

INTRODUCTION

Thank you to all of the reviewers for their thoughtful and valuable comments. Similar themes addressed by the reviewers were divided into three themes, my responses to which can be found below. A western blot study was added to SA1 to allow for analysis of TH in schizophrenia subjects off-drug vs. on-drug, the electron microscopy study became its own aim (SA2), and the rat study became SA3. SA3 will be using tissue from rats that have already been treated; no vertebrate animals will be involved in this study. Much of the Research Strategy has been extensively revised; major changes are highlighted using bracketed text.

Significance – *Reviewers expressed concern about what will be gained from the proposed research.*

I would like to emphasize that this research focuses on the ventral striatum for which only a handful of pathology studies have been done and which provided conflicting results. The importance of the ventral striatum, separate from the dorsal striatum, was updated in the Significance section of the Research Strategy. It is generally accepted that upregulated striatal dopamine is intrinsic to the disorder, but recent studies suggest that this may only be the case for the dorsal striatum. If the results from this study show that tyrosine hydroxylase is not elevated in the ventral striatum in schizophrenia, this could have implications for treatment strategies which currently block all striatal dopamine. Further, other hypotheses which implicate the nucleus accumbens, such as that proposed by Grace and colleagues, lack support from structural evidence. Their hypothesis, for example, suggests that reduced glutamatergic inputs from PFC, hippocampus, or amygdala lead to the disinhibition of phasic dopamine release in the nucleus accumbens. These types of inputs make asymmetric synapses on spines which will be analyzed in this study; the results will provide structural evidence for or against this hypothesis, as well as others. If differences in the glutamatergic-type input are found, the results would have implications for glutamate treatment strategies within the ventral striatum.

Training potential – *Reviewers 2 and 3 were unclear on the new approaches that will be learned.*

I regret that the initial submission was not clear on the training potential of this proposal. The technical and intellectual training to be gained, as well as the necessity of continued observed training has been emphasized in the Sponsor Information. While my previous time in Dr. Roberts' laboratory provided me with training for performing electron microscopy on postmortem human tissue, I will be learning a number of new methods for this research. I have a published paper that used many of the proposed techniques, however I joined the laboratory after many of them had already been performed and the samples were ready for analysis. Thus, my previous research provided me with training for performing data collection but none of the other techniques. This may have led to the confusion about new techniques to be learned. Further, new data collection and analysis techniques will be learned for specific aims 1 and 3, and training in rodent studies will be new. A list of new techniques to be learned has been provided in the Goals for Fellowship Training document.

Training plan – *Responses are to individual critiques regarding approach and methodology*

- Although treatment varies between patients with schizophrenia, treating rodents is a validated method to determine the effects of these drugs and how they may impact the brain. Changes regarding the antipsychotic drug treated rat methodology (including reason for using normal rats, rationale for 24-wk treatment paradigm, and support for using treated rats as a context for interpreting human data) can be found in SA3 Rationale. Additionally, an expanded explanation of how the rat data will be used to interpret the human data is provided in the Synthesis of postmortem human and rat analyses section.
- The marker PSD95 will no longer be used for determining spine counts. Spine density measures will instead be determined using electron microscopy.
- Variation due to tissue shrinkage will be minimized by matching cases for their years since death which corresponds to fixation time. Other measures taken to address this can be found in Approach.
- All cases with neuropathology are excluded from being used in the study. Methods for how cases were selected are clarified in the Methodology sections.
- Clarifications of method details and results of power analyses can be found in Methodology sections.
- Information on treatment response is not available for this study. Variability in patients, likely due to treatment response or symptom profile, is a common trend in studies of schizophrenia. However, with findings on dopamine in the striatum for example, the schizophrenia group still tends to have overall elevated levels, even within the variability. If there is variability in this study overlapping with the control group, cases will be individually assessed to determine if there is a trend based on more specific characteristics, such as diagnosis subtype.

SPECIFIC AIMS

Disruptions in the physical connections of striatal neural circuitry are unclear in schizophrenia (SZ), a severe mental illness affecting about 1% of the population, in which a lack of understanding results in the current treatment options effectively treating only a subset of the patients. Further, the subset of patients who do respond to treatment find relief for only their positive symptoms; the negative symptoms and the cognitive impairments, the most debilitating of SZ symptoms, remain untreatable. Many regions of the brain show evidence for aberrant functioning in SZ, however the primary region of pathology has yet to be determined. Further, the underlying circuitry of SZ has not been fully examined in some primary regions of interest, such as the nucleus accumbens. *The overall goal of this study is to examine the circuitry of the nucleus accumbens and determine the anatomical underpinnings of its pathways in SZ, which will provide a foundation that is fundamental for advances in treatment.*

Although there is convincing evidence for the role of the striatum in SZ, its pathology has not been widely studied in SZ research. A region of the ventral striatum called the nucleus accumbens (NAcc) is an integral part of the limbic system, and the major site of input into the basal ganglia from the mesolimbic dopamine (DA) system. It can be divided into core and shell subregions based on its structural, neurochemical, and functional features. The NAcc is a prime region of interest in SZ based on its circuitry; many regions that are disrupted in SZ, including prefrontal cortex, hippocampus, and thalamus, all converge with dopaminergic input on the medium spiny projection neurons within the NAcc. Further, neurons within the NAcc send projections to midbrain DA neurons which then project to the dorsal striatum. Importantly, this allows the NAcc to modulate the dopaminergic input that the dorsal striatum receives, and DA hyperfunction in the dorsal striatum is a hallmark characteristic of the disorder. Imaging studies have revealed changes in DA signaling in the striatum of patients with SZ, such as elevated presynaptic DA synthesis, release, and endogenous levels. The NAcc is also a prime region of interest based on its functional role; circuitry involving the NAcc core and/or shell mediates cognitive, motor, and emotional functioning, all of which are disrupted in SZ. To achieve their therapeutic effect, all currently available antipsychotic drugs (APDs) block DA receptors. Both first- and second-generation APDs exert effects on the firing of DA neurons in the ventral tegmental area (which provide input to the NAcc), and the ventral striatum is a common region of activation in response to both generations of APDs, providing support that this region may play a role in their therapeutic action. Further, activation in the ventral striatum during the early stage of treatment has been shown to predict treatment response.

Although the literature illustrates the importance of DA in SZ, as well as the significance of the NAcc, the anatomical pathology of the NAcc associated with SZ has been largely overlooked. Of the studies that have investigated the NAcc, it is often analyzed as one homogenous region, rather than by its distinct subregions. Moreover, in SZ the neuropathology of the NAcc has never been studied at the ultrastructural level in human postmortem tissue. A difficulty when studying human postmortem tissue is the confounding factor of chronic treatment with APDs. Studies in rodents have shown APDs have an effect in the NAcc, but longer treatment paradigms are necessary to correlate better with chronic treatment in patients. With the resources and expertise in our lab, it is possible to advance our knowledge by examining changes in the ultrastructural circuitry and dopaminergic innervation of the human NAcc in SZ subjects. Our lab has previously determined ultrastructural changes in the dorsal striatum of SZ subjects compared to normal controls, including an increase in the density of symmetric dopaminergic synapses and in asymmetric axospinous synapses. We hypothesize that the ventral striatum will also show changes in neural circuits in the disorder including increases in the density of dopaminergic innervation and in the number of dendritic spines receiving excitatory synapses. This work will be supplemented by studying the effects of chronic APD treatment in rodents.

Specific Aim 1 will test the hypothesis that subjects with schizophrenia have increased dopaminergic inputs in the nucleus accumbens core and shell compared to normal control cases by measuring the density of immunolabeling of tyrosine hydroxylase, a synthesizing enzyme of dopamine, using (**SA1.1**) optical densitometry, and (**SA1.2**) measuring protein levels using western blot assays.

Specific Aim 2 will test the hypothesis that subjects with schizophrenia have increased synapses, both symmetric (inhibitory) and asymmetric (excitatory), in the nucleus accumbens core and shell using stereology at the electron microscopic level to determine density of synapse types.

Specific Aim 3 will test the hypothesis that chronic antipsychotic drug treatment causes a decrease in dopaminergic innervation of the nucleus accumbens core and shell, by measuring the density of tyrosine hydroxylase immunolabeling in rodents that have been chronically treated with antipsychotic drugs.

The proposed research will provide insight into the abnormal circuitry that may be a core feature of SZ, further elucidate mechanisms of the illness, and offer insight into therapeutics which are not fully understood.

RESEARCH STRATEGY

SIGNIFICANCE

Importance

Schizophrenia (SZ) is a severe mental illness that affects about 1% of the population worldwide. The symptoms include hallucinations, delusions, cognitive and behavioral abnormalities. Most patients with SZ are completely incapacitated by the illness, at high risk for suicide, and unable to live happy or productive lives. Not all patients respond to currently available treatments, and in those who do, only psychotic symptoms are usually improved.¹⁷ There is an urgent need to improve treatment options for these patients which requires a better understanding of the underlying pathology than is currently known.

[Abnormalities have been found in many regions throughout the brain in SZ. The region of primary pathology however, remains unknown. A region of the ventral striatum, the nucleus accumbens (NAcc), is of significant interest in the disorder. The NAcc has been referred to as the “major site of integration between the limbic forebrain and the basal ganglia,”²⁴ as it receives input from numerous brain areas including prefrontal cortex, hippocampus, amygdala, thalamus, and midbrain dopamine (DA) neurons.²⁶ Importantly, all of these areas providing convergent input to the NAcc have been associated with SZ,²³ making the NAcc a prime region for integrating multiple disrupted areas to provide a comprehensive understanding SZ pathology. Reciprocal connections between the NAcc and substantia nigra indicate the importance of this region as well. Via these connections, the NAcc is able to modulate dopaminergic input to the dorsal striatum,²⁸ and striatal DA dysfunction is a hallmark characteristic of the disorder; elevated presynaptic DA functioning in the striatum of patients with SZ is intrinsic to the pathology of the disease and a risk factor for the illness (see ref. 33, 74).]

In addition to the support for the NAcc in disease pathology that is provided by its circuitry, numerous studies have also linked the NAcc to the treatment of SZ. The ventral and dorsal striatum contain the highest levels of DA D₂ receptors in the human brain,^{10,11,18,51} the primary receptor target antipsychotic drugs (APDs). Both 1st- and 2nd-generation APDs exert effects on the firing of DA neurons in the ventral tegmental area (VTA)^{16,92} which project to the NAcc.²⁷ Studies examining APD action in the NAcc have indicated changes in neuronal activity via altered mRNA expression,⁶⁴ the induction of immediate early genes,^{20,84} and increases in functional activation⁴⁹⁻⁵¹ in response to treatment. The ventral striatum is a common region of activation in response to both generations of APDs,^{49,51,84} providing support that this region may play a role in the therapeutic action of APDs (rather than in their side effects). Further, it has been shown that activation of the ventral striatum during early treatment stages is a predictor for treatment response,⁵⁰ emphasizing the need to further study this region in efforts to improve available treatment options for patients.

Despite the support for the role of the NAcc in SZ and its treatment, the neuropathology of the NAcc has largely been overlooked in SZ research. [Furthermore, the few anatomical studies of DA in the NAcc of SZ subjects have not investigated its distinct subregions,^{4,22,29,58,71,90} the core and shell, which are structurally, neurochemically, and functionally distinguishable.⁸⁹ Meredith et al.⁶⁷ showed that the core and shell have very different responses to DA depletion; within the core there was a loss of spines on projection neurons, while in the shell there was an increase in the tortuosity of dendritic trees. These profound differences in the structural consequences to DA depletion in the subregions of the NAcc show the necessity of studying disease-related effects and pharmacological consequences on a regional basis.]

Impact and improvement of scientific knowledge

[It is evident that gross structural deficits are not the primary cause of SZ, but that the underlying pathology more likely resides at a finer level, in brain circuitry and synaptic connections. Electron microscopy (EM) is the only way to study anatomy at this level, but it is rarely done in human postmortem brain outside of our laboratory. A novel feature of this proposal is that our lab is the only in the world doing quantitative studies in SZ subjects at the EM level in the NAcc. Since there are no complete animal models of SZ, limiting what can be gained from studies of pathophysiology in current models, studying postmortem tissue is crucial. Indeed, only a handful of postmortem studies have been done to analyze DA levels in the NAcc of SZ subjects directly, and these studies have found conflicting results and have not been replicated in the past 30 years with improved techniques, more standard boundaries of the NAcc, or examination of core and shell separately.

The proposed research will provide a comprehensive examination of the DA system in the core and shell of the NAcc of SZ: protein levels of tyrosine hydroxylase (TH), density of dopaminergic innervation, and EM of synaptic anomalies providing insight into what pathways may be affected. Results from our study will provide structural evidence to support or challenge current hypotheses regarding the pathology of DA in the NAcc in SZ. The NAcc is a crucial brain area in SZ, but has been largely overlooked in postmortem research. The improvement in scientific knowledge from these studies will be highly significant and will fill a large gap in our understanding of DA in this brain area in SZ.]

APPROACH

[Three distinct sets of SZ and normal control (NC) postmortem human subjects were selected for densitometry (SA1.1), western blot (SA1.2), and EM (SA2) analyses. APD drug effects will be assessed using off- and on-drug SZ subjects for the western blot (WB) analysis, as well as APD-treated rodents (SA3). Since the core and shell will not be individually analyzed using WB, the treated rats will allow for a more detailed assessment of drug effects by subregion. All human cases will be processed in matched pairs to minimize variation that could arise due to methodology. Similarly, tissue from each rodent group will be processed together. Data collection and analyses will be done blinded to the case diagnosis or rodent treatment group.]

Specific Aim 1 will test the hypothesis that subjects with SZ have increased dopaminergic inputs in the NAcc core and shell compared to NCs by measuring the density of immunolabeling of TH, a synthesizing enzyme of DA, [using (SA1.1) optical densitometry, and (SA1.2) measuring protein levels using WB assays.]

SA1 Rationale

DA hyperfunction in the striatum is a hallmark characteristic of the disorder, and the role of DA as central in SZ is one of the most accepted hypotheses of the illness.^{12,13,33} [There are many hypotheses regarding the primary pathology of SZ, including glutamate dysfunction,⁶⁹ prefrontal cortical hypofunction,³⁹ DA dysfunction,³³ cortical GABAergic inhibitory neuron dysfunction,⁵⁴ and hippocampal dysfunction.⁵⁶ Though they differ in the proposed origin of the disorder, one thing they all have in common is a disruption of the mesolimbic DA system. These hypotheses are primarily based on the role of DA and the mesolimbic system in the brain of healthy or addicted patients, but lack support from anatomical studies in SZ subjects. The strong evidence for the role of DA in SZ is largely from imaging studies which, until recently, have not had the fine resolution required to separately analyze the NAcc from the rest of the striatum. Furthermore, while few studies have analyzed DA specifically in the NAcc,^{4,22,29,58,71,90} none have analyzed DA in the core and shell separately, which have unique functional roles and distinct functional consequences when affected.^{19,67,89}]

SA1 Methodology

1A. Experimental Design The overall goal of SA1 is to determine the differences in DA innervation of the NAcc in SZ. Postmortem human tissue will be used to compare NC and SZ. [Two separate cohorts of tissue have been obtained; one for densitometry and one for WB analysis. The cohort for WB analysis contains both on- and off-drug cases, allowing for APD effects to be examined. SA1.1 will utilize immunohisto-chemistry for 1) calbindin (CalB), to delineate the division of core and shell subregions^{32,37,66}; and 2) TH, to identify dopaminergic terminals.⁷⁰ A series of sections will be processed for Nissl as well (Fig. 1). The fixed cohort does not include a useful number of off-drug cases. SA1.2 WB assays will be used to measure TH protein levels.]

1B. Brain Tissue Collection and Diagnosis Postmortem human tissue has already been collected for this study (Table 1). Brain tissue, collected from the Maryland Brain Collection, has been transferred to our laboratory at UAB with a non-human subjects IRB protocol (N110411002 and N110411003). DSM-IV diagnosis of SZ was confirmed by two psychiatrists based on medical history of the subject, family interviews, autopsy reports, and neuropathologic assessments. Subjects were considered off-drug if they had been untreated with APDs for at least 6 months prior to death. NCs had no history of neurological disease. Pairs of NC and SZ cases were chosen based on the best match of age, race, sex, postmortem interval (PMI), pH, and storage time (corresponds to time in fixative or freezer).

1C. Tissue Processing SA1.1. Brain Dissection and Tissue Processing: The entire striatum was immersed in a fixative of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4; PB), pH7.2-7.4. Tissue is first rinsed in 0.01% sodium azide in PB and cryoprotected in increasing concentrations of sucrose in PB, then stored in 30% sucrose and sodium cut on a sliding microtome in 12 series of 50µm thickness, collected

Table 1. Demographics of human tissue for light microscopy and western blot studies

	SA1.1			SA1.2			
	NC	SZ	P value	NC	SZ-off	SZ-on	P value
N	10	13	-	10	10	10	-
Age, years	46.2±10.41	48.85±14.21	0.641	43.5±13.13	42.5±9.85	46.3±10.32	0.736
Race (#AA, #C)	5AA, 5C	7AA, 6C	0.858	5AA, 5C	4AA, 6C	1AA, 9C	0.142
Gender (#M, #F)	8M, 2F	9M, 4F	0.569	8M, 2F	8M, 2F	8M, 2F	1.0
PMI, hours	11.9±3.75	12.31±5.82	0.926	16.5±5.60	16.7±6.11	16.4±5.76	0.993
pH	6.54±0.36	6.42±0.06	0.487	6.66±0.19	6.6±0.27	6.48±0.34	0.340
Years in storage	17.2±3.01	17.0±2.89	0.873	13.5±6.70	18.4±3.98	15.7±4.95	0.193
Type of APD	NA	8t, 1a, 1off, 3un	-	N/A	N/A	6t, 4a	-

Mean ± standard deviation for control and schizophrenia (SZ) tissue. For SA1.1, p-values for control vs. SZ (Mann-Whitney *t*-test). For SA1.2, p-values for control vs. SZ-on vs. SZ-off (ANOVA). Abbreviations: AA, African American; C, Caucasian; M, male; F, female; PMI, postmortem interval; APD, antipsychotic drug; t, typical; a, atypical; off, off-medication; un, unknown.

in antifreezing solution, and stored at -20°C until used.

Immunohistochemistry: Adjacent sections will be stained for Nissl, CalB, and TH (Fig. 1). Tissue sections are treated with 5% H_2O_2 , pre-incubated in 10% normal horse serum, and then in primary antibody [Anti-CalB d28K (Millipore), 1:1,000; Anti-TH (Millipore), 1:2,000]. Next, sections are incubated for 45 min in biotinylated horse-anti-mouse secondary antibody (Vector), then for 45 min in the avidin-biotin complex (1:100; Vector) using recommended dilutions and times.³⁵ Sections are then put in diaminobenzidine (6 mg/10 ml PB, Vector) for 5-15 min to visualize the reaction product.^{48,62} Washes (PB, 5 times, 5 min each) are performed between appropriate incubations. Specificity of the secondary antibody is verified by omitting the primary antibody, but otherwise performing an identical protocol. [SA1.2. Tissue Processing: Sections from one full series representing the rostro-caudal extent of the NAcc will be collected on a cryostat for protein extractions. Western Blot: Standard protocols will be used (e.g, see ref. 73). PVDF membranes will be incubated with the anti-TH antibody (1:10,000; Millipore). Duplicate samples will be analyzed for each subject and averaged. The bands will be visualized using a alkaline phosphatase chemiluminescence (Biorad, USA), exposing Kodak Biomax films. As an internal control, membranes of all cases will be reblotted for actin (1:40,000; Millipore), a protein not altered in SZ.]

1D. Data Collection Optical density measurements will be used for determining the density of TH labeling in the NAcc core and shell. **SA1.1.** Using the CalB stained sections, a pattern (mask) delimiting the regions of the core and shell will be generated and overlaid onto an adjacent TH stained section. Each section is digitized on a light box with a digital camera and analyzed using ImageJ software to determine the grayscale optical density of TH staining in each subregion. [Three voxels per subregion will be measured and averaged to determine the mean gray values for that section. Measurements will be done in three different sections and averaged between them, to obtain mean gray values of core and shell for each case. Similarly for **SA1.2**, optical density will be measured on the films.] Calibration will be performed using the step tablet feature of the software. For each aim, images of all sections will be taken using the same parameters of exposure.

1E. Statistical Analyses [Power Analysis: *A priori* power analyses from published postmortem studies were used to determine the minimum sample size necessary with power=0.80 and alpha=0.05. **SA1.1** Data from Perez-Costas et al.¹ which used optical densitometry of TH in substantia nigra of SZ and NC gave a sample size of $n=7$ per group using a two-tailed t -test (effect size=1.71). **SA1.2** A study from Mackay et al.⁵⁸ measuring DA concentration in the NAcc provided a total sample size of $n=12$ using a one-way ANOVA (effect size=1.27). Larger sample sizes than necessary were selected to prevent the difficulty of achieving large effect sizes.] **Data Analysis:** All data sets will first be assessed for normality using the Kolmogorov–Smirnov test, followed by the appropriate test: **SA1.1** unpaired t -test or Mann-Whitney to compare SZ and NCs. A separate analysis will be done for core and shell. **SA1.2** ANOVA or Kruskal-Wallis, followed by posthoc LSD t -tests to compare on-drug SZ, off-drug SZ, and NC.] The effect of possible covariates (shown in Table 1) on the outcome of each measure will be assessed using multiple regression analysis. Significance will be considered $p<0.05$ for a two-tailed test.

SA1 Expected and alternative outcomes

[Elevated presynaptic DA in the striatum is the most widely replicated dysfunction of the dopaminergic system in SZ,³³ but past imaging studies have not had the resolution to distinguish between dorsal and ventral striatum.^{2,8,30,31,53,68} Based on the circuitry of the NAcc and the implication of the mesolimbic DA system in SZ as discussed previously, I expect that SZ subjects will have an increase in the density of TH labeling in the NAcc compared to NCs. Also, based on the evidence that elevated presynaptic DA is present in off-drug and neuroleptic-naïve patients,^{8,30,31,34,42,53} and that chronic treatment with APDs reduces DA in the NAcc,^{5,15,36,88,93} I expect that off-drug subjects will have higher levels of TH than both on-drug SZ and NCs.

It is possible that SZ subjects will not have elevated TH in the NAcc. Recent studies using high resolution imaging to differentiate between striatal divisions^{60,61} found that elevated presynaptic DA functioning was present in the caudate of off-drug and neuroleptic-naïve patients, but not in the ventral striatum.^{34,42} This was an unexpected result, and an outcome of ‘no difference’ in this study would confirm these findings. Another alternative result is that the subjects with SZ instead have decreased TH in the NAcc. Past studies have shown that low presynaptic DA functioning correlates with the severity of negative symptoms.^{31,42} Either alternative hypothesis could suggest that the ventral striatum may play a role in the negative symptoms of SZ but, contrary to what has been typically thought, may not be implicated in psychosis due to elevated DA functioning.]

SA1 Potential problems

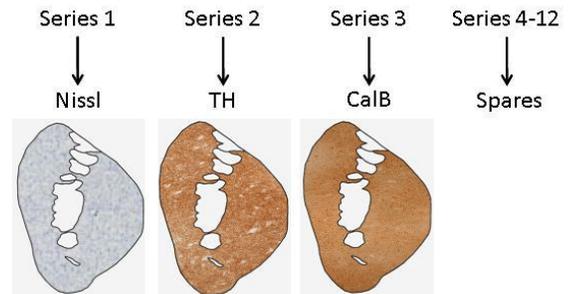


Figure 1. Staining plan uses 4 of the 12 series of tissue. Series 1: Nissl, series 2: tyrosine hydroxylase (TH), and series 3: calbindin (CalB) to use as a mask for the TH staining. Remaining series will be spares.

[The cohorts chosen for SA1 have been matched and statistical analysis has shown no differences (Table 1). Thus we do not expect such parameters to impact on our results. Studies in SZ always present the potential confound of APD treatment effects. Because of this, I have included two separate SZ groups in SA1.2, off-drug SZ and on-drug SZ. This will allow conclusions to be made regarding the effects of APD treatment on TH levels in the NAcc of subjects with SZ versus differences intrinsic to the disorder. Since these techniques and methodology have already been established in our laboratory, I do not anticipate any technical problems.]

Specific Aim 2 will test the hypothesis that subjects with SZ have increased synapses both symmetric (inhibitory) and asymmetric (excitatory), in the NAcc core and shell using stereology at the electron microscopic level to determine density of synapse types.

SA2 Rationale

[Pathology of SZ likely lies in the circuitry and synaptic connections linking brain regions. Though many studies have implicated the NAcc is SZ, none have been able to describe its circuitry. For example, Grace and colleagues have hypothesized, based on a MAM-treated rodent model of SZ, that reorganization of glutamatergic and dopaminergic inputs to the NAcc may be occurring in SZ (ref. 23). However, this is based on physiological phenomenon in healthy and MAM-treated rodents, and lacks the support of anatomical findings in SZ. Even though ultrastructure of the NAcc has been studied in rodents,^{65,67,85} it has never been studied in human. Thus, the proposed study will provide the first data on the ultrastructure of the human NAcc core and shell, as well as the aberrant circuitry that may be present in SZ.]

SA2 Methodology

2A. Experimental Design The overall goal of SA2 is to analyze the neurocircuitry present in the NAcc of subjects with SZ. Methods for performing comparative EM on human tissue have been established by our laboratory.^{47,80-83} Postmortem human tissue will be used for SA2 to compare NC and SZ.

2B. Brain Tissue Collection and Diagnosis A distinct cohort of postmortem brain tissue for SA2 (Table 2) has been obtained from the Maryland Brain Collection and was collected in the same manner described above (Methodology section 1.2B). All tissue in this cohort has a PMI of 8 hrs or less which allows for excellent preservation of the tissue suitable for EM.^{47,48,62,78-83}

2C. Tissue Processing Brain Dissection and Tissue Processing: Brains were fixed in 4% PFA and 1% glutaraldehyde in PB, pH 7.2-7.4, at 4°C. PFA-glutaraldehyde fixation followed by sodium borohydride treatment is an optimal technique for ultrastructural immunohistochemistry, as it preserves tissue ultrastructure and maintains antigenicity,²¹ and has been used in previous studies in our laboratory.^{47,48,62,78-83} A vibratome will be used to collect free-floating sections of the entire NACC at a thickness of 50µm in 12 series.

2D. Data Collection Electron micrographs will be taken of neuropil in core and shell. To determine the density of synapses in the neuropil, serial sections will be analyzed using the disector technique.^{25,73,86} This technique ensures that all parts of the region are sampled and that all synapses within the region have equal probability of being sampled, providing an unbiased estimate each synapse type. For each case, three sections across the rostrocaudal extent of the NAcc will be sampled and stereology will be used to collect a minimum of 100 synapses in 8-12 serial sections per sample. Average densities will be determined for each case using stereology counts over the total volume examined, as well as proportions of each synapse type. CalB-stained sections will be used as a reference for delimiting core and shell during data collection.

2E. Statistical Analyses [Power Analysis: Data from our laboratory on the density of asymmetric axospinous synapses in the dorsal striatum were used for an *a priori* power analysis to determine the minimum sample size necessary with power=0.8 and alpha=0.05. A two-tailed t-test of NC vs. SZ yielded a total sample size of $n=20$ (effect size=1.34).] [Data Analysis: There were no significant differences between the two groups when assessed for age, sex, race, PMI, brain pH, and years in storage (see Table 2). Statistical analysis will be as described in SA1.1. Separate analyses will be done for core and shell, and each dependent measure (subtypes of synapses based on postsynaptic density symmetry and postsynaptic structure).

SA2 Expected and alternative outcomes

Dopaminergic terminals form synapses with symmetric postsynaptic membrane specializations. Based on the robust evidence for elevated striatal DA in SZ discussed above, I expect that SZ subjects will have an increase in the density of symmetric synapses in the NAcc compared to NCs. [Glutamate was originally implicated in SZ due to the ability of NMDA receptor antagonists, such as PCP, to

Table 2. Case demographics for electron microscopy

	NC	SZ	P value
N	10	10	-
Age, years	50.3±17.19	51.4±12.92	0.871
Race (#AA, #C)	5AA, 5C	4AA, 6C	0.673
Gender (#M, #F)	5M, 5F	7M, 3F	0.388
PMI, hours	5.95±1.71	5.05±2.10	0.294
pH	6.91±0.33	6.73±0.49	0.410
Years in storage	12.8±5.43	14.5±5.10	0.450

Mean ± standard deviation for NC and SZ cases. P-values for control vs. SZ (Mann-Whitney *t*-test). Abbreviations: AA, African American; C, Caucasian; M, male; F, female; PMI, postmortem interval.

produce SZ-like symptoms.⁷ PCP causes an increase in spine density in the NAcc in rats.⁴⁶ Further, increased glutamate levels⁵² and increased glutamatergic-type synapses^{80,81,82} are present in the dorsal striatum in SZ. Thus, I also expect there will be a higher density of dendritic spines receiving asymmetric (glutamatergic-type) synapses in SZ.

Alternatively, the results could show no differences in synaptic organization between SZ subjects and NCs. It is possible that increased presynaptic DA in the striatum could be due to elevated levels within the existing terminals, rather than the sprouting of new dopaminergic terminals. The results from SA1 will help reconcile this, such that if an increase in the optical density of TH is seen but no difference in the number of terminals, I could conclude that the increase is due to elevated levels of TH within the existing terminals.]

SA2 Potential problems

[Since no clear demarcation exists between the core and the dorsal striatum, it will not be possible to determine total synapse numbers. Thus, results will be presented as average densities using the volume of the regions examined. To address possible tissue shrinkage, results will also be presented in proportions of synapse types. As discussed above, pairs of SZ cases and NCs were matched to minimize differences in fixation time. Additionally, the results could be confounded by the effects of APDs in the NAcc; studies in patients with SZ have shown that typical and atypical APDs have differential effects in the striatum.^{3,9,51} Because possible treatment effects are an inevitable consequence of studying subjects with SZ, several measures will be taken to aid in the interpretation of the postmortem results. These include the WB study on a cohort of subjects that were off medication prior to death, proposed in SA1.2, and the APD-treated rodent study proposed in SA3. Since these techniques are established in our laboratory, I do not anticipate any technical problems for SA2.]

Specific Aim 3 will test the hypothesis that chronic APD treatment causes a decrease in dopaminergic innervation of the NAcc core and shell, by measuring the optical density of TH immunolabeling in rodents that have been chronically treated with APDs.

SA3 Rationale

Nearly all patients with SZ have been chronically treated with APDs, which complicates the interpretation of results. One way to address this issue is to examine SZ subjects who were drug free prior to death, as will be done in SA1.2. However, this approach will not be possible for light or electron microscopy due to the limited number of these types of cases available. An alternative approach is to examine the effects of chronic administration of APDs in rats. Chronic treatment of rats with APDs is an important aspect of SZ research and has been widely used for understanding APD effects.⁵⁵

[Past studies have shown that treatment of rats with typical or atypical APDs have differential effects on the DA system within the NAcc.^{5,15,36,40,59,88,93} However, the length of treatment in these studies is typically 3 weeks, and studies by separate groups show profound differences in treatment effects in the dorsal striatum after treatment for 3 weeks^{44,91} compared to 6 months.^{43,63} Thus, to more accurately reflect the long-term treatment that patients receive, a 24-week chronic treatment paradigm was used. Further, although APD treatment administered through drinking water is a widely used and validated method, only one of the previous studies in the NAcc used this route of drug administration.³⁶ Administering APDs through drinking water provides many benefits over other commonly used methods, including decreased stress associated with treatment, accurate modeling of administration for patients, and provides more stable dosing in the rat than is achieved with other methods.^{41,72} The APD-treated rodent study will provide a model to determine the direct effects of APDs on TH levels in the NAcc, and provide a context for the interpretation of the findings in postmortem human NAcc.]

SA3 Methodology

3A. Experimental Design The overall goal of this aim is to determine the effects typical and atypical APDs have on dopaminergic innervation of the NAcc. Thirty-six Sprague Dawley male rats received either haloperidol (typical), olanzapine (atypical), or vehicle control in their drinking water for 24 weeks. Immunohistochemistry of TH and optical densitometry will be used to analyze TH levels. Any differences present at the light microscopic level can be further investigated with EM if time permits, or in future studies.

3B. Drug Treatment and Tissue Collection Animals were housed 2 per cage to avoid isolation stress and were weighed weekly to adjust drug dosages. At the time of euthanasia, rats were given a lethal dose of anesthesia (ketamine and xylazine) by IP injection, and blood was drawn when deeply anesthetized followed by intracardiac perfusion fixation with 4% PFA, 0.1% glutaraldehyde in 0.1M PB. Brains were hemisected, allowing one hemisphere to be used for light microscopy, and the other for future EM studies.

3C. Immunohistochemistry Tissue will be sectioned in 6 series of 50µm thickness on a vibratome, which yields 7-8 sections per series through the NAcc. Three separate series will be stained for Nissl, TH, or CalB, as shown in Figure 1. Dilutions of the primary antibodies are: anti-TH, 1:1000 (Millipore) and anti-CalB, 1:20,000 (Millipore).⁷⁶ The rest of the process will be done as detailed above (SA1 Methodology, section 1C).

3D. Light Microscopy Optical densitometry will be used to determine the density of TH labeling as detailed above (SA1 Methodology, section 1D). In addition to core and shell, whole region measurements will be done without division of subregions to allow for comparisons with past studies.

3E. Statistical Analyses Power analysis: Data from Perez-Costas et al,¹ which measured the effects of haloperidol and olanzapine on TH in the substantia nigra of treated rats using optical densitometry, was used for an *a priori* power analysis. With power=0.80 and alpha=0.05, a one-way ANOVA yielded a total sample size of $n=30$ (effect size=0.61). Data analysis: Data will be analyzed to determine significance of any differences found between the groups. Statistical analyses will be as described above (SA1 Methodology, section 1E).

SA3 Expected and alternative outcomes

Our lab has shown that chronic treatment of rats with haloperidol decreases total synapse density,^{43,77} including symmetric synapses in the striatum.⁷⁷ DA afferents form symmetric synapses in the striatum and NAcc,^{6,75} thus it is possible that chronic APD treatment causes a reduction in DA synapses. Since both typical and atypical APDs affect DA neurons of the VTA^{16,92} I expect to see a decrease in the density of TH levels after chronic treatment of rats with haloperidol and olanzapine. [Further, while VTA DA neurons do project to the entire NAcc, the core primarily receives its dopaminergic innervation from the substantia nigra,⁵⁷ which is not targeted by atypical APDs.^{16,87,92} Thus, I expect larger effects in the core with haloperidol than with olanzapine.

One alternate outcome is that chronic treatment does not result in reduced TH levels in the core or shell. A study from our lab found that chronic treatment with haloperidol did not result in significant changes in TH-labeled terminals in the dorsal striatum,⁷⁶ and Ingham et al.³⁸ found that a lesion of the dopaminergic nigrostriatal pathway did not reduce the density of symmetric synapses. Both of these findings were unusual, and authors of the lesion study suggest their result could be due to the small number and size of these synapses or to compensatory DA terminal sprouting. Similarly, it is possible that a negative result in the proposed study could be due to changes too small to measure, or to compensatory upregulation of dopaminergic innervation.]

SA3 Potential problems

[Since these techniques are established in our laboratory,⁷⁶ I do not anticipate any technical problems for the proposed research plan of SA3. However there are some conceptual issues that merit discussion. A potential problem with this aim is the disconnect between the rat treatment paradigm and the complexity of patient treatment, including varying dosages, durations, and drug types. Our choice of healthy rats, rather than ones partially modeling SZ, could be challenged because APD may have different effects in healthy vs. SZ-like rats. However, we chose to study a simple model to determine drug effects in the NAcc. There are no complete animal models of SZ and all current animal models rely on pharmacological, developmental, or physical manipulation to mimic aspects of the disorder and therefore may lack etiological validity. Thus, treating “diseased” rats in this study would only add another level of complexity to the interpretation of the APD effects. Direct comparison of rats to humans is difficult, but past studies using this approach have found similar changes in rats as those found by separate groups studying APD effects in SZ patients.³ Another issue is that our rats were treated with one APD, while current patients are often treated with a combination of APDs.] Importantly, the human cases that will be used in the present study were collected from an era when patients were usually on only one type of APD, rather than a combination, which reduces the complexity of potential drug effects. Chronic treatment APDs in rats has been shown to cause a small increase in striatal volume³ consistent with the same well established medication effect in patients with SZ.^{14,45} This change is small (<5%) and in previous studies from our laboratory was insignificant,^{76,77} so I do not expect this to impact the results.

Synthesis of postmortem human and rat analyses

If results of the analyses are as predicted, with an increase in TH levels and the density of spines in SZ subjects and decreases in treated rats, I could conclude that the increase seen in SZ subjects compared to NCs was intrinsic to the disorder rather than an effect of medication. [Results for the off-drug SZ subjects will allow these conclusions to be developed further. A finding of a larger increase in TH levels in the off-drug compared to on-drug subjects, along with reductions in the treated rats will provide strong evidence for increases in TH levels in the NAcc as intrinsic to the disorder. Without the evidence for chronic APD treatment causing reductions in TH levels in the rodents, this conclusion would require more speculation. Alternatively, if chronic treatment does not reduce TH levels in the NAcc of rats, it would suggest that any differences found between the off- and on-drug groups may be attributable to variation within the disorder rather than treatment. An example of what could cause this variation is the association found between lower dopaminergic activity and negative symptom severity discussed above (ref. 31,42). This would imply that variations in TH in the NAcc were not necessarily due to chronic treatment, and would warrant further study.] Lastly, if analyses show an increase in the density of TH levels in the treated rats, the results would suggest that any increased density of dopaminergic innervation in SZ subjects be a medication effect, perhaps a compensatory mechanism for the chronic blockade of DA D₂ receptors during treatment.

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SUMMARY STATEMENT
(Privileged Communication)

Release Date: 03/22/2013

Application Number: 1 F31 MH098566-01A1

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Review Group: ZRG1 F01-F (20)
Center for Scientific Review Special Emphasis Panel
Fellowships: Brain Disorders, Language, Communication and Related
Neurosciences

Meeting Date: 03/07/2013

Council: MAY 2013

PCC: AK-TNSF

Requested Start:

Project Title: Dopamine levels in postmortem human nucleus accumbens in schizophrenia

Requested: 2 years

Sponsor: Roberts, Rosalinda C
Department: Psychiatry & Behav Neurobiol
Organization: UNIVERSITY OF ALABAMA AT BIRMINGHAM
City, State: BIRMINGHAM ALABAMA

SRG Action: Impact Score: 30 Percentile: 16

Next Steps: Visit http://grants.nih.gov/grants/next_steps.htm

Human Subjects: 10-No human subjects involved

Animal Subjects: 10-No live vertebrate animals involved for competing appl.

1F31MH098566-01A1 McCollum, Lesley

RESUME AND SUMMARY OF DISCUSSION: This is a predoctoral fellowship application from an outstanding candidate who proposes to study the role of neuroanatomical changes and abnormal circuitry in schizophrenia. The applicant's academic grades, productivity record, and recommendation letters are excellent. The sponsor, Dr. Rosalinda Roberts, a professor of psychiatry at University of Alabama at Birmingham, is a well-funded, accomplished investigator with a solid mentoring record. The sponsor presents a comprehensive, detailed training plan specifically tailored to the applicant's needs. The revised research plan is substantially strengthened by the refocused specific aims, clearly presented rationale and power analysis. The training potential of the proposed work is high. Despite some remaining concerns, the revised application overall generates considerable enthusiasm.

DESCRIPTION (provided by applicant): Schizophrenia is a severe mental illness affecting about 1% of the population in which disruptions in the physical connections of neural circuitry are unclear. Our goal is to further understand the underlying changes to neural circuitry that occur in schizophrenia to provide a foundation that is fundamental for better treatment. The nucleus accumbens is a region of convergence for afferents of many brain areas disrupted in schizophrenia, and is the major site of input into the basal ganglia from the mesolimbic dopamine system, making it a key area of interest for schizophrenia; dopamine hyperfunction in the dorsal striatum is a hallmark characteristic of the disorder, and the role of dopamine as central in schizophrenia is one of the most accepted hypotheses of the illness. Although there is evidence for the role of the ventral striatum in schizophrenia, the anatomical pathology of the nucleus accumbens in schizophrenia has largely been overlooked. The proposed research will test the hypothesis that subjects with schizophrenia have increased dopaminergic inputs in the nucleus accumbens compared to normal control cases using postmortem human tissue. The density of tyrosine hydroxylase immunolabeling, the rate limiting synthesizing enzyme of dopamine, will be determined in control subjects compared to groups of off- and on-drug schizophrenia subjects using optical densitometry and western blot assays. The proposed research will also determine the ultrastructural abnormalities present in the nucleus accumbens neurocircuitry of subjects with schizophrenia using 3- dimensional stereological counting at the electron microscopic level. Lastly, chronically antipsychotic drug- treated rats will provide a context for the interpretation of results from the postmortem human studies. Results from the proposed research will provide insight into the abnormal circuitry that may be a core feature of schizophrenia, further elucidate mechanisms of the illness, and offer insight into therapeutics which are not fully understood.

PUBLIC HEALTH RELEVANCE: There is a need to improve treatment for patients with schizophrenia which requires a better understanding of the underlying pathology of the neural circuitry in the disorder than is currently known. This project will determine if there are underlying neural circuitry disruptions in the nucleus accumbens core and shell in schizophrenia to help fill the gaps in this necessary foundation of schizophrenia treatment.

CRITIQUE 1:

Fellowship Applicant: 1

Sponsors, Collaborators, and Consultants: 2

Research Training Plan: 3

Training Potential: 3

Institutional Environment & Commitment to Training: 2

Overall Impact/Merit: This revised application is from a highly motivated predoctoral candidate committed to an independent research career focusing on the neuropathology of mental illness. The main goal of the proposal is to further understand the aberrant neural circuitry found in the ventral

striatum of patients with schizophrenia, particularly at the ultrastructural level. Using light and EM immunohistochemistry in human postmortem tissue from schizophrenic subjects, the applicant proposes to assess changes in dopamine (via its biosynthetic enzyme tyrosine hydroxylase, TH) in axon terminals of the nucleus accumbens, a limbic structure often associated with schizophrenia. The applicant demonstrates valuable previous research experience, her scholastic record is outstanding, and she received strong letters of recommendation. The well-published sponsor has considerable and unique expertise in the ultrastructural analysis of normal and diseased human postmortem brain, including combined EM immunohistochemistry. The sponsor has formulated a comprehensive and detailed training plan and is committed to fostering the candidate's training and career development. The training potential for the applicant is excellent and the institutional environment and resources for predoctoral training are superb. When completed, the results of the proposed studies should provide new fundamental information on the neuropathology of schizophrenia. Only a few minor weaknesses remain in the proposal. Overall, the present applicant is an excellent candidate for a predoctoral NRSA fellowship.

1. Fellowship Applicant:

Strengths

- The applicant's previous research experience has been excellent, extending from her college days, briefly in industry, as a summer NSF REU fellow, to several valuable laboratory rotations in graduate school at the University of Alabama at Birmingham (UAB).
- The candidate has garnered one first-author publication (with the sponsor), one middle-author paper (from a laboratory rotation; new since previous submission) and numerous abstract presentations.
- The applicant's scholastic record has been excellent to outstanding at both the undergraduate and graduate levels. She earned numerous academic and professional honors and awards at her undergraduate institution.
- The strong reference letters described the applicant as "thoughtful, skillful, articulate, dedicated, focused, independent, enthusiastic, and a model student." With few exceptions, she was ranked in the upper 5% in all category ratings.

Weaknesses

- None noted.

2. Sponsors, Collaborators, and Consultants:

Strengths

- The sponsor, Dr. Rosalinda Roberts, a Professor of Psychiatry and Behavioral Neurobiology at UAB, is an expert in the study of human postmortem brain tissue at the ultrastructural level, and particularly with brains from subjects with neuropsychiatric disorders.
- The sponsor displays an excellent publication record.
- The sponsor's recent UAB Graduate Dean's Excellence in Mentorship Award bodes well for the training and mentorship of the applicant. Accordingly, the updated training plan crafted by the sponsor was extensive, detailed and specifically tailored to the needs and goals of the applicant.
- The sponsor appears to have sufficient funds to support the research activities of the candidate.
- The sponsor is the director of the Alabama Brain Collection which assures the ready availability of the human psychiatric brain tissue.

Weaknesses

- Minor: The sponsor has retained her recent predoctoral and 2 postdoctoral trainees in postdoctoral and Assistant Professor positions, respectively, in her laboratory, rather than encouraging them to establish independent careers.

3. Research Training Plan:

Strengths

- The proposed research is important in that it will add to our understanding of the neuropathology of schizophrenia by examining ultrastructural changes in the postmortem nucleus accumbens of schizophrenia subjects compared to controls.
- The applicant is very responsive to the previous critiques and, thus, this revised proposal is improved from the previous submission.
- Accordingly, the previously proposed two aims have now been rearranged into three aims, immunostaining for PSD95 has been omitted, schizophrenia cases with neuropathology have been excluded, additional rationale for certain aspects of the studies has been included, power analyses have been added, etc. The result is a more well-defined project in which more solid conclusions can be interpreted from the results.
- The aims remain straightforward and readily feasible within the context of the sponsor's previous work. In the rat study, the fact that the rats have already been treated with a regimen of antipsychotics, perfused and in storage will accelerate the completion of the aim.
- The research proposal continues to be well-written with good consideration of statistical analyses, expected outcomes, potential pitfalls and alternative approaches.
- Shadowing the clinician Dr. Lahti, who sees patients with schizophrenia, will be of substantial benefit to the candidate's training experience.

Weaknesses

- Although the applicant and sponsor have made clear that the proposed studies are novel in examining the nucleus accumbens, they are still not particularly innovative in that the same techniques, approaches and analyses used in previous studies of the sponsor are employed here.
- Questions of measuring other informative attributes of dendritic spines (e.g. length, shape) in addition to determining spine density in the experiments were not addressed.

4. Training Potential:

Strengths

- The training potential for the applicant is excellent. She will continue to enhance her research skills in light and electron microscopy in human material, immunohistochemistry, sectioning techniques and stereology. She has now enumerated the new skills to be learned, which include Western blots, light microscopy in rat material, and optical density measurements.
- The exposure to and training in human brain collection management is a relatively unique opportunity that could give her an advantage in attaining future faculty positions.
- Attending the week-long stereology course at Woods Hole is well-advised for the applicant and will certainly add to her training.

Weaknesses

- Although it is now clear that new skills will be attained, some methods appear to be variations of the techniques she already uses.

5. Institutional Environment & Commitment to Training:

Strengths

- The institutional research and training environment at UAB must be considered outstanding, particularly regarding the Department of Psychiatry and Behavioral Neurobiology (including the sponsor's EM facilities), the Neuroscience Graduate Program, and the Comprehensive Neuroscience Center.
- A notable strength is the first-year student "boot camp" at the Dauphin Island Sea Laboratory, which also provides upper-level students an opportunity to teach and mentor the entering class.

Weaknesses

- None noted.

Protections for Human Subjects:

Not Applicable (No Human Subjects)

Data and Safety Monitoring Plan (Applicable for Clinical Trials Only):

Not Applicable (No Clinical Trials)

Vertebrate Animals:

Not Applicable (No Vertebrate Animals)

Biohazards:

Not Applicable (No Biohazards)

Resubmission:

- The applicant has readily addressed the previous critiques. The result is an improved application that will be a strong training vehicle to put the applicant on track to achieving her career goals of becoming an independent investigator studying the neuropathology of mental illness.

Training in the Responsible Conduct of Research:

Acceptable

Comments on Format (Required):

- Numerous ethics courses consisting of a mixture of didactic, workshop, and online formats. The completed didactic course is commented on below.

Comments on Subject Matter (Required):

- Appears that all NIH-recommended RCR topics are covered.

Comments on Faculty Participation (Required):

- Led by the former editor and chair of the Publications Committee of the American Physiological Society.

Comments on Duration (Required):

- Semester-long.

Comments on Frequency (Required):

- Weekly

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2:

Fellowship Applicant: 2

Sponsors, Collaborators, and Consultants: 2

Research Training Plan: 3

Training Potential: 3

Institutional Environment & Commitment to Training: 1

Overall Impact/Merit: The applicant is applying for two years of support to complete PhD dissertation research. This is a revised application and the candidate and mentor did a good job of responding to prior criticism. Strengths of the application are in an excellent candidate, mentor, and overall research environment. Some minor weaknesses remain in the research plan and training plan as well as the response to prior critique. Overall, this is an excellent application with solid potential to prepare the student for postdoctoral studies and eventually an independent position as a research scientist.

1. Fellowship Applicant:

Strengths

- The applicant is excellent based on academic performance, two publications, letters of reference and this proposal.

Weaknesses

- None

2. Sponsors, Collaborators, and Consultants:

Strengths

- The mentor is an expert in EM and neuroanatomy and a leader in the field of brain banking.
- Funding appears to be adequate to support the student during the time frame proposed.

Weaknesses

- Some typos and missing experimental details are minor weaknesses in the proposal that could have been addressed by the mentor before submission.

3. Research Training Plan:

Strengths

- Ph.D. with special skills in neuroanatomy and EM stereology is rare and valuable.
- Understanding Brain Banking and limitations to use of human postmortem tissue is important.
- Detailed study of dopamine and neurotransmitter functionality in the ventral striatum with respect to SZ is warranted.

- Detailed study of dendritic spines at the ultrastructural level via EM is important and may provide valuable new information.
- Detailed study of the effects of chronic APD treatment on neurotransmitter function is reasonable.

Weaknesses

- Some details of the methods are lacking: i.e. student will do western blots and refers reader to ref. 73 with nothing about tissue preparation and no details provided on how EM is performed with respect to tissue processing, embedding and sectioning
- While it is clear the mentor is an expert in these methods, it is still useful to have the student demonstrate knowledge and proficiency by giving even brief description of experimental details: Is tissue stained en bloc? How much diffusion occurs? How much tissue is used for EM embedding?
- Power calculations for aim three suggest an N=30 is needed for power to detect significant differences between groups. However, student proposes to use 12 animals as controls, 12 with haloperidol and 12 with olanzapine.
- Stereology using only 100 dendrites in 8-12 sections not justified by power analysis and may be too few to give adequate power to detect meaningful biological differences.
- Difficult to compare western blots of APD on/off to just APD off with EM.

4. Training Potential:

Strengths

- Western blot is new technique for the student to develop.
- Cryosectioning is a new technique to be developed.
- EM, tissue processing, embedding, staining, ultramicrotome sectioning and use of the scope are all new to the student.

Weaknesses

- Lack of detailed methods is a minor concern. The applicant might have given better descriptions or cite specific research methods papers published by the mentor.

5. Institutional Environment & Commitment to Training:

Strengths

- Mentor demonstrates strong commitment to student
- Resources and laboratory support appear to be excellent
- Training plan is well described and specific to this student

Weaknesses

- None

Protections for Human Subjects:

- Not Applicable
- Not considered human research as post mortem tissue is deidentified

Vertebrate Animals:

Not Applicable (No Vertebrate Animals)

Biohazards:

Acceptable

- The mentor is expert in handling post mortem human tissues.

Resubmission:

- This is a revised application. Largest concern from the first review was that the student already knew how to perform EM, LM, IHC, and all associated techniques. The applicant and mentor clearly state that the early publication from the student was primarily image analysis done on images already generated by others and that he student did not have expertise in EM. Western blots are new and this requires cryosectioning, also new. The other major concern was the relevance of this detailed study on the ventral striatum given similar work already done on dorsal striatum from the same laboratory. This reviewer agrees that study of the ventral striatum in SZ is warranted. In general, the student could have done a better job in writing the response to the previous review, however, the response was adequate and alleviated the major concerns for this reviewer.

Training in the Responsible Conduct of Research:

Comments on Format (Required):

- Didactic course and web based

Comments on Subject Matter (Required):

- All 9 NIH required topics covered in these sessions

Comments on Faculty Participation (Required):

- Course is faculty taught

Comments on Duration (Required):

- Course was one time for 5 weeks

Comments on Frequency (Required):

- Students met once each week, web based training is taken annually

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3:

Fellowship Applicant: 2

Sponsors, Collaborators, and Consultants: 2

Research Training Plan: 4

Training Potential: 3

Institutional Environment & Commitment to Training: 3

Overall Impact/Merit: This is a revised application to study the dopamine innervation of the nucleus accumbens grossly and at the electron microscopic level in the postmortem brains of persons with schizophrenia. Although clear attempts were made to address the issues raised in the prior application, some concerns, including the training potential and research plan remain.

1. Fellowship Applicant:

Strengths

- The applicant is a strong candidate with a background in mathematics and physics.
- The applicant has 2 publications, one as first author in Synapse and 13 abstracts with 11 as first author.
- The applicant has excellent grades and strong letters of recommendation that place her in the top tier.

Weaknesses

- None noted.

2. Sponsors, Collaborators, and Consultants:

Strengths

- The sponsor, Dr. Roberts, is a highly accomplished neuroanatomist who is among the very few who perform electron microscopic studies of the postmortem human brain. As such she is an ideal sponsor for a trainee who wishes to specialize in the study of anatomic circuits in schizophrenia.

Weaknesses

- None noted.

3. Research Training Plan:

Strengths

- The study of the neuroanatomy and circuitry of the nucleus accumbens in the human brain is important and strength of the application.

Weaknesses

- Although some of the questions and issues raised in the prior application were addressed, few remain unanswered, including: the precise phenotypes of the cases to be used, the key questions in the neurobiology of schizophrenia that will be answered, the specificity of the hypotheses that will be tested, and the lack of sophistication of specific aims 1 and 3.
- DA has been studied in the nAcc for decades. The innovation here is to study it in the core and shell separately. However, the methods do not match the hypotheses in that no specific or differential predictions or hypotheses are made regarding these divisions. Given the known anatomy and efferent/afferent projects of nAcc, more sophisticated and discrete hypotheses could have been formulated.
- Aim 1- immune TH determination of shell vs. core - the methods proposed here (density measurement by camera from a light box view of the whole section) are not very sophisticated and provide limited training potential. In addition, the method has all of the disadvantages of ICC and none of the microscopic advantages. The same Calbindin delineation of shell vs. core could be used for much more quantitative Western analyses. Based on the sponsor's statement, it appears that the Western blot analysis will be performed from dissections performed on cryostat

sections. For example laser capture microscopy combined with Western blot or ELISA could be used to address the hypotheses with significantly greater precision.

- The description of the studies for Western blot analysis is sparse.
- The phenotypic information provided in Table 1 would have been more helpful if it included information on causes of death, presence or absence of illicit drugs and alcohol, other psychiatric comorbidities. This suggests a certain level of inattention to phenotypic issues that is contrary to current trends in postmortem human brain tissue studies.
- Although there is every reason to believe in the utility of descriptive ultrastructural studies of the nAcc, the interpretation of the findings and the implications for the pathophysiology of schizophrenia are as important. The application lacked sufficient emphasis on potential outcomes and their differential implications.

4. Training Potential:

Strengths

- Learning brain banking procedures as part of the training continues to be a significant strength.
- Similarly, learning the clinical features of schizophrenia by the shadowing experience described is a strength.
- Learning methods and procedures for the ultrastructural study of the human postmortem brain is a significant opportunity.

Weaknesses

- Despite the training potential of the EM studies, the general lack of sophistication in the hypotheses formulated and research design speaks to some shortcomings in the non-technical aspects of the training plan.
- The relative lack of sophistication of the immunohistological studies, and the lack of description of some of the fundamental procedures and interpretations may somewhat limit training potential in these areas.

5. Institutional Environment & Commitment to Training:

Strengths

- The institution is strong and the technical and intellectual environment for electron microscopy is a special strength.

Protections for Human Subjects:

Not Applicable (No Human Subjects)

Inclusion of Women, Minorities and Children:

Not Applicable (No Human Subjects)

Vertebrate Animals:

Not Applicable (No Vertebrate Animals)

Biohazards:

Acceptable

Resubmission:

- Many but not all of the concerns raised in the prior review have been addressed.

Training in the Responsible Conduct of Research:

Acceptable

Comments on Format (Required): Acceptable

Comments on Subject Matter (Required): Acceptable

Comments on Faculty Participation (Required): Acceptable

Comments on Duration (Required): Acceptable

Comments on Frequency (Required): Acceptable

Budget and Period of Support:

Recommend as Requested

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

ADMINISTRATIVE NOTE: The applicant's plan for training in the responsible conduct of research is acceptable.

NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-10-080 at <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-10-080.html>.

The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.

MEETING ROSTER

Center for Scientific Review Special Emphasis Panel CENTER FOR SCIENTIFIC REVIEW

Fellowships: Brain Disorders, Language, Communication and Related Neurosciences
ZRG1 F01-F (20) L

March 07, 2013 - March 08, 2013

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