RESEARCH PLAN

A. SPECIFIC AIMS

Alterations in specific subsets of γ-aminobutyric acid (GABA) expressing interneurons are one of the most consistent findings in schizophrenia. These changes are found in many brain regions, including the prefrontal cortex, hippocampus, and cerebellum. Specifically these interneurons express lower levels of the 67kDa isoform of GABA synthesizing enzyme glutamic acid decarboxylase (GAD_{67}) and the presynaptic GABA reuptake transporter GAT-1. Since GABAergic interneurons, such as chandelier cells in the prefrontal cortex and Golgi cells in the cerebellum, modulate the synchronicity and firing patterns of excitatory cells, dysfunction in these cells may lead to aberrant synaptic transmission such as that seen in schizophrenia. GABAergic interneurons are activated by glutamate sensitive N-methyl-D-aspartate (NMDA) receptors, another component proposed as dysfunctional in schizophrenia. The glutamate hypothesis of schizophrenia was developed because activity dependent NMDA receptor antagonists such as phencyclidine (PCP) produce schizophrenia-like symptoms in healthy adults and animal models. These antagonists preferentially block the NMDA receptors in GABAergic interneurons and potentially alter GABAergic function. Studies show that chronic intermittent exposure to PCP in animal models mimics some of the molecular, behavioral, and physiological dysfunction seen in patients with schizophrenia. Work in this proposal will test the **overall hypothesis that *an NMDA receptor hypofunction in specific subsets of GABAergic interneurons, specifically Golgi cells, underlies cerebellar abnormalities in schizophrenia.*** This hypothesis will be examined in the following specific aims:

Specific Aims:

1) Are the levels of GABAergic markers and NMDA receptor subunits altered in the cerebellum of patients with schizophrenia?

These studies will test the hypothesis that the cerebellum of patients with schizophrenia exhibits GABAergic expression deficits as seen in other brain regions and that this is associated with NMDA receptor hypofunction.

Quantitative real-time PCR (qRT-PCR) will be used to measure the levels of GABAergic markers and NMDA receptor subunits in cerebellar tissue from schizophrenic patients and control subjects. Results will be analyzed by using paired *t* tests. ANCOVA analysis will also be performed to co-vary for age, post-mortem interval, and brain pH. To further examine the effects of medication on the expression of these genes, rats will be treated with either haloperidol or clozapine and their gene expression levels will be evaluated using qRT-PCR.

2) Are the GABAergic and NMDA receptor gene expression changes seen in patients with schizophrenia replicated in an animal model?

These studies will test the hypothesis that chronic intermittent exposure to low levels of the activity dependent NMDA channel blocker PCP will elicit the same GABA related gene expression changes in an animal model as those seen in patients with schizophrenia.

qRT-PCR will be used to examine the expression of GABAergic markers and NMDA receptor subunits in cerebellar tissue from PCP treated rats. Results will be analyzed by comparing the values of PCP treated versus saline treated rats using *t* tests.

3) Do the changes in mRNA levels of GAD_{65} and GAD_{67} localize to a particular cell type in both schizophrenic patients and in the animal model?

These studies will test the hypothesis that Golgi cells, the major granule layer interneurons, are accountable for the decreases in mRNA levels of GABA markers seen in both schizophrenia and PCP treated rats.

Radio isotopic quantitative in situ hybridization will be used to measure GAD_{65} and GAD_{67} mRNA levels in Golgi cells and other cerebellar GABAergic neurons.
The number of cells and the number of grains within each cell type will be quantified and compared between experimental and control conditions.

B. BACKGROUND AND SIGNIFICANCE

Schizophrenia

Schizophrenia is a complex and devastating neuropsychiatric disorder affecting approximately 1% of the world's population. An estimated $58 million is spent annually dealing with the direct and indirect consequences of schizophrenia in the United States (Uhl and Grow, 2004). Both patients and their families are affected by the damaging constellation of symptoms caused by the disease. Schizophrenia has both genetic and environmental components and is characterized by positive (hallucinations, delusions, thought disorders), negative (flat affect, alogia, avolition) and cognitive (impairments in learning and specific areas of memory, i.e. working memory) symptoms (American Psychiatric Association, 2000; Lewis and Lieberman, 2000). These symptoms tend to manifest during late adolescence to the mid-twenties. They may be overwhelming and may contribute to suicide committed by 10% of schizophrenic patients. Classical treatments for schizophrenia involve the administration of typical (haloperidol or chlorpromazine) or atypical (clozapine, olanzapine, or risperidone) antipsychotics. While these medications manage positive symptomology effectively, further research needs to be performed in an effort to effectively treat the negative and cognitive symptoms.

Role of Glutamate and GABA in schizophrenia

While dopamine dysfunction in schizophrenia is widely accepted, a growing body of evidence suggests the involvement of glutamate and GABA alterations in this illness. The glutamate hypothesis of schizophrenia states that symptoms are related to hypofunction of NMDA receptor ion channels. Each channel consists of an obligatory NR1 subunit and co-assembles with any of the NR2A-D subunits, each conferring upon the channel distinct electrophysiological and biochemical properties. Channels containing NR2A and NR2B are thought to be high conductance channels while NR2C and NR2D containing channels are low conductance channels (Misra et al., 2000). NR2C and NR2D are more prone to NMDA receptor antagonism due to less Mg^{2+} block and longer open channel time than either NR2A or NR2B (Monyer et al., 1994). These subunits localize more to interneurons that express GABA in the hippocampus (Grunze et al., 1996). Other GABAergic interneurons express different combinations of NR2 subunits, such as seen in Golgi cells in the cerebellum, which express the NR2B and NR2D subunits (Misra et al., 2000; Brickley et al., 2003). Furthermore the NR2B subunit is more prone to antagonism than is the NR2A subunit (Chaperon et al., 2003). Decreased expression or selective block by NMDA receptor antagonists will alter the functional properties of channels containing specific subunits. These alterations may lead to downregulation of gene expression, diminished LTP, or aberrant development, all important in the dysfunction seen in schizophrenia.

NMDA receptor hypofunction has been characterized in different brain regions in schizophrenic patients. Receptor subunits have been shown to be altered in the prefrontal cortex (Akbarian et al., 1996) and single positron emission tomography (SPET) studies have shown decreased NMDA receptor binding in the hippocampus in schizophrenics (Pilowsky et al., 2006). Studies of NMDA receptor antagonists such as phencyclidine (PCP), ketamine, and MK-801 further implicate hypofunction of these channels in schizophrenia (Coyle, 2006). Administration of these agents elicits symptoms similar to that of schizophrenia in healthy adults, with negative and cognitive symptoms as well as overt psychosis (Adler et al., 1999), and exacerbation of symptoms in schizophrenic patients. NMDA receptor hypofunction, particularly on specific subsets of GABAergic interneurons, may be central to schizophrenia (Lewis and Gonzalez-Burgos, 2000; Coyle, 2006). These findings may be of great importance since GABAergic interneurons have been shown to be an essential component in modulating the synchronization of neuronal activity necessary for highly demanding cognitive tasks (Traub et al., 1996).

GABA is synthesized by the 65kDa and 67kDa forms of glutamic acid decarboxylase (GAD_{65} and GAD_{67}). Different genes code for each enzyme (Erlander et al., 1991). GAD_{65} protein is present in greater abundance in axon terminals while GAD_{67} localizes preferentially to the soma. However, mRNA for both is shown in the cell bodies of GABA positive cells (Esclapez et al., 1994). Additionally, GAD_{67} expression is more activity dependent than GAD_{65} as seen by increased levels in models of epilepsy (Sloviter et al., 1996; Freichel et al., 2006). Once synthesized, GABA is then released mainly by interneurons throughout the brain. These interneurons modulate activity by either synchronizing and/or inhibiting excitatory cells. The presynaptic GABA reuptake transporter (GAT-1) removes any remaining GABA from the synaptic cleft. Therefore, GAD_{65}, GAD_{67}, and GAT-1 are crucial to control GABA mediated transmission and overall brain function.

GABA dysfunction in certain subsets of GABAergic interneurons is one of the most consistent findings in the study of schizophrenia (Woo et al., 2004; Lewis et al., 2005; Torrey et al., 2005; Akbarian and Huang, 2006). Reductions in mRNA and protein levels of GAD_{67} were reported in the prefrontal cortex (PFC) (Volk et al., 2000; Hashimoto et al., 2003; Lewis et al., 2004), the hippocampus (Benes and Berretta, 2001; Heckers et al., 2002), and the cerebellum (Guidotti et al., 2000; Fatemi et al., 2005). Chandelier and basket interneurons in the PFC show decreases in the mRNA levels for GAT-1 as well (Volk et al., 2001). Postsynaptic changes such as increases in $GABA_A$ $\alpha 2$ receptor density and $GABA_A$ receptor radioligand binding in the PFC and anterior cingulate cortex were also observed (Benes et al., 1992; Benes et al., 1996; Volk et al., 2002). In addition to these findings, single nucleotide polymorphisms in the promoter region of the GAD_{67} gene have been found and were shown to be associated with reductions in grey matter in patients with childhood-onset schizophrenia (Addington et al., 2005). Considering the role of GABAergic interneurons in the modulation of excitatory output, it can be hypothesized that dysfunction in these cells may mediate some of the positive, negative, and cognitive symptoms seen in schizophrenia (Spencer et al., 2004).

Cerebellar contributions to schizophrenia

Traditionally most of the research performed in the field of schizophrenia has focused on brain regions directly implicated in the symptomology of the disease, mainly the prefrontal cortex and limbic areas. However Andreasen *et al* (1997) have implicated the cerebellum as an affected structure in schizophrenia through the cortico-cerebellar-thalamic-cortical circuit (CCTCC). Dysfunction in one area of this circuit will affect all other areas of the circuit. As a component of the CCTCC, the lateral hemispheres of the cerebellum have been implicated in cognitive and emotional functioning (Schmahmann and Sherman, 1998). Retroviral tracing and neuroimaging studies have shown cerebellum-prefrontal connections (Middleton and Strick, 2001) which may have a role in cognitive dysfunction in schizophrenia. These cognitive aspects were shown to localize to the lateral hemispheres of the cerebellum, particularly in crus I and crus II of lobule VII (Kelly and Strick, 2003; Ramnani, 2006). Therefore lateral cerebellar hemisphere connections link the cerebellum to cognitive functioning and subsequently in schizophrenia.

Clinically patients exhibit cerebellar neurological signs (Ho et al., 2004), deficits in eyeblink conditioning (Sears et al., 2000), and shortfalls in timing responses (Brown et al., 2005). Neuroimaging studies have shown increases in blood flow and in glucose consumption in the cerebellum of schizophrenic patients relative to that of other brain regions (Andreasen et al., 1997; Kim et al., 2000; Potkin et al., 2002; Malaspina et al., 2004). In accordance with increased cerebellar activity, our laboratory found that activity dependent genes expressed by granule cells, GAP-43 and BDNF, are upregulated in the cerebellum (Paz et al., 2006). Additional molecular studies have shown decreased expression of the development markers reelin and semaphorin 3A as well as protein for the GABA synthesizing enzymes GAD_{65} and GAD_{67} (Guidotti et al., 2000; Eastwood et al., 2003; Fatemi et al., 2005). This is interesting

considering the fact that GABA dysfunction in the cerebellum may lead to increases in granule cell firing. This, in turn, could account for the increases seen in blood flow, glucose utilization, and the expression of activity dependent genes. These changes ultimately impact cerebellar contributions to cognitive (and motor) functioning.

Apart from the cognitive aspects of the cerebellum, other factors make this region particularly interesting for the study of schizophrenia. The local circuitry of the cerebellum with the granule cell-Golgi cell interaction can be compared morphologically and physiologically with the pyramidal cell-chandelier cell interaction in the PFC implicated in schizophrenia (Lewis et al., 2005). The excitatory granule cell of the cerebellum receives input from pontine mossy fibers. Activation by mossy fibers excites granule cells and causes them to activate Purkinje cells, which are the sole output of the cerebellum, via parallel fibers. This interaction is modulated by the tonic inhibitory feedback mechanism inherent to the Golgi cell (Watanabe et al., 1998; Hirano et al., 2002) (**Figure 1**). Dysfunction of the GABAergic cell in this circuit leads to a dysregulation of firing by the glutamatergic cell and potentially to the increases in activity and the subsequent changes seen by neuroimaging and molecular studies. Golgi cells express GABA synthesizing enzymes GAD_{65} and GAD_{67} as well as GAT-1 (Takayama and Inoue, 2005) and the NMDA receptor subunits NR2B and NR2D (Misra et al., 2000; Brickley et al., 2003). These subunits are preferentially targeted by NMDA receptor antagonists such as PCP (Grunze et al., 1996; Rujescu et al., 2006). Altogether these data point to Golgi cells as being players in the proposed NMDA receptor-mediated GABAergic dysfunction in schizophrenia.

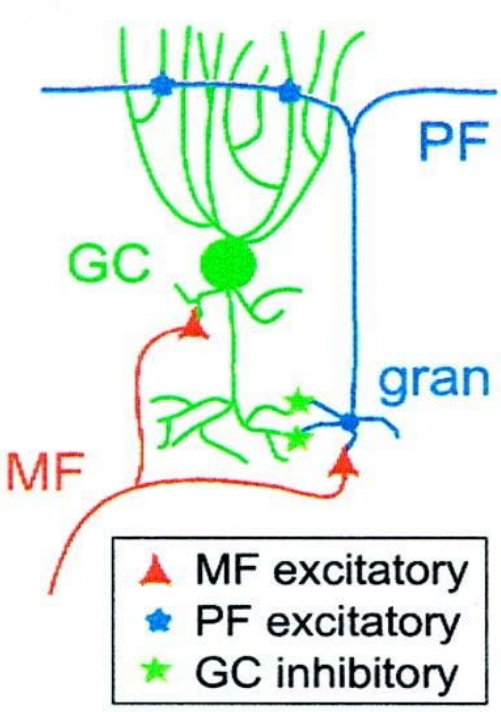

Figure 1: Golgi cell circuit. Granule cells are excited by pontine mossy fibers. Granule cells synapse on Purkinje, basket, stellate, and Golgi cells. The Golgi cells provide inhibitory feedback to the granule cell for synchronized activity (Vos et al., 1999).

In addition to Golgi and Purkinje cells, basket and stellate cells are the other major GABAergic interneurons in the cerebellum. Basket cells and stellate cells both provide inhibitory modulation to Purkinje cells. Basket cells synapse on the proximal dendrite, soma, and axon of the Purkinje cell while stellate cells synapse on the distal dendritic tree (Takayama and Inoue, 2005). Both cell types modulate Purkinje cell output (Midtgaard et al., 1993). Basket and stellate cells both express GAT-1 (Takayama and Inoue, 2005) while Purkinje cells only express GAT-1 transiently during development (Yan and Ribak, 1998). While Golgi cells influence Purkinje cell function through granule cell modulation, a decrease in basket and/or stellate cells activity directly contributes to unregulated Purkinje cell firing. This unregulated activity of Purkinje cells mediated by GABAergic interneuron dysfunction may contribute to cerebellar pathology in schizophrenia.

Of further interest, the lateral hemispheres of the cerebellum do not contain any direct dopaminergic input (Schweighofer and Doya, 2003), making it a more purely glutamate/GABA system than is seen in other brain regions with dopaminergic input such as the PFC. This is useful in determining the direct effects of schizophrenia with minimal effects from the medication. In addition, the cerebellum has intrinsic neuroprotective pathways whereby low level basal NMDA receptor activation and subsequent TrkB activation by BDNF dampen

excitotoxicity (Jiang et al., 2003; Wu et al., 2005; Xifro et al., 2005). This may be related to the increase in blood flow and glucose consumption seen in neuroimaging studies and in increased expression of activity dependent genes. Taken as a whole these findings indicate the cerebellum participates in the pathophysiology of schizophrenia.

Medication effects on gene expression

Antipsychotic medication by itself can differentially affect gene expression in distributed brain regions. Typical antipsychotics, such as haloperidol, were found to exhibit conflicting effects on the mRNA levels of GAD_{67} or GAT-1. Using *in situ* hybridization, Zink *et al* (2004) showed that haloperidol increased mRNA levels of GAD_{67} or GAT-1 in rats. Lipska *et al* (2003) and Laprade and Soghomonian (1995) also reported increased levels in the striatum, nucleus accumbens, and PFC in rats. Conversely Volk *et al* (2000, 2001) report no change in expression of these levels in non-human primates in the PFC. Furthermore, using magnetic resonance spectroscopy (MRS), Bustillo *et al* (2006) found that typical antipsychotics do not alter the levels of glutamate, glutamine, or N-acetylaspartate (NAA), a putative marker of cellular integrity. These findings are thought to be mediated through the proposed mechanism of D2 receptors blockade (Lipska et al., 2003). Interestingly these receptors are not present in the lateral hemispheres of the cerebellum. On the other hand, atypical antipsychotics have been shown to upregulate the GABAergic markers GAD_{67} and GAT-1 in prefrontal and limbic areas through unknown mechanisms (Zink et al., 2004a; Zink et al., 2004b). Also anti-apoptotic genes such as Bcl-2 were found to be upregulated by atypical antipsychotics, seemingly imparting neuroprotection (Bai et al., 2004). These same drugs also show alterations in NMDA receptor subunit expression (Hanaoka et al., 2003) and reversed the functional hyperactivity produced by NMDA receptor antagonists such as PCP (Ninan et al., 2003). In light of these data, medication effects seem to be significant contributors to the overall cellular and molecular changes observed. These neuroleptics need to be researched further in order to understand their effects in schizophrenia.

Phencyclidine

Phencyclidine (PCP) is a non-competitive NMDA receptor antagonist. Initially used as a surgical anesthetic, its use was discontinued because it caused symptoms very similar to schizophrenia (Jentsch and Roth, 1999; Morris et al., 2005; Hajszan et al., 2006). Like MK-801, PCP binds in the pore of open NMDA channels near the ion selectivity filter (Ferrer-Montiel et al., 1998), blocking cation flow though the channel. It selectively blocks NMDA channels located on GABAergic interneurons (Grunze et al., 1996). Thus PCP disrupts GABAergic interneurons from modulating the glutamatergic cells with which they synapse leading to increased glutamate release (Jackson et al., 2004). This potentially allows for downstream excitotoxic effects, such as dendritic damage and/or cell death. Because of this, areas more susceptible to excitotoxic damage, such as the PFC, are more vulnerable to the effects of PCP administration whereas areas such as the cerebellum, with its neuroprotective mechanisms, are not (Jiang et al., 2003; Wu et al., 2005; Xifro et al., 2005). NMDA receptor hypofunction may then be accountable for the neuroimaging findings of hypofrontality (Andreasen et al., 1992; Wolkin et al., 1992). Thus animal models utilizing NMDA receptor blockade through PCP may be an effective tool in studying the pathophysiological processes cardinal to schizophrenia.

Animal model

Animal models exploiting the GABA/glutamate system have proven useful in studying the underlying pathophysiology of schizophrenia. Models include picrotoxin induced antagonism of $GABA_A$ receptors in rats (Berretta et al., 2001), genetic knockdown of NMDA NR1 subunit in mice (Mohn et al., 1999), and NMDA receptor blockade by NMDA receptor antagonists in both rodents and non-human primates (Jentsch et al., 1997b; Jentsch et al.,

1997a; Jentsch and Roth, 1999; Cochran et al., 2003; Morris et al., 2005). All these models affect the GABA/glutamate balance in different manners, but only the PCP model has shown the GABAergic and NMDA receptor changes seen in schizophrenic patients (Lindahl and Keifer, 2004; Rujescu et al., 2006).

PCP causes many of the symptoms inherent to schizophrenia in humans. Studies are now being conducted focusing on administering the compound to rodents and primates to induce a schizophrenia-like phenotype in the animals. Acute and chronic dosing regimens show differential and often opposing effects in rodents (Jentsch and Roth, 1999). Immediately after administration of PCP neurons of the PFC show an initial excitation as seen by activation of early immediate genes (Gao et al., 1998). This is followed by a period of cortical depression as described in glucose utilization studies (Gao et al., 1993), presumably as a compensatory mechanism. Acute PCP also produces schizophrenia-like symptoms including social withdrawal (Sams-Dodd, 1997), impaired sensory motor gating (Mansbach and Geyer, 1989) and cognitive dysfunction (Jentsch et al., 1997b; Jentsch et al., 1997a). Chronic low dose administration of PCP in rodents showed decreases in metabolic activity in the prefrontal cortex, auditory cortex, hippocampus, and reticular nucleus of the thalamus (Cochran et al., 2003), all regions affected in schizophrenia. Along with this decrease in metabolic function, decreases in parvalbumin expression were also seen (Cochran et al., 2003). This mirrors the chandelier and basket cell dysfunction seen in the prefrontal cortex of schizophrenic patients (Hashimoto et al., 2003). Additionally levels of NAA and its metabolite NAAG are also altered with chronic PCP similar to that seen in schizophrenic patients (Reynolds et al., 2005; Bustillo et al., 2006). Taken together, the data suggest that the model Cochran *et al* (2003) proposed of chronic intermittent exposure to moderate levels of PCP is the most functionally and neurochemically relevant animal model based on NMDA receptor hypofunction.

Goals of the proposed study

GABAergic dysfunction and NMDA receptor hypofunction have been characterized in schizophrenia in multiple brain regions but have not been as extensively studied in the cerebellum. From alterations seen in specific subsets of GABAergic interneurons in the prefrontal cortex and limbic areas, I propose to test a hypothesis that an NMDA receptor hypofunction in GABAergic interneurons, specifically Golgi cells, underlies the pathophysiology of schizophrenia in the cerebellum. Specific Aim 1 involves characterizing both the levels of GABAergic marker and NMDA receptor subunits in the cerebellum of post-mortem schizophrenic patients versus pair matched controls. Any significant changes will be evaluated for typical and atypical medication effects. Experiments in Specific Aim 2 intend to compare the efficacy of chronic low dose PCP administration as an animal model of GABAergic dysfunction mediated by NMDA receptor hypofunction. Finally, Specific Aim 3 will attempt to localize changes in the GABAergic markers GAD_{65} and GAD_{67} to specific interneurons in the cerebellum of both post-mortem schizophrenic patients and PCP treated rats. Overall, the study will test the hypothesis that NMDA receptor dependent alterations in specific subsets of GABAergic interneurons underlie the abnormalities in cerebellar function in patients with schizophrenia.

Specific Aim 1: Are the levels of GABAergic markers and NMDA receptor subunits altered in the cerebellum of patients with schizophrenia?

SA1 Rationale

There is evidence that GABAergic interneurons in distributed brain regions are dysfunctional in schizophrenia. While many studies show altered levels of GABAergic markers and NMDA receptor subunits in post-mortem brain tissue, there has not been much evidence of these changes in the cerebellum. The few studies focusing on the cerebellum examined the vermis, not the lateral hemispheres where cognition is implicated. Additionally, the effects of

both typical and atypical neuroleptics on these markers have not been addressed. *Experiments in this aim will test the hypothesis that the expression levels of both GABAergic markers and NMDA receptor subunits are altered in the cerebellum of post-mortem schizophrenic patients and that typical and atypical medications exert differential effects on the expression of these genes.*

To address this hypothesis, human cerebellar tissue from the lateral hemispheres of post-mortem schizophrenic patients and age and post-mortem interval (PMI) matched control subjects was acquired from the Maryland Brain Collection. qRT-PCR will be performed to determine the relative expression levels of GABAergic markers and of NMDA receptor subunits. Furthermore, rodents will be administered either haloperidol or clozapine chronically. The same markers will be examined for gene expression changes due to medication effects.

Experimental Design

Evaluation of GABAergic markers

Initial studies will attempt to characterize the mRNA levels of GAD_{65}, GAD_{67}, and GAT-1 in post-mortem cerebellar tissue from crus I of VIIA. Total RNA will be isolated from 13 patients with schizophrenia and 13 control subjects and verified for integrity. Integrity of samples with an $OD_{260/280} \geq 1.8$ will be validated using a Bioanalyzer 2100 (Agilent Technologies; Santa Clara, CA). Samples with RNA integrity numbers (RIN) ≥ 8.0 will be considered intact. cDNA will be synthesized using M-MLV reverse transcriptase (Promega; Madison, WI). Primer Express 3.0 (Applied Biosystems; Foster City, CA) will be used to design primer pairs specific to each gene. Genetic sequence information will be acquired using the Ensembl Genome Browser (http://www.ensembl.org) for qRT-PCR primer pairs. The primers will span introns as a control for genomic DNA contamination. These genes will be normalized to the housekeeping gene β-actin. β-actin is chosen as the housekeeping gene for these experiments because it shows no change in our microarray analysis in contrast to GAPDH.

Each primer will be validated first according to Applied Biosystems SYBR Green protocol. Briefly, using an Applied Biosystems 7500 Fast quantitative Real-Time PCR machine, qRT-PCR employing SYBR Green Master Mix (Applied Biosystems; Foster City, CA) will be run to validate each gene using a pooled sample of cDNA. The change in the cycle threshold values, defined as $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\beta\text{-actin}}$ (Schmittgen et al., 2000; Livak and Schmittgen, 2001), for each dilution will be calculated versus β-actin. The ΔCt's for each primer pair will be plotted on a semi-log graph. These will be considered valid if the slope of their trendline is $\leq |0.1|$.

qRT-PCR for each of the 13 schizophrenic and 13 control subjects will be run in triplicate per plate using Applied Biosystem's (Foster City, CA) SYBR Green PCR Master Mix and validated primer sets for each gene. Each sample will also be run separately with β-actin on the same plate. Each set of triplicate Ct values will be averaged per plate and 3 plates will be run per sample. These logarithmic ΔCt values will be linearized by using the equation $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001). Linear values will be averaged per patient from three plates. The averaged linear values will be entered into Prism 4.0 (GraphPad Software; San Diego, CA) and analyzed using paired *t* tests. Values will be considered significant if $p \leq 0.5$. Also, an analysis of covariance (ANCOVA) will be performed to co-vary for age, PMI, and brain pH. Medication effects will be analyzed from the above data with atypical vs. typical neuroleptics in the schizophrenic patient population as well.

Pairwise analysis of the data shows significant decreases in GAD_{65} ($p=0.0122$), GAD_{67} ($p=0.0150$), and GAT-1 ($p=0.0008$) expression levels in the schizophrenic group versus control subjects (**Figure 2 A – C**). These data indicate GABAergic cells in the cerebellum of schizophrenic patients may be compromised. Additionally, patients on atypical antipsychotics

showed significant increases in GAD_{67} levels (p=0.0334) and near significant increases in GAD_{65} (0.0591; **Figure 2 D - F**). This indicates a difference in gene expression levels regulated by neuroleptic treatment that will be examined further in this aim.

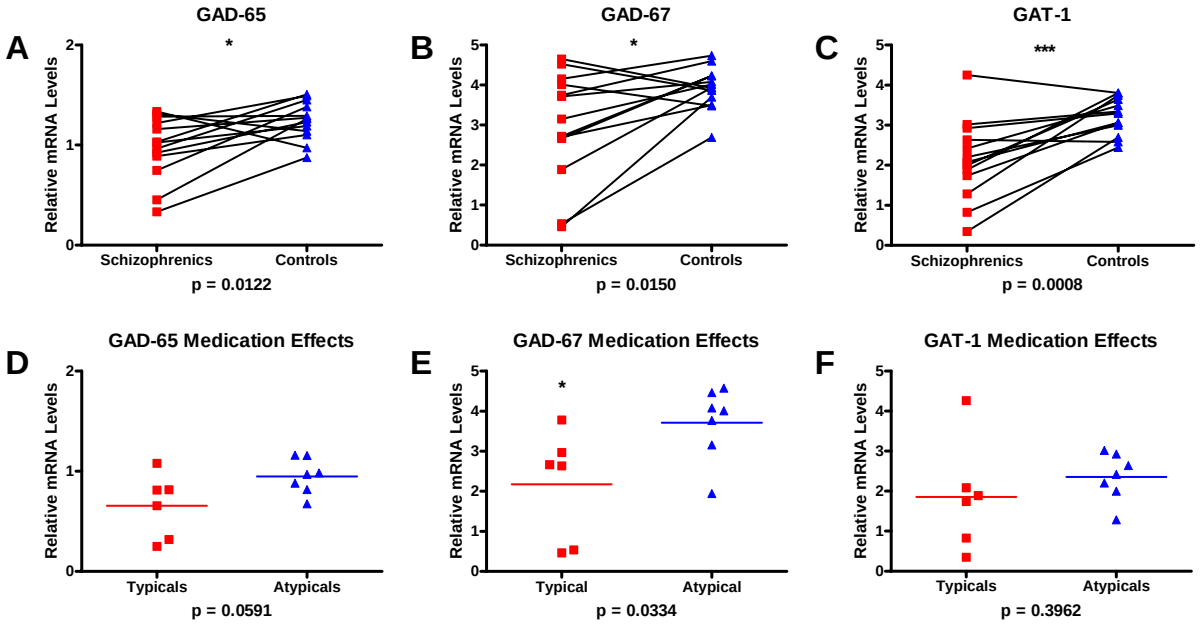

Figure 2: Relative mRNA levels of GABA markers and antipsychotic medication effects. The GABA markers (A) GAD_{65}, (B) GAD_{67}, and (C) GAT-1 show statistically significant differences between patients and controls. Additionally, significant differences are seen between patients on typical versus atypical antipsychotic medications for (E) GAD_{67} and trends for both (D) GAD_{65} and (F) GAT-1.
** $p \leq 0.5$; ** $p \leq 0.05$; *** $p \leq 0.01$.*

Evaluation of NMDA receptor subunits

NMDA receptor subunits in the cerebellum can be localized to specific cell types for distinct functions (Scherzer et al., 1997; Thompson et al., 2000). Of particular importance is the NR2B subunit which has been localized to Golgi cells (Scherzer et al., 1997; Misra et al., 2000). This subunit has been implicated in schizophrenia through genetic analyses and expression levels in postmortem tissue (Martucci et al., 2006). The NR2B subunit was found to be decreased in animal models of schizophrenia employing PCP (Lindahl and Keifer, 2004). In contrast to Golgi cells, basket and stellate cells mainly express NR2C and NR2D, Purkinje cells express mainly NR2A, and granule cells express mainly NR2C (Scherzer et al., 1997). Examining the mRNA expression levels of these subunits in the cerebellum of schizophrenic patients may indicate a dysfunctional cell type. This may suggest Golgi cells as affected based on previous studies.

The same cDNA samples used for GABAergic marker analysis will be used to analyze NMDA receptor subunits as well. Primers sets will be designed for NMDA receptor subunits NR1, NR2A, NR2B, NR2C, and NR2D. qRT-PCR and subsequent analysis will be performed as described previously for the GABAergic markers.

Data acquired using primer sets for all of the NMDA receptor subunits show non-significant changes in paired *t* tests (**Figure 3**). Interestingly, the NR2B subunit (**Figure 3c**) shows a near significant decrease. Analysis of typical versus atypical medications in the schizophrenic patients showed non-significant effects (data not shown).

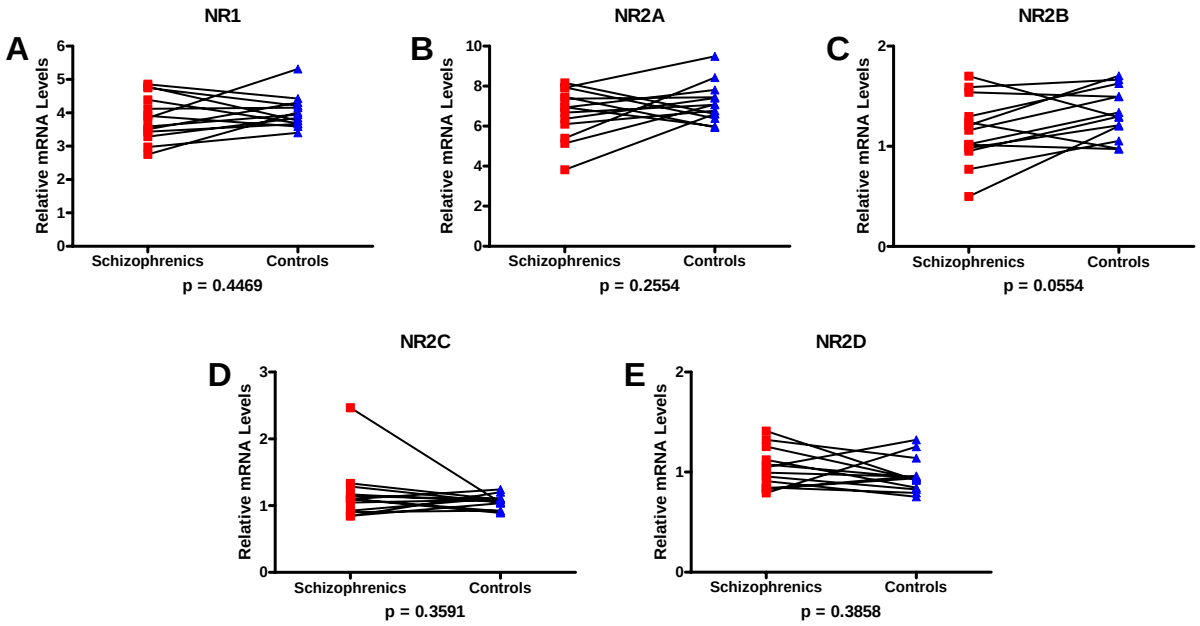

Figure 3: Relative mRNA levels of NMDA receptor subunits (A) NR1, (B) NR2A, (C) NR2B, (D) NR2C, and (E) NR2D in schizophrenic versus control subjects. No significant differences were found between patients and controls.

Medication effects on gene expression

Antipsychotic medication related gene alterations have been examined in some brain regions, but not in the cerebellum. These experiments aim to examine whether neuroleptic medications exert differential effects on cerebellar GABAergic markers and NMDA receptor subunit expression. mRNA levels from rats chronically treated with either haloperidol or clozapine will be analyzed by qRT-PCR as described before.

We acquired RNA that was isolated from the cerebellum of haloperidol treated rats in a previous experiment (Bustillo et al., 2006). Pair housed adult male Sprague-Dawley rats (n=16) were injected intramuscularly with either 38 mg/kg/month haloperidol-depo (Novaplus) or sesame oil (vehicle). These rats were injected once a month for 6 months and sacrificed one month after the last injection. The cerebellum was removed intact and frozen on dry ice. Samples were stored at -80^{o}C.

To examine the effects of clozapine on gene expression, pair housed adult male Long-Evans rats (n=20) will be injected with either 10 mg/kg/day clozapine (Novartis, Princeton, NJ) or 0.9% saline intraperitoneally daily for 21 days. This paradigm was chosen based on methods described by Bai *et al* (2004). The rats will be sacrificed 18 hours after the final injection. The lateral hemisphere of the cerebellum will be removed and frozen on dry ice. Samples will be stored at -80^{o}C.

cDNA will be synthesized from total RNA from the whole cerebellum (haloperidol) and the lateral hemisphere of the cerebellum (clozapine). qRT-PCR will be performed as previously described for the analysis of human GABAergic markers. Primer pairs will be designed for rat GAD_{65}, GAD_{67}, GAT-1, NR1, and NR2A – NR2D as previously described. Data will be evaluated as previously described comparing either haloperidol or clozapine treated rats versus saline controls in each experiment. These data will be analyzed using *t* tests with a value of $p \leq 0.5$ being considered significant. Preliminary data show no significant change in the expression levels of GAD_{67} in haloperidol treated rats (data not shown).

Expected outcomes and conclusions

Many studies have shown decreases in mRNA and protein levels of GABAergic markers and NMDA receptor subunits in distributed brain regions of patients with schizophrenia. The experiments proposed for Specific Aim 1 should complement these findings. Indeed, preliminary data shows significant decreases in the GABAergic markers GAD_{65}, GAD_{67}, and GAT-1 (**Figure 2 A – C**). These data point toward GABAergic expression deficits in the cerebellum of schizophrenic patients. Further analyses of these data show that patients on atypical neuroleptics have increased levels of these GABAergic markers. These changes were significant or close to significant when compared to typical antipsychotic treatments (**Figure 2 D – F**).

Previous studies have linked NR2B differences to schizophrenia in both human and animal models (Lindahl and Keifer, 2004; Martucci et al., 2006). NR2B is expressed predominantly by Golgi cells in the cerebellum (Misra et al., 2000). These findings would suggest that Golgi cells are affected in the cerebellum of schizophrenic patients. Additionally, Golgi cells express the NR2D subunit which may be altered as well (Misra et al., 2000; Brickley et al., 2003). Since the NR1 subunit is ubiquitous and Golgi cells do not express NR2A and NR2C, these mRNA levels may not be changed. Preliminary data show that all of the NMDA receptor subunits are not significantly altered in post-mortem tissue from patients with schizophrenia, but that the NR2B subunit shows a near significant decrease ($p=0.0554$; **Figure 3C**). Medication effects may account for the non-significance of the NR2B data. This will be studied further with analysis of haloperidol and clozapine treated rats.

Chronic treatment of rats with either haloperidol or clozapine will determine if either of these medications has an effect on gene expression of GABAergic markers and NMDA receptor subunits. Initial findings show that chronic haloperidol treatment has no significant effects on GAD_{67} expression. This finding is not surprising considering changes in GAD_{67} mRNA levels after haloperidol treatment are thought to be mediated through dopamine D2 receptor antagonism (Laprade and Soghomonian, 1995). This is a receptor that is absent in the lateral cerebellar hemispheres (Schweighofer and Doya, 2003). Despite this, the cerebellum may be affected indirectly by typical antipsychotic medications by changes in other brain regions that project to the cerebellum. Clozapine, on the other hand, binds to many different receptor subtypes (Kapur and Remington, 2001). Analysis of clozapine treated rats should show increased levels of the GABAergic markers based on our human data (**Figure 2 D - F**) and increases in the NR2B NMDA receptor subunit (Hanaoka et al., 2003).

Specific Aim 2: Are the GABAergic and NMDA receptor gene changes seen in schizophrenic patients replicated in an animal model?

SA2 Rationale

Molecular analysis of schizophrenia in humans *in vivo* is not possible with current technology. Therefore development of an animal model of schizophrenia is important for characterizing the neuropathological processes of schizophrenia. Analysis of an animal model may eventually lead to more effective treatment options for patients. Administration of the NMDA receptor antagonist PCP in rats has produced many of the behavioral, physiological, and molecular results found in schizophrenic patients (Sams-Dodd, 1997; Jentsch and Roth, 1999; Lindahl and Keifer, 2004; Morris et al., 2005). However, mRNA levels of GABAergic markers and NMDA receptor subunits in the cerebellum using qRT-PCR have not been measured in this model. Therefore, *experiments in this aim will test the hypothesis that chronic intermittent administration of low levels of PCP in rats alters GABAergic markers and NMDA receptor subunits as seen in schizophrenic patients*.

Experimental Design

To address this hypothesis, pair housed adult male Long-Evans rats (n=20) will be injected intraperitoneally with 2.58 mg/kg/day of either PCP or saline (Cochran et al., 2003). After an acclimation period of 7 days they will be injected once a day for the first five days, then injected on days 8, 10, 12, 15, 17, 19, 22, 24, and 26. The rats will be sacrificed after a washout period of 72 hours. The lateral hemisphere of the cerebellum will be removed and frozen on dry ice. Samples will be stored at -80^{o}C.

RNA isolation, cDNA synthesis, and qRT-PCR will be performed as previously described for analysis of GABAergic markers in human post-mortem tissue. qRT-PCR and subsequent analysis will be executed as described for analysis of the neuroleptic treated rats. Primer sets will be designed for GAD_{65}, GAD_{67}, GAT-1, and NMDA receptor subunits NR1, NR2A – NR2D.

Expected outcomes and results

In line with previous findings and our previously reported human data we expect to find similar decreases in GAD_{65}, GAD_{67}, and GAT-1. Additionally we should see a decrease in the NMDA receptor NR2B subunit since interneurons are 10 times more likely to be affected by PCP administration (Grunze et al., 1996) and since human levels in our data reached near significance. We should also see alterations in the NR2D subunit as well since it shows low Mg^{2+} block and has longer open channel times (Misra et al., 2000). NR1, the obligatory NMDA receptor subunit, NR2A, expressed primarily by Purkinje cells, and NR2C, expressed primarily by granule cells (Scherzer et al., 1997; Thompson et al., 2000), should not be altered.

Preliminary data show that treatment of rats with PCP does indeed reproduce the decreases in mRNA levels in the cerebellum of GAD_{65} ($p=0.0090$), GAD_{67} ($p=0.0003$), and GAT-1 ($p=0.219$) as seen in schizophrenic patients (**Figure 4**). Additionally, NMDA receptor subunit analysis shows that the mRNA levels of NR2B ($p=0.0020$) and NR2D ($p=0.0106$) are significantly decreased after PCP administration (**Figure 5**). Decreases in these subunits point to Golgi cells as the affected cerebellar cell type. Specific Aim 3 will attempt to examine this issue. Overall, our data suggest PCP administration is an effective model in reproducing the molecular changes in GABAergic markers and NMDA receptor subunits seen in schizophrenic patients.

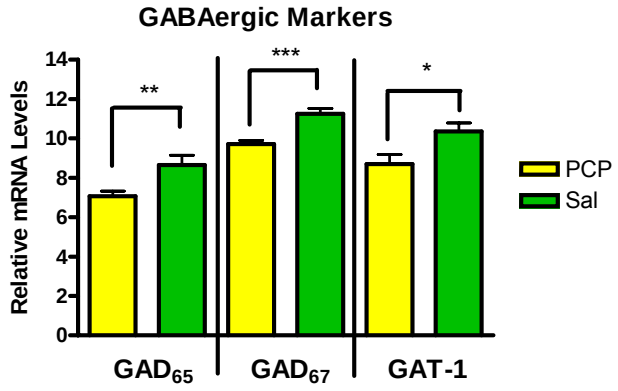

Figure 4: Relative mRNA expression levels of GABA markers (A) GAD_{65}, (B) GAD_{67}, and (C) GAT-1 in PCP treated rats versus saline controls. Statistically significant differences were found between PCP and saline for all of the GABA markers.
** $p \leq 0.5$; ** $p \leq 0.05$; *** $p \leq 0.01$.*

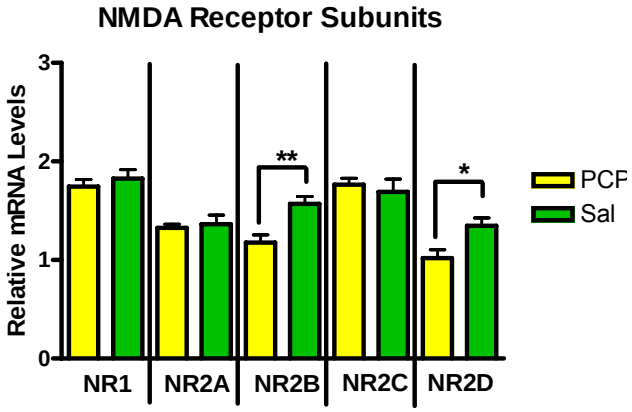

Figure 5: Relative mRNA expression levels of NMDA receptor subunits (A) NR1, (B) NR2A, (C) NR2B, (D) NR2C, and (E) NR2D in PCP treated rats versus saline controls. Significant differences were found in the expression of NR2B (p=0.0020) and NR2D (0.0106).
** $p \leq 0.5$; ** $p \leq 0.05$.*

<u>Specific Aim 3:</u> Do the changes in mRNA levels of GAD_{65} and GAD_{67} localize to a particular cell type in both schizophrenic patients and in the animal model?

<u>SA3 Rationale</u>

Comparing the local circuitry of the cerebellum to the PFC, the Golgi cell seems to be analogous to the chandelier cell as modulator of the excitatory output in the circuit. Golgi cells express the NR2B and NR2D subunits (Misra et al., 2000). We have shown NR2B to be decreased in schizophrenic patients (p=0.0554) and significantly decreased, along with NR2D, in PCP treated rats. Therefore, *experiments in this aim will test whether the changes seen in GAD_{65} and GAD_{67} mRNA levels in the cerebellum are localized to Golgi cells.*

In situ hybridizations will be performed on frozen sections from the lateral cerebellar hemispheres of both schizophrenic patients and PCP treated rats. S^{35}-radiolabeled riboprobes specific for GAD_{65} and GAD_{67} will be transcribed and hybridized to the sections. These will be exposed to film and then emulsion coated for quantitation of grains within each GABAergic cell type. All GABAergic cells will be quantitated in terms of cell number per area and area covered by grains per cell. This experiment will test whether one particular cell type in the cerebellum is affected in schizophrenia and in its animal model.

<u>Experimental Design</u>

Hematoxylin and eosin (H&E) staining of human tissue from the Maryland Brain Collection showed the presence of ice crystal artifacts, indicating that this tissue could not be used for *in situ* hybridizations. Therefore, additional tissue was acquired from the Harvard Brain Tissue Resource Center. This tissue was slow frozen and thus should not succumb to ice crystal damage. Total RNA from this tissue will be isolated and validated for integrity as described in Specific Aim 1. Preliminary analyses show that the RNA quality was good in 4 schizophrenic and 3 control cases (data not shown).

H&E staining of PCP treated rat cerebella from Specific Aim 2 also showed ice crystal artifacts. Therefore, an additional set of rats (n=6) will be treated with PCP as previously described. The forebrain and the left cerebellar hemisphere will be fast frozen in isopentane supercooled in methanol and dry ice for subsequent *in situ* hybridization analysis. The remaining cerebellar hemisphere and vermis will be frozen on dry ice. All samples will be stored at -80°C.

Frozen 10 μm sections will be taken from both human and rat tissue using a cryostat. Slides will contain either one schizophrenic and one control cerebellar section or one PCP rat and one saline rat section per slide. To verify the integrity of the tissue, 2 slides from each

species will be fixed using 4% paraformaldehyde (PFA) and stained using H&E. Preliminary data shows tissue from both species is intact (data not shown).

Purified plasmids containing both human and rat GAD_{65} and GAD_{67} (courtesy of Dr. Niranjala Tillakaratne, UCLA) were validated by sequencing at the UNM DNA sequencing facility and subsequent verification using NCBI's megablast BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). The plasmids will be linearized and stored at -20^{o}C until used for transcription of riboprobes.

Riboprobes will be transcribed from the linearized DNA in the presence of radioactive S^{35}-labeled UTP (Perkin Elmer). Hybridization solution containing riboprobe with a count of 1.5 x 10^6 c.p.m./100μL will be hybridized to each section. Slides will be treated with chloroform to delipidize and then hybridized overnight at 55^{o}C with either antisense or sense (control) riboprobes for both GAD_{65} and GAD_{67}. After hybridization the slides will be treated with RNase A and will be exposed to film to verify probe hybridization and specificity. Preliminary data show good signal for antisense human GAD_{67} and antisense for both rat GAD_{65} and GAD_{67}. Sense probes show very low levels of non-specific binding for human GAD_{67} and rat GAD_{65} and GAD_{67} (data not shown).

After the film has been developed, the slides will be dipped in NTB emulsion (Kodak). These will be exposed for 3, 6, and 9 days at 4^{o}C to determine proper exposure time. The slides will then be developed using D-19 developer (Kodak). Slides will be counterstained using H&E. The levels of GAD_{65} and GAD_{67} mRNAs will be quantitated in each layer using Image-Pro software (Media Cybernetics). Two pictures of each slide will be taken at 20X magnification. One picture will be in bright field to distinguish the cellular layers counterstained with H&E while the following picture will be taken in dark field to better visualize the silver grains. In each frame we will measure the density of GAD_{65} and GAD_{67} positive clusters per area. Since each cluster of grains represents a cell, the total number of clusters for Golgi, basket, and stellate cells will be counted per area (**Figure 6 A - B**) (Volk et al., 2000). For Purkinje cells, the cell number will be determined per Purkinje cell layer length. The number of Golgi cells in the granule layer, Purkinje cells in the Purkinje cell layer, and basket and stellate cells in the molecular layer will be measured. A total numbers of 300 – 600 cells will be counted, averaged, and compared to controls in both species. Additionally, the area per cell covered by grains will be measured using bright field at 60X magnification (**Figure 6 C)**. A circle will be drawn approximating mean of the 2D area of each cell type – 12μm diameter for basket/stellate cells and 25μm for Purkinje cells. Secondary labeling employing an mGluR2 antibody (mG2Na-s; Abcam) will be used to visualize Golgi cells colorimetrically since mGluR2 is expressed exclusively by Golgi cells in the cerebellum (Berthele et al., 1999; Simat et al., 2007) and Golgi cells are difficult to visualize in bright field with the H&E counter stain. Because of this we will trace the outline of the mGluR2 labeled soma and average the cell area for many cells to determine the size of circle to be drawn for analysis. Each image will be converted to grayscale using Adobe Photoshop 5.0 and analyzed using Image-Pro software. A circle will be placed over the soma and the number of area covered by grains per cell will be determined (Heckers et al., 2002) using the "Count/Size" feature. The range of measurable intensity will be determined manually and the area covered by grains will be quantitated (**Figure 6 D)**. Each measurement will be taken on three different days by three different people using coded samples. These will be decoded, averaged per condition, compared to controls, and analyzed statistically using a *t* test.

Expected outcomes and results

Since GAD_{67} has been shown to be decreased in specific subsets of GABAergic interneurons in the PFC (Volk et al., 2000) and the hippocampus (Heckers et al., 2002), we propose that similar cells in the cerebellum are affected. Golgi, basket, and stellate cells are the major GABAergic interneurons in the cerebellum. All these cells express GAD_{65} and GAD_{67} which have shown decreases in protein levels in the cerebellum (Fatemi et al., 2005). Of the

interneurons in the cerebellum, only Golgi cells express the NR2B NMDA receptor subunit (Misra et al., 2000).

Our previous data suggest that Golgi cells are primarily affected in the cerebellum of patients with schizophrenia. Thus, we expect to see decreased levels of GAD_{65} and GAD_{67} mRNA in Golgi cells through *in situ* hybridization techniques. This decrease will most likely manifest itself as a decrease in neurons expressing these markers instead of decreased expression per cell as has been found in the PFC (Volk et al., 2000). The other GABAergic interneurons, basket and stellate cells express mainly the NR2C and NR2D subunits. NR2C is not significantly decreased in either the human or rat experiments. Therefore, we should see decreases in cell numbers of Golgi cells for both GAD_{65} and GAD_{67}. Alternatively, we may see not see any change in GAD_{65} or GAD_{67} levels, or we may see decreases in basket/stellate and/or Purkinje cells as well. This will also be informative as indicating a more pronounced cerebellar GABA deficit. Ultimately, though, we expect to support our hypothesis that Golgi cells are the affected GABAergic interneuron in the cerebellum in patients with schizophrenia and in PCP treated rats.

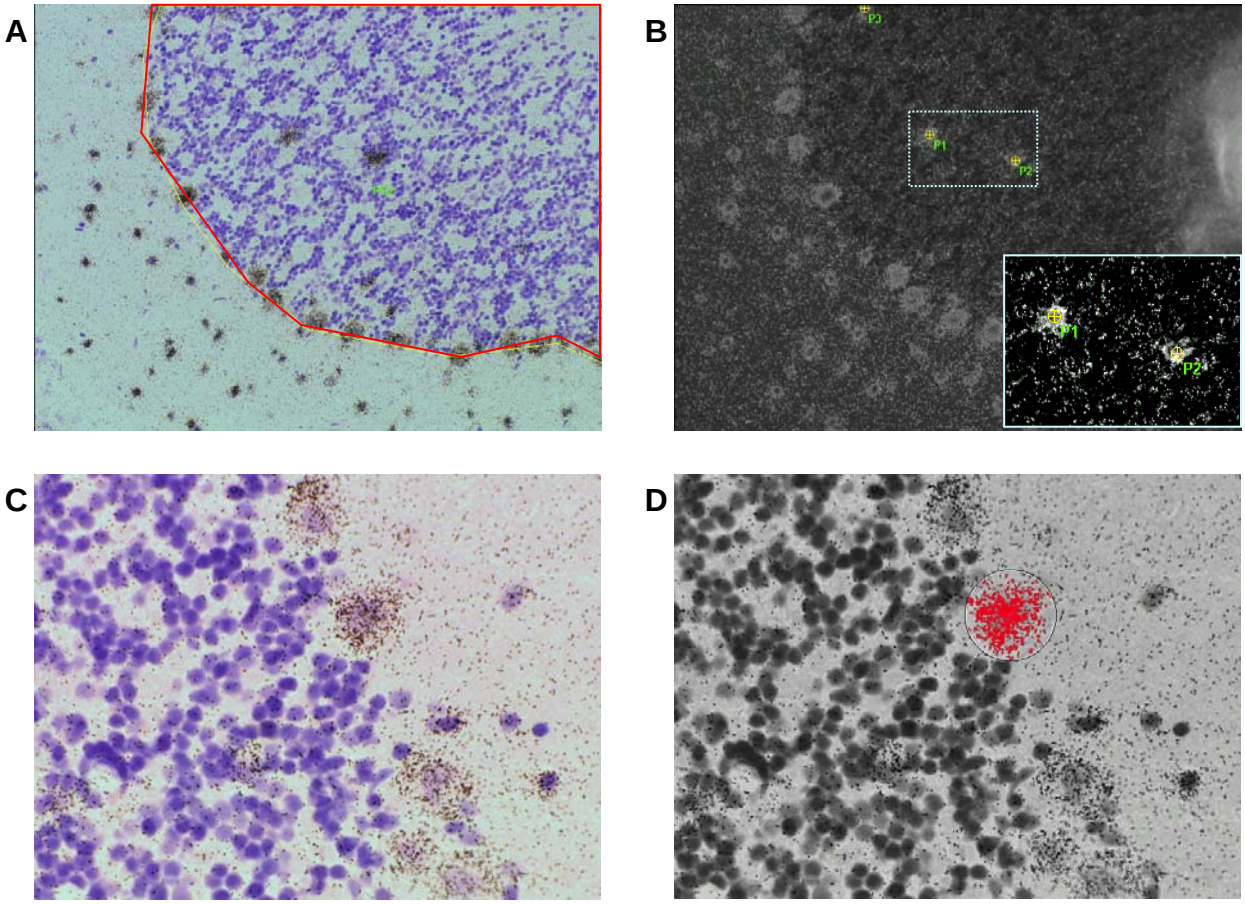

Figure 6: Rat cerebellum at 20X magnification (A, B) and 60X magnification (C, D). Bright field (A) shows tissue integrity is good and silver grains can be seen over the granule cell layer, Purkinje cell layer, and molecular layer. The area where Golgi cell clusters will be counted is outlined in red. Dark field (B) shows individual clusters to be counted. Image of Purkinje cells (C), which can be readily seen in bright field. This image is converted to grayscale (D) for analysis of area covered by grains per cell.

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