

REDUCED DENSITY OF CALBINDIN IMMUNOREACTIVE GABAERGIC NEURONS  
IN MAJOR DEPRESSIVE DISORDER: RELEVANCE TO NEUROIMAGING STUDIES  
AND FUTURE DIRECTIONS

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## ABSTRACT

JONATHAN CORWIN HUGHES: Reduced density of calbindin immunoreactive GABAergic neurons in major depressive disorder: relevance to neuroimaging studies and future directions  
(Under the direction of Carol Britson and Dorota Maciag)

**Background:** Several lines of evidence suggest dysfunction of the  $\gamma$ -aminobutyric acid (GABA)ergic system in major depressive disorder. Neuroimaging studies report reduced levels of GABA in the dorsolateral prefrontal and occipital cortex of depressed patients. Our previous postmortem study revealed a reduction in the density and size of calbindin-immunoreactive (CB-IR) GABAergic neurons in the prefrontal cortex in major depressive disorder. The goal of this study was to test whether the changes in CB-IR neurons can also be detected in the occipital cortex, where neuroimaging studies report a prominent GABA decrease.

**Methods:** A three-dimensional cell counting probe was used to assess the cell-packing density and size of CB-IR neurons in layer II of the occipital cortex in 10 major depressive disorder subjects and 10 psychiatrically healthy control subjects.

**Results:** The density of CB-IR neurons was significantly decreased by 28% in major depressive disorder subjects compared with the control group. The size of CB-IR neurons was unchanged in major depressive disorder subjects when compared with control subjects.

**Conclusions:** The reduction in the density of CB-IR GABAergic neurons in the occipital cortex in depression is similar to that observed previously in the prefrontal cortex. Deficits in cortical GABAergic interneurons may contribute to the low GABA levels detected in neuroimaging studies in major depressive disorder patients.

**Future Directions:** Experiments are proposed to determine whether or not decreases in calbindin-immunoreactivity are due to decreases in calbindin expression. Further experiments will be designed to investigate potential involvement of calbindin interactors in MDD.

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## **CHAPTER I. Reduced Density of Calbindin Immunoreactive GABAergic Neurons in Major Depressive Disorder: Relevance to Neuroimaging Studies**

### **INTRODUCTION:**

Major Depressive Disorder (MDD) is the world's leading cause of disability for people ages 15-44 years old, affecting more than 121 million people worldwide (Ustün et al., 2000). In addition, depression has been shown to have a significant economic burden, costing the U.S. alone an estimated \$53 billion dollars per year in cost from treatment, mortality and lost productivity (Wang et al., 2003). Despite this clinical and economic burden, treatment for this disease is largely ineffective, as seen in results from the recent STAR\*D (Sequenced Treatment Alternatives to Relieve Depression) trial. The study showed that initial remission rate in this large cohort of patients (n=4,041) was a mere 28% and that even after one year 30% of patients still had recurrent depressive episodes (Insel and Wang 2009). These disappointing figures underscore a frustrating lack of understanding of the pathophysiological processes that underlie MDD. While most research over the past fifty years has focused on the monoaminergic systems implicated in depression (Schildkraut and Kety 1967; see Hirschfield 2000 for an appropriate history of the monoamine hypothesis and its limitations), an accumulating body of evidence has shown amino acid neurotransmitters, especially the inhibitory GABAergic system, to also be involved in depression [see Sanacora and Saricicek 2007 for a comprehensive review].

Of particular note, the first <sup>1</sup>H-NMR (Proton nuclear magnetic resonance) studies of GABA in major depressive disorder by Sanacora et al. were performed in the occipital

cortex due to technical limitations and were found to be significantly reduced in depressed patients (Sanacora et al., 1999). These results were later replicated with a larger sample size and still found to be significant (Sanacora et al., 2004). Another group, Hasler et al., also found that GABA levels were decreased in the medial and dorsolateral prefrontal cortex (Hasler et al., 2007). These areas of the brain have been classically ascribed the psychological functions of executive thought, (i.e. planning, decision making, and discrimination) as well as personality and social behavior. Having previously demonstrated a reduction in size and cell packing density of Nissl-stained neurons in this area (Rajkowska et al., 1999), we sought to investigate two of the three non-overlapping subpopulations of GABAergic nonpyramidal neurons, calbindin and parvalbumin (calretinin having been previously studied). We found the density of calbindin-immunoreactive (CB-IR) neurons was significantly reduced in depressive subjects in the dorsolateral prefrontal cortex (dlPFC, Brodmann's area 9). In contrast, there was no difference in the density of parvalbumin-immunoreactive (PV-IR) neurons between depressed and control groups in the same brain region. Moreover, previous studies conducted by other groups have not identified changes in the density of calretinin-immunoreactive (CR-IR) GABA neurons in MDD (see Rajkowska et., 2007).

To this effect, the goal of this study was to investigate whether the morphometric differences in CB-IR GABA neurons between depressed and psychiatrically healthy subjects previously demonstrated in the prefrontal cortex can be also detected in the occipital cortex, Brodmann's area 17. Neuroimaging studies report a prominent GABA decrease; however, other measures of GABAergic function known to be altered in the PFC show no difference. After finding reductions in CB-IR neurons in the dlPFC, a follow up study

showed decreases in the same region of protein levels of glutamic acid decarboxylase, GAD-67, the GABA synthesizing enzyme in depressed subjects (Karolewicz et al., 2010). However, differences in levels of GAD-67 were not found to be statistically significant in the occipital cortex (unpublished data). In light of reports of altered GABA physiology in the occipital cortex, it was hypothesized that reductions in GABAergic neurons would be found, possibly explaining the GABA deficits observed in this brain region.

## **METHODS:**

### *Human Subjects*

Postmortem brain samples were collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, Ohio, from 20 subjects. Informed written consent was obtained from the legal next-of-kin of all subjects. Next-of-kin were interviewed and retrospective psychiatric assessments were conducted in accordance with Institutional Review Board policies, as described in Rajkowska et al. (1999). Research on the psychological autopsy method has revealed that diagnoses from structured clinical interviews with family members are in good agreement with diagnoses based on reviewing the subject's medical records (Deep-Soboslay et al., 2005; Kelly and Mann 1996). Ten subjects met clinical criteria for MDD, and the other 10 subjects (termed normal control subjects) did not meet criteria for an Axis I diagnosis based on DSM-IV (Table 1). A trained interviewer administered either the Schedule for Affective Disorders and Schizophrenia: Lifetime Version (SADS-L) or the Structured Clinical Interview for DSM-IV (SCID) to knowledgeable next-of-kin of all subjects, as previously described in Spitzer and Endicott (1978) and First et al. (1996). Diagnoses for Axis I disorders were assessed independent-



ly by a clinical psychologist and a psychiatrist and consensus diagnosis was reached in conference, using all available information from the knowledgeable informants, the coroner's office, and any available previous hospitalizations and doctor's records. In addition, strong inter-rater concurrence has been obtained when a structured clinical interview was used to collect information from depressed patients versus information collected from next-of-kin (McGirr et al., 2007). Of the 10 subjects that met DSM-IV criteria for MDD via retrospective assessment, there was no evidence that 4 ever sought mental health treatment, another 2 were seen by primary care physicians, and the remaining 4 were seen by psychiatrists. Six of the 10 subjects received a premortem diagnosis of MDD. Responses from the subjects evaluated with the SADS-L were also recorded in the SCID, and these subjects met DSM-IV criteria for MDD using information collected with either structured diagnostic interview.

Toxicology assays were performed by the coroner's office using gas chromatography with mass selective spectrometry or high-performance liquid chromatography to detect the following classes of compounds in blood and urine: antidepressant or antipsychotic drugs, barbiturates, benzodiazepines, sympathomimetic amines, cocaine and its metabolites, opiates, phencyclidine, cannabinoids, and antiepileptic drugs. According to the medical records, 5 out of 10 MDD subjects had a prescription for antidepressants and 3 out of these 5 subjects had a prescription for antidepressants in the last month of life. However, only one MDD subject had detectable levels of an antidepressant medication in postmortem toxicology screening (Table 1). Six of the 10 MDD subjects died by suicide. The control subjects were group matched with the depressed subjects for age, gender, postmortem interval (PMI), time in formalin (TF), and brain tissue pH (Table 1).

### *Tissue Preparation*

Tissue was collected at autopsy and fixed in phosphate-buffered formalin (10%), as described previously (Rajkowska et al., 1999). Blocks of tissue from the occipital cortex of each subject were embedded in 12% celloidin. Morphometric parameters were measured within the calcarine sulcus of Brodmann's area 17 (the primary visual cortex [V1]). Brodmann's area 17 was chosen, as previously published magnetic resonance spectroscopy (MRS) studies measuring GABA in depressed patients were centered primarily on the V1 region.

The tissue blocks were sectioned at 40  $\mu\text{m}$  and stained for either Nissl substance, which stains all neurons and glia, or immunohistochemistry using antibodies to calbindin-D28K, staining the calbindin-expressing GABAergic neurons. Nissl-stained sections were used to identify cytoarchitectonic features of Brodmann's area 17 and to draw the borders between individual cortical layers. These laminar borders were then imposed on the adjacent (200  $\mu\text{m}$  apart from Nissl section) immunostained sections to determine the laminar distribution of immunoreactive cells.

### *Immunocytochemistry*

Celloidin-embedded sections were immunostained after the removal of celloidin (Miguel-Hidalgo and Rajkowska 1999). The sections were incubated with a rabbit polyclonal anti-calbindin-D28K antibody at 1:750 dilution (Millipore/Chemicon, AB1778, Billerica, Massachusetts). Binding of these antibodies was detected with a secondary anti-rabbit antibody according to the Avidin Biotin Complex (ABC) method (ABC Kit, Vector Laboratories, Burlingame, California). The immunostained sections were adjacent to or within 200  $\mu\text{m}$  of the Nissl-stained sections used for the identification of the relevant

area. To minimize the variability in the intensity of staining, sections from depressed and control subjects were stained simultaneously. For each subject, three coronal sections were used for morphometric analyses.

### *Morphometric Analyses*

The estimation of cell-packing density and size of CB-IR neurons were carried out by the investigator naive to the diagnoses. Calbindin-IR neurons were analyzed in cortical layer II, as most CB-IR neurons were located in this layer. The density of CB-IR neurons was estimated with a  $\times 40$  oil-immersion objective (1.0 numerical aperture) using the Optical Fractionator probe of Stereo Investigator Software (version 8.21.4 32-bit, MicroBrightfield, Inc., Williston, Vermont). In each section, 40 to 60 three-dimensional (3-D) counting boxes ( $70 \times 70 \times 14 \mu\text{m}$ ;  $1 \mu\text{m}$  guard zone from the top) were placed randomly within the contour outlining layer II. The packing density of immunopositive cells was calculated in each section by dividing the total number of cells counted in all boxes by the combined volume of all counting boxes. The size of CB-IR neurons was estimated by measuring the volume of immunoreactive cell bodies with the Nucleator probe of the Stereo Investigator Software.

### *Statistical Analyses*

Mean values for cell density and neuronal size (somal volume) obtained from the three sections of each subject were compared between the groups using analysis of covariance (ANCOVA) with age, PMI, brain tissue pH, and TF as covariates. Pearson correlation analysis was used to assess the influence of confounding factors such as age, age at onset of depression, PMI, pH, and TF on neuronal density and size (SPSS, version 16.0, SPSS Inc., Chicago, Illinois).

## RESULTS

The density of CB-IR neurons was significantly reduced by 28% in the MDD subjects [ $21.2 \pm 2.7 \times 10^3$  neurons /mm<sup>3</sup>] compared with the age-matched control group [ $29.5 \pm 2.2 \times 10^3$  neurons /mm<sup>3</sup>; ANCOVA,  $F(1,14) = 7.58$ ,  $p = .016$ ] (Figures 1 and 2). The reduced density in MDD subjects does not appear to be specifically related to death by suicide because there was almost no difference in the mean density of CB-IR neurons between the six MDD suicide ( $20.8 \pm 3.0 \times 10^3$  neurons /mm<sup>3</sup>) and four MDD nonsuicide ( $21.6 \pm 5.5 \times 10^3$  neurons /mm<sup>3</sup>) subjects (Figure 3).

Correlation analyses revealed no association between the density or size of CB-IR neurons and confounding variables such as age at the time of death, PMI, brain pH, and storage time in formalin (for statistics, see Table 2). The lack of influence of confounding variables on calbindin (CB) neuron density or size was observed whether all subjects (MDD + control subjects) were combined for analysis or each of the diagnostic groups was tested separately. Similarly, there was no correlation between density or size of CB-IR neurons and age at onset of depression in the MDD group (Table 2).

## DISCUSSION

The present study demonstrates marked [28%] reductions in density of CB-IR neurons in layer II of the occipital cortex in MDD subjects compared with control subjects. However, size of CB-IR neurons was not significantly different between the two groups. All but one of our MDD subjects were free of antidepressants at the time of death as revealed by postmortem toxicology screening, suggesting this was largely a medication free population. The deficit in GABA neurons is consistent with multiple proton magnetic resonance spectroscopy studies showing prominent reductions in GABA levels

in the occipital cortex of antidepressant-free, living depressed patients (Sanacora et al., 1999; Sanacora et al., 2004; Price et al., 2009; and Benes and Berretta 2001). Moreover, a 2007 neuroimaging study found significant reductions in dorsomedial and dorsolateral prefrontal cortex GABA content in MDD patients, although GABA levels were unchanged in the frontal polar and ventromedial prefrontal regions in the same patients (Hasler et al., 2007). This is in strong agreement with our previous postmortem analyses of calbindin-IR and parvalbumin-IR populations of GABAergic neurons in different regions of prefrontal cortex (Rajkowska 2007) wherein a marked 50% reduction in the density of CB-IR neurons were detected in Brodmann's area 9 of the dlPFC but not in Brodmann's area 47 of the ventral orbitofrontal cortex [ORB] in MDD subjects. Thus, both postmortem and neuroimaging studies suggest that GABA pathology in MDD is widespread but regionally specific. The findings suggest that a decreased density of CB-IR GABA neurons in the occipital cortex [present study] and dlPFC (Rajkowska 2007) in MDD subjects may contribute to the low cortical GABA content observed in these cortical regions in depressed patients (see Sanacora and Saricicek 2007, Sanacora et al., 2004; Hasler et al., 2007; Price et al., 2009; Benes and Berretta 2001).

Although parvalbumin-IR was not specifically examined in this study, the subpopulation of parvalbumin-IR GABAergic neurons was not affected in either dlPFC or ORB in previous studies (Rajkowska 2007). The calbindin-IR and parvalbumin-IR neurons belong to two distinct subpopulations of GABA neurons. Calcium binding protein, calbindin, is mainly expressed by double bouquet neurons, which make synapses on dendrites and synaptic spines of pyramidal neurons and have physiological features of non-fast-spiking interneurons. In contrast, GABA neurons expressing parvalbumin correspond

to basket or chandelier cells and form synaptic connections on somata and axonal initial segment of pyramidal neurons, as well as have physiological properties of fast-spiking interneurons (Benes and Berretta 2001). This suggests a specific loss of a physiologically unique cell type may be associated with the pathology underlying MDD. Based on our postmortem findings, the CB-IR subpopulation of GABAergic neurons is affected in MDD, whereas other subpopulations have yet to be studied.

The present study, to our knowledge, provides the first postmortem evidence for GABA neuron pathology in the occipital cortex in depression. Although visual function is not commonly studied in association with depression, intriguing subjective complaints of visual deficits (Friberg and Borrero 2000) and neurofunctional alterations in the occipital lobe have been reported in patients with MDD. Specifically, differences in visual evoked potential response amplitudes (Vasile et al., 1992; Coullaut-Valera et al., 2007) and stimulus-induced plasticity of visual evoked potential [VEP] responses (Normann et al., 2007) were previously observed between healthy control subjects and in subjects with severe MDD. Moreover, contrast discrimination thresholds were found to be altered in patients with MDD compared with comparison subjects (Bubl et al., 2009), and a recent study by Golomb et al., (2009) demonstrated that spatial suppression for high contrast stimuli [postulated to be mediated by GABAergic interneurons] is abnormal in MDD subjects compared with healthy comparison subjects. These studies suggest pathophysiological processes within the occipital cortex may, in fact, be associated with MDD. Interestingly, the parieto-occipital cortex is also one of the brain regions recently identified in the processing of sustained anxiety (Hasler et al., 2007).

This preliminary study demonstrates a reduction in the density of CB-IR neurons in the occipital cortex of individuals suffering with MDD. These findings are consistent with several reports of decreased GABA content in the occipital cortex and further suggest that the underlying pathology associated with MDD may be more widespread in the brain than commonly conceived. The present study, however, has some limitations. The sample size used in our study is relatively small and further investigations with increased number of subjects could provide better understanding of the influence of potentially confounding variables [medication use, smoking status, or alcohol abuse] on our results and demonstrate the consistency of the finding.

Additionally, we have not yet investigated whether cell loss of GABAergic neurons or decrease in the concentration of calcium binding proteins within individual cells account for the observed reductions in the density of cells expressing calcium binding proteins.

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## TABLES AND FIGURES

**Table 1.** Demographic Characteristics of Control and MDD Subjects. (Maciag et al., 2010).

Parameter	Controls (n=10)	MDD (n=10)
Age (years)	40.2 ± 4.9	44.8 ± 5.3
Age range (years)	17 – 65	19 – 81
PMI (hrs)	22.5 ± 2.1	24.2 ± 1.3
PMI range (hrs)	13 – 35	17 – 31
pH	6.6 ± 0.09	6.49 ± 0.08
pH range	5.93 – 7.0	6.06 – 6.82
TF (months)	44.2 ± 3.5	53.7 ± 4.8
TF range (months)	22 – 57	38 – 82
Gender (F:M)	4:6	4:6
Medication history*	none	n = 3
Toxicology:		
Clean	n=8	n=3
Antidepressant drugs	none	n=1 (sertraline)
Other	n=2 (propoxyphene, oxycodone, n=1; ethanol, n=1)	n = 6 (morphine, codeine, hydrocodone, diphenhydramine, n=1; lidocaine, n=1; tramadol, n=1; cannabinoids, n=1; ethanol, CO, alprazolam, n=1; ethanol, n=1)
Cause of death	Cardiovascular disease, n=10	Suicide, n=6 (shot gun, n=3; CO poisoning, n=1; hanging, n=1; drowning, n=1), Other causes, n=4 (cardiovascular disease, n=3; homicide, n=1)
Diagnosis	none (n=10)	MDD, n=8; MDD and alcohol abuse, n=1; MDD and cannabis abuse, n=1
Duration of MDD (years)	not applicable	6 ± 2
Duration range	not applicable	1 month – 20 years
Smoking	Smokers, n=1	Smokers, n=6

Data represent the mean ± SEM. The average age, PMI, TF, and pH of MDD subjects were not statistically different from the control subjects.

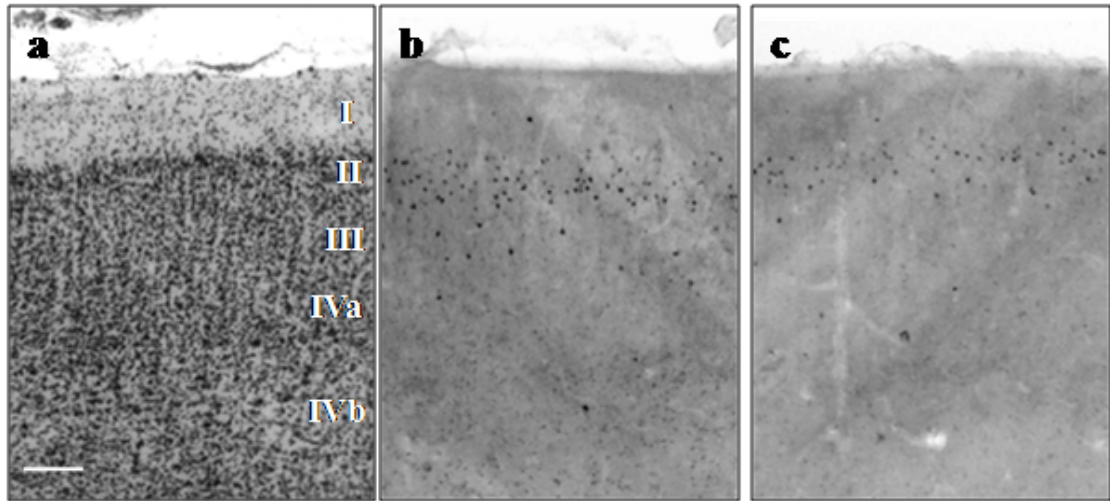
CO, carbon monoxide; F, female; M, male; MDD, major depressive disorder; PMI, post-mortem interval; TF, time in formalin.

\*Treatment with antidepressants within 4 weeks before death

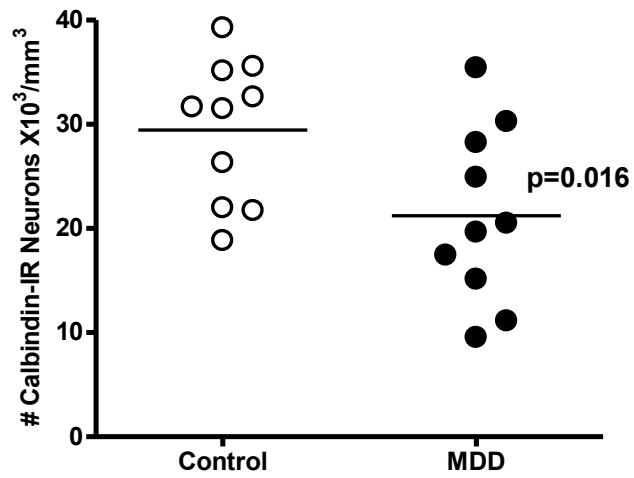
**Table 2.** Summary of Correlations (Pearson Analyses) Between the Density and Size of CB-IR Neurons and Confounding Variables in Control and MDD Groups. (Maciag et al., 2010).

Variable	Density				Size			
	Control		MDD		Control		MDD	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age at the Time of Death	-.073	.841	-.356	.313	.147	.686	.276	.44
Age at Onset of Depression	—	—	.481	.16	—	—	.213	.556
PMI	.539	.108	-.193	.144	-.178	.624	.091	.803
pH	.36	.307	.15	.659	-.326	.358	.427	.219
TF	.262	.463	.497	.144	-.002	.996	.026	.942

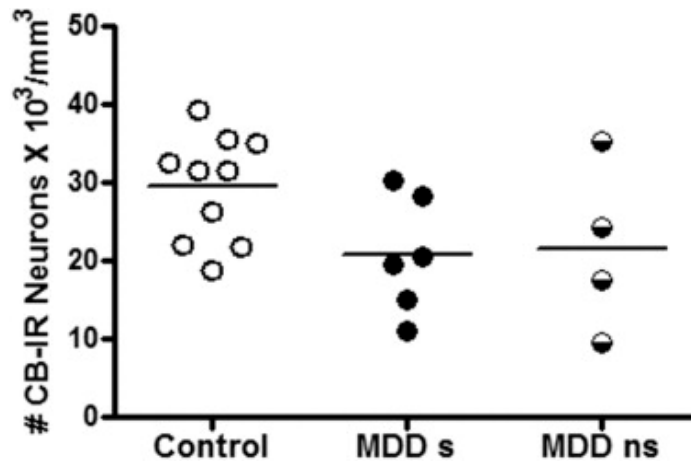
CB, calbindin; IR, immunoreactive; MDD, major depressive disorder; PMI, postmortem interval; TF, time in formalin.



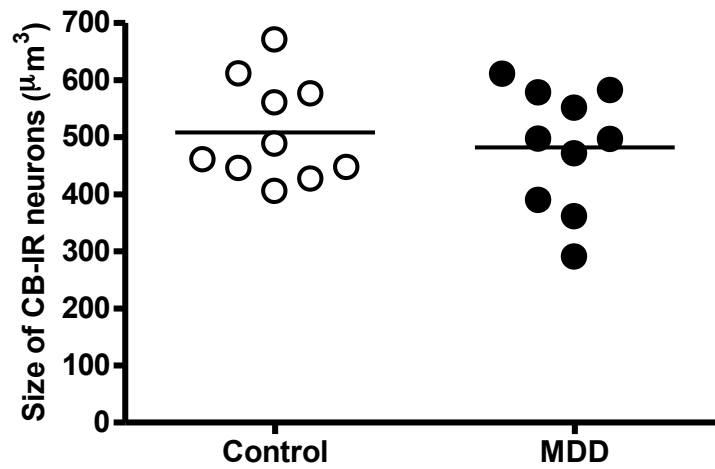
**Figure 1 a:** Nissl stained section of a non-psychiatric control subject showing the typical cytoarchitectonic features of area 17. **b:** CB immunoreactive neurons in control subject (adjacent section to Nissl). **c:** CB immunoreactive neurons in MDD subject. Note that the majority of CB-IR neurons are localized to layer II and in a smaller number to the upper part of layer III. Images were obtained using the x4 objective, scale bar = 125  $\mu\text{m}$ . (Maciag et al., 2010).



**Figure 2** Cell packing density of CB-IR neurons in the occipital cortex (Brodmann's area 17) of control (open circles) and MDD subjects (closed circles). Values for the individual subjects (circles) and mean values (horizontal lines) are presented. (Maciag et al., 2010).



**Figure 3.** Cell-packing density of CB-IR neurons in the occipital cortex (Brodmann's area 17) of control (open circles), MDD suicide (MDD s, closed circles), and MDD non-suicide (MDD ns, half-closed circles). Values for the individual subjects (circles) and mean values (horizontal lines) are presented. Note that the mean values for CB-IR neuron density are comparable between MDD suicide and MDD nonsuicide (unpaired  $t$  test,  $t = .048$ ,  $df = 8$ ,  $p = .96$ ; Mann-Whitney test,  $Z = .0$ ,  $p = 1$ ). CB, calbindin; IR, immunoreactive; MDD, major depressive disorder. (Maciag et al., 2010).



**Figure 4** Size of CB-IR neurons in the occipital cortex (Brodmann's area 17) of control (open circles) and MDD subjects (closed circles). Values for the individual subjects (circles) and mean values (horizontal lines) are presented. (Maciag et al., 2010).



## **CHAPTER II. Are Reductions in Calbindin Immunoreactive GABAergic Neurons in Major Depressive Disorder Due to Reduced Calbindin Expression?**

### **INTRODUCTION AND SPECIFIC AIMS**

**Involvement of GABAergic Interneurons in Major Depressive Disorder.** The two amino acids, glutamate and  $\gamma$ -aminobutyric acid (GABA), are the primary neurotransmitters responsible for excitation and inhibition in the brain, respectively. The role of GABA in particular is becoming an increasingly important player in the pathophysiology of major depressive disorder (MDD). Support for its involvement stems from many lines of evidence, such as direct clinical correlations between proton magnetic spectroscopy observed GABA deficits in depressed patients (Sanacora et al., 1999, 2004; Hasler et al., 2007) as well as subsequent GABA recovery after treatment with antidepressants (Sanacora et al., 2002, 2003). In addition, large-scale gene array studies report altered GABAergic and glutamatergic neurotransmission in MDD (Sequeira 2007, 2009), especially in those committing suicide. Finally, histological studies have reported reductions in density and size of cortical neurons in MDD subjects (Rajkowska 1999), and have most recently identified these reductions in GABAergic interneurons in the dorsolateral prefrontal and occipital cortex (Rajkowska et al., 2007; Maciag et al., 2010).

**Reductions in Calbindin-Immunoreactive GABAergic Neurons in Major Depressive Disorder.** Studies of human and primate cerebral cortex have shown three distinct subpopulations of nonpyramidal GABAergic neurons based on the calcium-binding protein expressed, namely calbindin (CB), calretinin (CR), or parvalbumin (PB) (Beasley et al.,

2002; Condé et al., 1994; Cotter et al., 2002; DeFelipe, 1997; Uylings et al., 2002; Zaitsev et al., 2005). Studies by our group and others found deficits in calbindin-immunoreactive (CB-IR), while there were no significant differences in PV-IR or CR-IR expressing neurons (Rajkowska et al., 2007, Maciag et al., 2010, Daviss and Lewis, 1995; Reynolds and Beasley, 2001). DeFelipe (1997) found decreased mRNA transcript levels in MDD patients of somatostatin, a protein which has been shown to selectively colocalize with calbindin; in contrast, neither calretinin nor parvalbumin transcript levels were decreased (Sibille et al., 2011). However, one problem common to these studies is an inability to ascribe the observed deficits to cell loss of CB-IR GABAergic neurons or a decrease in the concentration of calbindin within individual neurons. Identifying which is the cause of observed deficits will undoubtedly be indispensable in understanding how GABAergic alterations may affect the pathophysiology of the disorder.

**Aim 1. To determine whether observed deficits in CB-IR neurons in the dlPFC (Brodmann's area 9) in MDD are due to reduced calbindin expression in individual cells.** *Hypothesis: Calbindin-D28k mRNA transcripts are not decreased in depressed patients and thus observed deficits in CB-IR neurons are not due to a decrease in expression.*

**Aim 2. To investigate possible alterations in downstream targets of and interactors with calbindin-D28K in MDD post-mortem brain tissue.** *Hypothesis: While calbindin-D28K expression is not decreased in depressed patients, calbindin function is altered, such that CB-IR neurons from depressed patients show evidence of activation of proteins or pathways interacting with calbindin that may be important in MDD.*

- a) Investigate activation of apoptosis by caspase-3, an executioner caspase and inhibited target of calbindin, in depressed and healthy subjects.
- b) Measure levels of myo-inositol, whose levels have been observed to be decreased in depression and which is a product of inositol monophosphatase 1 (IMPA1), an activated target of calbindin, in depressed and healthy subjects.

## **SIGNIFICANCE**

**Importance: individual and societal burden of major depressive disorder.** Major Depressive Disorder (MDD) is the world's leading cause of disability for people ages 15-44 years old, affecting more than 121 million people worldwide (Ustün et al., 2000). In addition, depression has been shown to have a significant economic burden, costing the U.S. alone an estimated \$53 billion dollars per year in cost from treatment, mortality and lost productivity (Wang et al., 2003). Despite this clinical and economic burden, treatment for this disease is largely ineffective, as seen in results from the recent STAR\*D (Sequenced Treatment Alternatives to Relieve Depression) trial. The study showed that initial remission rate in this large cohort of patients (n=4,041) was a mere 28% and that even after one year 30% of patients still had recurrent depressive episodes (Insel and Wang 2009).

**Barriers: to treatment and development of novel antidepressant targets.** These disappointing figures underscore a frustrating lack of understanding of the pathophysiological processes that underlie MDD. While most research over the past fifty years has focused on the monoaminergic systems implicated in depression, the monoaminergic theory of depression has been found inadequate in explaining the pathophysiology of the disorder (Hirschfeld 2000, Krishnan and Nestler 2008). While many alternative theories

have been proposed, including altered neuroendocrine and neuroimmune function in depressed patients, a comprehensive neurobiological theory of depression is still lacking, and likely will not emerge until more is understood about the cellular and molecular changes that take place in the depressive disease state. More discoveries in the field will at the very least provide novel drug targets for pharmaceutical companies to pursue.

**Improvement of Scientific Knowledge: Understanding Etiology of and Cellular/Molecular Pathways Involved in Observed GABAergic Alteration in MDD.** As shown in the preceding section, the inhibitory GABAergic system has been shown to be strongly involved in depression. Indeed, many experiments seem to be converging specifically on calbindin-expressing GABAergic interneurons as responsible for histological changes seen in depression. However, none of the experiments performed heretofore have been able to distinguish between gross cell loss of the calbindin-expressing subpopulation of GABAergic neurons and/or a decrease in the calbindin protein itself. The experiments proposed herein will determine whether or not these deficits are due to a decrease in calbindin expression (**Aim 1**) and explore two possible pathways by which calbindin deficits may negatively impact an affected neuron (**Aim 2**). Calbindin interacts with several proteins implicated in MDD, perhaps the two most promising being caspase-3 (Cristakos and Liu, 2004; Wann et al., 2006) and inositol monophosphatase 1 (Levine, 1995; Harwood, 2005). Regardless of the findings in **Aim 1**, exploring these pathways will be fruitful, as alterations in either mRNA levels or calbindin conformation could be expected to cause changes in these pathways.

## **APPROACH**

**Aim 1.** To determine whether observed deficits in CB-IR neurons are due to reduced calbindin expression in individual cells.

**Background/Preliminary Studies.** Immunocytochemistry is a common method to measure the presence of protein by the use of a labeled antibody complex which is specific only to the protein of interest. In previous studies (Rajkowska et al., 2007; Maciag et al., 2010), we have reported decreases in calbindin-D28K immunoreactive GABAergic non-pyramidal neurons in MDD. However, because immunocytochemistry only measures presence of protein, these decreases in immunoreactivity may be due to loss of neurons in the calbindin-D28K expressing neuron subpopulation of GABAergic interneurons or due to a decrease in calbindin-D28K expression. In order to determine calbindin expression at the cellular level, we will use laser capture microscopy (LCM) to capture GABAergic nonpyramidal neurons immunoreactive for calbindin-D28K in both depressed and psychiatrically healthy patients. While measurement of calbindin protein would be the most direct assessment of calbindin expression, protein levels for the number of neurons possible to obtain by LCM are too low to measure. In place of this, we will perform real time PCR on cDNA clones synthesized from mRNA isolated from the laser captured neurons and compare mRNA transcript levels between MDD and control patients. The methods described herein are adapted from Goswami et al., 2010.

## **Methods**

**A. Subjects.** The same subjects used in our previous studies will be used in these experiments in order to draw stronger correlations between the two studies. Human

brain specimens will be collected from a total of twelve subjects meeting criteria for MDD according to the Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 1994) from our existing brain repository along with twelve controls matched as closely as possible for age, gender, postmortem interval (PMI), and race. Subjects will be screened for presence of antidepressant or any psychotropic drug which may confound the study.

**B. Tissue Sampling.** The study will be carried out on frozen tissue blocks of the dorsolateral prefrontal cortex, the same area studied previously (Rajkowska 2007). Tissue sections 10 um thick will be collected on Silane-Prep glass slides in a cryostat, along with 20 um reference sections collected every 240 um used for Nissl staining. All parts of the microtome used to obtain the sections will be wiped with 95% ethanol and RNase wipes to avoid cross-contamination and RNase contamination. Additionally, the tissue mounted slides will be kept on dry ice during sectioning and then stored at -80°C for storage.

**C. Immunofluorescence Staining and Laser Capture Microscopy.** Three sections from each subject will be stained using previously described methods (Goswami et al., 2010) with anti-calbindin-D28K antibody conjugated to a fluorescent secondary antibody and stored in a dessicator until ready for LCM processing. The calbindin immunofluorescent-stained neurons will be visualized by a fluorescence filter and captured using the infrared laser of the Veritas microdissection system (Molecular Devices, Sunnyville, CA, USA), allowing us to capture a pure population of calbindin-expressing GABAergic neurons. Laser capture parameters will be optimized and roughly 500 calbindin-positive neurons will be obtained from each section for a

total of 1500 neurons per subject. Each MDD and control matched pair will be processed in parallel in order to avoid variation due to reagents and handling.

**D. RNA Isolation and cDNA Synthesis.** Total RNA from captured cells will be isolated using the PicoPure RNA isolation kit (Molecular Devices) and purified from DNA contamination using RNase-free DNase I (Qiagen, Valencia, CA, USA), using the manufacturer's protocol for both. RNA integrity will be assessed by using the Agilent Bioanalyzer 2100 with Agilent Lab-on-a-Chip Picochip RNA kit (Agilent Technologies, Foster City, CA, USA). After isolation of RNA, all samples will be immediately processed for reverse transcriptase reaction to avoid potential RNA degradation, using a mixture of random and oligo dT primers to avoid 3' bias. The obtained cDNA will be stored at -20°C until further use.

**E. Primer Design and Real Time PCR.** Sequences for calbindin-D28K and control genes of interest will be downloaded from the NCBI nucleotides database and primers for these genes constructed. Before performing, quantitative PCR, the specificity of the primers will be confirmed by purifying the PCR product and DNA sequencing. Quantitative PCR will be performed in a 96 well format using a similar protocol as Goswami et al., 2010.

**F. Statistical Analysis.** For real time PCR data, mRNA level data from each of three slides will be averaged together for a single measurement for each subject and then compared between MDD and control pairs using a paired t-test with a result considered significant at  $p < 0.05$ . Confounding variables such as age, pH, PMI and cause of death will also be taken into consideration by Pearson correlation analysis. Furthermore, a power analysis test will be performed as soon as preliminary data has been

collected in order to determine the number of subjects needed to detect a significant result.

**Expected Outcomes, Potential Problems and Solutions, Future Directions.** Sibille et al. (2011) have reported that levels of somatostatin mRNA, but not parvalbumin or calretinin, were decreased in MDD using whole tissue homogenate of dIPFC. Somatostatin heavily co-localizes but does not interact with calbindin to our knowledge. Thus, the most plausible explanation for a decrease in the two colocalizing, but not interacting proteins is actual loss of the neuron subpopulation expressing these proteins, and not necessarily a decrease in calbindin or somatostatin expression. Since Sibille et al. could not normalize their mRNA measurements by number of neurons as we propose to do, their decreases could very well be due solely to a direct neuronal loss. Thus, we expect mRNA levels of calbindin to show no significant difference between MDD and control pairs.

We do not anticipate any problems with this approach as it has been performed with success by our close collaborators (Goswami et al., 2010). We do not foresee any problems which will cause a dramatic abandonment of this procedure. The results of these experiments will better direct our experiments in Aim 2.

**Aim 2.** To investigate possible alterations in downstream targets of and interactors with calbindin-D28K in MDD post-mortem brain tissue.

**Background/Preliminary Studies.** Whether decreases in CB-IR neurons in the dIPFC and the occipital cortex in MDD are due to a decrease in calbindin expression or otherwise (presumably by direct neuronal loss), how the protein is specifically affected in our depressive subjects still remains unanswered. Calbindin is a calcium-binding protein, which has classically been described a calcium buffer, but because of new interactions



with other proteins is being described more as a calcium sensor (Schwaller et al., 2002). Through its interaction with several proteins, calbindin plays a role in apoptosis (by interaction with cleaved caspase-3 and possibly procaspase-3 (Christakos and Liu, 2004)) and calcium homeostasis (by virtue of its four calcium binding pockets *and* its ability to activate inositol monophosphatase 1 (Berggard et al., 2002)), as well as a few less well-defined interactions with proteins such as Ran binding protein 9 and inhibitor of nuclear factor kappa-B kinase subunit gamma. We propose to study the two former pathways for evidence of alterations in the depressed subjects. While postmortem studies are unable to assess protein activity and interactions as they were during the patient's lifetime, we contend that it is still possible to identify evidence of alterations by examining the molecules and pathways suspected interactors affect. Obviously, any mechanism for a pathway by which calbindin is proposed to have an effect in depression will have to be validated in *in vitro* and *in vivo* models. Until then, we tentatively propose two mechanisms by which either of the two outcomes possible in **Aim 1** may alter the activity of calbindin's downstream interactors, caspase-3/procaspase-3 (CASP3/pro-CASP3) and inositol monophosphatase 1 (IMPA1):

1. **Outcome 1: Calbindin expression is not decreased in MDD.** If, as we hypothesize, calbindin mRNA transcripts are not found to be decreased in MDD subjects, we propose that in MDD a change in the cellular environment, such as altered calcium levels or other conditions known to affect calbindin's interaction with other proteins (Berggard et al., 2002), cause calbindin to lose normal interaction with the proteins described below.

2. **Outcome 2: Calbindin expression is decreased in MDD.** If, on the other hand, calbindin mRNA transcript levels *are* found to be decreased in **Aim 1**, we propose that the pathways investigated herein are still pertinent, as a decrease in calbindin protein may well be assumed to affect the normal functioning of calbindin's interactors.

Proposed mechanisms of action for each individual protein will be explained in the following sections.

**Aim 2a.** Investigate activation of apoptosis by caspase-3, an executioner caspase and inhibited target of calbindin, in depressed and healthy subjects.

**Background.** One of calbindin's most well-studied interactors, caspase-3 is an executioner caspase involved in apoptotic cell death. Having previously reported a decrease in size and density of Nissl-stained neurons in the pre-frontal cortex and having most recently observed a deficit of calbindin-immunoreactive neurons in both the dlPFC and the occipital cortex, caspase-3 makes an interesting object of study, especially considering that it has been shown to be inhibited by calbindin (Christakos and Liu, 2004). To examine the possibility of this scenario, we propose to do a double-immunofluorescence study labeling both calbindin and cleaved caspase-3 in depressed and control patients.

#### **Methods.**

- A. **Subjects.** The same subjects used in our previous studies will be used in these experiments in order to draw stronger correlations between the two studies. Human brain specimens will be collected from a total of twelve subjects meeting criteria for MDD according to the Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 1994) from our existing brain repository

along with twelve controls matched as closely as possible for age, gender, post-mortem interval (PMI), and race. Subjects will be screened for presence of antidepressant or any psychotropic drug which may confound the study.

**B. Tissue Sampling.** The study will be carried out on frozen blocks of the dorsolateral prefrontal cortex, the same area studied previously (Rajkowska 2007). Tissue sections 20  $\mu\text{m}$  thick will be collected on Silane-Prep glass slides, along with 40  $\mu\text{m}$  reference sections collected every 200  $\mu\text{m}$  used for Nissl staining.

**C. Immunocytochemistry and Imaging.** Three sections per subject will be stained using existing protocols with anti-calbindin and anti-cleaved caspase-3 primary antibodies (of different organismal origin), followed by staining with two different secondary antibodies (e.g. Green Alexa Fluor  $\text{\textcircled{R}}$  488 and Red Alexa Fluor  $\text{\textcircled{R}}$  647) specific to the relevant primary antibody. Sections will be visualized using a confocal microscope and fifteen images representative of the section will be taken from each slide, for a total of 45 images per subject.

**D. Analysis.** Images will be processed in Photoshop CS4 using a color range function which excludes any color besides the green or red pixels desired to be measured. Following this procedure, images will be processed by ImageJ and measured for area fraction, giving an area fraction for both total cleaved caspase-3 and total calbindin. These measurements will be compared between MDD and control subjects by normalizing the area fraction of cleaved-caspase 3 colocalized with calbindin for area fraction of calbindin (i.e.  $AF_{\text{colocalized}}/AF_{\text{calbindin}}$ ). Results will be compared using a paired t-test with a result considered significant at  $p < 0.05$ . Confounding variables such as age, pH, PMI and cause of death will also be taken

into consideration in our analysis. Furthermore, a power analysis test will be performed as soon as preliminary data has been collected in order to determine the number of subjects needed to detect a significant result.

**Expected Outcomes, Potential Problems and Solutions, Future Directions.** Since we hypothesize in Aim 1 that observed decreases in CB-IR neurons are due to neuronal loss and not a decrease in calbindin expression, we expect that cleaved caspase-3 will be increased in MDD patients relative to their matched pair controls. This would provide a feasible mechanism by which these neuronal losses may have occurred. Should both hypotheses be supported, we intend to propose a mechanism of action between calbindin and caspase-3, as well as propose experiments validating this mechanism. We would propose that calbindin changes its conformation dramatically in response to a cellular distress, such as loss of calcium, which in turn lowers its ability to inhibit caspase-3. A recent study showed that when incubated with BAPTA, a calcium chelator, the extent of calcium decrease was highly correlated with expression of activated caspase-3 and calcium binding proteins such as calbindin and parvalbumin, and furthermore, that these BAPTA-induced calcium depletions had less of an effect in brain regions that had been previously shown to robustly express these calcium binding proteins (Turner et al., 2007).

If, on the other hand, we find in Aim 1 that calbindin expression is decreased in our MDD subjects, our model in which cleaved caspase-3 is more abundant in those patients may still be valid, only more directly explained by a special interaction with the caspase. Indeed, it would be expected that with less calbindin protein present in the cell, its ability to inhibit caspase-3 would be lowered. In this scenario, we would propose further experiments to validate this mechanism in *in vitro* and *in vivo* models.

**Aim 2b.** To indirectly investigate activity of IMPA1, an activated target of calbindin-D28K, in depressed and control patients.

**Background:** Another well-studied interactor with calbindin, inositol monophosphatase 1 is an enzyme important in the recycling of inositol monophosphate into myo-inositol, which can then be used to produce new inositol 1,4,5-triphosphate, an important secondary messenger in the cell (McAllister et al., 1992). Inositol has been implicated in major depressive disorder as far back as 1978, where levels of the myo-inositol were found to markedly decreased in the cerebrospinal fluid of depressed patients (Barkai et al., 1978). Treatment of depressed patients with inositol dietary supplements were found to significantly improve symptoms (Levine 1995), but use as an adjuvant is not widely accepted (Sarris et al., 2009). Zheng et al. (2010) report that high-frequency rTMS (repetitive transcranial magnetic stimulation) treatment increased myo-inositol levels in young patients with treatment-resistant depression.

In addition to studies of myo-inositol, the involvement of IMPA1 in depression is further supported by the now known mechanism of action of lithium, whose salts have been used to treat depression as early as 1886 (Schioldann 2011). IMPA1 is widely accepted to be the therapeutic target of lithium, especially in studies of bipolar disorder (Berridge et al., 1982). Lithium works via an uncompetitive inhibition mechanism wherein it binds the enzyme-substrate complex, making its inhibition most effective at high substrate concentrations (Hallcher and Sherman 1980). The result of IMPA1 inhibition is myo-inositol depletion, which leads to an attenuation of IP<sub>3</sub> signalling since recycling of myo-inositol to IP<sub>3</sub> is inhibited and cell uptake of exogenous myo-inositol is blocked by a Li<sup>+</sup>-induced rise in intracellular pH and subsequent decrease in affinity of H<sup>+</sup>-dependent

myo-inositol transporter (HMIT) (Song et al., 2008). Agam et al. (2009) showed that IMPA1 knockout mice showed decreased immobility in the Porsolt forced-swim test (Porsolt et al., 1977), an indication of antidepressant-like effect.

Finally, Berggard et al. (2002) discovered by affinity purification with a random 12-mer peptide library that IMPA1 was an interactor with calbindin. Through further cell-free experiments (ibid), the authors showed IMPA1 was an activated target of calbindin and that this activation was most pronounced in conditions that would lead to low IMPase activity, that is, low pH and low substrate concentration. Furthermore, the authors reported that the activation effect of calbindin increased dramatically with lower concentrations of  $\text{Ca}^{2+}$ . Most of the psychiatric studies to date investigating the link between IMPA1 and calbindin in human post-mortem tissues regard bipolar disorder. Most notable is the finding by Shamir et al. (2005) that supplementation of human recombinant calbindin to crude brain homogenate increased the activated of IMPase by 3.5 fold and that bipolar patients who had been treated with Li had a two-fold increase in calbindin over bipolar patients without treatment. While studies in mice by the same author could not replicate the observed increase after lithium treatment, the authors noted that the animal studies only lasted up to 30 days whereas the human subjects had been treated for years (ibid).

Based on these studies, we pose the questions 1) What is the biological function of calbindin's activation of IMPA1 and 2) Is IMPA1 activity different in our depressed subjects? While the former question will take much more investigation, we speculate that when a neuron is overactive (perhaps in times of behavioral stress), continuous activation of signaling pathways in the neuron such as  $\text{IP}_3$  signaling causes a demand for  $\text{IP}_3$  utiliza-

tion, increasing inositol monophosphate, a metabolite of IP<sub>3</sub>, and decreasing myo-inositol, a precursor of IP<sub>3</sub>. IP<sub>3</sub> causes release of Ca<sup>2+</sup> from the endoplasmic reticulum through IP<sub>3</sub> receptors, which then in turn are used in other cellular processes such as further cell signaling. Conceivably, continued neuronal activation would cause a drop in intracellular calcium, and for downstream effects to continue, more Ca<sup>2+</sup> must be released from the ER. This could be effected by sustaining IP<sub>3</sub> production, which would be made possible by calbindin's activation of IMPA1, which is most pronounced at lower Ca<sup>2+</sup> levels and other conditions of cellular stress (Berggard et al., 2002). We could further speculate that if calbindin expression or function is negatively altered in MDD as we propose in Aim 1, then sustained IP<sub>3</sub> production and use would stagnate as IMPA1 became saturated with the maximum load of inositol monophosphate it could handle. Obviously this hypothesis is highly speculative; as our first step towards testing it, we propose to measure the activity of IMPA1 in our depressed and control subjects.

## **Methods**

- A. **Subjects.** The same subjects used in our previous studies will be used in these experiments in order to draw stronger correlations between the two studies. Human brain specimens will be collected from a total of twelve subjects meeting criteria for MDD according to the Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 1994) from our existing brain repository along with twelve controls matched as closely as possible for age, gender, postmortem interval (PMI), and race. Subjects will be screened for presence of antidepressant, lithium, or any psychotropic drug which may confound the study.

- B. **Tissue Sampling.** Because we propose assaying the activity of IMPA1 directly in regard to its interaction with calbindin within CB-IR GABAergic neurons, we will use cells obtained from Aim 1/Method C to perform our studies on. Cells from each of the three sections per subject will be homogenized and total protein level normalized across subjects and sections.
- C. **IMPA1 Activity Assay.** We will use the assay described by Nemanov et al. (1999) to assay the activity of IMPA1. In short, this assay uses a phosphate-sensitive malachite green color reagent whose absorbance changes with release of inorganic phosphate. Substrate (inositol monophosphate) would be added to the cell homogenate and stopped by addition of the malachite green reagent. This assay can be performed at high-throughput with a plate reader.
- D. **Statistical Analysis.** Activities for each of the three slides per subject will be average for a single activity per subject and then compared between depressed and control groups using a paired t-test with a result considered significant at  $p < 0.05$ . Confounding variables such as age, pH, PMI and cause of death will also be taken into consideration by Pearson correlation analysis. PMI will be particularly monitored in analysis and screening of subjects as it can be particularly important in postmortem protein function. Furthermore, a power analysis test will be performed as soon as preliminary data has been collected in order to determine the number of subjects needed to detect a significant result.

**Expected Outcomes, Potential Problems and Solutions, Future Directions.** We expect IMPA1 activity to be decreased in depressed patients relative to controls, presumably because of a decrease in calbindin expression or functioning (of course, this premise will be



answered in Aim 1 before completion of these experiments). Should this hypothesis be supported, we would continue piecing together a potential mechanism for involvement of calbindin-regulated inositol signaling alterations in MDD. Possible experiments could include measurement of intracellular calcium and inositol metabolites such as IP<sub>3</sub>, inositol monophosphate, and myo-inositol, as well as *in vivo* experiments with mice investigating the effect of acute and chronic stress on inositol signaling. One potential problem that should be addressed is the possibility of IMPA1 being too degraded in our subjects to detect any activity, perhaps due to a lengthy PMI. In this case, we would propose measuring myo-inositol levels directly by LC/MS/MS as described by Kindt et al., (2004). These and other inositol metabolite levels could be measured to “forensically” assess the state of inositol signaling in the subjects before death. Another possible problem could be insufficient levels of IMPA1 enzyme recovered from LCM experiments. In this case, we will consider upscaling to gray matter tissue punches from relevant brain regions (Brodmann’s area 9).

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