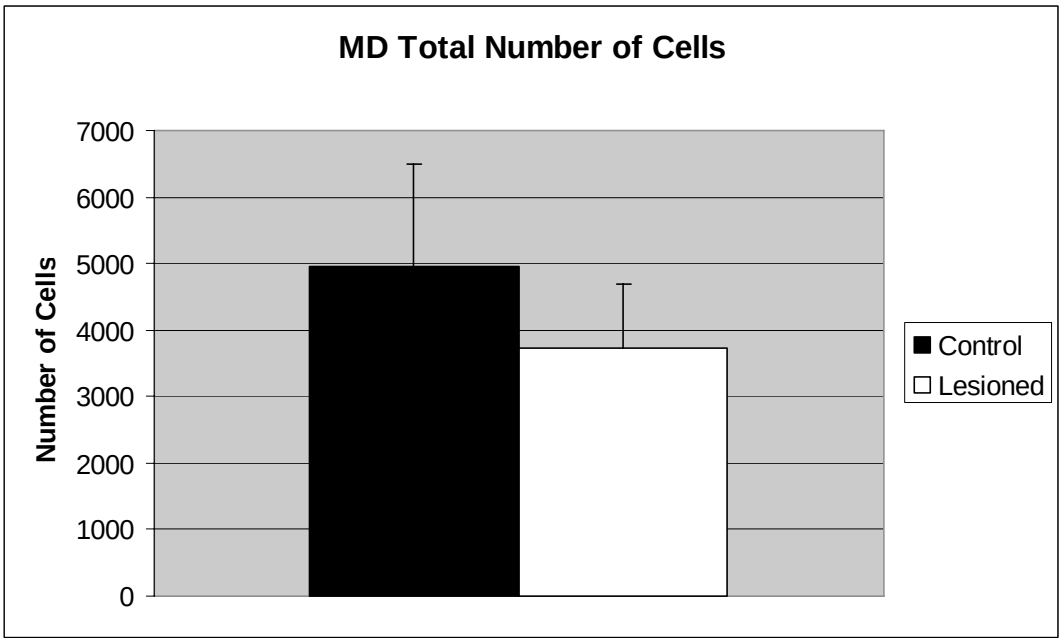


Figure 3. Histogram showing the average decrease in cell number in the MD at P60 following a lesion of the MD at P4. There was an averaged decrease in total cell number of 23.43%. Control: 4947.5± 1539.7. Lesion: 3719.4±976.4 (p=0.0044, t=4.92).

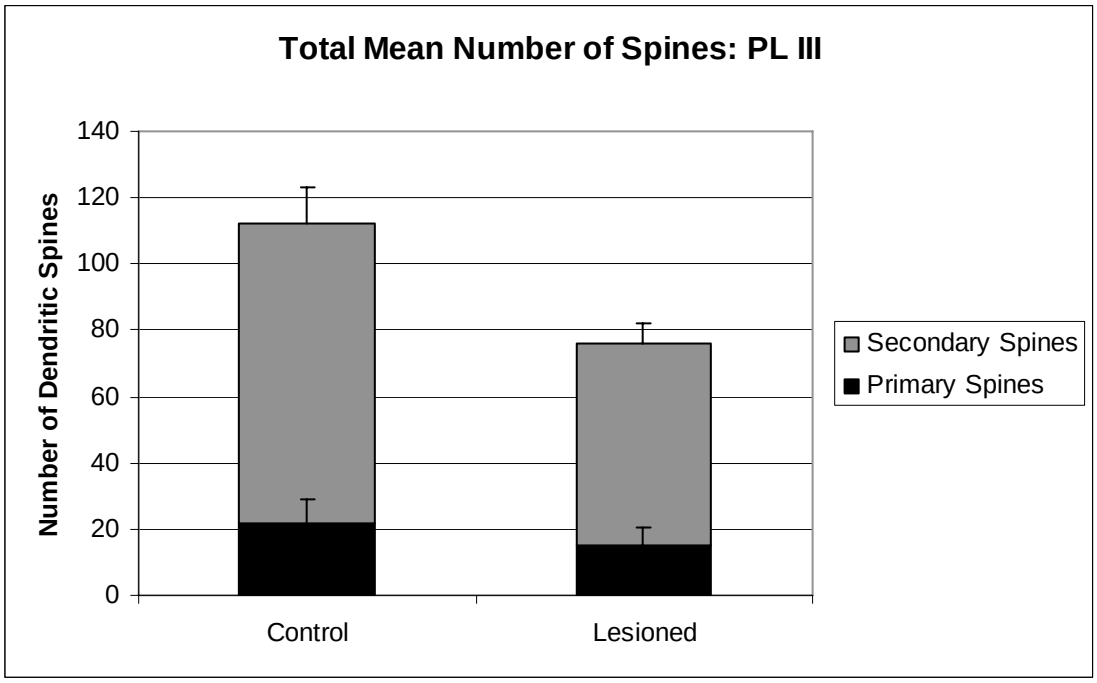


Figure 4. The average number of spines along primary dendrites showed no significant difference between control and lesioned hemispheres of the superficial PL (control: 21.78± 7.4. Lesion: 15.04± 5.2. p=0.1183, t=1.88). The number of spines along secondary was decreased in the cells of the lesioned side (control: 90.52± 11.04, lesion: 61.17± 5.7. p=0.0003, t=9.16).

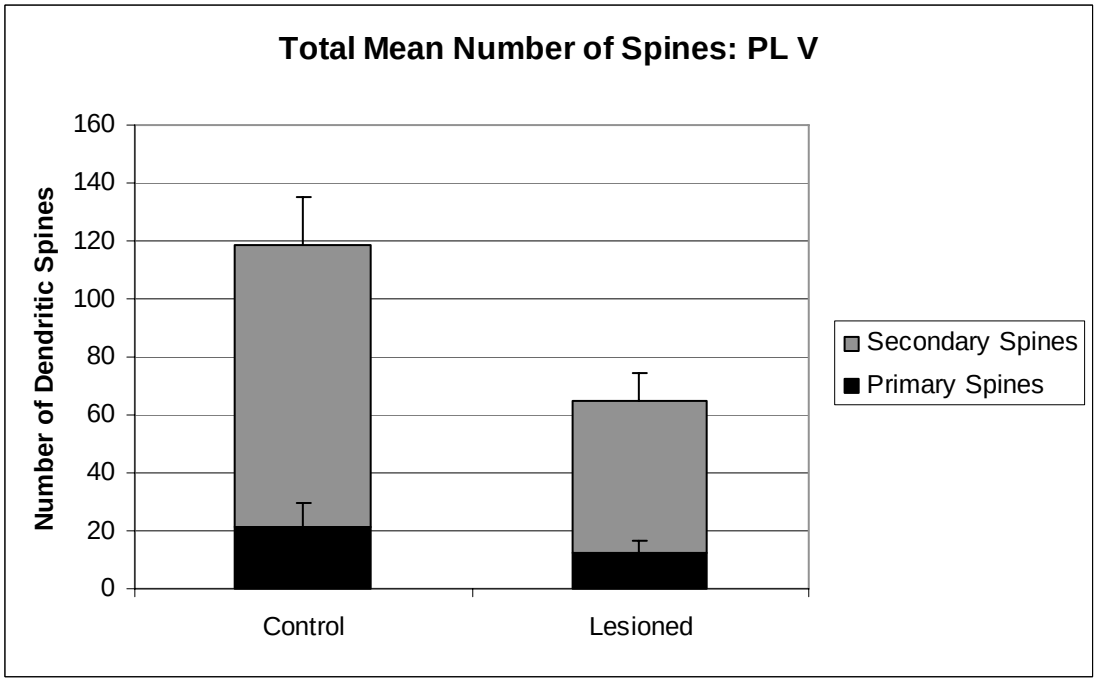


Figure 5. There is no significant difference in primary spines between the two groups for the deep PL. (control: 21.35± 8.4, lesion: 12.5± 4.1. p=0.08, t=2.21). Secondary spines decreased substantially in the lesioned side (control: 97.10± 16.9, lesion: 52.22± 9.9. p=0.0006, t=7.81).

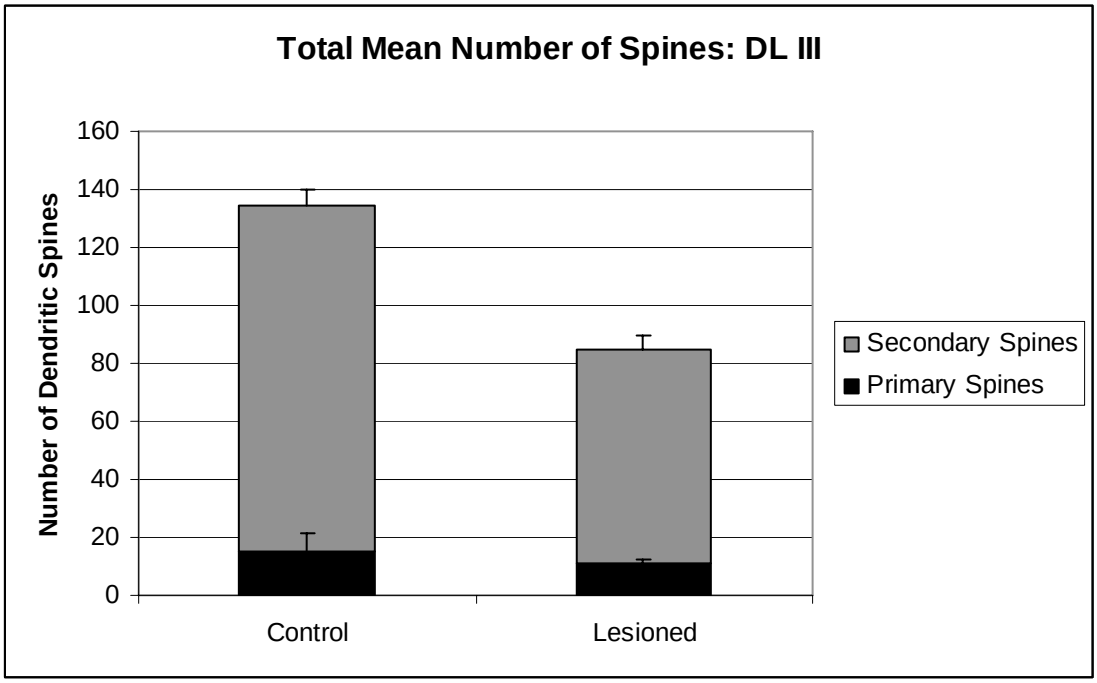


Figure 6. Pyramidal neurons in the superficial DL of the lesioned sides showed no significant decrease in primary spines (control: 15.40±5.9, lesion: 10.95±1.3. p=0.06, t=2.43), but did show a robust reduction in secondary spines (control 118.80±5.7, lesion: 74.08±4.7, p=0.00007, t=12.1).

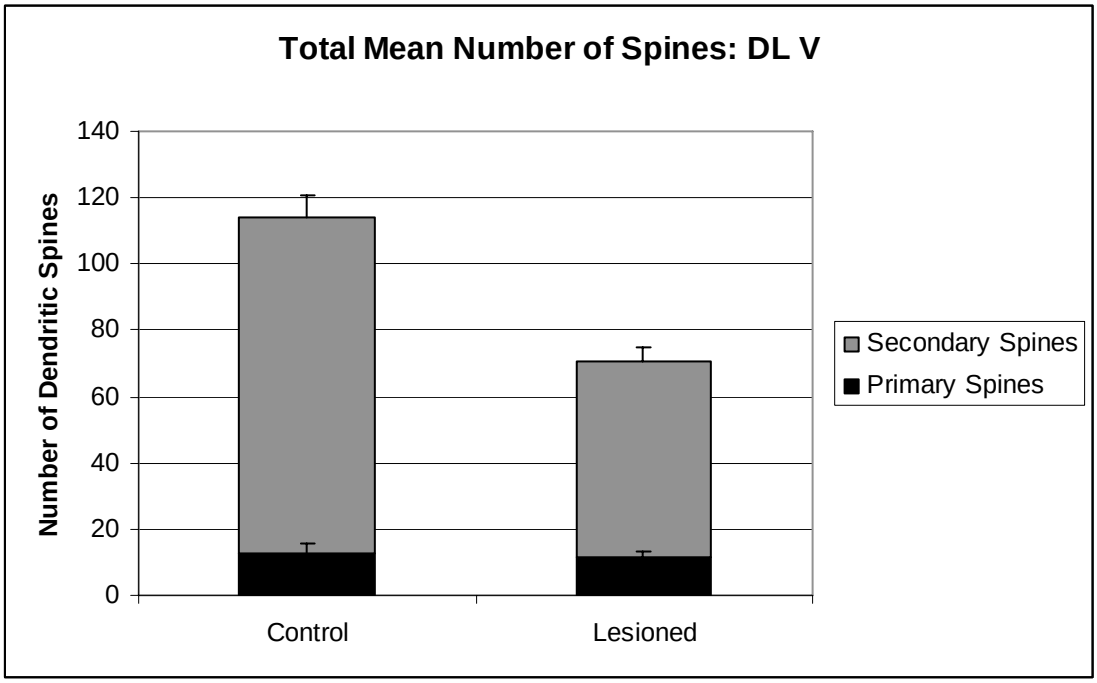


Figure 7. The total average number of spines along primary dendrites in the deep DL did not show a difference between the lesion and control groups (control: 12.92±2.9. lesion: 11.4±2.1. p= 0.207. t=1.45). However, the total mean number of spines along secondary dendrites did show a profound decrease for the lesioned side (control: 101.1±6.8, lesion: 59.28± 4.02. p=0.00016, t=10.17).

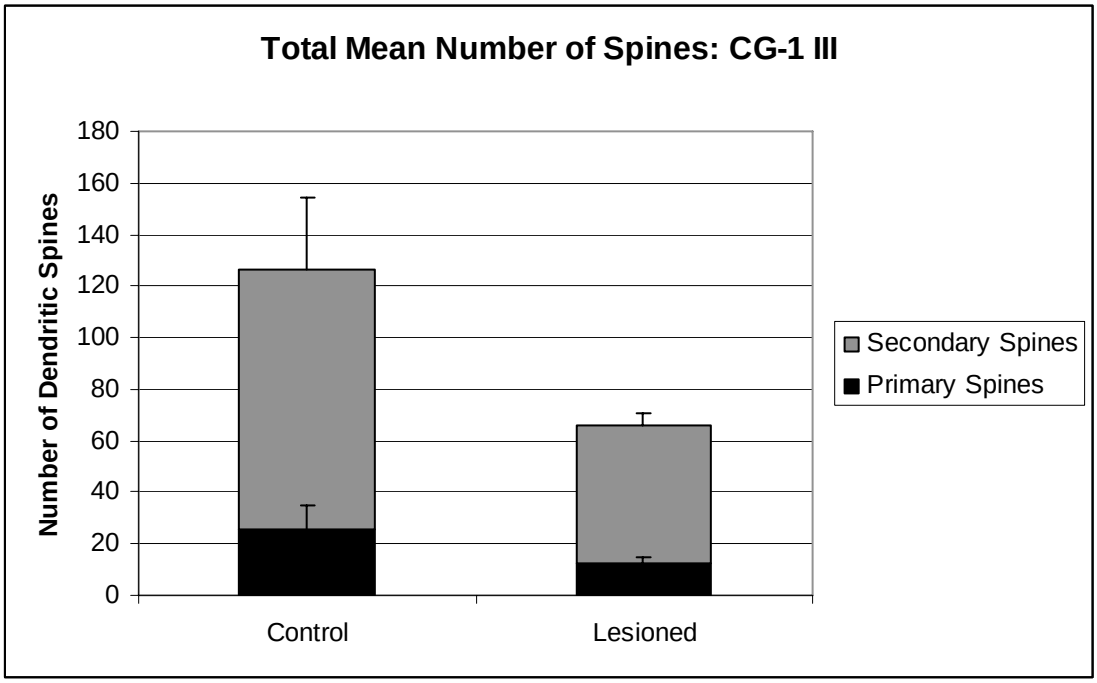


Figure 8. Pyramidal cells in the lesioned CG-1 superficial layer had significant reductions in spines on both primary dendrites (control: 25.98± 9.3, lesion: 12.16± 2.7. p=0.03, t=3.31) and secondary dendrites (control: 100.72± 27.9, lesion: 53.6± 4.7. p= 0.01, t= 4.2).

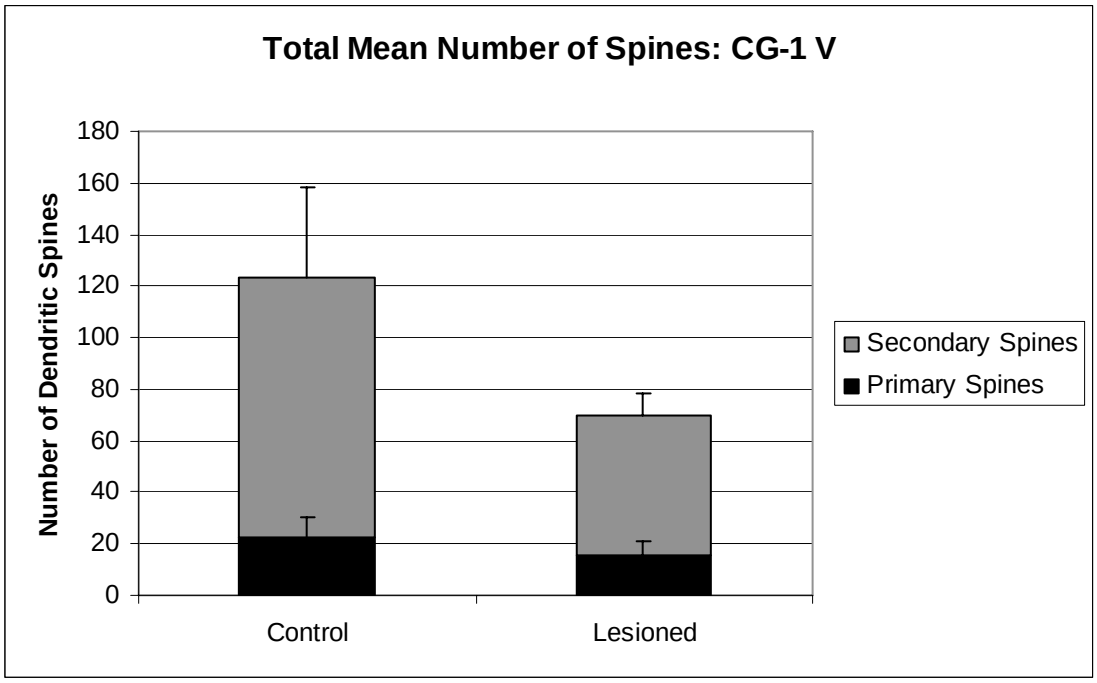


Figure 9. Dendritic spines were decreased in cells of the lesioned hemisphere in deep CG-1 as compared to controls. Primary spines control: 22.26±7.8, lesion: 15.9±5.1, p=0.048, t=2.8. Secondary spines control: 101.4±34.6, lesion: 53.88±8.4, p=0.013, t=4.25.

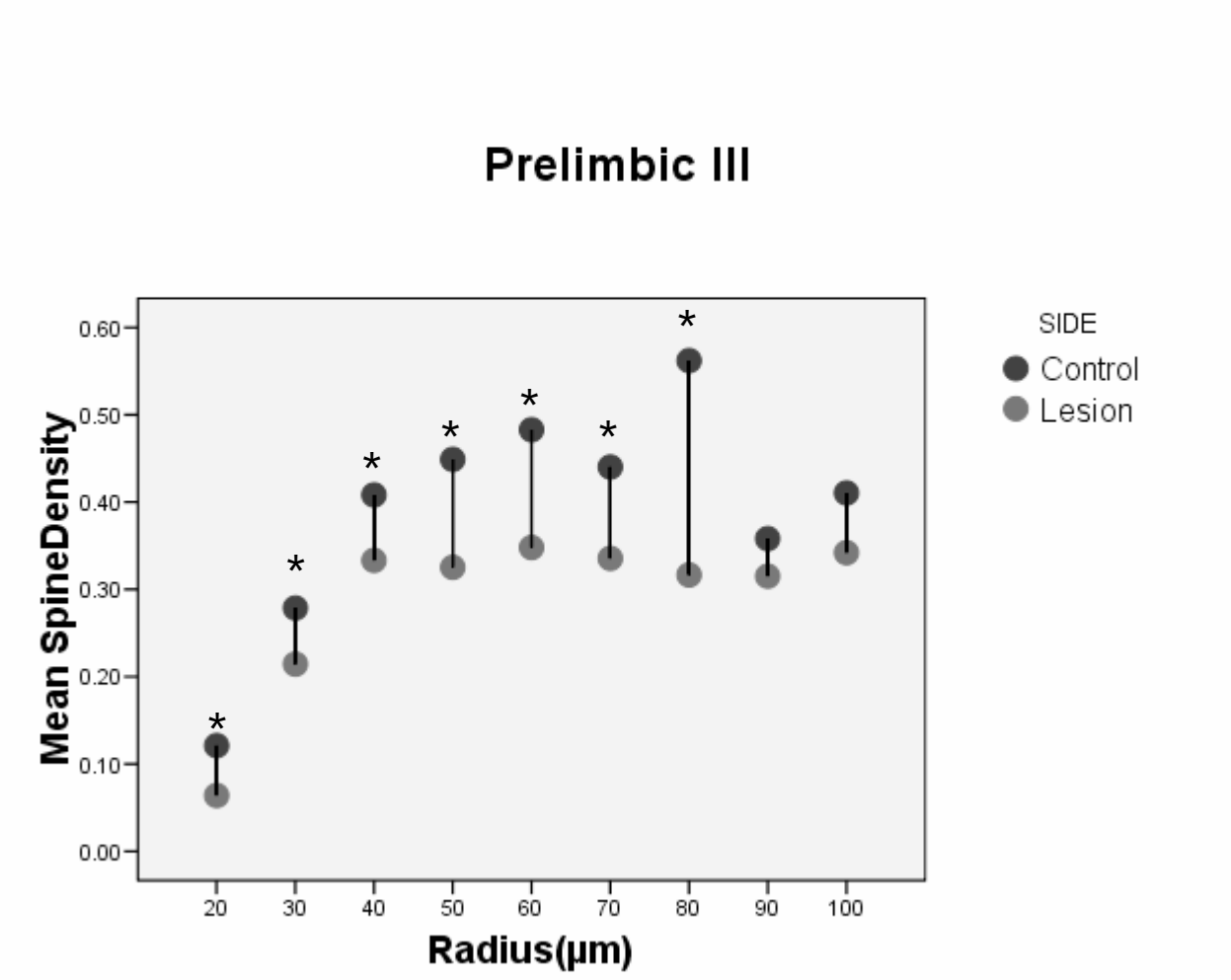


Figure 10. In Prelimbic cortex, superficial layer, spine density analysis revealed decreased density levels for pyramidal cells in the lesioned hemisphere for the proximal dendritic segments (when the radius of the concentric shell intersecting the cell was 20µm) and along the more distal segments as well. There was no significant difference in spine density for the most distant segments (radius ≥ 90).

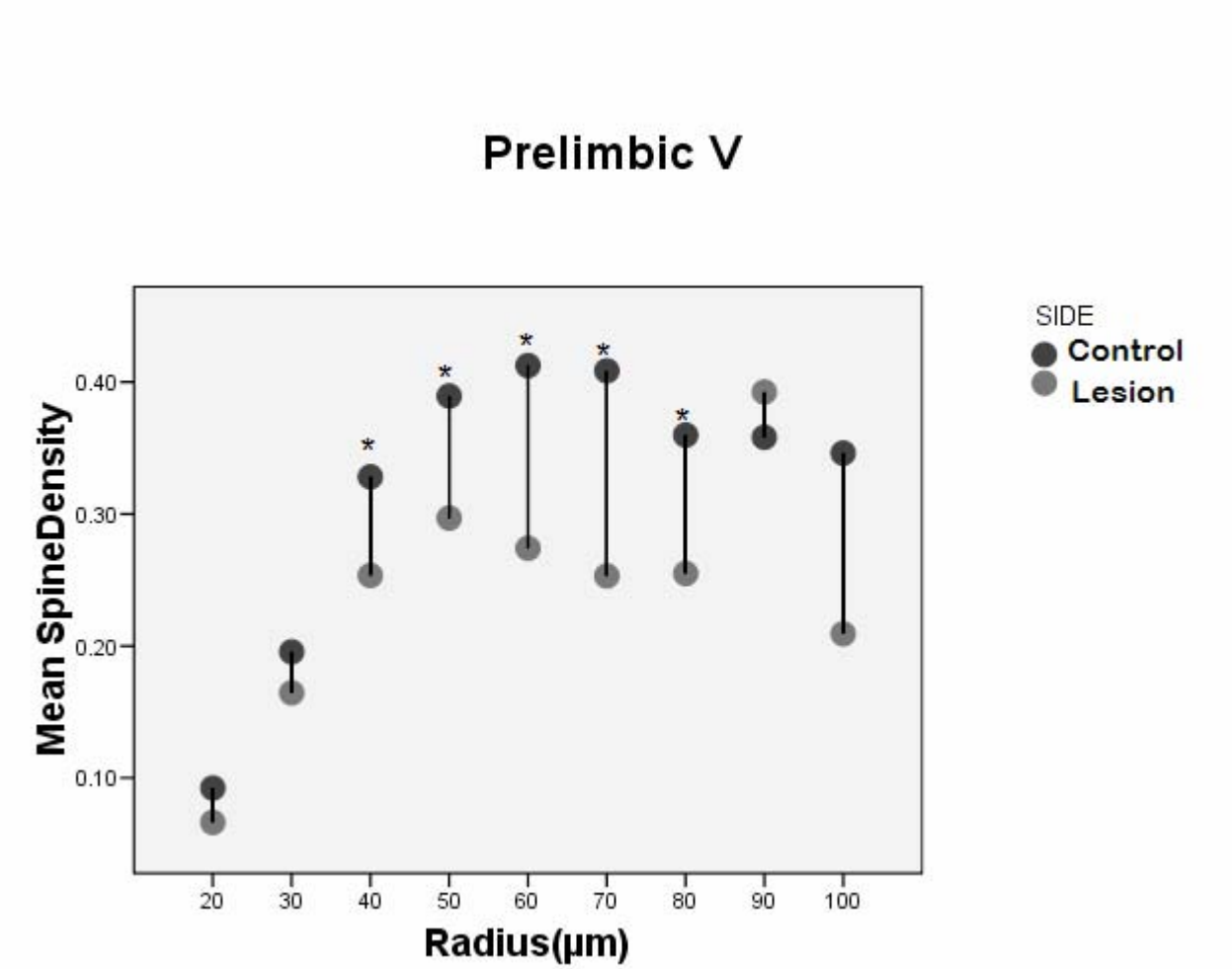


Figure 11. The mean spine density for proximal segments suggested a decreasing trend in the lesioned side, but not to a significant degree. For the more distal segments, neurons in the lesioned side did show significantly reduced spine densities as compared to controls, except at the most distant points (radius ≥ 90µm).

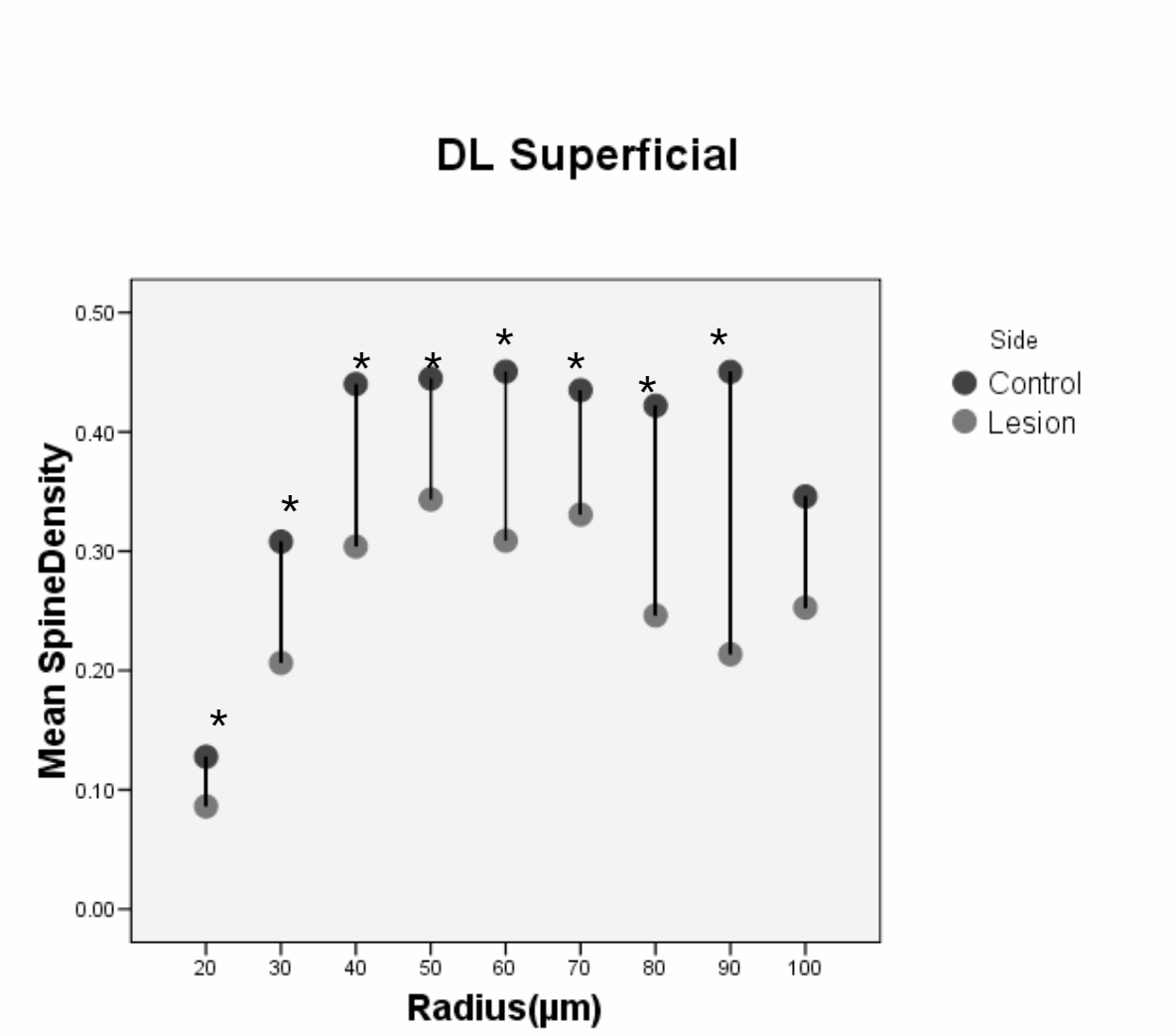


Figure 12. Data showed a reduction in spine density for pyramidal cells in the superficial Dorsolateral anterior cingulate cortex, which was evident along dendritic segments close to the soma, as well as with increasing distance away from the soma. The difference in spine density was not significant only when the radius of the intersecting shell was 100µm.

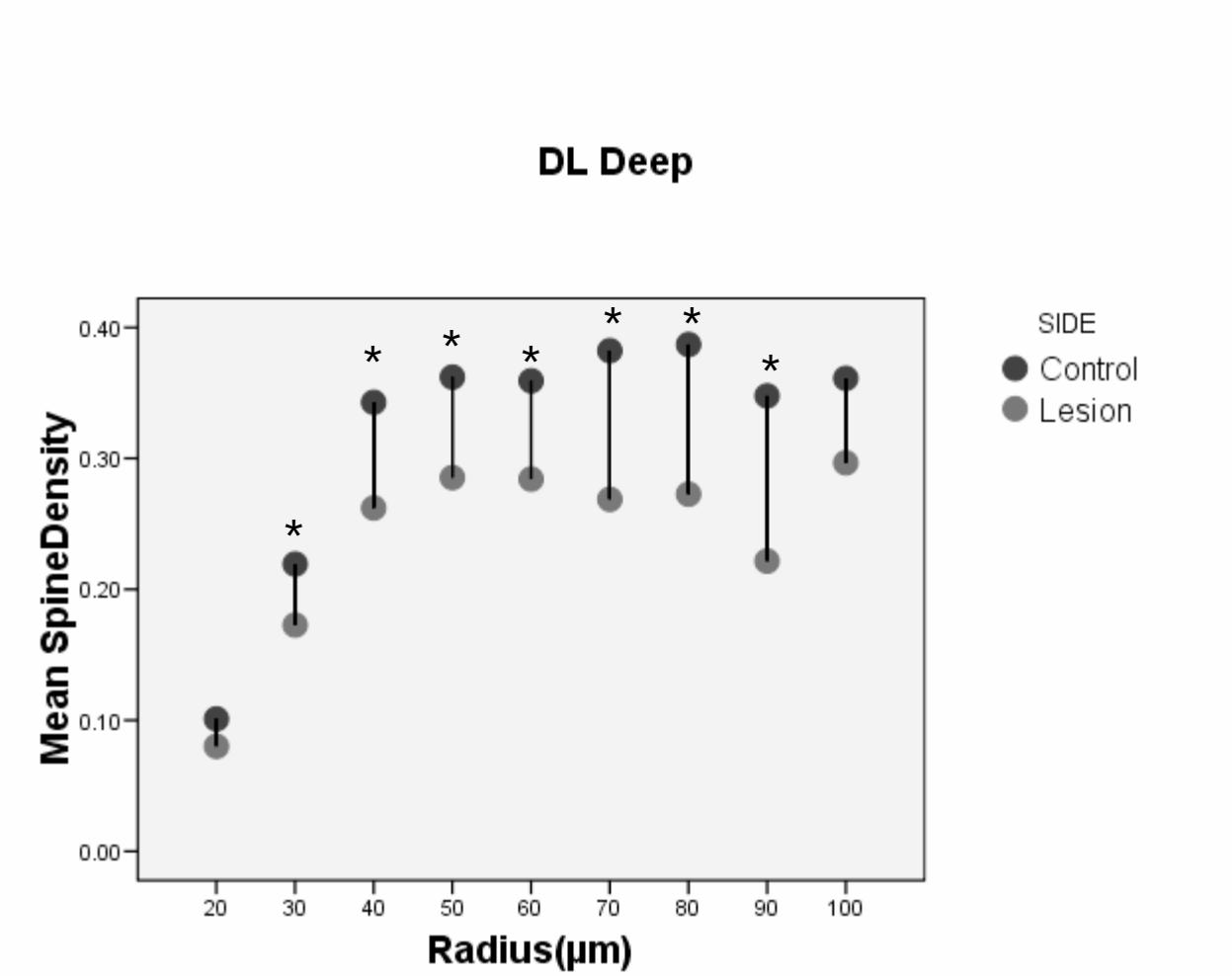


Figure 13. Proximal dendritic segments (radius=20µm) of pyramidal cells in the DL deep cortex did not show a significant difference in the mean spine density between control and lesion sides. The difference in mean spine density increased with increasing distance from the soma, except when radius=100µm.

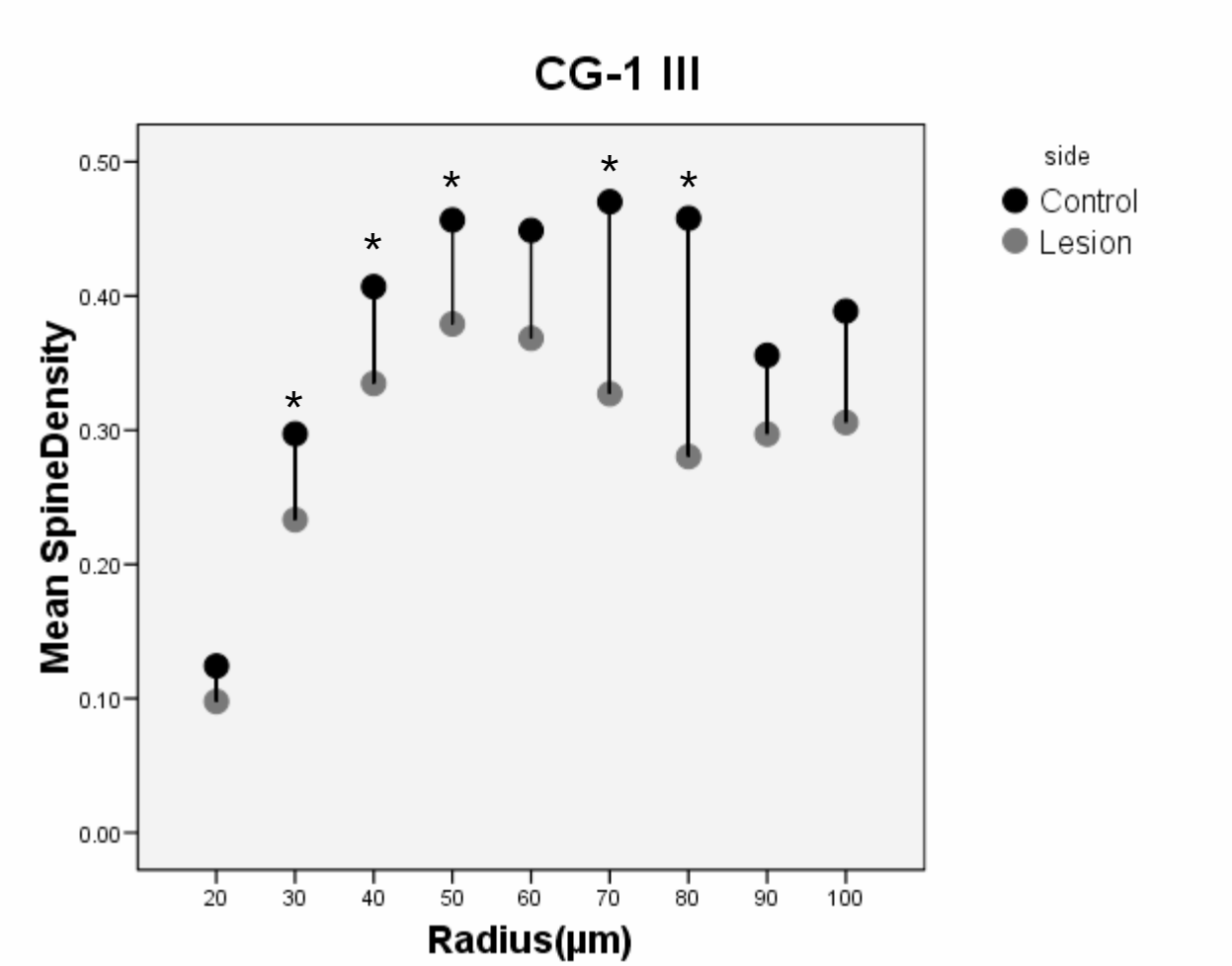


Figure 14. In CG-1 superficial layer, the mean spine density for pyramidal cells in the control hemisphere is higher than for cells in the lesioned side. Significant difference in density is reached at certain distances from the soma (radius: 30, 70, 80µm), however, the lesion side exhibits a lower trend in density.

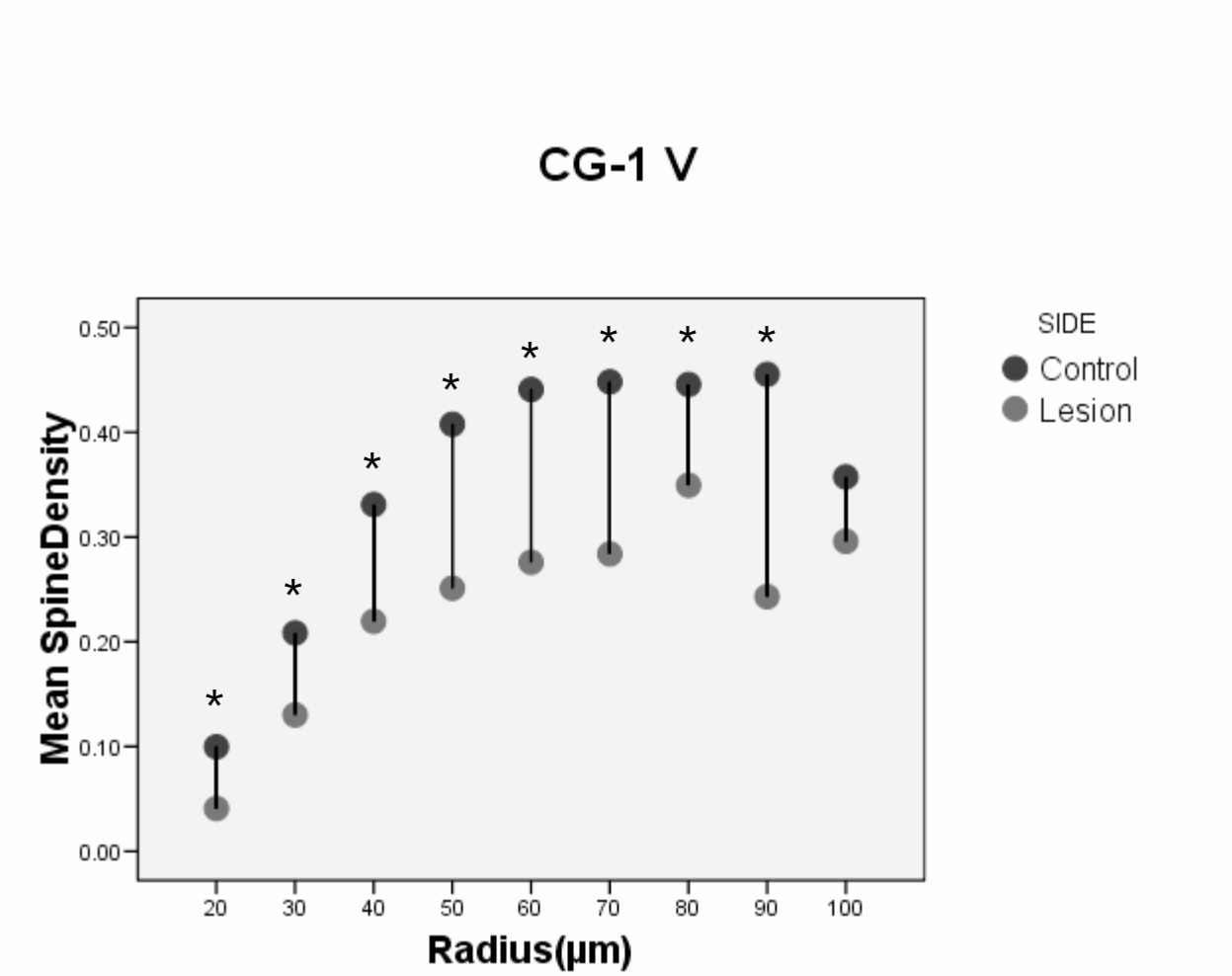


Figure 15. In the deep CG-1 subregion, mean spine densities were decreased for neurons in the lesioned side as compared to neurons in the control side. The reduction was significant for all proximal and more distal dendritic segments, except for the most distant point (radius=100µm).

Chapter 5: Discussion

This project was undertaken to create an animal model for schizophrenia by focusing on the special circuitry and close connection between the mediodorsal nucleus of the thalamus (MD) and the prefrontal cortex (PFC). These two regions were chosen as targets of investigation because several lines of evidence have shown they are intimately linked (Barbas et al., 1991; Pirot et al., 1994; Kuroda et al., 1995; Kuroda et al., 1998; Negyessy et al., 1998; Wang and Shyu 2004), and furthermore, both areas are affected in schizophrenia (Pakkenberg 1990; Pakkenberg 1992; Rajowska et al., 1998; Stone et al., 1998; Bunney and Bunney 2000; Conklin et al., 2000; Kalus et al., 2000; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Perry et al., 2001; Broadbelt et al, 2002; Byne et al., 2002; Jones et al., 2002; Selemon et al., 2003; Black et al., 2004; Broadbelt et al., 2006). The intricate connectivity between these two regions is evident since early development, when thalamic afferents influence the development and differentiation of the PFC in an activity dependent manner (Kuroda et al., 1995; Van Ooyan et al., 1995; Kossel et al., 1997). The late myelination of this circuitry, coinciding with the onset of symptoms (Benes 1989; Benes 2002) offers further evidence of the involvement of these two regions in schizophrenia. It was hypothesized that an early thalamic lesion could result in morphological alterations of pyramidal cells in the PFC, and these morphological changes were assessed by immunostaining profiles for microtubule-associated protein-2 (MAP2), as well as by the number of basilar dendrites and spine densities for pyramidal neurons in the PFC.

Area fraction analysis of MAP2 immunostaining showed that an average MD volume decrease of 12% was sufficient to cause a significant reduction in the staining profiles of this

protein in prefrontal cortical areas. The effect was similar across all three cortical regions and for both cortical layers without an apparent change in pyramidal cell density. MAP2 is used as a marker of dendritic integrity; it increases the spacing between microtubules in nascent dendrites leading to branching (Hely et al., 2001); consequently, a decrease in MAP2 during development could result in a loss of dendritic branching and sprouting for growing pyramidal cells.

Furthermore, early thalamic lesions showed an effect on the number of basilar dendrites in the PFC neurons. A mean MD volume decrease of 14.8% accompanied by a 23.4% loss of cells led to a substantial reduction of primary and secondary basilar dendrites across all three PFC regions. The effect was more substantial for secondary dendrites. Primary dendrites are formed early in development with no new ones formed after postnatal day 7, while the rest of the dendritic field remains plastic into adulthood (Petit et al., 1988), so that a loss of afferents after postnatal day 4 creates a more pronounced impact on the still pliable secondary dendrites. Since dendritic branches are induced by afferent innervation (Kossel et al., 1997), a decrease in the number of basilar dendrites may reflect diminished input from the thalamus. A loss of dendrites in superficial pyramidal neurons may primarily disrupt the transfer and integration of information between ipsilateral and contralateral cortical regions, while a deficit in the basilar dendrites of deep pyramidal cells would affect the exhange of information between cortical and subcortical regions, including feedback to the thalamus.

The alterations in the connection between the MD and the PFC were further investigated by examining pyramidal dendritic spines. An average MD volume decrease of 14.8% led to significant changes in the spine population for pyramidal cells in the PFC. Spines were analyzed in two ways; the averaged total number of spines along primary and secondary dendrites was estimated, and spine density (number of spines over dendritic length) was also calculated.

Analysis of mean spine populations showed a lesion effect that was weak for spines along primary dendrites, but prominent for spines along secondary dendrites where the reduction was statistically significant in all three cortical subregions and across superficial and deep layers. The more detailed spine density measurements showed a lesion effect for proximal as well as distal dendritic segments. Dendritic spines are induced and influenced by afferent activity (Berry 1974; Drakew et al., 1995; Kossel et al., 1997; Kirov and Harris 1999), and they are the predominant postsynaptic elements receiving input from thalamic afferents (Berry 1974; Kuroda et al., 1995). Hence an early lesion resulting in volume and cell loss in the MD affected the postsynaptic elements of this circuit, the dendritic spines on pyramidal neurons of the PFC.

Morphological effects were evident in the three prefrontal subregions studied. dorsolateral anterior cingulate (DL), prelimbic (PL) and anterior cingulate (CG-1) cortices were chosen as study targets because of the intricate interconnectivity with the MD. The medial MD sends projections to medial prelimbic and dorsal insular cortices, while the lateral MD sends afferents to anterior cingulate and medial precentral (dorsolateral anterior cingulate) cortices (Pirot et al., 1994; Paxinos 1985). The morphological alterations investigated, including the levels of MAP2, the number of basilar dendrites, and spine population and densities, were similar across all three PFC subregions. Because the medial and lateral MD project differentially (Pirot et al., 1994; Paxinos 1985), suggests that the lesions were precise and large enough to encompass the entire nucleus. The effects on MAP2 immunostaining, as well as the number of basilar dendrites did not show variation between the two cortical layers studied (see chapters 2, 3). A slight disparity between superficial and deep layers was seen only for the number of spines along secondary dendrites: 32.4% and 37.2% fewer spines in the superficial leayers of PL and DL respectively, versus 41.6% and 47.6% fewer spines in the deep layers of the same

subregions. No difference between the two layers was evident in CG-1. MD afferents terminate mainly on layer III of the PFC (Kuroda et al., 1995, Herkenham 1990); in turn, corticothalamic fibers arise predominantly from layers V and VI (Kuroda et al., 1995). The greater impact for spines in the deep layer may suggest a large degree of communication between the layers; an impact in layer III neurons exacerbating the effects for layer V neurons, which could further influence subcortical communication. The slight discrepancy between the two layers was only seen for spine population, and this may reflect the pliable nature of spines.

Previous investigations in schizophrenia employed histochemical protocols on postmortem tissue or used imaging techniques on diagnosed subjects. However, evidence suggests that rather than a degenerative disorder, schizophrenia is likely a developmental disorder (Benes et al., 1986; Pakkenberg 1990; Cannon et al., 2000; Falk et al., 2000; Popken et al., 2000; Young et al., 2000; Niemi et al., 2003; Rapoport et al., 2005). Schizophrenia is frequently diagnosed post pubescence, when most developmental milestones have passed. Scientists are challenged to investigate the developmental factors of this devastating disorder using these techniques. Therefore research steers towards animal models that can be manipulated to investigate particularities of this disorder and its developmental components.

The general drawback of an animal model is precisely the contention that schizophrenia is a uniquely human disorder. It is impossible to capture the entire complexity of this disorder in one type of animal model. It is possible, however, to create a relevant animal model by focusing on one aspect of the disorder, such as the connection between two specific regions. The rat is an efficient medium for a developmental animal model for several reasons: significant brain development and synaptogenesis occurs perinatally (Wise et al., 1979; Van Eden et al., 1986; Petit et al., 1988; Molnar et al., 1998); the nervous development of this animal is well

documented, and it possess similar cortical regions with reciprocal connections to subcortical structures homologous to human anatomy (Paxinos 1985). Therefore, this animal model created by a lesion performed on the MD of rat pups during the first week of life is a viable option to investigate the complex relationship between the MD and the PFC, its developmental components, and its suspect role in the occurrence of schizophrenia.

It may be argued that a unilateral lesion may yield confounding results from the risk of commissural communication, and from the absence of MD medial separation by ventricles (Paxinos 1985). It is unlikely in this specific circuitry since MD innervation of the PFC is unilateral (Pirot et al., 1994; Negyessy et al., 1998). Furthermore, dendrites of MD neurons are confined to the nucleus, and even to within the segment of the MD where they arise (Kuroda et al., 1998). And although some thalamic afferents synapse onto callosal cells (Kuroda et al., 1998), only 3% of corticothalamic afferents project contralaterally to anterior cingulate areas (Negyessy et al., 1998), so that the threat of crossed fiber contamination may be negligible.

A unilateral lesion diminished variability between the experimental and control groups across the subjects; the ipsilateral hemisphere served as the experimental group, while the contralateral hemisphere was used as control. It may be argued that inherent laterality hinders this model, for it is unknown whether the results seen were a product of the experimental manipulations, or merely a reflection of naturally occurring interhemispheric differences. Structural asymmetries have not been reported in the rat thalamus itself, and although investigations have found cortical thickness asymmetries in the medial and orbital prefrontal cortices of the rat (Van Eden et al., 1984), no research has found cytological differences in these regions as pertaining to neuronal numbers or neuronal structure. Furthermore, we studied rat brains not subjected to electrolytic lesions and found no volumetric differences between the right

and left MD thalami. Area fraction analysis of the prefrontal cortical regions of nonlesioned animals showed no difference in immunostaining profiles between the hemispheres, and a multivariate analysis of variance showed no hemispheric difference in the number of basilar dendrites for pyramidal cells ($p=0.803$, $F=0.20$,) in any of the three prefrontal cortical subregions studied (data not shown). Therefore, the changes seen in pyramidal cells of the PFC likely resulted from the early MD lesions, and not from a naturally occurring asymmetry.

The loss of volume and cells in the MD in schizophrenic brains has been well documented (Pakkenberg 1990; Pakkenberg 1992; Popken et al., 2000; Young et al., 2000; Byne et al., 2001; Lewis et al., 2001; Byne et al., 2002), but the meachanism leading to such loss remains unknown. Schizophrenia is thought to be a multifactorial disorder because of the existence of subtypes, variety of prognoses, presence of genetic background, and variety of drug reactions. It is possible that the loss of cells in the MD of schizophrenics may result from disrupted apoptotic mechanisms ensuing polygenic mutations and environmental triggers. Future studies need to determine the possible cause for the loss of cells in this thalamic nucleus.

The data obtained in this project mirrors the results obtained from human schizophrenic brains (Garey et al., 1998; Broadbelt et al., 2002; Jones et al., 2002; Kalus et al., 2000) (see table 1). The similarity in the data obtained from postmortem material and that obtained in this project suggests this model may be an efficient vehicle to further investigate schizophrenia related effects on this circuit. The data shows that the pyramidal cells of the PFC display a compromised dendritic arbor, specifically a decrease in dendritic material. This is suggestive of a loss of synaptic surface area, which may translate into problematic information processing capabilities for these cells. The reported MD volume and cell loss was suspected to play a role in the morphological alterations of the PFC cells described above. And although this circuitry has

been suspect in schizophrenia research, the directionality of the effects has been impossible to decipher when using human substrate. The present animal model was successful in this effort and provides evidence that an early loss of MD cells and their afferents can cause morphological alterations on the pyramidal neurons of the PFC. The mechanism mediating these physical modifications in the pyramidal arbor is yet unknown, but calcium is a likely suspect. Calcium cascades play a pivotal role in activity dependent development of neurites (Chakravarty et al., 1974; Hely et al., 2001; Ramakers et al., 2001). Elevated calcium in the cytosol participates in the direct or indirect activation of calcium-dependent protein kinases (Bito et al., 1997) including calmodulin protein, CamKII, MAP2, neurogranin, among others. Some of these protein kinases activate transcriptional factors, which induces de novo protein synthesis (West et al., 2001). Examination of schizophrenic brains has shown reductions in immunostaining profiles for several calcium dependent proteins, including MAP2 (Jones et al., 2002), neurogranin (Broadbelt et al., 2006) and calmodulin (submitted manuscript). Implying there is an alteration in the calcium cascades associated with schizophrenia. This may be the future direction for this animal model, to further investigate the role of calcium cascades on the development of dendritic arbors by examining neurogranin, and calmodulin levels in the PFC after early thalamic damage.

SCHIZOPHRENIC			RAT	MODEL	
MAP 2	III	V		III	V
Area 9	40%	45%	Dorsolateral	28%	26%
Area 32	32%	44%	Prelimbic	26%	34.7%
Dendrites	Area 32		Dendrites	Prelimbic	
Primary	17.4%	29%	Primary	25%	24.8%
Secondary	15%	46%	Secondary	39.7%	31.8%
Spines	40.6%		Significant	Decrease	All areas

Table 1. Comparison of morphological alterations reported in postmortem schizophrenic brains to those structural changes in the PFC following an early thalamic lesion. The resemblance of the results suggests that early damage to the MD is sufficient to cause the structural alterations reported in the PFC of schizophrenics, and is hence an efficient model for the disorder.

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