Cortical Cells, Circuits, Connectivity and Cognition in Schizophrenia

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CENTER SPECIFIC AIMS

Long-term functional outcome in individuals with schizophrenia and related disorders is primarily determined by the degree of impairment in certain core cognitive abilities, such as working memory and attention[1,2]. Working memory depends upon the proper temporal activation of networks of neurons distributed across cortical regions. For example, visual working memory requires both 1) the communication of information from the primary visual cortex (**V1**) across the hierarchy of visual cortical regions to the posterior parietal cortex (**PPC**) and dorsolateral prefrontal cortex (**DLPFC**), and 2) the sustained activity of, and synchronous interactions among, neurons in the PPC and DLPFC[3-6]. Similarly, visual attention, whether bottom-up, salience detection or top-down, cognitively-guided searching, is implemented via these same distributed cortical circuits[7-9]. Subjects with schizophrenia exhibit impairments in working memory and attention, and altered patterns of activity in DLPFC and PPC during visual working memory and attention tasks[10-15]. These findings highlight an unanswered critical question: What are the cortical cellular, circuitry and connectivity bases for the impairments in visual working memory and attention in schizophrenia and related disorders?

In primates, ascending visual information is conveyed across cortical regions via excitatory projections that arise predominantly from layer 3 pyramidal cells (**PCs**) and that principally target PC dendritic spines in layers 3-4[16]. In addition, descending projections from DLPFC to PPC also predominately (~75%) originate in layer 3 PCs[17-19] and terminate with highest density in layer 3 of PPC[19,20]. The molecular, morphological and functional properties of layer 3 PCs exhibit regional specificity in the primate brain. For example, layer 3 PCs differ in morphology between V1, PPC and DLPFC in monkeys[21,22], in intrinsic excitability between V1 and DLPFC in monkeys[23], and in morphology between V1 and DLPFC in humans[24]. In addition, the gray matter of the human neocortex shows a general caudal-to-rostral topography of regional differences in gene expression, with V1 and prefrontal regions at opposite ends[25].

In schizophrenia, PCs in layer 3 of the DLPFC exhibit morphological abnormalities, such as smaller cell bodies, shorter dendrites and fewer dendritic spines[26,27]. Furthermore, in studies of the same subjects, these disease-related abnormalities exhibit laminar specificity (e.g., PCs in layers 5-6 are unaffected) and regional differences in magnitude (e.g., DLPFC>>area V1)[28,29]. These findings, in concert with evidence of altered expression of genes regulating dendritic spines in DLPFC layer 3[27,30,31], support the idea of an intrinsic molecular abnormality (i.e., cell type-autonomous disturbance) in layer 3 PCs that is moderated by factors that differ across cortical regions.

Thus, the complementary studies proposed in this application are designed to test the following integrative **Central Hypothesis:** ***Cell type-autonomous molecular disturbances in layer 3 PCs give rise to morphological abnormalities in these neurons. The severity of this cellular pathology is moderated across cortical regions as a function of normal regional differences in the properties of layer 3 PCs, such that measures of pathology are smallest in area V1, intermediate in PPC and greatest in DLPFC. This cellular pathology alters cortical circuitry within and between regions, impairing functional connectivity across regions, and resulting in disturbances of both bottom-up and top-down processes during visual working memory and attention in individuals with schizophrenia.***

This hypothesis provides a detailed accounting of impaired visual working memory and attention in schizophrenia which integrates abnormalities in distinct cell types that form specific cortical circuits with the resulting impairments in functional connectivity that underlie the emergence of cognitive deficits. The five inter-related projects (**P**) of the proposed Center provide convergent tests of this hypothesis 1) at the molecular, cellular, laminar and local circuitry levels in postmortem brain samples from individuals with schizophrenia or schizoaffective disorder (**P1**&**P2**), and 2) at the regional and distributed circuitry levels through imaging and neurophysiological studies of cognitive processes in medication-naïve subjects in a first-episode of psychosis (**P5**); these studies are both informed and constrained by parallel studies in monkeys (**P3**&**P4**). A key innovation of this approach is the integration of studies with multiple levels of resolution, from molecules to behavior, that provides a translational assessment of both bottom-up and top-down explanations of cortical and cognitive dysfunction. In addition, the combination of molecular-cellular-circuit level analyses and in vivo indices of brain function offer a platform for future identification of novel, pathologically-based targets for therapeutic interventions accompanied by pathophysiologically-informed biomarkers that can be used to predict and monitor the efficacy of such interventions.

1. CENTER OVERVIEW

At the University of Pittsburgh, we have a well-established milieu in which basic and clinical neuroscientists collaborate productively using a translational strategy and a multidisciplinary approach to investigate the disease process of schizophrenia and related disorders. The over-arching goals of our investigations are 1) to identify novel molecular targets for therapeutic interventions that are based on the underlying pathophysiology of specific clinical features, and 2) to develop sensitive biomarkers of that pathophysiology that can be used to assess the efficacy of new compounds with activity at those targets. The investigations proposed in this new application for a Silvio O. Conte Center for Translational Mental Health Research reflect the synergistic scientific interactions among Center investigators and the resulting development and implementation of innovative experimental designs and research tools to achieve these goals. The investigators include well-established schizophrenia researchers (Drs. Lewis, Haas, Salisbury, Sampson and Sweet), accomplished senior scientists in other fields who have been attracted to schizophrenia research by the scientific challenges and collaborations described in this application (Drs. Colby, Luna, Olson, Sibille and Tseng), and productive, earlier career-stage investigators (Drs. Chen, Cho, Fish, Ghuman, González-Burgos and Volk), whose development will be fostered by the environment and activities of the proposed Center.

The focus and investigators of the proposed Center are distinct from the University of Pittsburgh Conte Center for the Neuroscience of Mental Disorders (MH084053) funded July 1, 2008-June 30, 2013. Although in its first 5 year period of funding, MH084053 was not eligible for competing renewal due to the timing of funding criterion in the new program announcement for Conte Centers for Basic or Translational Mental Health Research. In the proposed Center, the Central Hypothesis, scientific strategy and research methods are new, as is the investigative team; only Drs. Lewis and Olson served as Project leaders in MH084053.

The proposed Center offers a highly interactive scientific environment that integrates the basic and clinical research activities of multiple investigators from the University of Pittsburgh and the adjacent Carnegie Mellon University. Collectively, the proposed Center represents a broad array of expertise that spans molecular, cellular, systems, cognitive, and clinical neuroscience. In addition to its specific research objectives, the proposed Center provides 1) a rich environment for training and career development in which undergraduate, graduate and medical students, postdoctoral fellows and psychiatry residents can become involved in studies that bring the methods and knowledge base of basic neuroscience to address critical questions in clinical research, and 2) a mechanism for disseminating the importance of, and the knowledge gained from, translational studies of schizophrenia and related disorders to the broader scientific and lay communities.

As described below, this Center is built around complementary approaches to testing a **Central Hypothesis** regarding the neural substrate for a core clinical feature of schizophrenia. The 5 proposed projects provide an integrated test of this hypothesis in the following manner: Postmortem studies of subjects with schizophrenia or schizoaffective disorder (**P1**&**P2**) will reveal molecular, morphological and local circuitry abnormalities in layer 3 PCs that increase in severity from V1 to PPC to DLPFC. This pattern of pathology will directly parallel measures of the pathophysiology of regional synchrony and functional connectivity associated with impaired attention and working memory in medication-naïve subjects in a first-episode of psychosis (**P5**). The correspondence between these measures of pathology and pathophysiology will be supported by findings that layer 3 PCs 1) increase in complexity of molecular, morphological and neurophysiological properties across the V1-PPC-DLPFC network (**P1**-**P3**), and 2) provide connectivity across regions that supports the synchronous activity necessary for attention and working memory (**P4**). ***The synergism and bi-directional interactions of these projects creates a translational strategy to incisively test the Central Hypothesis and to facilitate subsequent studies leading to transformative discoveries***. Specifically, cross-project integration provides an innovative approach for examining cell type- and circuit-specific pathology that may account for both top-down and bottom-up explanations of functional disturbances in core cognitive processes in schizophrenia. In addition, combining molecular-cellular-circuit level analyses with in vivo indices of brain function offers the opportunity to identify novel, pathologically-based targets for therapeutic interventions accompanied by pathophysiologically-informed biomarkers that can predict and monitor the efficacy of such interventions.

The approach of the proposed Center also offers a ***novel strategy that is responsive to the NIMH Research Domain Criteria (RDoC) initiative***. Specifically, **P5** employs a naturalistic strategy to recruit and study all medication-naïve subjects who present in a first-episode of psychosis (focusing on schizophrenia, schizophreniform and schizoaffective disorder), and the postmortem studies of **P1**&**P2** focus on subjects with schizophrenia or schizoaffective disorder. The latter choice was made based on power calculations (see **Stats/DM Core**) and current uncertainties regarding how to select cases from a brain bank that mirror the

clinical sample (personal communications with Dr. Bruce Cuthbert, 2013). However, based on the outcome of **P5**, future studies identical to those proposed in **P1**&**P2** can be conducted employing samples from subjects with other psychotic disorders. In addition, within the proposed sample size for **P1**&**P2** we have the capacity to conduct exploratory clustering analyses[32] to determine if measures of layer 3 PC pathology identify distinct subsets of subjects. ***Because the best means of implementing the RDoC approach is an active area of investigation[33], and the existing literature principally utilizes categorical diagnoses, the following sections refer to studies of schizophrenia (including schizoaffective disorder in some studies)***. In addition, in **P1**, **P2** & **P5**, schizophrenia is used to refer, in aggregate, to the affected subjects of interest.

2. BACKGROUND AND SIGNIFICANCE

2.1 The burden of schizophrenia and related disorders

2.1.i) Schizophrenia is a major public health problem. Schizophrenia is a devastating illness that afflicts 0.5-1% of the world's population[34]. Affected individuals frequently come to clinical attention during late adolescence or early adulthood, many suffer from co-morbid depression and substance abuse, 5-10% eventually die by suicide, and most experience a lifetime of disability[34]. As a result, schizophrenia is associated with a substantial emotional burden for the family and incurs tremendous economic costs for society in terms of medical expenditures and lost productivity[35]. Indeed, schizophrenia ranks as one of the leading causes of years of life lost to disability and premature mortality in market economies[36].

2.1.ii) Current treatments for schizophrenia have limited effectiveness. The principal pharmacological treatment for schizophrenia, antipsychotic medication, reduces the severity of positive symptoms (e.g., hallucinations, delusions and thought disorder). Although antipsychotics have made it possible for many individuals with schizophrenia to live outside of hospital settings, limitations in both the effectiveness and tolerability of currently available antipsychotics leave many affected individuals with little or no remission of symptoms[37]. Furthermore, antipsychotics have little or no effect on the cognitive impairments that are the major determinants of long-term social and occupational outcome[38]. Although recent work offers promise for new types of interventions[39], ***the development of new approaches for remediating or preventing the pathophysiology underlying the cognitive deficits in schizophrenia remains a critical unmet need.***

2.2 A research strategy for addressing the therapeutic challenge in schizophrenia

2.2.i) The rational development of new treatments requires an understanding of the disease process. In addition to their limited effectiveness, all medications currently used to treat schizophrenia and related disorders were discovered by serendipity. These problems emphasize the need for a new approach to treatment development similar to that used in other domains of medicine where drug development begins with the identification of molecular targets based on their role in the pathophysiology of an illness[36]. Although the need for a shift in strategy is compelling, its implementation depends upon knowledge of the underlying disease process (Fig.0.1). In this view of a disease process, the etiology or cause of a brain illness unleashes pathogenic mechanisms that produce a pathological entity, a conserved set of molecular and cellular disturbances in the brain. This pathological entity so alters the brain's normal circuitry and function that the resulting pathophysiology gives rise to the emergent properties recognized as the clinical features of the illness[40]. Thus, rational therapeutic interventions normalize function at molecular and cellular levels in order to improve the physiological functions of the affected neural circuits so that the clinical features are ameliorated (Fig.0.1).

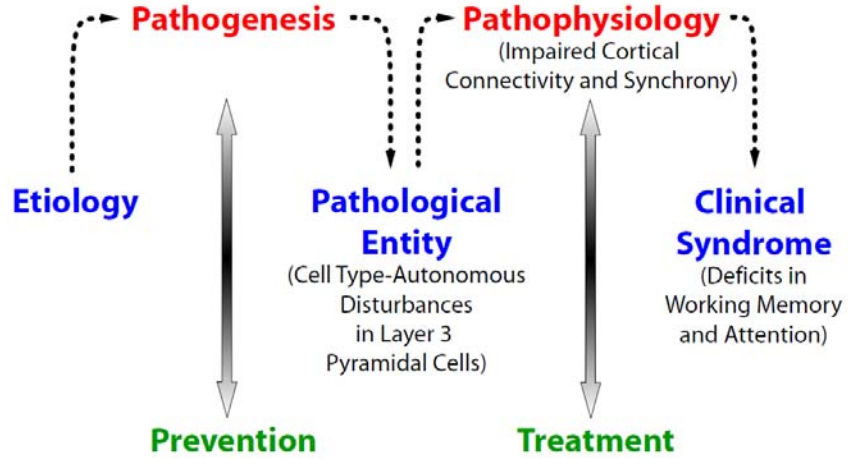

Fig.0.1. Schematic diagram illustrating the disease process model of schizophrenia and related disorders and the specific components of this process that will be examined in the studies proposed in this Center.

Understanding this disease process is, of course, complicated by the heterogeneity associated with the diagnosis of schizophrenia. Etiologically, most cases of schizophrenia appear to be the consequence of the

complex interplay of a large number of genetic liabilities[41] and environmental risk factors[42] that alter the developmental trajectories of neural circuits[43]. At the other end of the disease process, the broad range of clinical features found in individuals who meet DSM criteria for schizophrenia suggests that multiple brain systems are affected. Thus, the etiological and clinical heterogeneity of schizophrenia likely represents both diversity (the existence of different disease entities within the population meeting DSM criteria) and variability (variance of particular parameters within a disease entity due to genetic background and other differences). Consequently, a critical challenge in schizophrenia research is the development of an investigative strategy that is robust to etiologic and clinical diversity and sufficiently powered to account for both biological and experimental variability. Below we outline a set of research approaches (e.g., a focus on specific dimensions of symptoms, on a common pathology downstream from etiological factors and on conserved proximal pathophysiological mechanisms) that we believe will allow us to deal productively with such heterogeneity.

2.2.ii) An innovative strategy for dissecting the disease process guides the research proposed in this Center. Given the number of different genetic liabilities and environmental exposures associated with an increased risk of schizophrenia, different combinations of these causal factors probably induce distinct pathogenic events that differ across affected individuals and that are distal from the pathophysiology of a given clinical feature. However, these alterations are likely to converge upon a more limited set of proximal molecular and cellular changes—a final common pathway that directly gives rise to the pathophysiology underlying a given clinical feature, and that would be expected therefore to be conserved across individuals who share that clinical feature. Consequently, in the proposed studies we have elected not to focus on the expanding number of putative risk genes for schizophrenia or on their potential pathogenic consequences, although components of the proposed studies are likely to provide novel information regarding pathogenesis (e.g., cell type-specific gene expression profiling in **P1**). Instead, we look downstream in the disease process at the clinical features and pathological markers that appear to be relatively common across individuals diagnosed with schizophrenia. At the clinical level (**2.3**), we focus on well-established, core, cognitive deficits in schizophrenia that are dependent upon the circuitry of the V1-PPC-DLPFC network. At the pathological level (**2.4**), we focus on alterations in PCs that furnish the projections among the cortical regions that mediate visual working memory and attention. The proposed studies are designed to test the idea that these abnormalities are linked by the pathophysiology (**2.5**) of altered functional connectivity that subserve both top-down and bottom-up information processing. Thus, the Center structure provides convergent and multidisciplinary probes of the robustness of this model. However, we recognize that disturbances in other domains of information processing and in other brain regions and circuits are present in the illness. Thus, the proposed studies provide a proof-of-concept approach that will provide a strategic model for examining these other phenomena.

The rationale for this strategy is based upon our two-fold goal of 1) identifying novel pathologically-based treatment targets that can be used to guide the development of rational interventions, and 2) developing novel, pathophysiologically-based biomarkers that can be used in clinical trials to assess the extent to which an intervention normalizes the pathophysiology of the illness. Achieving the first goal requires recognizing that molecular alterations observed in association with the disease state could represent any of the following four "Cs": 1) *Cause*, an upstream factor related to the disease pathogenesis; 2) *Consequence*, a deleterious effect of a cause; 3) *Compensation,* a response to either cause or consequence that helps restore homeostasis; or 4) *Confound*, a product of factors frequently associated with, but not a part of, the disease process[26]. The molecular alterations most likely to be useful treatment targets are 1) the downstream consequences that are closely tied to, and the direct and powerful determinants of, the brain pathophysiology that mediates the clinical feature of interest; and 2) the homeostatic compensations that could be augmented. Thus, **P1**-**P3** employ an integration of postmortem human investigations and studies in non-human primates to provide pathological and physiological bases for identifying and validating potential novel treatment targets. In parallel, **P4**&**P5** use comparable cognitive tasks and complementary measures of functional connectivity and synchrony in monkeys and in medication-naïve subjects with a first episode of psychosis; this strategy provides a robust assessment of potential biomarkers that reflect (as a consequence of indexing molecular-cellular-circuitry pathology) the underlying pathophysiology of the clinical features of interest.

To explicate the empirical basis for the proposed tests of this model, the following sections briefly summarize the rationale for our focus on specific clinical features (**2.3**), the pathophysiology of the affected neural circuits that gives rise to those features (**2.4**), and the pathology of the neurons forming those circuits (**2.5**).

2.3 Clinical feature focus: Impairments in core cognitive domains

2.3.i) Cognitive deficits are the core features of schizophrenia. Although positive symptoms are usually the presenting and most striking clinical feature of schizophrenia, disturbances in cognition have been regarded as central to the illness since its initial description as dementia praecox. A variety of cognitive abnormalities have been described in schizophrenia, including disturbances in attention[44-46] and working memory[47-50]. These impairments are thought to be the core features of the illness[51] for the following reasons. First, cognitive deficits occur with high frequency, are relatively stable over time and are independent of the psychotic symptoms of the illness[52]. Second, cognitive abnormalities are present prior to the initial onset of psychosis[53-56]. Third, the unaffected relatives of individuals with schizophrenia also exhibit similar, although milder, cognitive deficits[57,58]. Finally, the degree of cognitive dysfunction is the best predictor of long-term functional outcome for affected individuals[1,2]. Thus, the development and implementation of effective treatments for cognitive deficits remains a major goal in schizophrenia research.

2.3.ii) Impairments in working memory and attention represent prototypic cognitive deficits in schizophrenia. Of the demonstrated types of cognitive impairments in schizophrenia, substantial research has focused on working memory, typically defined as the ability to transiently maintain and manipulate a limited amount of information in order to guide thought or behavior[59]. A large literature[13], confirmed by meta-analyses[14], indicates that large deficits in working memory are present in subjects with schizophrenia, including in visuospatial working memory. These deficits cannot be explained by effects of IQ, duration of illness, or antipsychotic medications[14]. Similarly, impairments in selective attention have been widely replicated in subjects with schizophrenia[44-46]. More recently, both types of disturbances have been conceptualized as disturbances in 'cognitive control', the ability to maintain a set of rules needed to guide response selection toward a common goal, which is thought to depend on a distributed neural network including the DLPFC[60]. Consistent with this idea, performance on tasks requiring only bottom-up attention typically is intact in schizophrenia[61] but other bottom-up processes, notably early sensory processing, are affected[62].

2.4 Pathophysiology focus: Distributed neural circuitry mediating visual working memory and attention

2.4.i) Working memory impairments in schizophrenia are associated with dysfunction of the DLPFC. Working memory impairments in schizophrenia are accompanied by altered activation of the DLPFC, a brain region known to be associated with working memory[63]. Under appropriate conditions of cognitive demand, altered activation of the DLPFC is a robust finding in schizophrenia[15,64-66]. Most interpretations of these results converge on the idea that DLPFC dysfunction in schizophrenia is task-dependent and related to working memory impairment[67-69]. Studies examining neural activity using fMRI indicate that during working memory tasks, subjects with schizophrenia exhibit an altered relationship between working memory load, behavioral performance and DLPFC activation[70]. Importantly, deficits in activation of the DLPFC during working memory tasks predict the severity of cognitive disorganization symptoms in subjects with schizophrenia[71]. In addition, reduced working memory capacity has been suggested to be rate limiting in the performance of other cognitive tasks in schizophrenia[72]. Thus, working memory deficits seem to be a central feature of schizophrenia, and identifying the neural alterations in the DLPFC and its connections with other cortical regions that produce these functional alterations is essential for understanding the underlying disease process.

2.4.ii) Working memory depends upon a distributed cortical network that is altered in schizophrenia. Although working memory impairments in schizophrenia have been strongly associated with dysfunction of the DLPFC, working memory processes depend upon the proper temporal activation of networks of neurons distributed across certain cortical regions. For example, visual working memory requires the communication of information from the primary visual cortex (**V1**) through regions in the occipital cortex and posterior parietal cortex (**PPC**) to the DLPFC (Fig.0.2A). In particular, working memory involves the sustained activity of, and synchronous interactions among, neurons in PPC and DLPFC[3-6], and subjects with schizophrenia exhibit altered patterns of PPC and DLPFC activity and functional connectivity during working memory tasks[10,13].

2.4.iii) Working memory and attention are mediated by overlapping cortical networks. Both visual working memory and visual attention use the same neural substrate, with different cortical regions contributing to specific aspects of each function. For example, in primates, neurons in both PPC and DLPFC fire during periods when the monkey is allocating attention to a particular location in visual space[7-9] or is holding information in working memory[73,74]. Although task-related activity varies slightly from area to area within PPC and DLPFC[75,76], neurons in both locations convey similar signals related to working memory and attention. Thus, although working memory and attention are operationally distinguishable, they both depend on an overlapping ensemble of cortical regions including PPC and DLPFC[77].

2.4.iv) Cortical regions mediating visual working memory/attention have distinctive circuitry.

In primates, ascending visual information is conveyed across cortical regions via excitatory projections that arise predominantly from layer 3 pyramidal cells (**PCs**) and principally target the dendritic spines of PCs in layers 3-4 (Fig.0.2B)[16]. Descending projections from DLPFC to PPC also predominately (~75%) originate in layer 3 PCs[17-19] and terminate with highest density in layer 3 of PPC[19,20] (Fig.0.2B). Thus, the projections furnished, and received, by layer 3 PCs are thought to be key neural elements of the circuitry that mediates the functional connectivity from which the cognitive processes of working memory and attention emerge.

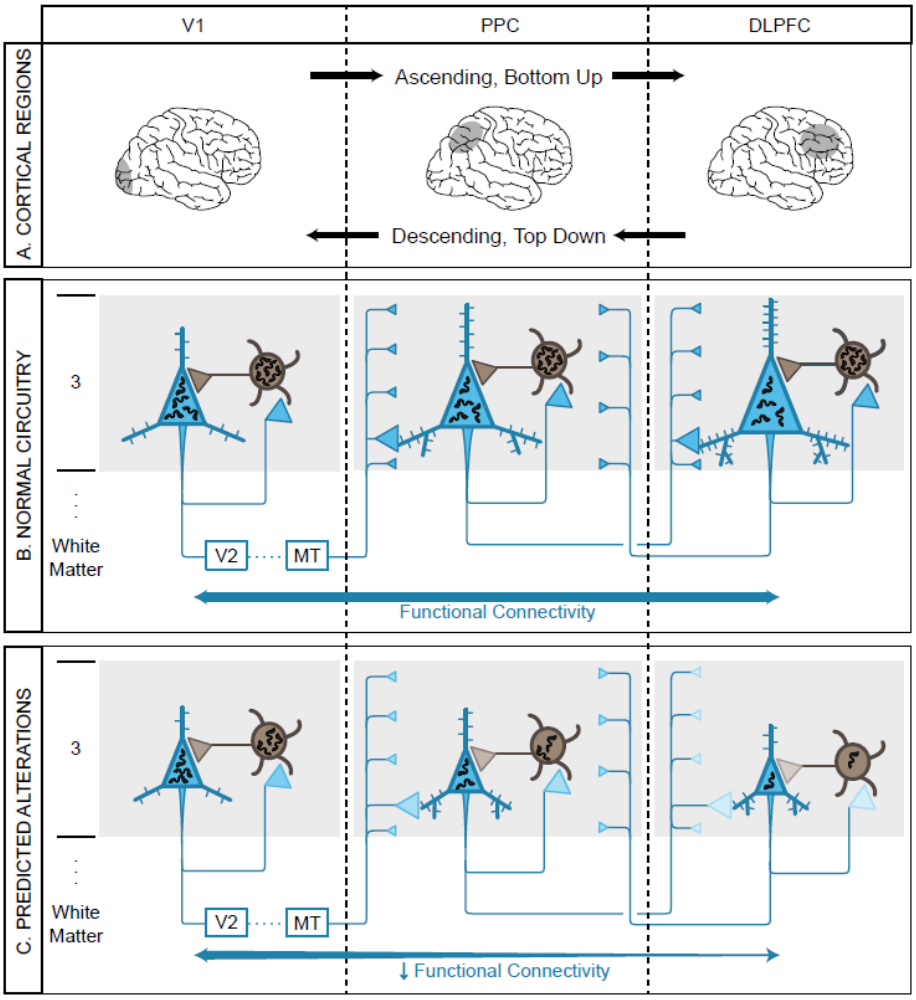

Fig.0.2. Cortical circuitry studied in this Center.

A) Cortical regions of interest. Shaded areas indicate approximate locations of the three cortical regions of interest on the lateral surface of the human brain. V1 = primary visual cortex in all projects. PPC = human inferior parietal lobule (IPL) and supramarginal gyrus (SMG) in the human imaging studies of **P5** or cytoarchitectonic BA40 in the human postmortem studies of **P1&P2**. Human BA40 is the homologue of macaque area 7a studied in **P3&P4**. DLPFC = area 46 in all projects, and includes area 9 in **P5**.

B) Normal patterns of circuitry. Ascending projections conveying visual information originate in the cortex from layer 3 PCs (blue) in V1, are conveyed through a series of intermediate regions (e.g., V2 and MT), and terminate in layers 3 and 4 of higher order visual regions in PPC which project to DLPFC. In addition, layer 3 PCs in DLPFC provide descending projections to PPC, which terminate most heavily in layer 3. Layer 3 PCs normally exhibit regional differences in morphological features, such as somal size, dendritic complexity and spine density. Layer 3 PCs are reciprocally connected with PV basket cells (gray). Hash lines to left indicate the borders of layer 3.

C) Predicted alterations in schizophrenia and related disorders. The **Central Hypothesis** predicts that layer 3 PCs have a cell-autonomous abnormality, evident in altered gene expression (squiggly lines), altered morphology (smaller soma, shorter dendrites, fewer dendritic spines), and altered synaptic proteins (shading intensity of blue triangles). The severity of this pathology is moderated by normal regional differences in the properties of layer 3 PCs such that measures of molecular and morphological pathology are least in area V1, intermediate in PPC and greatest in DLPFC. This regional pattern of pathology is mirrored in gene expression and synaptic protein changes in layer 3 PV cells (gray cells and triangles). As a result, functional connectivity is differentially impaired across regions (DLPFC>PPC>V1), compromising both bottom-up and top-down processing, and giving rise to impairments in visual attention and working memory.

2.5 Pathology focus: Layer 3 PCs as a locus of pathology

2.5.i) Morphological and molecular alterations of PCs in schizophrenia exhibit laminar specificity. In subjects with schizophrenia, convergent lines of evidence indicate that a subset of PCs in the DLPFC, and to varying degrees in other cortical regions, exhibit a distinct set of morphological abnormalities[28,78-82]. For example, PCs in layer 3 of the DLPFC have a smaller cell body, shorter dendrites, and fewer dendritic spines (Fig.0.2C) in subjects with schizophrenia relative to healthy comparison subjects and subjects with major depression[28]. Similarly, in the only direct comparisons across layers within the same region of the same subjects, PCs in layers 5 or 6 did not exhibit morphological alterations in schizophrenia[29,82].

Consistent with these findings, expression of genes that regulate dendritic spines is altered in layer 3 of the DLFPC[27]. Some of these transcripts are expressed exclusively in layer 3[31], and for others the level of expression correlates with dendritic spine density only in layer 3[30]. In addition, our preliminary studies of

individually laser-dissected PCs in the DLPFC showed that layer 3 PCs had markedly lower expression of gene products involved in the mitochondrial regulation of energy production (see Fig.1.2 in **P1**).

This laminar specificity in the morphological and molecular abnormalities of PCs in schizophrenia is accompanied by a similar laminar pattern of alterations in the parvalbumin (**PV**)-containing basket class of GABA neurons that are reciprocally connected with layer 3 PCs (Fig.0.2C)[83]. For example, in schizophrenia subjects 1) PV mRNA levels are selectively lower in DLPFC layers 3-4 [84]; 2) mRNA levels of the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD67), are markedly lower in DLPFC layer 3 PV neurons[84]; and 3) GAD67 protein levels are lower in the axon terminals of PV basket neurons in layer 3[85]. Since the expression of both PV and GAD67 are activity-dependent[86], these findings support the idea that a deficit in excitatory output from layer 3 PCs, which heavily innervate PV neurons through their local axon collaterals[83], leads to the lower levels of PV and GAD67 in schizophrenia[87].

In concert, these data suggest that ***in schizophrenia layer 3 PCs have an intrinsic molecular pathology that results in morphological abnormalities, contributes to other changes in gene expression that impair cellular function, and gives rise to alterations in excitatory output that impair both local and distributed circuits***[27,87].

2.5.ii) Morphological alterations of layer 3 PCs in schizophrenia exhibit regional specificity. In the only direct comparisons across regions within the same subjects, layer 3 PCs in V1 had only 50% of the spine deficit present in the DLPFC[28], and did not have smaller cell bodies or shorter dendrites relative to healthy comparison subjects[28,80]. Interestingly, this pattern parallels regional differences in the morphological and functional properties of layer 3 PCs in the primate brain. For example, in monkeys layer 3 PCs exhibit marked differences in structure and excitability between areas V1 and DLPFC; relative to DLPFC, layer 3 PCs in V1 are 1) smaller, have less extensive dendritic arbors, and far fewer dendritic spines; and 2) higher input resistance, depolarized resting membrane potential and action potential firing rates[23]. Similarly, layer 3 PC dendrites in monkey and human neocortex become increasingly more complex in a caudal-to-rostral progression from V1 to DLPFC[24]. In addition, the transcriptome of the human brain shows a general caudal-to-rostral pattern, with gene expression in the frontal cortex enriched relative to primary sensory and motor cortices[25]. ***Thus, regional differences in the normal properties of layer 3 PCs are likely to moderate the apparent severity of their molecular (P1) and morphological (P2) pathology in schizophrenia (Fig.0.2C), and consequently to influence the functional impact of this pathology on working memory and attention through the connections between regions furnished by layer 3 PCs (P5).***

2.6 Central Hypothesis

The findings summarized above indicate that the molecular and morphological abnormalities of PCs in schizophrenia exhibit regional and laminar specificity in presence or severity. Given the critical roles of the affected layer 3 PCs in furnishing connections between cortical regions, these patterns suggest that inter-regional information flow is altered in the illness. However, most molecular studies of the cortex in schizophrenia to date have utilized gray matter homogenates that could not identify the types of cells contributing to the gene expression alterations, and most cellular studies have focused on cell types within a single cortical region. Those studies that have examined more than one region within the same subjects have done so without a circuitry-based construct for understanding the relationship between alterations in each region, and without the layer and cellular levels of resolution required for interpreting findings in the context of that construct. Furthermore, in vivo studies of functional connectivity in schizophrenia have generally done so without reference to the laminar, cellular and circuitry substrates for that connectivity. Although interesting proposals have attempted to cross these divides[60], they point to the need for the type of convergent, multidisciplinary studies capable of rigorous hypothesis testing that are proposed in this Center application. The projects proposed in this application will provide convergent tests of the following **Central Hypothesis:**

Cell type-autonomous molecular disturbances in layer 3 PCs gives rise to morphological abnormalities in these neurons. The severity of this cellular pathology is moderated across cortical regions as a function of normal regional differences in the properties of layer 3 PCs, such that measures of pathology are smallest in area V1, intermediate in PPC and greatest in DLPFC. This cellular pathology alters cortical circuitry within and between regions, impairing functional connectivity across regions, and resulting in disturbances of both bottom-up and top-down processes during visual working memory and attention in individuals with schizophrenia.

This **Central Hypothesis** posits that a core cortical pathology in schizophrenia involves abnormalities intrinsic to layer 3 PCs (i.e., cell type-autonomous) that furnish connections among cortical regions (Fig.0.2C). The severity of this pathology differs across regions due to region-specific properties of layer 3 PCs. That is, normal regional differences in layer 3 PCs (Fig.0.2B) moderate the severity of molecular, morphological and local circuitry measures of pathology (**P1**&**P2**) such that alterations in layer 3 PCs are greatest in DLPFC, intermediate in PPC, and least in area V1 (i.e., DLPFC>PPC>V1). The resulting disturbances in both local and distributed neural circuits lead to functional alterations in the timing and nature of inter-regional functional connectivity that produce the cortical dysfunction present during working memory and attention tasks in individuals with schizophrenia. We use "***functional connectivity***" to refer broadly to oscillatory phase synchrony and correlated hemodynamic responses across cortical regions. Functional connectivity is detectable by correlational vs. causal approaches. Neurophysiological measures (**P4**&**P5**) will use phase coherence and Granger Causality, while fMRI studies (**P5**) will use beta-series correlation and dynamic causal modeling. Thus, the **Central Hypothesis** provides a novel and substantial accounting for cortical dysfunction in schizophrenia by examining the neural substrate of this dysfunction in distinct cell types that form specific distributed circuits and by examining the functional integrity of these circuits.

For each component of the **Central Hypothesis**, potential alternative explanations may need to be considered. For example, regional differences in the magnitude of pathology might not reflect region-specific effects on layer 3 PCs, but a compounding or amplification of the effects of altered outputs from layer 3 PCs in one region to another across levels of the V1-PPC-DLPFC network. In this scenario, the severity of pathology would be predicted to be greater in DLPFC and PPC than in V1, with PPC very comparable to DLPFC (i.e., DLPFC=PPC>V1) because both DLPFC and PPC represent high levels of the visual stream hierarchy that are directly interconnected. This, and other potential alternatives, are considered in the interpretation of each project, and represent one way in which the findings of a given project may influence the design and interpretation of other projects. However, given the available data, the **Central Hypothesis** provides a cogent and rational accounting across molecular-cellular-circuitry-connectivity-cognitive levels of analysis.

Thus, the **Central Hypothesis** guides the work conducted within each project, and is subject to modification as informed by the results obtained from each of the projects. Through our extensive interactions, all Center investigators are made aware of the data that suggest modifications to the **Central Hypothesis**, and they are then able to make appropriate adjustments in their study designs or experimental models. Thus, ***our objective is to operate in a truly bidirectional fashion such that individual projects both attend to and contribute to the Central Hypothesis***. The proposed approaches for testing the **Central Hypothesis** will provide a rational basis for the identification and validation of novel therapeutic targets and for the implementation of pathophysiologically-based means for assessing the efficacy of novel compounds with activity at those targets.

We recognize that schizophrenia and related disorders are multifaceted, that the Center addresses only one aspect of this diversity and variability, and that the proposed projects reduces complex systems and brain functions to tractable questions and testable predictions. In addition, due to the fiscal constraints of the Center mechanism, we have elected not to, at this time, examine important related topics (e.g., integrity of white matter tracts connecting areas; developmental changes that predate or emerge during psychosis; etiology and pathogenesis of layer 3 PC pathology). Thus, the direct application of any individual finding to a comprehensive understanding of schizophrenia and related disorders may prove overly simplistic. However, the **Central Hypothesis** integrates projects and interactions among investigators to provide a powerful vehicle for translating findings from well-controlled individual studies into the broader significance of understanding certain components of the disease process (Fig.0.1). In this respect, the Center provides a mechanism for modifying interpretations that are too reductionist and for fostering contact between tractable experimental studies and clinical realities. The proposed studies are part of a longer term process in which the combined information from each project of the Center synergizes and leads to the design of more targeted future studies.

3. IMPACT AND INNOVATION

Transforming the state-of-the-art in schizophrenia research

The program announcement for Conte Centers specifies that the proposed research must address innovative, creative and high risk/high impact research questions. In this application, we have emphasized the use of innovative approaches to address critical questions that provide direct tests of our **Central Hypothesis** and that have broader implications for a neural circuitry understanding of psychiatric disorders. The Center as a whole is innovative in that it focuses on the mechanisms linking pathology, pathophysiology and clinical features of schizophrenia (Fig.0.1) due to the integrative and cross-fertilizing strategies of each project.

3.1 Conceptual and strategic innovations. The proposed Center directly advances translational schizophrenia research in the following ways:

- We focus on 1) specific domains of the clinical syndrome (**P5**), 2) a conserved pathology (**P1**&**P2**) downstream from etiology, and 3) the neural substrate (**P3**&**P4**) for the pathophysiological mechanisms (**P5**) that link pathology and clinical features.
- Cross-project integration provides a means to systematically move across levels of analysis from regional activation and synchrony (**P5**), to activity of ensembles of neurons preferentially found in certain cortical layers (**P4**), to the inter-and intra-regional circuitry among specific cell types that comprise these ensembles (**P3**), to morphological (**P2**) and molecular (**P1**) features that determine the functional properties of these cell types.
- We apply a conceptual perspective which recognizes that brain connectivity is highly dynamic, that neural network effects reflect both bottom-up and top-down processing, and that a disease process approach to schizophrenia needs both to incorporate these realities and to provide the means to test them.
- Use of a monkey model system provides the capacity to make controlled and direct assessments of the same cells, circuitry and connectivity that are altered in the illness. For example, **P3** is the first study in primates to determine the distinctive molecular, morphological and neurophysiological properties of PCs based on their connectivity, and **P4** is the first study to ask whether measures of functional connectivity based on coherence and causality analysis depend differentially on particular cortical layers.

3.2 Methodological innovations. We employ the following novel methods that provide resolution at the levels of specific cell types, circuits, and connectivity:

- We have used laser microdissection (**LMD**) of cortical layers and mRNA analyses to demonstrate striking laminar differences in gene expression and to identify alterations in gene expression in schizophrenia not detected in gray matter homogenates[88,89]. In **P1**, we extend this approach to the cellular level, capturing specific classes of cortical neurons located within the same layer. This approach offers the opportunity to detect novel cell type-specific abnormalities in transcripts.
- In **P1,** analyses of mRNAs and regulatory miRNAs, in the context of cell type-specific coexpression networks, represent clear advances over prior studies of target identification performed in whole tissue and with limited knowledge of interacting gene products.
- **P2** employs a novel segmentation imaging method we developed[90] that permits quantification of fluorescence intensity of immunoreactivity for proteins at specific pre- and post-synaptic sites[91].
- **P3** combines retrograde transport and cell type-specific gene expression profiling to identify molecular markers distinguishing subclasses of PCs that differ in the projection target of their principal axon. These markers may be used to study in human brain PCs with these particular projections. This advance will permit an unprecedented analysis of schizophrenia at the level of cells with known connectivity.
- **P4** is the first study to use linear array electrodes as a means for assessing the contributions of particular cortical layers to functional connectivity in monkeys.
- **P5** combines multimodal imaging with state-of-the-art source reconstructions of electrophysiological scalp recordings, allowing sophisticated spectral analytic methods to be applied to localize activity, and generating frequency band-specific findings regarding regional and functional connectivity disturbances in cortical networks subserving visual working memory and attention.

3.3 Experimental design and analysis innovations. We use innovative statistical designs, clustering approaches and gene network analyses in postmortem brain research and novel techniques for studying functional connectivity (**Stats/DM Core**).

- Our statistical models for **P1**&**P2** account for subject clinical characteristics and comorbid factors, and for the effects of our pair-wise, blinded processing designs. This approach enhances the detection of disease-related differences and their relationships to cortical region by controlling for biological and measurement variability unrelated to the disease process, and by taking into account the correlations across cortical regions and repeated measures within a region.
- We utilize statistical clustering and mixtures of distributions techniques to determine whether specific findings hold true generally or more so for specific subsets of affected subjects (**P1**&**P2**)[92].
- We employ novel methods we developed for analyzing gene co-expression networks (**P1**&**P3**)[93].
- We will develop new statistical techniques to enhance the yield of functional connectivity studies (**P4**&**P5**).

4. UNIQUE VALUE OF THE CENTER APPROACH

4.1 Scientific interactions among projects and cores

The research projects in the proposed Center all converge on the Central Hypothesis (Fig.0.3). In addition, they are also complementary (Fig.0.3) and interactive (Table 2) in the following ways:

4.1.i) The projects are complementary in orientation. Three projects are clinical in orientation, directly investigating the disease process of schizophrenia, and two are basic investigations in monkeys of the neural substrate for that process. The interplay between clinical and basic orientations makes it possible for the neurobiological mechanisms underlying clinical observations to be examined in model systems and for hypotheses generated by basic studies to be tested in clinical investigations.

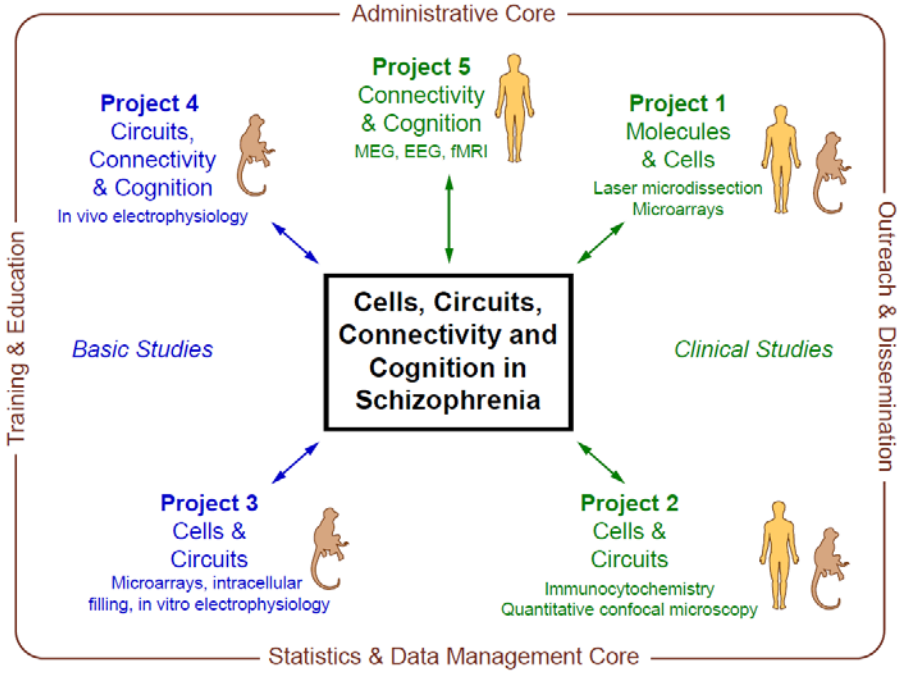

Fig.0.3. Schematic diagram illustrating the type of study, level of analysis, principal technical approaches, species studied and interactions with the cores of the 5 projects in the proposed Center. The bi-directional arrows indicate that each project tests aspects of the Central Hypothesis and is in turn informed and constrained by the findings of the other projects.

4.1.ii) The projects utilize different disciplines. Investigations of gene products and proteins in the context of neural circuitry, neurophysiology (in vitro or in vivo with behavioral analyses), and multimodal imaging techniques (fMRI, EEG and MEG) combined with cognitive paradigms are all employed to investigate the function of cortical cells, circuits and connectivity.

4.1.iii) The projects use clinical resources that offer transformative insights into the disease process. Two complementary approaches are used in the selection of subjects for study. First, the ***study of medication-naïve subjects in their first episode of psychosis*** (**P5**) offers the following strengths: 1) the opportunity to assess the pathophysiology present at clinical symptom onset; 2) the ability to dissociate pathophysiology from changes that develop over time, either as consequences or compensations to the disease process; and 3) isolation of the pathophysiology from the effects of medications and the social and health factors that are secondary to the disease process. In addition, the focus of **P5** on teens and young adults offers insight into sensitive periods of development that confer heightened vulnerability to the disease process.

Second, **P1&2** use brain specimens (Table 1; Appendix 1 contains detailed information on each subject) ***from subjects with schizophrenia (n=32) or schizoaffective disorder (n=18) from an existing collection provided by the Pittsburgh Brain Tissue Donation Program*** directed by Dr. Lewis. Each subject is individually matched to a healthy comparison subject for sex, and as closely as possible for age, PMI and RIN. All tissue samples have RIN≥7.0 and brain pH≥6.1 which indicates excellent RNA quality (**P1**), and PMI<28 hours which is consistent with excellent protein preservation (**P2**). DSM diagnoses for all subjects (affected and healthy comparison) are based on structured interviews with family/significant other informants, medical records and consensus conference confirmation by experienced research clinicians[94]. With the **Stats/DM Core**, we are able to investigate whether any significant findings are associated with greater illness severity using available measures from psychological autopsy (i.e., factors predictive of disease severity such as family history of schizophrenia or earlier age at illness onset; and factors that indicate illness severity including suicide, no history of marriage, lower socioeconomic status, and living dependently at time of death)[32,85,95]. We can also assess whether any findings are associated with manner, cause or time of death; nicotine, cannabis or alcohol use; or use of antipsychotic, antidepressant, anxiolytic, or mood stabilizer medications.

Thus, ***the use of a naturalistic recruitment strategy focused on all medication-naïve subjects who present in a first-episode of psychosis (P5), and the use of postmortem samples from subjects with schizophrenia or schizoaffective disorder (P1&P2), provide complementary approaches that are not constrained by a single DSM diagnosis.*** The design of **P5** will inform the potential value of potential future studies under **P1**&**P2** (e.g., studies of bipolar or major depression with psychosis), and the design of **P1**&**P2** will inform the neural substrate of the subjects with schizophrenia or schizoaffective disorder studied in **P5**. In concert, ***this approach represents a unique opportunity to advance the NIMH RDoC initiative in a novel fashion by crossing DSM diagnoses, disease stage and level of resolution of brain measures***.

Table 1. Summary characteristics of human subjects for postmortem studies in P1 and P2.

Parameter	Healthy Comparison	Schizophrenia
N	50	50
Sex	36M / 14F	36M / 14F
Race	41W / 9B	35W / 15B
Age (yrs)	49.4 ± 14.2	47.9 ± 13.5
PMI (hrs)	17.9 ± 5.6	16.8 ± 6.7
Brain pH	6.7 ± 0.2	6.6 ± 0.3
RIN	8.2 ± 0.6	8.1 ± 0.6

Values are group means ± SD. PMI = postmortem interval. RIN = RNA Integrity Number

4.1.iv) The proposed projects are complementary in terms of the species investigated. Many of the critical questions regarding the role of cortical dysfunction in the working memory and attention abnormalities in schizophrenia cannot be addressed in humans either for obvious ethical reasons or because the necessary level of analysis requires more tractable experimental systems. To maximize our ability to address these questions, we employ experiments in monkeys (**P3**&**P4**) to complement the proposed postmortem (**P1**&**P2**) and living (**P5**) human studies. Monkeys provide an appropriate species in terms of feasibility, relevance and scientific yield to answer specific questions with generalizability to humans. In addition, because we seek to integrate findings across cells, circuits, connectivity and cognition, it is essential to employ an animal model that best captures the features of the human brain for each level of analysis. Thus, three projects focus on studies in humans and two focus on studies in monkeys.

P1&**2** also examine existing brain tissue collections from young adult, male macaque monkeys exposed to oral doses of haloperidol, olanzapine or placebo (n=6 per group) twice daily for ~2 years which mimics long-term treatment of schizophrenia[96]. The final trough drug plasma levels were within the range associated with clinical efficacy in humans (~1.5 ng/ml for haloperidol; ~15 ng/ml for olanzapine)[96]. If warranted by the findings in humans, similar studies can be conducted in a second group of adolescent male monkeys exposed to acutely intoxicating doses of tetrahydrocannabinol (THC) or vehicle (n=7 monkeys per group) administered intravenously via vascular access port once daily, 5 days/ week, for 12 months[97].

4.1.v) Core scientists have a history of facilitating research by project investigators. Drs. Sampson and Tseng of the **Stats/DM Core** have played key roles in the design and analysis of numerous studies conducted by many of the investigators in the proposed Center as reflected in their extensive history of joint publications with Center investigators (see Biographical Sketches). This relationship has been particularly strong with Drs. Lewis, González-Burgos-Burgos, Sibille, Sweet and Volk.

4.1.vi) Multiple interactions within the Center provide powerful tests of the Central Hypothesis. The proposed projects converge on the Central Hypothesis along complementary vectors (Table 2). First, multiple studies across projects are linked via planned ***experimental*** interactions; that is, the projects share resources, expertise or information that is essential for the conduct of the proposed research protocols. Second, through designated ***conceptual*** interactions, the findings from one project will influence the design, conduct, and/or interpretation of findings in other projects. Third, anticipated ***convergent*** interactions will serve as the basis for novel future investigations. Finally, the experimental outcomes of the proposed projects will also lead to a set of novel studies focused on the identification and validation of pathophysiology-informed targets for novel therapeutic interventions whose efficacy can be monitored by biomarkers of the underlying disease process.

4.2 Summary of interactions and integration in the Center

The proposed integrated program of research comprises five projects and two cores that 1) are interdependent; 2) are complementary in orientation, in the scientific disciplines employed and in the species studied; 3) employ innovative research strategies, experimental designs and technical methods; and 4) converge upon the testing of a single **Central Hypothesis**. In addition, the sharing of resources, coordination of efforts, and focus on a mutually influential set of studies offers an economy of costs and synergism of outcome that we believe are the hallmarks of an effective Center. ***This integrative approach to the study of schizophrenia and related disorders provides for bi-directional movement along pathways from***

primates to patients, from molecular mechanisms to local and distributed neural circuits, and from functional connectivity to cognitive functions. The multiple inter-relationships among Center investigators facilitate the exchange of information and ideas so that the conduct of individual studies is influenced by the dynamism of the entire Center. Thus, ***the effectiveness of the Center as a whole is greater than the sum of its parts because of the convergence and interactions of its components and because of the opportunities for extensions beyond the proposed projects.*** Most importantly, the resulting synergism of the Center's activities promotes a translational program in schizophrenia research that effectively transfers information from the clinic to the laboratory and back to the clinic.

Table 2. Examples of Center Interactions (see Project descriptions for additional examples)

INTERACTIONS BETWEEN PROJECTS	**P1**&**P2** will provide the first within-subjects, cell type-specific correlation of disease-related transcriptome and morphology pathology, providing an unbiased identification of specific genes that may contribute to reductions in somal volume and dendritic spines.
	Analysis of COX4I1 mRNA (**P1**) and protein (**P2**) levels within the same subjects and cell type will inform to what extent COX4I1 protein in layer 3 PCs is under transcriptional control, or disease-specific factors affect mRNA stability, translation, or trafficking. The latter set of possibilities may then be informed by the miRNA studies in **P1**.
	Data regarding the regional magnitude of somatodendritic pathology (**P2**) will constrain the interpretation (or provide novel predictions) regarding how normal functional connectivity (**P4**) is altered in disease (**P5**).
	P2 will assess the density and protein content of thalamocortical, VGlut2 containing, boutons. These findings will inform alternative interpretations that a common source of afferents contributes to normal PPC-DLPFC functional connectivity (**P4**) or its alterations in schizophrenia (**P5**).
	P1&**P3** will use comparable approaches to test the idea that regional transcriptional differences in layer 3 PCs are conserved between monkeys and humans, supporting the use of monkey studies (**P4**) to interpret findings in humans (**P5**).
	Discovery of molecular markers that selectively identify PCs that interconnect PPC and DLPFC (**P3**) will be used in future studies of the properties of these specific populations of PCs in human health and disease (**P1**&**P2**).
	Findings from **P3** regarding regional differences in the molecular, morphological and neurophysiological properties of layer 3 PCs will reveal the cellular basis for the predicted moderating effect of region on the severity of pathology (**P1**&**P2**) and function (**P5**) in schizophrenia.
	P3 will use slice recording to characterize the electrophysiological properties of DLPFC-PPC layer 3 PCs that predispose them to oscillate at low or high frequencies. **P4** will use in vivo recording to determine whether layer 3 PCs exhibit cross-area phase coherence at low or high frequencies in tasks requiring attention and working memory. The results will allow relating pathology of layer 3 PCs, as revealed by **P1-2**, to disorders of functional connectivity in low or high frequency bands, as revealed by **P5**.
	The use of analogous tasks in **P4**&**P5** will permit informed inferences regarding the cellular and circuit mechanisms that underlie functional connectivity findings in humans.
	Scalp-recorded monkey potentials (**P4**) will be compared with scalp-recorded human potentials (**P5**) on attention and working memory tasks. This direct comparison of macro-potentials serves as a cross-species validation of cortical information processing, and as an intermediate link between intracranial monkey recordings and human model-based intracranial source reconstructions.
	P4 findings that functional connectivity at certain frequencies or under certain task conditions depend on layer 3 will enable **P5** to infer that functional connectivity disturbances in schizophrenia at those frequencies or under those task conditions is due to the cellular and circuitry pathology localized to layer 3 (**P2**).
	Concurrence in the relative regional magnitudes of molecular (**P1**) and morphological (**P2**) pathology in layer 3 PCs with the functional abnormalities in **P5** will provide strong evidence for the proposed cellular and circuitry mechanisms underlying working memory and attention impairments in schizophrenia.
	P5 will provide a systems-level functional link to cellular and local circuitry pathology (**P1**&**P2**) in schizophrenia, thereby identifying pathophysiologically-informed biomarkers to guide novel diagnostic and therapeutic approaches.

5. TIMELINE FOR MILESTONES

Table 3 provides a year-by-year breakdown of milestones for each project and for some of the milestones that emerge from cross-project and core interactions. As described in the **Administrative Core**, these milestones will be used to assess the progress of individual projects and the Center as a whole at the annual meetings of the External Scientific Advisory Board.

Table 3. Milestones

Year	Project 1-Sibille	Project 2-Sweet	Project 3-Lewis	Project 4-Olson	Project 5-Luna & Salisbury	Center
1	Complete LMD of layer 3 PCs and assay mRNA and miRNA expression in V1, PPC & DLPFC for entire cohort (Aim 1).	Complete tissue sectioning (V1, PPC & DLPFC) for all subjects. Complete analyses of PC somal volume and COX4I1 content in first 25 subject pairs (Aims 1&2).	Perform Retrobead injections in monkeys 1&2. Conduct microarray analyses on all layer 3 PCs and those interconnecting PPC and DLPFC (Aim 1). Begin Exp 3.1 (Aim 3).	Train 2 monkeys on tasks requiring attention and working memory in preparation for recording in areas 7a-46.	Record EEG, MEG & fMRI from 20 patients and 10 controls during attention (Aim 1) and working memory (Aim 2) tasks. Perform 6 month follow up on 10 patients.	Stats/DM create database structures for **P1-P3**. Create Center website. Recruit and train cohort of undergraduate student researchers (this applies for each year).
2	Analyze mRNA and miRNA expression data for layer 3 PCs across regions and conduct covariate analyses (Aim 1). Begin LMD of layer 3 PV cells in V1, PPC & DLPFC for entire cohort (Aim 2).	Complete analyses of dendritic spine density and glutamate bouton density and content in first 25 subject pairs (Aims 1&3).	Perform Retrobead injections in monkeys 3-5. Begin microarray analyses on all layer 3 PCs and those interconnecting PPC and DLPFC (Aim 1). Complete Exp 3.1 (Aim 3)	Carry out recording in areas 7a-46 during performance of attention and working memory tasks.	Record EEG, MEG & fMRI from 20 patients and 10 controls during attention (Aim 1) and working memory (Aim 2) tasks. Perform 6 month follow up on 20 patients.	Conduct within subject correlations of molecular (**P1**) and cellular (**P2**) findings in layer 3 PC for healthy and schizophrenia subjects.
3	Assay mRNA and miRNA expression in layer 3 PV cells in V1, PPC & DLPFC of entire cohort (Aim 2). Conduct qPCR confirmation studies for Aim 1.	Complete analyses of PV basket cell bouton content in first 25 subject pair (Aim 2). Complete analyses of PC somal volume and COX4I1 content in second 25 subject pairs (Aims 1&2).	Perform Retrobead injections in monkeys 6-8. Conduct microarray analyses on all layer 3 PCs and those interconnecting PPC and DLPFC (Aim 1). Begin Exp 3.2 (Aim 3).	Complete recordings and analyze data for areas 7a-46 study. Train 2 monkeys on tasks requiring attention and working memory in preparation for recording in LIP-FEF.	Record EEG, MEG & fMRI from 20 patients and 10 controls during attention (Aim 1) and working memory (Aim 2) tasks. Perform 6 month follow up on 20 patients. Preliminary analysis of data for local and connectivity measures.	Compare abnormalities in **P5** to layer 3 PPC-DLPFC connectivity in **P4**. Compare frequency bands for regions/layers across **P3**&**P4.** Compare frequency bands for connectivity analysis (**P5**) with layer 3 findings for monkey areas 7a and 46 (**P4**).
4	Analyze mRNA and miRNA expression data for layer 3 PV cells and conduct covariate analyses (Aim 2) Conduct qPCR confirmation studies for Aim 2.	Complete analyses of dendritic spine density and glutamate bouton density and content in second 25 subject pairs (Aims 1&3).	Perform Retrobead injections in monkeys 9&10. Complete Exp 3.2 (Aim 3). Analyze microarray data (Aim 1). Reconstruct layer 3 PCs that interconnect PPC and DLPFC (Aim 2).	Carry out recording in LIP-FEF during performance of attention and working memory tasks.	Record EEG, MEG & fMRI from 20 patients and 10 controls during attention (Aim 1) and working memory (Aim 2) tasks. Perform 6 month follow up on 20 patients.	Compare microarray data for layer 3 PCs between **P1** and **P3**. Conduct within subject correlations of molecular (**P1**) and morphological (**P2**) alterations in layer 3 PV neurons.
5	Complete gene network analyses across subject groups, regions and cell types (Aim 3). Assess disease specificity of Aims 1&2 data (Aim 4).	Complete analyses of PV basket cell bouton content in second 25 subject pairs (Aim 2). Assess disease specificity in antipsychotic-exposed monkeys (Aim 4).	Reconstruct and analyze morphology of layer 3 PCs that interconnect PPC and DLPFC (Aim 2). Integrate findings across Aims 1-3.	Complete recordings and analyze data for LIP-FEF connectivity.	Perform 6 month follow up on 10 patients. Recruit any additional needed subjects during the first 6 months, with patient follow up during the last 6 months. Complete final analysis of data for local and connectivity measures.	Use PC-type specific markers from **P3** in **P1**&**P2** studies. Compare abnormalities (**P5)** to layer 3 LIP-FEF connectivity in **P4**. Compare frequency bands for connectivity analysis (**P5**) with layer 3 findings for monkey areas LIP and FEF (**P4**).

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SPECIFIC AIMS

This project (**P1**) provides molecular tests at the cell type-specific level for two key components of the Center's **Central Hypothesis**: 1) a core pathology in schizophrenia involves cell-autonomous abnormalities in cortical layer 3 pyramidal cells (**PCs**); and 2) the impact of this pathology on gene expression is moderated by factors that differ across cortical regions. A unique strength of **P1** is a focus on cellular level analyses which have superior sensitivity for detecting both larger and novel transcript alterations relative to typical postmortem studies conducted in tissue homogenates. Thus, the first goal of **P1** is to characterize the expression profiles of gene products (**mRNA**) and gene regulatory micro RNA elements (**miRNA**) in layer 3 PCs in three regions of the human neocortex: primary visual cortex (**area V1**), posterior parietal cortex (**PPC**) and dorsolateral prefrontal cortex (**DLPFC**) (Aim 1). Sets of layer 3 PCs will be individually collected using laser microdissection (**LMD**) in schizophrenia and healthy comparison subjects. Within-region analyses will inform the molecular substrate for the cell-autonomous pathology of layer 3 PCs in schizophrenia. Across-region analyses will test the ideas that 1) mRNAs and miRNAs are normally differentially expressed depending on brain region; and 2) these regional differences interact with the disease process operative in layer 3 PCs to produce region-specific differences in the severity of molecular pathology in schizophrenia (see Fig.0.2C in **Center Plan**).

The second goal of **P1** is to identify potential targets and therapeutic strategies for restoring functional balance in V1, PPC and DLPFC, regions that mediate visual working memory and attention. Accordingly, since layer 3 PC alterations in schizophrenia are predicted to induce compensatory transcriptional responses in the parvalbumin (**PV**)-containing GABA neurons that they innervate, a molecular perspective on both PC and PV neuron alterations is necessary for a comprehensive approach that can guide future drug target discovery. Hence, a similar molecular characterization will be performed in individually-collected PV neurons (Aim 2). Moreover, the coordinated regulation of gene transcripts, as measured by coexpression, allows for the identification of groups of key functionally-related genes that are relevant to specific cell types, regions, or conditions. Thus, analyses of coexpression networks will be performed for each cell type in V1, PPC and DLPFC, with the idea that disease-related network changes will identify potential therapeutic targets (Aim 3). Finally, whether the observed alterations are specific to the disease process, rather than due to medications or comorbid conditions, will be investigated using appropriate contrasts among human subjects and by the use of non-human primates exposed to antipsychotic medications (Aim 4).

Aim 1. Examine the expression of mRNAs and miRNAs in layer 3 PCs of V1, PPC and DLPFC. LMD-collected PCs from subjects with schizophrenia or schizoaffective disorder (denoted here together as "schizophrenia"; see Table 1 in **Center Plan**) and matched comparison subjects (n=50 pairs) will be processed for hybridization on Affymetrix Gene 2.0 and Nanostring miRNA arrays, and analyzed for deregulated mRNAs, biological pathways and regulatory miRNAs. *We predict that 1) disease-related molecular abnormalities in layer 3 PCs are present in all three cortical regions; 2) the severity of these abnormalities matches the regional pattern of layer 3 PC morphological pathology (**P2**), so that the regional magnitude of the abnormalities is DLPFC>PPC>V1; and 3) these abnormalities relate to morphological features (e.g., spines, synapses) and activity level (e.g., energy metabolism).*

Aim 2. Examine the expression of mRNAs and miRNAs in layer 3 PV cells in V1, PPC and DLPFC. LMD-collected PV cells will be analyzed as in Aim 1. *We predict that disease-related abnormalities in layer 3 PV cells reflect compensatory responses that parallel in effect size those of layer 3 PCs in the same subjects.*

Aim 3. Build gene coexpression networks for layer 3 PCs and PV cells in V1, PPC and DLPFC and identify alterations in network characteristics in schizophrenia. mRNA and miRNA expression datasets from Aims 1 and 2 will be used to build coexpression networks within each cell type, cortical area and subject group. Biological information (e.g., gene function, miRNA targets, cellular pathways, differential expression from Aims 1 and 2) will be overlaid onto the mRNAs and miRNAs that form the nodes of the networks. Differences in nodes, connectivity and other features will be investigated between control- and disease-based networks. *We predict that 1) biological networks reveal cell type- and regional-specificity in the coordinated use of mRNAs and miRNAs; and 2) specific measures of coexpression networks are altered in schizophrenia, moderated by regional effects, and identify key genes of potential therapeutic interest.*

Aim 4. Determine if the alterations found in Aims 1-3 are specific to the disease process. In addition to covariate analyses within each aim, we will address whether findings are attributable to medications or to comorbid conditions (e.g., alcohol or other substance use, death by suicide, etc) using appropriate contrasts among human subjects and by the use of postmortem samples from an available cohort of non-human primates exposed to antipsychotic medications, as previously performed in multiple studies in our labs[3-8].

OVERVIEW

The **Central Hypothesis** posits that layer 3 PCs have a cell type-autonomous molecular pathology that 1) leads to morphological abnormalities in these neurons (**P2**); 2) differs in severity across V1, PPC and DLPFC; and 3) impairs functional connectivity across regions, giving rise to disturbances in both working memory and attention in individuals with schizophrenia (**P5**) (**Center Plan,** Fig.0.2). The primary goal of **P1**, shared with **P2**, is 1) the characterization at the cellular level of the core pathology of cortical layer 3 PCs in schizophrenia; and 2) the determination of how this pathology is moderated by regional differences in mRNA and miRNA expression. The second goal of **P1** is to identify possible molecular targets for restoring functional balance across the affected cortical regions. This goal will be pursued 1) by determining the mRNA and miRNA changes in layer 3 PCs and those in PV-containing GABA neurons that may be induced by the alterations in layer 3 PCs, and 2) by the investigation of changes in gene coexpression networks formed by layer 3 PCs or PV cells across V1, PPC and DLPFC. Results from **P1** will inform all other Center projects on the cellular and molecular bases in support of the **Central Hypothesis** that regional differences moderate the pathology of layer 3 PCs. **P1** will provide potential molecular leads for protein marker studies in **P2.** Comparison of regional differences in gene expression patterns in control subjects with **P3** studies in monkeys will both inform and constrain the interpretation of the cellular basis for the comparisons of functional connectivity across cortical regions in humans (**P5**) and monkeys (**P4**). Finally, the analysis of potential therapeutic strategies will provide a translational platform to inform future studies in schizophrenia and related disorders (**P5**).

SIGNIFICANCE

Investigating the cell type-autonomous molecular pathology in layer 3 PCs in schizophrenia.

Prior findings revealed morphological and molecular alterations in schizophrenia that exhibit laminar and cellular specificity (**Center Plan,** Fig.0.2). For instance, layer 3 DLPFC PCs have a smaller cell body, shorter dendrites, and fewer dendritic spines in subjects with schizophrenia relative to healthy comparison subjects[9-10]. Efforts to characterize the molecular details of this laminar- and cellular- specific pathology using unbiased large scale gene arrays have reported alterations in the expression of genes encoding proteins involved in synaptic function[11], mitochondrial and energy metabolism[12-13], immune function[14] and other systems[15-18]. Meta-analyses, performed without a focus on affected neural networks, have not provided more specific leads[19]. However, these prior studies were limited by the use of gray matter tissue homogenates that do not distinguish mRNA expression across cell types. miRNAs are small non-coding RNAs that regulate the expression of genes[20], and that have been implicated in disease processes, including schizophrenia (e.g. miR-137/189/212/219; reviewed in[20]). Since miRNA regulate genes in cell- and region specific manners[20], these prior studies have been similarly limited by the use of gray matter tissue samples. Indeed, the major source of variability in gene profiles is due to cellular admixture. This contributes in masking subtle differences in cell subtypes and disease-related changes localized to subgroups of PCs. Together, these limitations highlight the need for approaches with better sampling specificity.

Technical improvements in single cell harvesting and in molecular approaches for handling minute amounts of collected biological material now make it possible to investigate expression profiles in specific cortical layers or cell types. For instance, a study in LMD-collected supragranular (layers 2-3) and infragranular (layers 5-6) layers identified both laminar-specific patterns of expression and novel schizophrenia-related gene changes[21]. Studies of circumscribed cell populations with known markers, such as PV neurons, have been performed but are limited to 1-2 molecular targets at a time (e.g., PV and GAD67[15,22-33]) by in situ hybridization. Here our cell-specific approach will provide an in-depth characterization of cell and regional specificities in layer 3 PC and PV cell mRNA and miRNA molecular profiles. In schizophrenia, these studies will reveal the intrinsic alterations of layer 3 PCs, the induced changes in PV cells, and the deregulated properties of the PC/PV local circuit. Results will show at the cell type level the mRNAs and miRNAs that contribute to morphological changes of layer 3 PCs across areas (**P2**), with a disease effect that DLPFC>PPC>V1 (**Center Plan, 2.6**). This will also test the hypothesis of an interaction between layer 3 PC pathology in schizophrenia and cortical area specificity. Finally, as it is not feasible in humans, **P3** will use an approach of retrograde labeling of layer 3 PCs to further refine the cell type-specific studies by investigating profiles of the layer 3 PCs that interconnect PPC and DLPFC, with the goal of testing whether the regional differences are conserved across species and present in those layer 3 PCs that interconnect PPC and DLPFC.

Coexpression network as an analytical tool to identify complex disease-related biological alterations and molecular targets for therapeutic purposes.

Changes in expression levels are investigated in Aims 1 and 2 for multiple gene mRNAs and miRNAs (Fig.1.1.a). An important derivative aspect of expression datasets is the study of coexpression networks. Two mRNA or miRNA transcripts are defined as coexpressed if their patterns of expression are correlated across samples (Fig.1.1.b); for example, across layer 3 PC samples from different regions in healthy controls or in subjects with schizophrenia. Coexpression can identify genes whose functions participate in the same cellular pathways or that share common regulatory factors[34]. Coexpression is represented by a *link* between two mRNA or miRNA (defined as *nodes*), and these links are used to build networks of genes and miRNAs with shared function (Fig.1.1.b). Biological information can be superimposed onto those networks, such as the cell type of origin (See node shades in Fig.1.1.b). We have developed custom Matlab and R-based analytical methods to construct and investigate gene networks based on coexpression links using expression datasets from human brain[1,35-40] or targeted coexpression network[1]. In Aim 3, we will overlay gene functional annotations and miRNA targets onto networks built using expression datasets from PCs or PV cells separately from controls (control network) and subjects with schizophrenia (disease network). We will build separate networks across 2 cell types, 3 regions, and 2 states (disease/control) for a total of 10 networks, and differences in network structures and properties will be investigated.

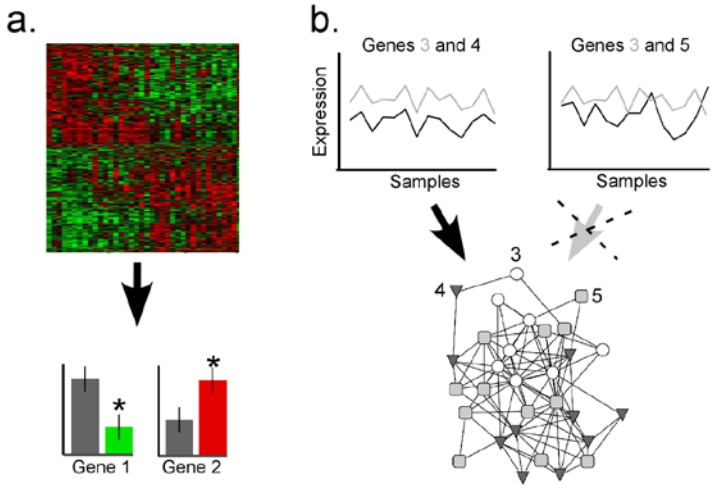

Fig.1.1. a) Differential expression extracted from large-scale datasets. In the cluster plot, genes are in rows and samples in columns. Green and red indicate low and high expression. **b)** Schematic of coexpression links used to build coexpression networks. Genes 3 and 4 show correlated expression levels across samples from different subjects (X-axis), and this correlation forms a **link** in the network between the **nodes** formed by genes 3 and 4. Genes 3 and 5 are not correlated and the link is not included. Highly connected nodes are called **hubs**. Overlay of transcript origin is as follow: white for oligodendrocyte-enriched genes, dark grey for neuronal-enriched genes, and light gray for genes expressed in both cell types. Data are from[1-2].

Together, the network-based approach 1) allows the inference of functional information (i.e., how mRNAs and miRNAs work together) using postmortem brain samples, and 2) can identify changes in critical genes, (i.e., highly connected nodes or *hub*, receptor or enzymes at key network nodes) as targets for therapeutic-oriented follow-up studies designed to restore the functional balance across V1-PPC-DLFPC network in schizophrenia.

INNOVATION

Technical innovation. *Region, layer and cell type specificity.* The proposed studies will provide cell type-specific profiles of altered mRNA and miRNA expression in schizophrenia and healthy comparison subjects, hence mitigating gray matter sample dilution problems. The innovative aspects of single cell type studies in human postmortem tissue are made possible by extensive testing of the methods in our laboratories, and due to improvement in genome characterization and novel mRNA and miRNA array tools. This level of analysis is predicted to reveal larger, more specific and novel transcript alterations in comparison to tissue homogenates.

Conceptual and strategic innovations. 1) *Network approach.* Conceptual innovation is provided by the systematic and integrative analyses of mRNA and miRNA regulatory elements at the level of cell types. These novel integrated analyses are made possible by the recent application of network theories to gene-based datasets[41], which are now being applied to the human postmortem brain[38]. 2) *Unprecedented targeted analysis*. These proposed molecular analyses will be applied to specific cell types within the relevant cortical layer and neural network that are deregulated in schizophrenia. This integration of cell, region and network represents a major conceptual innovation compared to prior studies using tissue homogenates in single regions. 3) *Greater potential for therapeutic discovery.* The analyses of mRNAs and regulatory miRNAs, in the context of cell type-specific coexpression networks represent clear advances over studies of target identification performed in whole tissue and with limited knowledge of interacting gene products.

PRELIMINARY STUDIES

We have substantial expertise in analyzing changes in gene functions using gene arrays in human postmortem samples[1,11-12,14,40,42-44]. We provide here preliminary results for the proposed cell-based expression analyses (Aims 1 and 2). In collaboration with Dr. George Tseng (**Stats/DM Core**), we have developed and published

methods to construct and investigate gene networks based on coexpression links using datasets from human brain specimens and have applied these techniques to psychiatric disorders[1,35-40,45], as proposed in Aim 3.

Layer 3 PC large scale gene expression analysis: deficits in mitochondrial-related genes in schizophrenia. An initial pilot study using LMD collections of layer 3 PCs in human DLPFC demonstrated stable gene array signal between 50 and 100 PCs, compared to 25 PCs, as measured by average correlation of gene signals between replicate samples (i.e. the average r-values between 25, 50, and 100 PC collections were 0.87, 0.92, and 0.94 respectively). Next, LMD was used to collect 100 PCs per subject from Nissl-stained sections (Fig.1.2.A) in DLPFC of schizophrenia and healthy comparison subjects. Extracted RNA was processed onto Affymetrix U133 gene arrays for gene expression studies. Signal enrichment due to LMD is shown in Fig.1.2.B. A pathway analysis revealed a highly significant overrepresentation of differentially-expressed transcripts related to mitochondrial functions (e.g. Fig.1.2.C). The majority of differentially-expressed transcripts were under-expressed in schizophrenia, including decreased (and qPCR-validated) expression of COX4I1. COX4I1provides robust immunohistochemistry signal in human postmortem brain and is thus used in **P2** as a marker for reduced mitochondrial function in schizophrenia. These mitochondria-related changes were not attributable to the effects of antipsychotic medications or other factors frequently comorbid with schizophrenia. In the context of reductions in PC volume and dendritic spines, the array findings reflect reduced energy requirement and mitochondrial transcript expression.

A. LMD of Nissl-stained PC in Human DLPFC

B. Altered Expression of Mitochondrial mRNAs is Enriched in Layer 3 PCs

	Gray Matter		Pyramidal Cell	
Gene	% Δ	p	% Δ	p
COX7A1	-9.4	0.23	-34.5	0.003
UQCRQ	-2.3	0.21	-25.0	0.001
COX5B			-19.2	<0.001
COX7B			-25.7	<0.001
COX4I1			-15.8	0.026

C. Lower Expression of Genes Regulating Energy Production in Layer 3 PCs

Mitochondrial Gene Pathways	Q-values
Reactome Electron Transport Chain	$<10^{-7}$
Kegg Parkinsons Disease	$<10^{-7}$
Reactome Glucose Regulation of Insulin Secretion	$<10^{-5}$
Kegg Oxidative Phosphorylation	$<10^{-5}$

Fig.1.2. A) LMD of layer 3 PC. **B)** Altered expression of specific transcripts is enriched in LMD collected PCs relative to gray matter. Lower section includes additional mitochondrial genes of interest with low expression in layer 3 PCs. **C)** P-values of pathways implicated in mitochondrial *functions.*

Given that somal size differs across the cortical regions of interest, to determine whether somal size alone is a determinant of gene expression, we conducted microarray analyses of the largest 25% versus the smallest 25% of LMD samples of layer 3 PCs in PPC from 4 control subjects. Neither RNA quality nor expression levels of multiple housekeeping transcripts differed between large and small PCs, confirming that somal size is not a general confound. However, as expected, certain transcripts (e.g., NEHF-neurofilament heavy peptide - see **P2**) were enriched in large PCs, whereas others (e.g., CALB1, GLRA3, HPCA, CTNNB1) were enriched in small PCs.

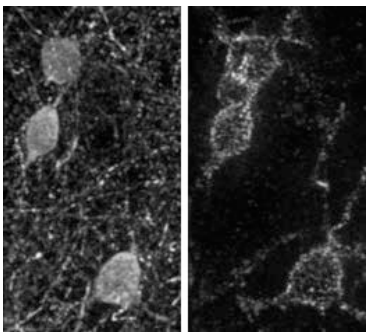

Fig.1.3. Aggrecan is a robust marker of PV cells for LMD. 60x projection image of immune-fluorescence dual-labeling for PV (left) and aggrecan (right) in layer 3 of human DLPFC.

Perineuronal net immunostaining and PV cell LMD. Due to its cytosolic location, immunodetection of PV protein is problematic for LMD. However, markers of perineuronal nets (PNNs), which are almost exclusively found around PV basket cells[46] (Fig.1.3), are detectable in LMD tissue. PV cells were detected using antibody for aggrecan, a key component of the PNN, which in our studies is well-preserved in subjects with schizophrenia unlike VVA or WFA staining of PNNs. QPCR from 200 aggrecan-positive cells collected by LMD in DLPFC layer 3 from 6 schizophrenia and 6 matched comparison subjects showed that PV cell markers (GAD67, PV and GAD65) were highly expressed in LMD aggrecan-positive cells, not detected in Nissl-stained PCs, and decreased (GAD67 and PV) in schizophrenia (-25% and 27% respectively, $p<0.05$, paired T-test); these data demonstrate our ability to collect layer 3 PV cells and detect gene expression changes in schizophrenia.

APPROACH

Aim 1. Examine the expression of mRNAs and miRNAs in layer 3 PCs in V1, PPC and DLPFC.

Hypotheses. 1) Region differences reflect layer 3 PC morphological differences in V1, PPC and DLPFC. 2) Disease-related molecular abnormalities in layer 3 PCs are present in all three areas and the regional magnitude of the abnormalities is DLPFC>PPC>V1. 3) Abnormalities in genes and regulatory elements relate to structural features (e.g., spines, synapses) and activity level (e.g., energy metabolism).

Methods. LMD on fresh frozen sections. LMD-captured PCs from subjects with schizophrenia or

schizoaffective disorder (defined here as "schizophrenia") matched to healthy comparison subjects (n=50 pairs; **Center Plan**, Table 1) will be processed for hybridization on arrays, and analyzed for deregulated mRNAs and miRNAs. Upon brain collection, coronal blocks are cut in ~2 cm blocks through the rostro-caudal extent of the brain and stored at –80C. 12 µm cryostat sections are obtained from V1, PPC (area 40) and DLPFC (area 46) and mounted on LMD slides. Nissl-stained layer 3 PCs are identified based on their distinctive morphology and laminar and regional location, and LMD-captured using a LEICA LMD 6500 system. Two 100 cell samples will be collected per subject. All PCs within the sampling window are collected, and the LMD user is blind to the sample label, hence avoiding collection bias. The two samples are pooled, providing high sampling reliability[21]. RNA and array processing rely on standard array quality controls. *For gene mRNA expression*, we will use the Genechip Gene ST 2.0 from Affymetrix. This array covers >30,000 coding transcripts, >11,000 long intergenic non-coding transcripts, and individual probes per exon. Multiple probes per exon allow us to distinguish regulation of gene isoforms and exon usage. Gene-level expression is also obtained, where multiple exon probes are summarized into an expression value. *For miRNA expression*, we will use the Nanostring nCounter miRNA assay, for a comprehensive 800 human miRNA survey. Samples will be processed by the University of Pittsburgh Genomics Core facility. The study will include 600 arrays [(50 schizophrenia, 50 controls) x 3 regions (areas V1, 40, 46; **Center Plan,** Fig.0.2.A and **P2**) x 2 (Gene mRNA and miRNA) arrays]. Quantitative real time PCR (qPCR) will be used to validate key results, as routinely performed in our lab[42,47-49].

Analytical methods. Data preprocessing and candidate marker detection (for both mRNA and miRNA). Array scans will be assessed for quality control. mRNA or miRNA differential expression between control and schizophrenia subjects will be determined by standard linear model and corrected for potential confounding co-factors (e.g., sex, age, postmortem interval, brain pH, RIN, manner/cause/time of death, smoking, substance use, psychotropic medications). Since the same patients are used for all three regions, repeated measures will be adopted to correct the dependence and increase statistical power. Also, since the number of potential confounders is large (n=10) relative to the sample size (n=50 pairs), we will adopt a model of variable selection method[36], where only variables that affect gene expression are retained, using Bayesian Information Criterion (BIC) and dataset-wide permutation analyses (details in **Stats/DM Core**). Our studies show feasibility and improved accuracy of this strategy in detecting candidate markers in major depressive disorder[36,50]. Functional annotations and analysis. By including annotations on the function, localization and molecular features (i.e. enzyme, receptor, structural elements) of each gene, we will investigate the types of biological pathways that are the most represented in sets of differentially-expressed genes. A focus will be placed on genes relating to energy metabolism and neuronal structures corresponding to a priori hypothesis for alterations in layer 3 PCs in schizophrenia. miRNA target analysis. For each identified miRNA, we will apply a new ComiR system[51] that integrates and improves four popular miRNA target gene prediction databases (PITA, miRanda, mirSVR and TargetScan). miRNA predicted target genes will be compared to the set of differentially-expressed genes and analyzed for over-representation of biological pathways. Integrative analysis. We will apply Ingenuity Pathway Analysis (IPA) for mRNA-miRNA interaction analyses to obtain a systems view of the genomic results. IPA integrates public databases on gene annotations and interactions, and provides enhanced knowledge about subcellular location, functional gene family, and association with drugs and multiple canonical biological pathways. The IPA visualization and network graphing tools provide support for the assessment of results, and together facilitate mechanistic understanding. Networks integrative studies will also be performed in Aim 3.

Data analysis, interpretation and alternatives. Regional differences. Increased mRNA and miRNA expression and increased relative representation of structural (dendritic branches and spines) and functional (i.e. mitochondria, metabolism) elements in DLPFC compared to PPC and to V1 would provide molecular support for the increasing morphological complexity of PCs from V1 to PPC and DLPFC. Changes in transcriptional/translational regulators (transcription factors and miRNAs) will identify factors mediating these regional differences. Schizophrenia-related differences. The primary dataset-wide and FDR-controlled analyses will reveal the mRNAs and miRNAs that form the basis for the morphological abnormalities and downregulation of energy production in schizophrenia. We predict that prior findings showing structural changes in schizophrenia in layer 3 PCs will be confirmed and of greater magnitude. For instance, prior reports on genes implicated in spine formation (i.e. DUO, CDC42 genes; 12-20% changes[52-53]) may show greater differences due to the enrichment in LMD-collected layer 3 PCs. However, changes in other genes and miRNAs implicated in those pathways will provide for the first time an in-depth perspective on molecular mediators of the reduced somal volume, dendritic complexity and energy requirement of layer 3 PCs. Deregulated miRNAs known to target genes in those pathways will further confirm the energy and structural a

priori hypotheses. Identification of the full set of genes targeted by deregulated miRNA will provide perspectives on miRNA-dependent changes in coordinated translation programs. Disease by region changes. Straightforward results may be 1) identification of signaling and/or mitochondria/energy-related genes that show regional differences in healthy comparison subjects and disease effects within regions; 2) interactions between region and disease, resulting in a magnitude of changes in schizophrenia that is DLPFC>PPC>V1; and 3) the identification of regionally-enriched and disease-affected miRNAs that target genes within the same pathways. Alternatively, disease changes may show similar effect sizes across regions (or PPC>DLPFC)[54], with interacting miRNA factors showing regional differences. This would shift the focus on protein changes for specific genes targeted by deregulated miRNA, which would be investigated in **P2**. If DLPFC layer 3 PCs show the largest alterations in schizophrenia, in the absence of regional differences in healthy comparison subjects, this could result from a cell type-autonomous layer 3 PC pathology interacting with the compounding effects of altered inputs from layer 3 PCs across the ascending pathway of V1-PPC-DLPFC network.

Comments. **1)** Hemispheric lateralization was not observed in systematic, large scale, gene expression analyses of multiple regions in the human brain[55], so we do not expect that our focus on the right hemisphere (due to Brain Bank protocols for frozen tissue specimens) will be a confound. Moreover we have previously found that layer 3 PC spine density in the left hemisphere[9] correlated within subjects with spine-related transcript expression in layer 3 in the right hemisphere[52-53]. **2)** Subject clustering. With the proposed sample size (n=50 pairs), we have the capacity to conduct exploratory clustering analyses[56] to determine if measures of layer 3 PC pathology identify subsets of subjects, within and/or across DSM diagnoses (see Design and Statistical Methodology in **Stats/DM Core**). 3) **P2** will assess measures of morphology and proteins in layer 3 PCs, including the subset containing NNFP. If they find unique alterations in those cells, we will conduct LMD experiments targeted to NNFP-labeled cells. **4) P3** may identify markers for layer 3 PCs that interconnect PPC-DLPFC that will enable us to specifically assess gene expression changes in these cells in schizophrenia. **5)** Although directed at determining cell type-specific regional patterns of molecular pathology, our findings may also inform mechanisms of PC pathology (**Center Plan, *2.2.ii***). **6)** Note that direct sequencing (RNAseq) of LMD has yet to be established as sufficiently reliable, quantitative and cost-effective, which has guided our choice of mRNA/miRNA arrays, although we are closely monitoring technical developments. RNAseq data from area 46 gray matter for all 50 subject pairs will be available through our participation in the CommonMind Consortium. **7)** For **timeline** for completion of this and all other **P1** aims, see Table 3 in **Center Plan.**

Aim 2. Examine the expression of mRNAs and miRNAs in layer 3 PV cells in V1, PPC and DLPFC.

Hypotheses. Disease-related molecular abnormalities in layer 3 PV cells reflect compensatory responses that parallel in effect size those of layer 3 PCs in the same subjects.

Methods. The same cohort and similar molecular biology and analytical methods from Aim 1 will be used to collect PV cells in layer 3 of V1, PPC and DLPFC, with the only difference that LMD of PV cells will be performed by PNN immunostaining using an anti-aggrecan antibody (Fig.1.3).

Data analysis, interpretation and alternatives. Changes in PV cell function (e.g., lower PV and GAD67 expression) have been suggested to arise as a consequence of reduced excitatory input from layer 3 PCs[57]. Hence the prediction is that downstream PV changes in energy metabolism will correlate with similar changes in layer 3 PCs. These results would be consistent with prior reports of cell type-specific homeostatic reductions in GAD67 and PV RNAs which are reported to be activity-dependent[58]. Changes in miRNA and target genes will provide insights into biological mechanisms regulating the PV cell changes. Fewer regional differences are expected in PV gene and miRNA profiles in control subjects, reflecting the more homogeneous structure and functions of PV cells across regions compared to PCs. However, we predict changes in mitochondrial and metabolic functions that parallel those observed in PCs across regions. This would provide robust correlative evidence for active participation of PV neurons in matching layer 3 PC changes in function.

Aim 3. Build gene coexpression networks for layer 3 PC and PV cells in V1, PPC and DLPFC and examine changes in network characteristics in schizophrenia.

Hypotheses. 1) Biological networks reveal cell type- and regional-specificity in the coordinated use of mRNAs and miRNAs. 2) Measures of coexpression networks are altered in schizophrenia, are moderated by regional effects, and identify key genes of potential therapeutic interest.

Methods. mRNA and miRNA datasets will be used to build coexpression networks within cell types and areas. Multiple levels of biological information are assigned to mRNA and miRNA that form the nodes (e.g. what is the function of this gene? Is it a target of a miRNA from the dataset? Is that node identified as differentially-

expressed in Aim 1 or 2?). Using terminology from the field of network biology, network quantitative measures will include “degree” (number of links for a particular node), “clustering coefficient” (number of connection between neighboring nodes), “hub” (highly connected nodes), “assortativity” (hub/hub, and hub/non-hub connection ratio) and “path length” (average number of nodes traversed across the network). This collective knowledge is referred to as “information transfer”, and individual measures are used as quantitative features for statistical analyses between control and schizophrenia-based networks. Reference networks are random networks where the same numbers of nodes are randomly selected 100 times and the distribution of random measures is used to derive p-values for control versus schizophrenia comparisons. We will then restrict the scope of the analyses to areas of the networks that are enriched in genes and miRNAs identified as differentially expressed in Aims 1 and 2. This novel “hybrid approach” combines coexpression with differential expression analysis[38], and can be viewed as “guided network analysis” with a priori information on key nodes.

Data analysis, interpretation and alternatives. Region differences in gene network structure have not been investigated. Here, molecular members of specific network modules that vary across regions will reveal differences in the use of molecular pathways that characterize those brain regions. The region specificities will interact with disease effects, and manifest as changes in network features across regions in schizophrenia. Schizophrenia-related changes and identification of targets with potential therapeutic interest. We predict changes in the efficiency of “information transfer” of existing networks, such as reduced clustering coefficient or fewer links to hubs affecting specific genes, including mitochondrial and morphology-related genes. This decreased synchronization in molecular functions would be consistent with reduced capacity to adapt to biological changes, and would provide a molecular level analysis of reduced neural network flexibility that is not achievable by single mRNA/miRNA analyses. For instance, an important and critical feature of biological networks is their putative vulnerability to disease effects on highly-connected hub nodes[59], since disruption of highly connected mRNAs/miRNAs would affect integration of molecular processes. At the gene level, we have however shown that hubs are relatively protected in brain disorders[38], consistent with evidence that multiple genes, rather than few centrally-located ones, are causal in psychiatric disorders[60]. We predict that 1) nodes (mRNAs and miRNAs) that are strategically located to regulate the function of hubs (i.e. off-hub), rather than the hubs themselves, may show greater probability of changes in networks built from schizophrenia expression datasets; 2) these findings are restricted to PC-based networks (i.e. primary intrinsic pathology); and 3) the nature of the nodes (e.g., receptor, channel, enzymes) may inform strategies for future drug target studies**.** An alternative result is that of increased network efficiency, which would be interpreted as reflecting the presence of dominant biological effects. For instance, decreased energy requirement (as predicted to occur in PC and PV cells) could result from the dominant role of negative feedback systems due to structural or signaling changes. Finally, a lack of coordinated changes between PC and PV-based networks in schizophrenia could suggest independent pathologies in those cell types. We have shown that brain gene networks are resilient[38], so we do not expect any global change in the structure of mRNA/miRNA coexpression networks.

Aim 4. Determine if the alterations found in Aims 1-3 are specific to the disease process.

Hypothesis. Findings from Aims 1-3 reflect disease-related molecular abnormalities in layer 3 PC and PV cells rather than the effects of medications or comorbid conditions.

Methods. Similar methods used above and in **P3** (Aim 1) will be applied to collect and process layer 3 PC and PV cells in the non-human primate cohort exposed to antipsychotic medications (**Center Plan, *4.1.iv***).

Data analysis. The statistical models applied in Aims 1 and 2 include analyses of covariance for multiple factors. Nonetheless, and as performed in multiple prior studies in our labs[3-8], the putative effects of medications or comorbid conditions (e.g., alcohol or other substance use, death by suicide) will be addressed by appropriate contrasts among human subjects (see **Center Plan, *4.1.iii*** for other potential covariates). We acknowledge that not all comparisons may be possible due to cohort size and inter-dependency structure of the potential confounds. Therefore, we will also use an available cohort of non-human primates that were exposed to antipsychotic medications to investigate drug-induced changes in layer 3 PC and PV cells. We can also investigate whether any significant findings are associated with greater illness severity using available measures from psychological autopsy (i.e. factors predictive of disease severity such as male sex, family history of schizophrenia, and earlier age at illness onset; and factors that indicate illness severity including suicide, no history of marriage, lower socioeconomic status, and living dependently at time of death).

Predictions and alternative outcomes. We predict that the alterations observed in Aims 1-3 reflect the disease process of schizophrenia and not confounding or comorbid factors. The alternative outcome of specific effects of potential confounds will be evaluate as needed and in concert with results from **P2.**

HUMAN SUBJECTS

This project will use postmortem brain specimens (see Table 1 in **Center Plan** and Appendix 1) that have already been procured by the Pittsburgh Brain Tissue Donation Program under the direction of David A. Lewis, MD (Center Director for this proposal). All of the procedures for the next-of-kin consent for brain tissue donation and diagnostic interviews with informants were approved by the University of Pittsburgh's Institutional Review Board (IRB) and Committee for Oversight of Research Involving the Dead (CORID). ***No new subjects will be involved in this project,*** but we provide below the relevant human subjects information.

Risks to human subjects.

Human subject involvement, characteristics and design. The subject interviewees are the relatives and friends of individuals who died in Allegheny County and met the subject criteria for this proposal.

Sources of materials. Brain samples are obtained through the Allegheny County Medical Examiner's Office. Informed consent is obtained from the next-of-kin for the removal of brain tissue prior to the autopsy. Permission to review medical and psychiatric records of the subjects is obtained by use of a release of information consent form. Conduct of structured interviews with the interviewees involves informed consent.

Potential risks. The main risks are that the interviews are time-consuming and may be upsetting for the family and friends of the victims. However, we have observed no untoward effects of these interviews in our previous experience with psychological autopsies, and in fact, the informants frequently find the experience to be helpful, and appreciate the opportunity to talk about their loved ones. If we detect a psychiatric disorder in the interviewee, then we will make any clinical concerns known to the interviewee, and facilitate a psychiatric referral as indicated.

Adequacy of protection against risks.

Recruitment and informed consent. A trained research associate is in daily contact with the Medical Examiner's office in order to identify cases of interest as soon as possible. The Medical Examiner's office provides us with names, addresses and phone numbers of the next-of-kin of the cases, and brain specimens are obtained as noted above. Approximately two months after the death, we contact the next-of-kin expressing our condolences, and asking them to participate in the structured interview. Based on our previous experience, this interval appears to be the appropriate and humane time to wait before interviewing the family. Other informants (e.g., other family members, peers, friends) are nominated by the next-of-kin. If the informant is younger than 18, then permission to contact the informant is sought from the parent first. All participants are given, both verbally and in writing: 1) the phone numbers and addresses of the investigators, 2) a description of the project, 3) a description of the payments, risks, and benefits, and 4) a commitment to protect the information obtained by treating it confidentially.

Protections against risk. In order to compensate families for time involved in the interviews, we pay $100 for each completed interview. If the interviewee becomes upset during the interview, the interviewer who is a licensed and experienced clinical psychologist will attempt to explore what is upsetting and try to return to the interview format. If that is not possible, then the interview will be terminated. If the subject is willing, a follow-up interview will be set up.

Confidentiality will be assured by storing all hard copies of research data in locked files in a locked office. Questionnaires, interviews, tapes, and brain material will be labeled by ID number only. Only licensed psychologists Drs. Sue Johnston and Mary Ann Kelly and David Lewis, MD (Director of the Brain Tissue Donation Program) have access to the code that links data sets to relatives' names. No individual is identified through any scientific communications.

Any interviewees who show evidence of psychiatric disorder will be referred for treatment. All interviewers are experienced clinicians with extensive experience in the assessment of psychiatric disorders and suicidal risk, who are knowledgeable about current referral resources in the community. Interviewees with psychiatric difficulties will be offered referrals at WPIC, other mental health facilities (such as their local MH/MR), or private practitioners. Interviewers have continuous back-up through Dr. Lewis or his associates, Drs. Robert Sweet and David Volk.

Potential benefits of the proposed research to the subjects and others.

Potential benefits to the interviewees include the opportunity to participate in a study which may further our understanding of the pathogenesis of schizophrenia. Furthermore, many individuals may find participation

personally helpful; that is, they appreciate the opportunity afforded by the interviews to talk about their loved ones. Additionally, participants may have psychiatric difficulties identified, and are referred for further help. If they prefer to meet off campus, we travel to their homes or another location. Finally, we compensate interviewees for their time. In contrast to these benefits, the potential risks of this study are minimal, and no instances of emotional distress related to participating in the psychological autopsy process have occurred to date.

Importance of the knowledge to be gained.

The proposed studies seek to understand the pathogenetic processes that contribute to dysfunction of cortical inhibitory circuitry in a subset of schizophrenia subjects which may help inform preventative treatment strategies, novel diagnostic approaches, and individualized treatment strategies for the disorder. Consequently, the minimal risks associated with conducting interviews with surviving family members and friends of study subjects are reasonable in relation to the importance of characterizing the disease process of schizophrenia.

VERTEBRATE ANIMALS

All non-human primate tissue specimens to be used in this project are already available. No additional animals will be used for this project. All procedures for obtaining the tissue had been approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

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SPECIFIC AIMS

The **Central Hypothesis** posits that layer 3 pyramidal cells (**PCs**) in subjects with schizophrenia have a cell-autonomous abnormality that is 1) reflected in altered morphology and 2) differs in severity across cortical regions including primary visual (**V1**), posterior parietal (**PPC**) and dorsolateral prefrontal (**DLPFC**) cortices, which are part of the visual working memory and attention network (**V1-PPC-DLPFC**)[1-4]. This project will test the following components of the **Central Hypothesis**: 1) Altered layer 3 PC morphology (e.g., reduced dendritic spine density) is present in subjects with schizophrenia; 2) these alterations include layer 3 excitatory boutons; and 3) the lower local excitatory drive that results from these alterations is reflected in reduced markers of activity in layer 3 PCs and in their reciprocally connected layer 3 parvalbumin (**PV**) expressing basket cells[5-8]. In contrast, morphology is not altered in layer 5 PCs in subjects with schizophrenia[4,9,10].

The substantial data supporting this model is summarized in the **Center Plan** (Fig.0.2) and **P1**&**2** Significance. Importantly, this model leads to a number of novel predictions that are tested in the proposed studies. First, the model predicts that deficits in layer 3 PC somal volume and dendritic spine density are present in V1-PPC-DLPFC, but moderated by region-specific factors. To date, only two limited studies have directly compared layer 3 PCs across V1 and DLPFC in schizophrenia[1,10], and PPC has not been examined. Second, our preliminary data also clearly indicate that the DLPFC exhibits lower markers of local network activity, including reduced mRNA expression of cytochrome c oxidase subunit IV isoform 1 (**COX4I1,** see **P1**) within PCs, and reduced levels of the 67 kDa isoform of glutamate decarboxylase (**GAD67**) and parvalbumin (**PV**) within PV basket cells[6,11-14]. It is not known whether similar PC abnormalities in V1 and PPC are present. However, functional neuroimaging data indicate impaired activity within V1 and PPC as well as DLPFC during visual tasks in subjects with schizophrenia[15,16]. Accordingly, our model predicts that layer 3 PC abnormalities in V1 and PPC are associated with lower molecular markers of neuronal activity in these regions. Finally, reductions in pre-synaptic proteins, such as synaptophysin (**SYP**) and synapsin 1 (**SYN1**), have been reported in schizophrenia[21-24], and reduced SYP and SYN1 expression results in impaired glutamatergic bouton function, behavior and cognition[17-20]. Our model predicts that these reductions predominate in intracortical glutamatergic boutons within layer 3 of V1-PPC-DLPFC and are positively correlated with the magnitude of the underlying morphologic abnormalities in each region. We will examine these predictions in an integrated set of aims, studying V1, PPC and DLPFC concurrently in the same subjects.

Aim 1. To determine the relative magnitude of PC morphologic alterations in layer 3 of V1, PPC and DLPFC in schizophrenia. In Exp.1.1, we will use design-based stereology[2-4] to quantify PC somal volume in layer 3 of all three regions. In Exp.1.2, we will quantify the density of dendritic spines, identified based on colocalization of the f-actin binding toxin, phalloidin[25,26], and spinophilin[27-29] fluorescence, in all regions. *We predict that PC somal size and dendritic spine density are reduced in subjects with schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Aim 2. To determine if protein markers of neuronal activity are altered in layer 3 of V1, PPC and DLPFC in schizophrenia. In Exp.2.1, we will evaluate the levels of COX4I1 protein in layer 3 PC somata in all three regions. In Exp.2.2, we will measure intra-bouton levels of GAD67 and PV in layer 3 PV basket cells in all three regions. *We predict that somal levels of COX4I1 protein and intra-bouton levels of GAD67 and PV are reduced in subjects with schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Aim 3. To determine the nature and magnitude of alterations in excitatory cortical projections in layer 3 of V1, PPC and DLPFC in schizophrenia. We will quantify levels of SYP and SYN1 immunoreactivity in axonal boutons immunoreactive (**IR**) for the vesicular glutamate transporter (**VGlut**) 1, which selectively labels glutamatergic boutons of cortical origin[30-32], in all three regions. Specificity of changes for glutamatergic boutons of cortical origin will be established by comparisons to the same measures in VGlut2-IR boutons, which arise primarily from thalamus[30-32]. *We predict that VGlut1 bouton densities and intra-bouton protein levels of SYP and SYN1 are reduced in subjects with schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Aim 4. Determine if the alterations found in Aims 1-3 are specific to the disease process of schizophrenia. Understanding the potential contribution of the alterations observed in Aims 1-3 to impairments in V1-PPC-DLPFC in schizophrenia depends upon knowing whether these changes are specific to the disease process. Consequently, we will use the same experimental approaches to determine whether positive findings from Aims 1-3 are attributable to medications or to comorbid conditions (e.g., alcohol or other substance use, death by suicide, etc). As in our prior studies[27,33-37], these questions will be addressed by appropriate contrasts among human subjects and by the use of non-human primate models.

OVERVIEW

The **Central Hypothesis** posits that layer 3 PCs have a cell-autonomous abnormality that is reflected in altered morphology and that differs in severity across regions in V1-PPC-DLPFC[1-4]. These alterations of layer 3 PCs result in locally reduced excitatory drive, reflected in reduced markers of metabolic activity in layer 3 PCs and reduced activity-dependent markers in reciprocally connected layer 3 PV-expressing basket cells[5-8] (**Center Plan**, Fig.0.2). This project provides key tests of these predictions by using approaches with cellular and circuit specificity to determine the nature and magnitude of alterations in selected proteins and structures in schizophrenia as a function of their regional location within V1-PPC-DLPFC.

By studying proteins and structures within postmortem human brain from individuals with schizophrenia, **P2** serves as an essential link between disease-related molecular findings in these same neurons, layers and regions (**P1**) and abnormal information processing in the disease (**P5**). Specifically, findings from **P2** will: 1) indicate whether regional and disease-specific alterations in the PC transcriptome (**P1**) result in altered PC morphology and protein levels; 2) constrain the interpretation of how normative functional connectivity (**P4**&**5**) is altered in disease (**P5)** by defining the nature of the disease-associated alterations in PCs, the markers of their local circuitry and activity, and the regional magnitude of these effects; and 3) guide predictions for future forward translational studies that further characterize these circuits through the use of markers specific for PCs projecting between PPC and DLPFC that may be identified in **P3**.

SIGNIFICANCE

Morphological alterations, present in cortical layer 3 PCs of subjects with schizophrenia, contribute to information processing deficits. Substantial evidence indicates reduced layer 3 PC somal volume and dendritic spine density in multiple regions in schizophrenia and schizoaffective disorder[1-4,10,27,38] (these two diagnoses will be referred to hereafter, for brevity, as schizophrenia, see **Center Plan 1.0**). PCs in layer 3 of the DLPFC (both areas 9 and 46) have a smaller cell body[2,10], shorter dendrites[1,39] and fewer dendritic spines[1] in schizophrenia relative to healthy comparison subjects. Interestingly, in the only direct comparisons across regions within the same subjects, layer 3 PCs in V1 had only 50% of the spine deficit present in the DLPFC, and did not have smaller cell bodies or shorter dendrites relative to healthy comparison subjects[1,10]. In contrast to layer 3 PCs, those located in layer 5 in DLPFC do not manifest these morphological alterations in schizophrenia[9,10]. Studies in other regions have provided mixed results regarding the morphological integrity of layer 5 PCs, and these neurons have not been evaluated in V1 or PPC[4,39,40].

The conserved morphological deficits in layer 3 PCs in schizophrenia are likely to alter cortical information processing (**P4**). For instance, spines play important roles in segregating inputs encoding distinct information[41] and serve to equalize the magnitude of dendritic responses to inputs at different distances from the soma[42]. In addition, the reduction in dendritic length and branching in the cortex of individuals with schizophrenia would be expected to alter excitatory input summation, EPSP magnitude[43], summation of responses at the soma[44], and neuron firing rates[45]. The proposed studies will determine whether these alterations result in evidence of reduced markers (**Aim 2**), and *in vivo* measures (**P5**), of local network activity.

Impairments of the glutamatergic outputs of layer 3 PCs may also be present in schizophrenia and contribute to dysfunction. In subjects with schizophrenia, mRNA and protein levels of pre-synaptic proteins that affect glutamate release, such as SYP and SYN1, are lower[21-24,46]. In addition, at least for SYP, protein level deficits are greater in DLPFC than in V1[22], raising the hypothesis that regional differences in layer 3 PC morphologic alterations in schizophrenia may also be reflected as axonal pathology within these cells. Importantly, within a cortical region, most glutamate boutons arise from the local axon collaterals of PCs in that region[47], and thus these boutons provide a robust measure of the local circuitry. It is not known if reports of lower SYN1 and SYP protein in schizophrenia result from frank loss of boutons or lower within-bouton protein levels, as the techniques used in these earlier reports can not differentiate among these two alternatives. But either scenario would be associated with lower excitatory activity in the local circuitry. In the case of a normal complement of boutons but lower protein levels per bouton, cultured hippocampal neurons from mice lacking SYP have pronounced synaptic depression during sustained activity and have slower recovery of recycling vesicle pools after depletion.[17] Similarly, the elimination of SYN1 protein results in reduced glutamate release and delayed recovery of synaptic transmission after high frequency stimulation[18]. Our measurement approach, which uses multiple-label quantitative fluorescent microscopy, allows us to determine both bouton density and relative intra-bouton protein levels[37,48,49], providing for a robust interpretation of findings (Aim 3).

PCs and reciprocally connected PV basket cells create a local circuit, the activity of which is essential

to information processing. Layer 3 PCs are reciprocally connected with PV basket cells whose cell bodies are typically located in the same layers[7,8,50]. These PC-PV basket cell circuits are essential for the generation of gamma oscillations that are correlated with cognitive performance, including working memory, and are impaired in schizophrenia[51]. Several lines of evidence suggest that markers of activity are reduced in layer 3 PC-PV circuits in schizophrenia, at least in DLPFC. For example, a pathway analysis (see **P1** Preliminary Studies) of DLPFC layer 3 PCs revealed a highly significant overrepresentation of differentially-expressed transcripts related to mitochondrial functions. The majority of differentially-expressed transcripts, including COX4I1, were under-expressed in schizophrenia. Similarly, down-regulation of GAD67 and PV mRNA, widely replicated findings in subjects with schizophrenia[5], can result from blockade of excitation to PV cells[6]. PV mRNA is reduced in both V1 and DLPFC in schizophrenia[13], and in DLPFC is markedly reduced in layer 3 but not in layer 5[12]. Recently, we reported that levels of GAD67[14] and PV[52] proteins were 49% and 23% lower, respectively within the boutons of PV basket cells in DLPFC layers 3-4 in schizophrenia. Since expression of GAD67 and PV are both activity-dependent[6], these findings suggest that reduced PV basket cell inhibition may provide compensation for loss of local excitatory drive, at least at the level of the DLPFC, in schizophrenia; this prediction will be tested in each investigated region of V1-PPC-DLPFC in Aim 2. Whether regional GAD67 and PV reductions in schizophrenia are correlated with measures of impairment within the excitatory boutons that serve as the local substrate of PC to PV cell drive will be tested in Aim 3.

INNOVATION

1) The proposed studies will provide the first comprehensive comparison, *within the same subjects*, of protein and structural measures *in the same local circuitry* across cortical regions of the V1-PPC-DLPFC network in schizophrenia. 2) In contrast to earlier studies that relied on laminar location of single makers to selectively identify circuit components, we will use concurrent labeling with multiple markers to enrich our measures for the neurons and projections of interest (e.g. Fig.2.1 & Fig.2.3). 3) We have developed a highly innovative approach capable of evaluating levels of specific proteins embedded within cellular microdomains that form cortical circuits[53]. Specifically, stereologic sampling is combined with multiple-label, spinning disk confocal fluorescence microscopy, followed by image processing using deconvolution and iterative intensity and morphologic segmentation[48]. This combination enables detection of small synaptic structures with high spatial resolution, with biochemical selectivity, and with the ability to assess the relative expression levels of multiple proteins within the identified structures. 4) The integrated study of mRNA (**P1**) and protein levels within the same subjects, regions, layers and cells will inform on the role of transcriptional control in any observed disease-related protein alterations.

PRELIMINARY STUDIES

Overview. We have substantial published findings in support of the proposed hypotheses, cited throughout the application, indicating that in schizophrenia: 1) Morphological impairments are present in layer 3 PCs in DLPFC and other cortical regions, although relatively understudied in V1 and not previously assessed in PPC[1-4,27]; 2) The magnitudes of these impairments are greater in layer 3 of DLPFC than in V1[1]; 3) Expression of pre-synaptic mRNAs and proteins is downregulated in DLPFC[21,54]; 4) mRNA and protein levels of GAD67 and PV are reduced in layer 3 in DLPFC[11,12,14].

With regard to technical feasibility we also have published data indicating our ability to: 1) Apply stereologic sampling in combination with multi-label fluorescence confocal microscopy and custom intensity/morphological segmentation algorithms to quantify the density of, and relative protein levels within, pre- and postsynaptic structures in postmortem human and monkey cortex[14,32,37,48,49,52,53]; 2) Apply design-based stereology to the determination of PC somal volume, including in studies utilizing confocal fluorescence microscopy[2-4,55]; 3) Label the specific excitatory[32] and inhibitory[14,37,49,52] bouton populations proposed in this application, and extract information on their relative protein content.

Table 2.1. Planned experimental assays

Exp	Assay	Primary Measures	Antibody (species;wavelength)
1.1	1	PC somal volume	NeuN (g. pig; 488), SMI-32 (mouse; 647), COX4I1 (rabbit; 568)
1.2	2	Dendritic spine density	spinophilin (rabbit; 488), phalloidin (568)
2.1	1	PC somal COX4I1 content	NeuN (g. pig; 488), SMI-32 (mouse; 647), COX4I1 (rabbit; 568)
2.2	3	PV basket cell bouton PV content; PV basket cell bouton GAD67 content	vGAT (g. pig; 405), PV (rabbit; 488), GAD67 (mouse, 568), GAD65 (goat; 647)
3	4	VGlut1 bouton density; VGlut1 bouton SYP content; VGlut1 bouton SYN1 content	VGlut2 (rabbit; 405), SYP (goat; 488), VGlut1 (g. pig; 568), SYN1 (mouse; 647)

Thus, our presentation below is limited to providing additional preliminary evidence in support of the feasibility of those assays planned in our experiments (Table 2.1) and not established by our prior published studies (Assays 1, 2 and 4).

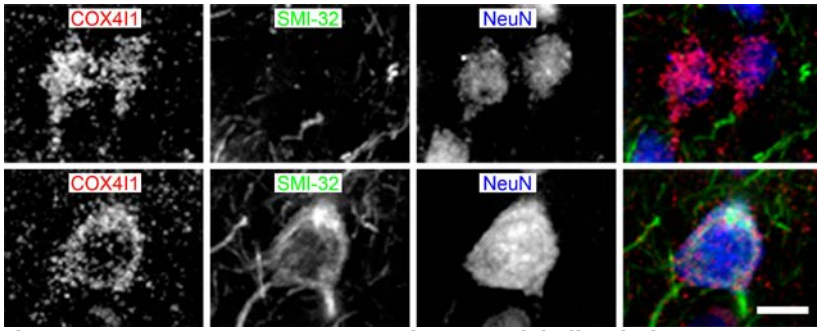

Fig.2.1. Assay 1- NeuN, SMI-32, and COX4I1 labeling in human cortex. COX4I1 is readily detectable in neurons labeled by NeuN (Top), including the subset that are also NNFP+ (SMI-32+) (Bottom). Scale bar 10 um.

Assay 1 (Exp.1.1 and 2.1). Assessment of PC somal volume and PC somal COX4I1 content. Antibodies (species; wavelength): Neuronal nuclei (NeuN; guinea pig; 488), COX4I1 (rabbit; 568) and SMI-32 (mouse; 647)**.** We have established our ability to label COX4I1 in all neurons (colabeled by NeuN[56]) in postmortem human DLPFC (Fig.2.1, rabbit anti-COX4I1, Bethyl Labs, #A301-899A). We will also include labeling with antibody SMI-32, directed against the non-phosphorylated form of neurofilament protein (NNFP), that labels long-range projection PCs[57]. This will enhance our interpretation of findings by allowing analysis of whether somal volume reductions are restricted to NNFP- (i.e. PCs providing short range projections) or NNFP+ (i.e. PCs including long-range projection neurons, a subset of which connects PPC and DLPFC (**P3**).

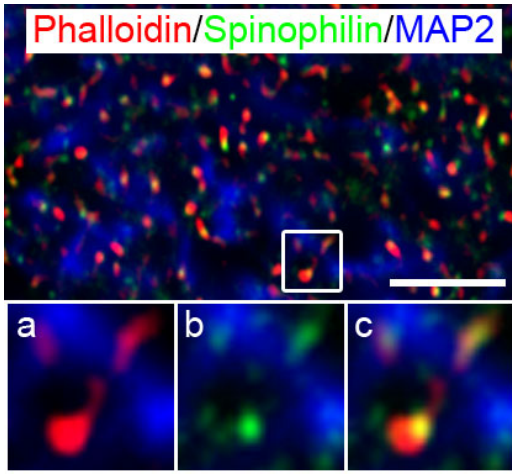

Fig.2.2. Assay 2- Dendritic spine labeling in a single z-plane of human cortex (top). Inset shows (a) phalloidin-labeled spines (red) emerging from a MAP2-labeled dendrite (blue) with (b) spinophilin (green) and (c) the merged image showing colocalization of phalloidin and spinophilin in the spine head (yellow). Scale bar 5µm.

Assay 2 (Exp.1.2). Quantification of dendritic spine density. Antibodies (species; wavelength): Spinophilin (rabbit; 488), Phalloidin (568). Spinophilin is a protein enriched in dendritic spines[28,29]. By quantifying spinophilin-labeled punctae, we previously showed lower dendritic spine density in schizophrenia[27]. Because spinophilin-IR punctae may also be present in dendritic shafts[29], we have developed a dual label approach; including the f-actin binding protein, phalloidin increases specificity for the identification of spines by requiring the colocalization of the two labels[25,26] (Fig.2.2).

Assay 4 (Exp.3). Assessment of excitatory bouton populations. Antibodies (species; wavelength): VGlut2 (rabbit; 405), SYP (goat; 488), VGlut1 (guinea pig; 568), SYN1 (mouse; 647). We have previously established that VGlut1 (marker of glutamate boutons of cortical origin) and VGlut2 (marker of glutamate boutons of thalamic origin) are present in distinct boutons in human cerebral cortex[32] (Fig.2.3). In pilot assays, we found that VGlut1 and VGlut2 boutons had similar relative mean ± SD levels of SYP (3271 ± 850 and 2983 ± 294, respectively). In contrast, VGlut1 boutons had nearly 3-fold higher SYN1 levels (3195 ± 674) than VGlut2 boutons (1014 ± 863).These data indicate our ability to determine SYP and SYN1 levels in distinct bouton populations. They also suggest that VGlut1 and VGlut2 boutons differ in their dependence on SYP and SYN1, and thus may differ in vulnerability to schizophrenia-related alterations in these synaptic proteins.

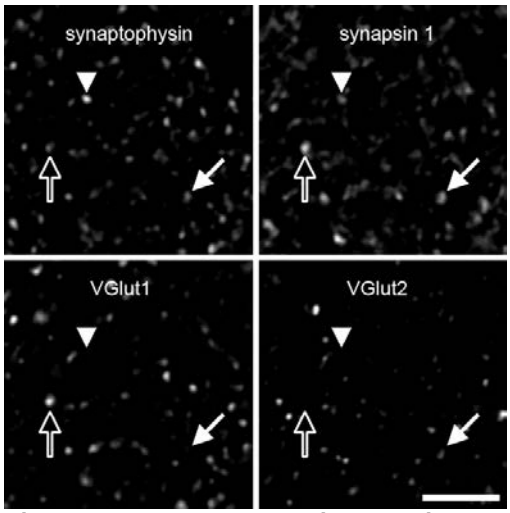

Fig.2.3. Assay 4: Excitatory bouton subpopulations in human cortex. Independent VGlut1 (open arrow) and VGlut2 (filled arrow) colocalization with SYP and SYN1. SYP and SYN1 also label other boutons (e.g. GABAergic) lacking VGlut expression (arrowhead). Bar = 5µm.

Further comment on technical issues applying to all assays. 1) Specificity. For all proposed experiments (Assays 1-4), the specificity of the primary antibodies for the structures and proteins of interest was confirmed by a variety of methods including Western blot[14,32,37,49,58-62] (Bethyl Labs unpublished data), evaluation of colocalization[14,25,26,29,37,49,53,57], and studies in knock-out mice[37,58]. In addition, exclusion of the primary antibodies resulted in the complete absence of immunoreactivity. 2) Autofluorescence. To minimize autofluorescence, sections are pre-treated with 1% sodium borohydride, which reduces fixative-induced fluorescence[63,64]. We address age-related lipofuscin accumulation in neuronal soma[65,66] by imaging and masking it in an unused channel (e.g., excitation

350 nm, emission 460 nm) in order to exclude it during analyses[14,67]. 3) Protein quantification. We have verified the sensitivity and linearity of our approach to quantification of relative fluorescence intensity by embedding in human tissue sections 1.0 µm fluorescent microspheres of varying intensities[48]. We have also demonstrated that this approach can effectively identify intra-bouton protein level differences between cell types and disease groups[14,37,49,53].

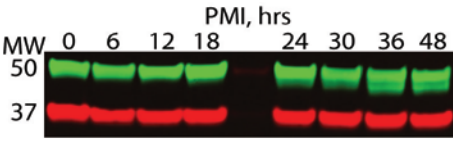

Fig.2.4. Example of SYP stability in an animal model of PMI. SYP Red, Tubulin control Green.

APPROACH

Overview. 1) An overview of the proposed aims and experiments can be found in Table 2.1. The timeline is presented in the **Center Plan** Table 3. 2) Subjects for Aims 1-3 will be 50 pairs. Each subject with schizophrenia or schizoaffective disorder [referred to below as schizophrenia (see **Center Plan 1.0**)] is matched to a comparison subject for sex and as closely as possible for age, postmortem interval and other characteristics (**Center Plan** Table 1 & **Appendix 1**) to reduce biological variance across groups. All subjects have a postmortem interval <28 hours, within the range associated with good preservation of the studied proteins (see[14,32,37,58,68] and unpublished findings for SYP, SYN1 and COX4I1, in which all have ≥80% preservation up to 48 hours PMI (e.g., Fig.2.4). 3) We will conduct all assays in two stages, each consisting of 25 pairs. 4) Analysis of PPC is performed on area 40 lateral to the intraparietal sulcus. This choice is based on evidence that it is affected in schizophrenia,[69] and is located identically to monkey area 7a studied in **P3**&**P4** (see Fig.0.2). 5) Specificity of findings for layer 3 will be established by identical studies in layer 5 of all regions. 6) Statistical tests and power estimation for Aims 1-4 are described in the **Stats/DM Core**.

Aim 1. To determine the relative magnitude of PC morphologic alterations in layer 3 of V1, PPC and DLPFC in schizophrenia.

Hypothesis. *PC somal size and dendritic spine density are reduced in schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Methods. Immunocytochemistry and spinning-disk confocal microscopy with stereologic sampling[14,32,37,48,49,53] are used in multiple-label experiments (Table 2.1) to assess differences between subject groups in PC somal volume (Exp.1.1) and dendritic spine density (Exp.1.2) in all regions. NeuN labels all neurons,[56] and colocalization of phalloidin and spinophilin fluorescence is used to identify dendritic spines. The inclusion of NNFP will enhance our interpretation of findings by allowing further analysis of whether any observed changes include long-range projection PCs (NNFP+), a subset of which serve to connect the regions of interest, providing a bridge to the study of these neurons in **P3**&**P4**.

Tissue. Coronal sections (40 µm) evenly spaced throughout the rostral to caudal extent of blocks containing areas V1, 40 and 46 from the immersion-fixed left hemisphere of matched pairs of schizophrenia and control subjects are used. Within sections, areas V1, 40 and 46 are identified using established cytoarchitectonic criteria[22,70-72], as done in our prior studies of these and other regions of human neocortex[1,2,4,55].

Tissue Processing. Sections from each pair are processed together throughout all assay and imaging procedures to reduce experimental variance. Slides are coded to blind subject number and diagnosis. Sections are pretreated with 1% sodium borohydride and processed for fluorescence immunocytochemistry using procedures that permit labeling throughout the z-axis with limited post-processing shrinkage in this axis[34,48,73]. Sections are multi-labeled as described for Assays 1 and 2, Table 2.1 (exemplified in Fig.2.1 & Fig.2.2).

Imaging. Multi-labeled sections are imaged using an Olympus BX51WI microscope equipped with an Olympus DSU spinning disk confocal and a 60X 1.4 NA oil immersion super-corrected objective. The Stereo Investigator program is used for systematic, random sampling[53]. Adjacent Nissl-stained sections are used to trace the pial surface and border between gray and white matter of areas V1, 40 and 46. A contour containing layer 3 and one containing layer 5 are outlined. Contours are aligned with the immunolabeled sections using pial surface fiduciaries and a sampling grid is randomly placed over the contour. At each sampling site, tissue thickness is measured and sequential image planes 0.25 µm apart are collected as 3-D data sets.

Image Processing. Image processing and fluorescence intensity/morphological segmentation are conducted as described,[14,32,37,48,49,53] tailored to the individual assessment. For Exp.1.1 we will create 3-D object masks of PC soma. Using the unbiased associated point rule (see[37,53]), PC soma are selected and somal volume quantified with the nucleator probe as in our prior studies[2-4,55]. For Exp.1.2, we require colocalization of phalloidin and spinophilin fluorescence to identify dendritic spines, which enhances specificity relative to the

use of either marker alone. Using the associated point rule, phalloidin puncta 3-D object masks that overlap a spinophilin puncta 3-D object mask are quantified.

Predictions and alternative outcomes. We predict that layer 3 PC somal volumes and dendritic spine densities will be lower in all regions in schizophrenia, with the magnitude of the disease-associated differences DLPFC>PPC>V1. We can then evaluate convergence with functional measures of impairment in the three regions made in **P5**. It is possible that the magnitudes of disease differences do not follow the predicted regional order. If for example, we find PPC to be more severely affected,[74] we can infer whether this may reflect a greater intrinsic molecular deficit (**P1**) or a greater degree of morphologic complexity (demonstrated by greater somal volumes and spine densities in control subjects) in PPC. Regardless, finding a different pattern of magnitude of morphologic alterations will cause us to modify our hypotheses in Aims 2 & 3 to anticipate that the magnitude of alterations in local activity markers and glutamate boutons will track with morphology. Because we will also label PCs for NNFP, we will evaluate whether somal volume reductions are restricted to NNFP- (i.e. PCs providing short-range projections) or NNFP+ (i.e. PCs including the long-reange projection neurons that interconnect our regions of interest). Finding changes limited to one population would lead us to modify our hypothesis for Exp.2.1 to predict that COX4I1 levels are reduced selectively in those PCs. Importantly, our methods for single cell mRNA capture in **P1** work in sections immunolabeled for detection of NNFP, allowing us to examine molecular changes specifically within NNFP-defined subsets of PCs if indicated.

Comments. **1.1)** Although measures of relative protein levels in PC soma or spines (i.e., NNFP, f-actin, spinophilin) are not being tested in this aim, our approach generates quantitative fluorescence intensities in addition to measures of object number. Thus, we can distinguish whether observed changes in object densities (e.g., reduced spine density) represent structural alterations or altered detectability due to changes in protein levels[32,37,48]. *Thus, our approach offers a distinct advantage over alternatives such as brightfield counts of objects, wide field optical densities, or western blot of tissue homogenates, none of which can disentangle structure number and protein expression per structure*. **1.2)** We have chosen to use fluorescence labeling of PCs for determination of somal volume rather than DAB-based labels because in a prior study we determined that the latter approach created a disease-specific confound that masked differences in somal size[55]. **1.3)** Recently, a new approach (CLARITY) that allows much thicker (e.g., 1 mm), fluorescently-labeled sections to be assessed has been developed[75]. We are actively piloting this approach and if feasible will implement it in the proposed studies where applicable. 1.4) We will conduct exploratory clustering analyses (**Stats/DM Core**)[76] to determine if layer 3 PC pathology identifies subsets of subjects within, or across, DSM diagnoses.

Aim 2. To determine if protein markers of neuronal activity are altered in layer 3 of V1, PPC and DLPFC in schizophrenia.

Hypothesis. *Somal levels of COX4I1 protein and intra-bouton levels of GAD67 and PV are reduced in schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Methods. In the same subjects as in Aim 1, we will evaluate PC somal levels of COX4I1 protein in all regions (Exp.2.1). In Exp.2.2 we will measure levels of GAD67 and PV in PV basket cell boutons (PV-IR, vesicular GABA transporter (**vGAT**)-IR and GAD65-IR[14,49]) in all regions. Tissue, tissue processing, imaging and image processing are as described above for Aim 1. 3-D object masks of PC soma will be created and selected as described in Aim 1 and mean fluorescence intensity of COX4I1 in these mask objects will be extracted for analysis. Mean fluorescence intensity of GAD67 and PV in PV basket cell bouton mask objects will be determined as previously described[14,49].

Predictions and alternative outcomes. We predict that COX4I1 levels in PC soma and PV basket cell intra-bouton GAD67 and PV levels are reduced in schizophrenia, and will interpret such findings as reflecting reduced activity within the layer 3 PC-PV cell circuit. This interpretation would be supported by evidence that the impairments in COX4I1, GAD67 and PV correlate within subjects. We further predict that the regional ordering of reduction magnitudes will follow that predicted for PC morphology in Aim 1 (Fig.0.2C). Findings consistent with this prediction would support a causal connection between structural alterations and network activity. It is possible that we will find a greater morphologic deficit in one region (e.g., PPC) with a greater activity marker deficit in another (e.g. DLFPC). This could indicate that DLPFC activity is reduced, in part, by lower feedforward excitation from PPC (i.e., there is a compounding effect of altered connectivity, as discussed in the **Center Plan**). This prediction could be subject to convergent testing in **P5**, and by evaluating whether reduced COX4I1 in PPC is present in NNFP+ PCs (i.e. PCs that provide long-range corticocortical projections). If we find no change in COX4I1 protein levels, we will confirm that COX4I1 mRNA is reduced in the same subjects (**P1**). If so, there may be disease-specific effects on mRNA stability, translation or trafficking, which

may be informed by the miRNA studies in **P1**.

Comments. **2.1)** Our preliminary data has found evidence for alterations in multiple COX transcripts (see **P1**). We have initially focused on COX4I1 because of the availability of an antibody yielding high-quality labeling of human post-mortem tissue (Fig.2.1) and can pursue additional markers of mitochondrial activity as indicated by the findings of **P1** (e.g. Fig.1.2). **2.2)** We have chosen to focus on PV basket cell boutons because of our strong preliminary data that PV basket cell intra-bouton GAD67 and PV levels are reduced in DLPFC in schizophrenia. However, our labeling approach would also allow us to identify chandelier cell boutons (PV-IR, vGAT-IR and GAD67-IR but not GAD65-IR[49]) and quantify GAD67 in this population. **2.3)** If **P1** finds reduced COX transcripts in PV cells in schizophrenia, we will extend our study of COX4I1 protein levels to PV basket cell somas (distinguished from PV chandelier cells by labeling peri-neuronal nets, see Fig.1.3 in **P1**)[77].

Aim 3. To determine the nature and magnitude of alterations in excitatory cortical projections in layer 3 of V1, PPC and DLPFC in schizophrenia.

Hypothesis. *VGlut1 bouton densities and intra-bouton protein levels of SYP and SYN1 are reduced in schizophrenia in layer 3, and the regional magnitude of the reductions is DLPFC>PPC>V1.*

Methods. In the same subjects as in Aim 1, we will use immunoreactivity to VGlut1 to determine the density of excitatory boutons of cortical origin[30-32], as well as intra-bouton levels of SYP and SYN1, in layer 3 of all regions. Specificity for projections arising from within the cortex will be established by comparison to boutons labeled for VGlut2, which is present in glutamate boutons of subcortical, primarily thalamic, orign[30-32]. Tissue, tissue processing, imaging and image processing are used, as described for Aim 1, to generate object masks of VGlut1- and VGlut2- labeled puncta. Mean fluorescence intensity of SYP and SYN1 in these object masks will be extracted for analysis.

Predictions and alternative outcomes. We predict that bouton densities and intra-bouton levels of SYP and SYN1 will be lower in schizophrenia, with the magnitude of reductions decreasing from DLPFC to PPC to V1. Prior studies on total tissue SYP and SYN1, however, leave open the possibility that only density or intra-bouton protein levels are affected, a distinction our approach allows us to make (**Comment 1.1**). Findings as predicted will lead us to test if glutamate bouton impairments are correlated with the magnitude of reduced local activity markers in Aim 2. We will further test whether findings are selective for VGlut1 boutons in contrast to boutons expressing VGlut2 (predominantly of thalamic origin). We may find that VGlut2 boutons are affected. We would then similarly ask whether these changes are correlated with local activity markers from Aim 2, which would suggest that thalamocortical impairments contribute to the observed pathology. This interpretation could be subject to convergent testing in functional connectivity analyses in **P5**, as well as providing critical information for the interpretation of parallel primate studies in **P4**.

Aim 4. Determine if the findings from Aims 1-3 are specific to the disease process of schizophrenia.

Hypothesis. *The findings from Aims 1-3 are not due to medications or attributable to comorbid conditions.*

Methods. To determine whether positive findings in Aims 1-3 are due to antipsychotic medications, we will use our prior approach[1,2,14,27,33-37] (see also **P1**): 1) Compare subjects with schizophrenia who were on or off antipsychotic medications at the time of death; 2) Conduct parallel studies in nonhuman primate models of antipsychotic drug exposure developed to mimic human patient exposures to these agents (see **Center Plan 4.1.iv** for details of animals). For the latter approach, two equally-spaced coronal sections (40 µm) from the region of interest will be assayed per monkey and imaged as in Aims 1-3.

Predictions and alternative outcomes. We predict that the alterations observed in Aims 1-3 reflect the disease process of schizophrenia and not confounding or comorbid factors. We may, however, detect associations with other potential confounds, and can evaluate them as needed. For example, if an association with history of cannabis abuse is detected, we have available tissue from a cohort of tetrahydrocannabinol exposed monkeys[78]. Similarly, our brain tissue collection includes subjects with death by suicide or alcohol dependence without other psychiatric illness that can be tested to evaluate the effects of these and other comorbidities (e.g., suicide)[1,2,14,27,37].

Comments. **4.1)** We are also able to investigate whether any significant findings are associated with greater illness severity using available measures from psychological autopsy (i.e., factors predictive of disease severity such as family history of schizophrenia and earlier age at illness onset; and factors that indicate illness severity including no history of marriage, lower socioeconomic status and living dependently at time of death)[14,32,37,76].

HUMAN SUBJECTS

This project will use postmortem brain specimens (see Table 1 in **Center Plan** and Appendix 1) that have already been procured by the Pittsburgh Brain Tissue Donation Program under the direction of David A. Lewis, MD (Center Director for this proposal). All of the procedures for the next-of-kin consent for brain tissue donation and diagnostic interviews with informants were approved by the University of Pittsburgh's Institutional Review Board (IRB) and Committee for Oversight of Research Involving the Dead (CORID). ***No new subjects will be involved in this project,*** but we provide below the relevant human subjects information.

Risks to human subjects.

Human subject involvement, characteristics and design. The subject interviewees are the relatives and friends of individuals who died in Allegheny County and met the subject criteria for this proposal.

Sources of materials. Brain samples are obtained through the Allegheny County Medical Examiner's Office. Informed consent is obtained from the next-of-kin for the removal of brain tissue prior to the autopsy. Permission to review medical and psychiatric records of the subjects is obtained by use of a release of information consent form. Conduct of structured interviews with the interviewees involves informed consent.

Potential risks. The main risks are that the interviews are time-consuming and may be upsetting for the family and friends of the victims. However, we have observed no untoward effects of these interviews in our previous experience with psychological autopsies, and in fact, the informants frequently find the experience to be helpful, and appreciate the opportunity to talk about their loved ones. If we detect a psychiatric disorder in the interviewee, then we will make any clinical concerns known to the interviewee, and facilitate a psychiatric referral as indicated.

Adequacy of protection against risks.

Recruitment and informed consent. A trained research associate is in daily contact with the Medical Examiner's Office in order to identify cases of interest as soon as possible. The Medical Examiner's Office provides us with names, addresses and phone numbers of the next-of-kin of the cases, and brain specimens are obtained as noted above. Approximately two months after the death, we contact the next-of-kin expressing our condolences, and asking them to participate in the structured interview. Based on our previous experience, this interval appears to be the appropriate and humane time to wait before interviewing the family. Other informants (e.g., other family members, peers, friends) are nominated by the next-of-kin. If the informant is younger than 18, then permission to contact the informant is sought from the parent first. All participants are given, both verbally and in writing 1) the phone numbers and addresses of the investigators, 2) a description of the project, 3) a description of the payments, risks, and benefits, and 4) a commitment to protect the information obtained by treating it confidentially.

Protections against risk. In order to compensate families for time involved in the interviews, we pay $100 for each completed interview. If the interviewee becomes upset during the interview, the interviewer who is a licensed and experienced clinical psychologist will attempt to explore what is upsetting and try to return to the interview format. If that is not possible, then the interview will be terminated. If the subject is willing, a follow-up interview will be set up.

Confidentiality will be assured by storing all hard copies of research data in locked files in a locked office. Questionnaires, interviews, tapes, and brain material will be labeled by ID number only. Only licensed psychologists Drs. Sue Johnston and Mary Ann Kelly and David Lewis, MD (Director of the Brain Tissue Donation Program) have access to the code that links data sets to relatives' names. No individual is identified through any scientific communications.

Any interviewees who show evidence of psychiatric disorder will be referred for treatment. All interviewers are experienced clinicians with extensive experience in the assessment of psychiatric disorder, suicidal risk, and up-to-date knowledge about referral resources in the community. Interviewees with psychiatric difficulties will be offered referrals at WPIC, other mental health facilities (such as their local MH/MR), or private practitioners. Interviewers have continuous back-up through Dr. Lewis or his associates, Drs. Robert Sweet and David Volk.

Potential benefits of the proposed research to the subjects and others.

Potential benefits to the interviewees include the opportunity to participate in a study which may further our understanding of the pathogenesis of schizophrenia. Furthermore, many individuals may find participation personally helpful; that is, they appreciate the opportunity afforded by the interviews to talk about their loved

ones. Additionally, participants may have psychiatric difficulties identified, and are referred for further help. Finally, we compensate interviewees for their time and travel to their homes if they wish. In contrast to these benefits, the potential risks of this study are minimal, and no instances of emotional distress related to participating in the psychological autopsy process have occurred to date.

Importance of the knowledge to be gained.

The proposed studies seek to understand the pathogenetic processes that contribute to dysfunction of cortical inhibitory circuitry in a subset of schizophrenia subjects which may help inform preventative treatment strategies, novel diagnostic approaches, and individualized treatment strategies for the disorder. Consequently, the minimal risks associated with conducting interviews with surviving family members and friends of study subjects are reasonable in relation to the importance of characterizing the disease process of schizophrenia.

VERTEBRATE ANIMALS

All non-human primate tissue specimens to be used in this project are already available. No additional animals will be used for this project. All procedures for obtaining the tissue had been approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

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SPECIFIC AIMS

Visual working memory and attention depend on the flow of information to the posterior parietal cortex (**PPC**) and dorsolateral prefrontal cortex (**DLPFC**), as well as coordinated neural activity in PPC and DLPFC. These two regions are directly linked by projections from layer 3 pyramidal cells (**PCs**)[2,3] that are predicted to mediate their functional connectivity (**P4**). Consequently, cell-autonomous abnormalities in the neurons furnishing these projections are posited to impair functional connectivity between PPC and DLPFC in schizophrenia (**P5**), leading to deficits in working memory and attention (**Central Hypothesis**). A key element of this hypothesis is that region-specific properties of layer 3 PCs moderate the severity of the molecular (**P1**) and morphological (**P2**) pathology of these neurons in schizophrenia; specifically, the pathology of layer 3 PCs is predicted to be more severe in DLPFC than in PPC, and thus the projections from DLPFC-to-PPC are predicted to be more impaired than those from PPC-to-DLPFC (**P5**).

P3 serves as a critical bridge between the **P1**&**P2** studies of all PCs in layer 3 of human PPC and DLPFC and the **P4** studies that focus on the layer 3 connections between PPC and DLPFC in monkeys as follows: Aim 1 provides two key assessments of our translational strategy for testing the **Central Hypothesis**. First, Exp.1.1 tests the idea that regional differences in layer 3 PC gene expression between human PPC and DLPFC are conserved in monkeys. Second, Exp.1.2 focuses on the layer 3 PCs that interconnect PPC and DLPFC. This experiment provides a robust test of the idea that region of origin accounts for differences in gene expression by controlling for length of, and type of brain region targeted by, the principal axon, factors known to be associated with differences in certain molecular features[4] or dendritic morphology[5] of PCs in monkeys. Thus, Aim 1 provides a key transition from **P1**&**P2** studies in humans of all layer 3 PCs (which cannot currently be divided into subsets based on their principal axon projection) to studies of the subset that interconnect PPC and DLPFC in monkeys. These region-defined differences in gene expression are predicted to be associated with differences in morphological (Aim 2), and neurophysiological (Aim 3) properties of layer 3 PCs that provide the circuitry substrate for the functional connectivity between monkey PPC and DLPFC studied in **P4**.

Aim 1. Determine gene expression in layer 3 PCs of PPC and DLPFC and in the subset that interconnect these regions. The molecular pathology of layer 3 PCs in schizophrenia (**P1**) is predicted to be moderated by normal regional differences in gene expression patterns. To determine if such regional differences are conserved in monkeys, Exp.1.1 will compare gene expression patterns in layer 3 PCs of monkey PPC and DLPFC, using the same approach described in **P1**. To determine if the layer 3 PCs that interconnect areas PPC and DLPFC differ in gene expression, Exp.1.2 will use in vivo retrograde transport of inert fluorescent beads to identify selectively these layer 3 PCs, and laser microdissection and microarrays to assess differences in gene expression. *We predict that gene expression patterns for all layer 3 PCs in monkey PPC and DLPFC mimic those present in human brain. We also predict that gene expression patterns differ between layer 3 PCs that interconnect PPC and DLPFC in a manner that parallels regional differences in morphology (Aim 2).*

Aim 2. Determine the dendritic tree morphology of layer 3 PCs interconnecting PPC and DLPFC. The morphological pathology of layer 3 PCs in schizophrenia is predicted to be moderated by regional differences in their normal somatodendritic properties (**P2**). To compare the dendritic tree morphology of layer 3 PCs interconnecting PPC and DLPFC in monkeys, we will perform quantitative morphometry of PCs retrogradely-labeled in vivo and intracellularly-filled during recordings in cortical slices (Aim 3). *We predict that the complexity and spine density of layer 3 PC dendritic trees are greater in DLPFC than in PPC.*

Aim 3. Determine the cellular neurophysiology of layer 3 PCs interconnecting monkey PPC and DLPFC. Regional differences in layer 3 PC dendritic morphology (Aim 2) may determine differences in cellular excitability. However, inter-regional synchrony of local oscillatory activity (**P4**&**P5**) is facilitated if the PCs interconnecting PPC and DLPFC share functional properties important for production of local oscillations such as membrane resonance (low frequency) and synaptic inhibition from parvalbumin (**PV**)-positive GABA neurons (high frequency). We will combine in vivo retrograde transport with in vitro electrophysiology in brain slices. *We predict that layer 3 PCs interconnecting PPC and DLPFC differ in intrinsic excitability but share properties relevant for low (membrane resonance) and high (synaptic inhibition) frequency local oscillations*.

These aims provide a rigorous assessment of how region-specific cellular and circuitry properties of layer 3 PCs in PPC and DLPFC contribute to normal PPC-DLPFC functional connectivity (**P4**) and to the molecular (**P1**), morphological (**P2**), and connectivity (**P5**) substrates for impaired visual working memory and attention in schizophrenia.

OVERVIEW

In the **Central Hypothesis**, a cell type-autonomous pathology in cortical layer 3 PCs is predicted to be moderated by region-specific factors, such that among the cortical regions of interest in this Center, pathology is greatest in DLPFC, intermediate in PPC and least in V1 (Fig.0.2, **Center Plan**). This pathology is postulated to contribute to the neural circuitry substrate for altered cortical functional connectivity that leads to impairments in visual working memory and attention in schizophrenia. The link between the cellular and circuitry level tests of the Central Hypothesis in **P1**&**P2**, and the connectivity and cognitive tests in **P5**, can only be examined through translational studies in experimental animals, such as non-human primates, that share similar cellular, circuitry, connectivity and cognitive properties with humans. Thus, the goal of **P3** is to provide, in concert with **P4**, the essential information from studies in monkeys that is needed to integrate the postmortem findings from **P1**&**P2** with in vivo findings from **P5** in a robust and comprehensive test of the Center's **Central Hypothesis**.

The idea that region-specific factors moderate the severity of layer 3 PC pathology in schizophrenia is supported by previous postmortem human studies demonstrating regional differences in pathology, and by evidence that 1) patterns of gene expression normally differ across primate cortical regions[6] and 2) that morphological and neurophysiological properties of layer 3 PCs exhibit regional differences[7]. However, no studies have directly compared these properties across all layer 3 PCs in monkey PPC and DLPFC, or in the subset of layers 3 PCs that interconnect these regions. Thus, the goal of **P3** is to conduct studies to determine if the layer 3 PCs interconnecting monkey PPC and DLPFC differ in molecular and morphological properties that are region-dependent, but share neurophysiological properties required for inter-regional synchrony.

SIGNIFICANCE

This project brings several critically important advances to understanding the cortical cellular and circuitry abnormalities underlying cognitive dysfunction in schizophrenia. First, via labeling with non-toxic retrograde tracers, we can identify and study the specific populations of layer 3 PCs predicted to mediate the functional interactions between PPC and DLPFC that are thought to be essential for visual working memory and attention (**P4**). Second, the transcriptional, morphological and neurophysiological characterization of these PCs will provide information crucial to understanding the normal role of these cells in PPC-DLPFC interactions (**P4**), and consequently for understanding how their alterations could contribute to functional connectivity disturbances underlying visual working memory and attention impairments in schizophrenia (**P5**). Third, transcriptional profiling of layer 3 PCs that interconnect PPC and DLPFC may reveal cell type-specific molecular markers that could be used to identify these specific PC subpopulations in postmortem human brain samples, and thus to study the pathology (**P1**&**P2**) of neurons with known connectivity in schizophrenia.

A key feature of **P3** for understanding the disease process of schizophrenia is the use of the macaque monkey as a model system. The neocortices of macaque monkeys and humans have very similar cytoarchitectonic features, including the presence of higher-order association areas (e.g., DLPFC and PPC), that are not well-represented in rodents[8]. Moreover macaque and human genomes are 93% homologous[9,10] and give rise to very similar neocortical transcriptional architectures[6]. Thus, the macaque represents the best available animal model system for characterizing the transcriptional, morphological and neurophysiological properties of the layer 3 PCs that interconnect PPC and DLPFC. The results of these studies are essential for obtaining, with the required specificity at both the cellular and circuitry levels, the information needed to guide and interpret studies of the homologous cortical connectivity in humans (**P1**&**P2**&**P5**).

INNOVATION

Most approaches to understanding the disease process (Fig.0.1, **Center Plan**) underlying cortical dysfunction in schizophrenia have depended on the following general strategy: Relate alterations in brain function and behavior obtained in vivo to pathology obtained postmortem via experimental manipulations in rodent models that recapitulate some aspect of the latter and measure surrogate markers of the former. Although useful in many respects, this strategy is limited for studies of cortical dysfunction due to the multiple differences in cortical organization, complexity, and cellular properties between rodents and primates. To overcome this limitation **P3** project offers the following innovations:

• Layer 3 PCs are studied in PPC and DLPFC in both monkeys (**P3**) and humans (**P1**&**P2**) at the same levels of resolution.

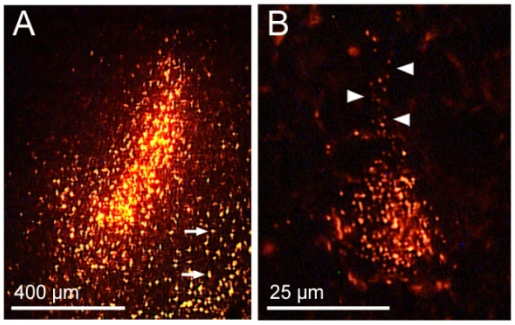
• The specific subset of layer 3 PC neurons that interconnect PPC and DLPFC are studied to examine the cellular and circuitry substrates for synchrony at various frequency bands between these regions in monkeys (**P4**) and humans (**P5**).

• These innovations are made possible by the use of inert retrograde transport techniques to characterize, for the first time in monkey neocortex, the molecular, morphological and neurophysiological properties of specific populations of PCs defined by region, layer and target of principal axon projection.

• Due to these design and technical innovations that permit the interrogation of the specific populations of cortical PCs mediating core cognitive functions that are altered in schizophrenia, **P3** provides a strategic advance in translational approaches to understanding the disease process of the illness.

PRELIMINARY STUDIES

Use of Retrobeads to label specific populations of layer 3 PCs. The proposed studies are the first to combine in vivo transport of Retrobeads to label specific populations of PCs in monkey neocortex with the selective study of these neurons using molecular, morphological and neurophysiological methods. The feasibility of this approach is demonstrated by the following findings. In a pilot study, red and green Retrobeads were injected in layer 3 in separate locations of monkey DLPFC area 46 (Fig.3.1A). As expected, 14 days later, retrogradely-labeled layer 3 PCs were present in the DLPFC in the same and opposite hemispheres and in ipsilateral PPC (area 7a). Labeled neurons were visualized (Fig.3.1B) and captured by laser microdissection (**LMD**), and RNA was isolated and subjected to quantitative PCR. PCR results (Fig.3.1C) indicate that the captured PCs 1) expressed PC-specific (e.g., VGLUT1; **P2**) and layer 3 PC-specific (e.g., CUTL2[11]) transcripts; 2) were uncontaminated by glia-selective transcripts; and 3) were not altered in gene expression by the presence of Retrobeads. In addition, retrogradely-labeled neurons were readily visualized in living slices prepared for electrophysiological studies (Fig.3.2A) and had normal functional properties (Fig.3.2B,C).



C

Expression in labeled cells relative to gray matter	Beads	Nissl
	Fold Difference	
Pyramidal cell marker VGLUT1	1.58	1.60
Layer 3 pyramidal cell marker CUTL2	1.48	1.76
Glial cell marker MBP	0.03	0.09

Fig.3.1. A) Injection site of red Retrobeads in monkey area 46. Arrows mark retrogradely-labeled PCs near the injection site. **B)** Retrobeads fill and clearly outline the soma and proximal apical dendrite (arrowheads) of a layer 3 PC. **C)** qPCR data demonstrating that, relative to total gray matter, RNA obtained by LMD of Retrobead-labeled neurons is enriched for PC-specific (VGLUT1) and layer 3 PC-specific (CUTL2) transcripts, and contains very low levels of a glial cell marker (MBP). Importantly, the fold differences relative to gray matter in Retrobead-labeled PCs are comparable to those of Nissl-stained layer 3 PCs from other tissue, confirming that the presence of Retrobeads does not alter gene expression.

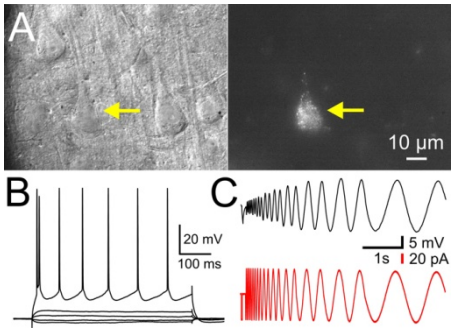


Fig.3.2. A) Infra-red DIC (left) and epifluorescence (right) images of an layer 3 PC (arrow) in contralateral area 46 labeled by green Retrobeads. **B)** Membrane properties of a green Retrobead-labeled layer 3 PC in ipsilateral area 7a. **C)** Response (black) of a layer 3 PC to injection of sinusoidal current (red, 50 pA; 30 to 1 Hz) used to measure membrane resonance properties (Aim 3).

Morphometric analysis of layer 3 PC properties. Aim 2 compares dendritic properties of layer 3 PCs interconnecting PPC-DLPFC. For this purpose, during electrophysiological recordings, PCs are filled with biocytin and visualized as in our previous studies[1,12]. Using Neurolucida software, digital 3D neuron reconstructions (Fig.3.3A,B) are performed for quantitative morphological analysis. Dendritic complexity is determined by Sholl analysis, computing the total dendritic length and the number of dendritic intersections per Sholl ring as a function of distance from the soma (Fig.3.3C). Spine density is determined as previously described[12] in DIC images of the biocytin-filled layer 3 PCs (Fig.3.3D).

Studies of synaptic inhibition onto layer 3 PCs. Studies of functional connectivity between monkey DLPFC and PPC in **P4** employ coherence and causality analysis in the frequency domain. These studies (and parallel human studies in **P5**) seek to determine phase locking of oscillatory activity across areas. Exp.3.1 in Aim 3 will assess membrane resonance in layer 3 PCs interconnecting PPC and DLPFC (Fig.3.2C) as resonance may contribute to phase locking at lower frequency. As inhibition from PV neurons to PCs is crucial for local gamma oscillations[13,14], Exp.3.2 in Aim 3 will study PV-mediated inhibition in layer 3 PCs interconnecting PPC and

DLPFC. Recordings of inhibitory postsynaptic currents (IPSCs) from a DLPFC layer 3 PC typically show high variability in amplitude and rise time (Fig.3.4A), reflecting heterogeneity in the strength and somatodendritic location of the underlying synapses[15]. 2D histograms of IPSC amplitude and rise time (Fig.3.4B) are used to compare the variability in IPSC properties between the layer 3 PCs interconnecting PPC and DLPFC. This approach does not directly identify synapses, but generates a profile of the inhibitory inputs characteristic of layer 3 PCs in each area. Exp.3.2 also compares the probability that retrogradely-labeled layer 3 PCs in each area share inputs from the same PV neurons. Shared inputs may contribute to the generation of high frequency oscillations, and thus to phase locking at gamma frequency (**P4**&**P5**). The presence of shared inhibitory inputs is tested in simultaneous recording from PC pairs (Fig.3.4C,D). Preliminary data show that a fraction of the IPSCs are synchronized across PCs in a pair (synch-IPSCs, Fig.3.4C). The probability of finding synch-IPSCs was markedly reduced when firing activity was blocked with tetrodotoxin (Fig.3.4D). This finding suggests that the synch-IPSCs likely originate in the same inhibitory neurons (Fig.3.4C), because stochastic GABA release in the absence of GABA neuron firing cannot produce a meaningful proportion of synch-IPSCs.

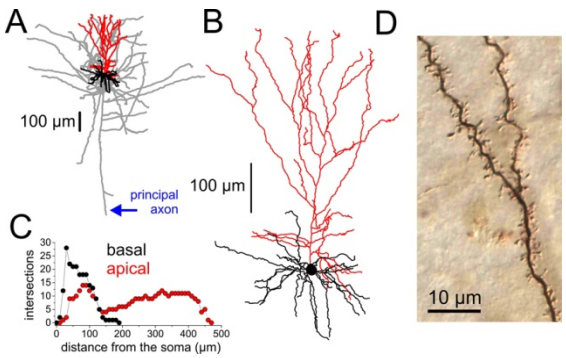


Fig.3.3. A) 3D reconstruction of a biocytin-filled layer 3 PC in DLPFC. Axon, basal and apical dendrites shown in gray, black and red. Note the principal axon entering the white matter. **B)** Detailed view of dendritic tree shown in A. **C)** Sholl analysis of the apical and basal dendrites. **D)** Dendritic spines visualized with DIC in a biocytin-filled layer 3 PC.

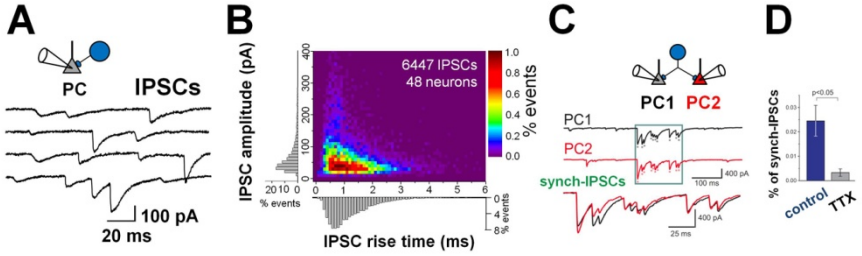


Fig.3.4. A) IPSCs, recorded after glutamate synapses are pharmacologically blocked[1] from a layer 3 PC in monkey DLPFC, show large variability in rise time and amplitude. **B)** 2D histogram of IPSC rise and amplitude built for a sample of monkey DLPFC layer 3 PCs. **C)** Scheme representing common input from a PV neuron onto a PC pair. **D)** Examples of synchronous IPSCs (synch-IPSCs, interval between cells <125 µs) recorded from layer 3 PC pair. The bottom panel shows at an expanded time scale, the synch-IPSCs found in the green box at the top. **E)** The proportion of synch-IPSCs decreases significantly by blocking action potential firing with 1 µM tetrodotoxin (TTX).

APPROACH

Aim 1. Characterize gene expression in layer 3 PCs of monkey PPC and DLPFC and in the subset that interconnect these regions.

Rationale. The **Central Hypothesis** posits that region-specific properties of layer 3 PCs moderate the severity of the molecular pathology (**P1**) of these neurons in schizophrenia, and that this pathology contributes to disturbances in functional connectivity between these regions in schizophrenia (**P5**). The use of monkeys as a translational bridge across the unavoidable gap between cellular (**P1**&**P2**) and functional (**P5**) studies of subjects with schizophrenia requires knowledge of whether the patterns of gene expression across all layer 3 PCs in human PPC and DLPFC are conserved in monkeys, and whether the subsets of layer 3 PCs that interconnect these two regions differ in gene expression. The latter question is important because in primate neocortex most PCs furnish a principal axon which projects to a single cortical target region[2,3,16-18], suggesting the existence of different subtypes of layer 3 PCs. This interpretation is supported by evidence that molecular[4] and morphological[5] properties of layer 3 PCs in monkeys differ in relation to the length of, and type of brain region targeted by, their principal axons. Thus, the goal of Aim 1 is to provide a critical "pivot" between the studies of all layer 3 PCs in **P1**&**P2** and the studies of those PCs that specifically interconnect PPC and DLPFC in Aims 2 and 3 below and in **P4**.

Hypotheses. We predict that gene expression patterns for all layer 3 PCs in monkey PPC and DLPFC mimic those present in human brain. We also predict that gene expression patterns differ between layer 3 PCs that interconnect PPC and DLPFC in a manner that parallels regional differences in morphology (Aim 2).

Methods. Injections of red or green fluorescent latex microspheres (Retrobeads®, Lumafluor Inc.)[19,20] in PPC (area 7a; homologous to area 40 in humans, **P1**&**P2**) or DLPFC (area 46) of the opposite hemispheres of macaque monkeys are used to retrogradely-label layer 3 PCs that project to areas 46 or 7a, respectively, in the

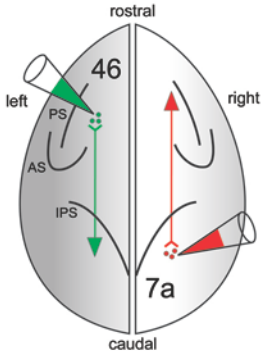


Fig.3.5 Schematic diagram of the dorsal surface of monkey brain showing the locations of the injections for green and red Retrobeads in areas 46 and 7a, respectively, and the regional locations of the retrogradely-labeled layer 3 PCs (triangles) to be studied. The injection sites and bead colors will be reversed across monkeys. AS, arcuate sulcus; PS, principal sulcus; IPS, intraparietal sulcus.

same hemisphere (Fig.3.5). Importantly, the retrobeads are inert and do not alter the properties of retrogradely-labeled neurons as demonstrated by a large number of studies in rodents[21-30], and our preliminary findings. Each young, adult male rhesus monkey (n=10; see **Stats/DM Core** for sample size calculations) receives injections (100 nl each at 600, 1,000 and 1,400 µm below the pial surface, a distance which spans the densest termination zones of the axons projecting between areas 7a and 46[31,32]. In each region, injections are made at 15 sites spaced at 1 mm intervals across the cortical surface. Five monkeys are injected as shown in Fig.3.5, and in the other 5 monkeys the hemispheres are reversed in order to assess, and control, for any effects of laterality. Following a 14 day survival period, animals are deeply anesthetized, perfused with cold artificial cerebrospinal fluid (ACSF), and the brain removed[12,33]. Adjacent coronal tissue blocks containing the non-injected areas 7a or 46 (e.g., left 7a and right 46 in Fig.3.5) are fresh-frozen (Aim 1) or immersed in cold ACSF (Aims 2-3). From the frozen blocks, tissue sections are prepared as described in **P1**. Alternate adjacent sections are either stained for Nissl substance (Exp.1.1) or left unstained (Exp.1.2). In Exp.1.1, Nissl-stained PCs in layer 3 are visualized under brightfield illumination and captured by LMD, blind to Retrobead content. For Exp.1.2, fluorescence illumination is used to identify Retrobead-containing PCs in layer 3 and to capture them by LMD. In both Exp.1.1 and 1.2, random samples (n=3 per region per monkey) of 100 layer 3 PCs are obtained. Samples of this size in human PFC show little cross-sample variance within subjects. Following RNA isolation, microarray analyses are conducted using the GeneChip® Rhesus Macaque Genome Array which simultaneously interrogates >47,000 *M. mulatta* transcripts. The sequence information for this array was selected from public data sources on the rhesus macaque whole genome and GenBank® STSs, ESTs and mRNAs, with additional probe sets designed to interrogate rhesus transcripts orthologous to the human transcripts in the GeneChip® Human Gene 2.0 Array used in **P1**. Analyses of gene expression patterns are conducted as described in **P1** to determine which transcripts are differentially expressed in layer 3 PCs between areas 7a and 46. Comparisons of regional gene expression profiles between humans and monkeys are conducted as described in the **Stats/DM Core**.

Expected and alternative outcomes. We predict that gene expression patterns for all layer 3 PCs in monkey PPC and DLPFC mimic those present in human brain (**P1**). This prediction is strongly supported by existing studies demonstrating that the neocortices of macaque monkeys and humans have very similar cytoarchitectonic features, highly homologous genomes[9,10] and very similar transcriptional architectures[6]. This finding will validate the use of monkeys as a model system for testing the **Central Hypothesis** regarding the role of layer 3 PCs in the disease process of schizophrenia across **P1**-**P5**.

We also predict that gene expression patterns differ between the layer 3 PCs that interconnect PPC and DLPFC. This prediction is supported by existing data on differences in gray matter measures of gene expression across cortical regions[6]. Such differences would be expected to be clearly evident in our findings given that our study design eliminates “noise” contributed by gene expression differences across cortical layers, and between neurons and glia, and PCs and interneurons. The design of Exp.1.2 provides a robust test of the effect of region on gene expression since the retrogradely-labeled neurons studied differ only in regional location, and not in length of principal axon or type (e.g., ipsilateral cortical association area) of target. In particular, we predict that the layer 3 PCs in DLPFC will show greater expression of genes that regulate dendritic complexity and spine number, and energy metabolism (as in **P1**), given the predicted differences between DLPFC and PPC in dendritic complexity (Aim 2) and intrinsic excitability (Aim 3) of layer 3 PCs.

If differences in gene expression are not found between PPC and DLPFC, then the idea that normal regional differences in layer 3 PCs moderate the regional severity of pathology in schizophrenia may need to be reconsidered by **P1**&**P2**. For example, the increasing severity of pathology from V1 to DLPFC in schizophrenia could reflect a compounding of pathology due to alterations in inputs from layer 3 PCs across the ascending projections of the V1-PPC-DLPFC network. Alternatively, the combination of no gene expression differences in layer 3 PCs between areas 7a and 46, and similar levels of layer 3 PC pathology in PPC and DLPFC in schizophrenia (**P1,** Aim 1 alternative outcomes), would predict that the severity of functional connectivity disturbances in schizophrenia does not differ in a top down versus bottom up fashion (see **P5**).

Comments. **1)** In monkeys, subsets of layer 3 PCs in PPC and DLPFC furnish principal axons that project to the same cortical regions[34], suggesting that layer 3 PCs in each region may share those properties related to having a common projection target. Thus, the studies proposed in **P1**&**P2** are able to assess region-specific effects on layer 3 PC pathology because any target-specific effects are likely to be similar in both PPC and DLPFC. However, as noted above, the direct study of layer 3 PCs that interconnect these regions in **P3** will provide a robust test that region, and not axon target, influence regional differences in gene expression. **2)** Qualitative differences in gene expression profiles may reveal specific markers of PC neurons that are distinctive for the layer 3 PCs that furnish projections between PPC and DLFPC, providing a "molecular identity" for those neurons. This outcome may provide unprecedented tools to study PC pathology in schizophrenia (**P1**&**P2**) at the level of individual neurons with known connectivity. **3)** About 25% of the PCs that interconnect areas 7a and 46 are located in layer 5. We previously demonstrated transcriptional differences between layers 3 and 5 in human brain[11]; we will preserve tissue for future gene expression studies of retrogradely-labeled layer 5 PCs to compare to **P2**. **4)** A goal of **P1** is to examine underlying disease mechanisms using mRNA/miRNA/coexpression networks; **P3** is focused solely on regional differences that are best assessed by mRNA differences, but miRNA studies can be conducted as indicated by results of **P1**.

Aim 2. Characterize the dendritic tree morphology of layer 3 PCs interconnecting PPC and DLPFC.

Rationale. The **Central Hypothesis**, that regional differences moderate the magnitude of pathology in layer 3 PCs in schizophrenia, is based, in part, on regional differences in the dendritic tree morphology of layer 3 PCs across the V1-PPC-DLPFC network in primates[7,35-37]. In addition, the functional specialization of the DLPFC relative to other cortical areas is thought to be due, in part, to its inputs from a richer set of cortical connections[37]. Thus, the integration of such inputs may explain the larger dendritic tree complexity and greater spine density of DLPFC layer 3 PCs. However, no previous studies have compared dendritic tree complexity and spine density between the layer 3 PCs that interconnect areas DLPFC and PPC.

Hypothesis. The complexity and spine density of layer 3 PC dendritic trees are greater in DLPFC than PPC.

Methods. Intracellular filling with biocytin during electrophysiological recordings (Aim 3) and morphometric analyses are conducted in retrobead-labeled PCs in areas 7a and 46. After recordings, slices are processed to visualize biocytin for reconstruction and quantitative dendritic tree morphometry[1,12]. Complexity is measured by dendritic length and number of intersections per ring in the Sholl analysis of basal and apical dendrites (Fig.3.3B,C). Dendritic spine density is measured as previously described[12]. See **Stats/DM Core** for analyses.

Expected and alternative outcomes. Consistent with the **Central Hypothesis**, previous studies found that dendritic tree complexity and spine density increases from posterior to frontal cortical regions in primates and is highest in DLPFC[7,36,37]. We thus expect to find more complex dendritic trees and greater spine density in the layer 3 PCs that project from area 46 to 7a, than in those that furnish the reciprocal projection. Alternatively, it is possible that layer 3 PCs interconnecting areas 7a and 46 do not differ in these measures. This outcome would suggest that increasing severity of morphological pathology from V1 to DLPFC in schizophrenia (**P2**, Aim 2 alternative outcomes) could reflect an amplification of pathology due to the progressive decay of information as it is relayed across regions in the ascending V1-PPC-DLPFC network. Alternatively, the combination of no differences in dendritic tree morphology between the layer 3 PCs interconnecting areas 7a and 46 in monkeys, and similar levels of morphological pathology in layer 3 PCs in PPC and DLPFC in schizophrenia (**P2**), would predict that the severity of functional connectivity disturbances in schizophrenia does not differ in a top down versus bottom up fashion (see **P5**).

Comments. Quantitative morphometry may reveal differences in the laminar distribution of dendritic tree segments indicative of differential sampling of inputs by PPC and DLPFC neurons. Projections from layer 3 PCs interconnecting PPC and DLPFC terminate in supragranular layers in both regions[31,32,38], suggesting that PCs interconnecting PPC and DLPFC reciprocally innervate each other. However, overlap of dendritic tree segments with axonal projections within a layer does not demonstrate synaptic targeting or connection strength[39].

Aim 3. Characterize the cellular neurophysiology of layer 3 PCs interconnecting PPC and DLPFC.

Rationale. Greater dendritic tree complexity and spine density in DLPFC layer 3 PCs reflect integration of a larger number of excitatory inputs. If layer 3 PCs in DLPFC also receive higher mean levels of excitatory input, then the balanced activity of layer 3 DLPFC PCs may require homeostatic adjustments of intrinsic excitability and/or greater control via synaptic inhibition. However, coherence of layer 3 activity between PPC and DLPFC (**P4**) may be facilitated if the PCs interconnecting these areas share functional properties relevant for

oscillatory synchronization. In particular, similar membrane resonance may facilitate low frequency (theta, alpha and beta) synchronization[26,40,41], whereas similar inhibition from PV cells may induce high frequency activity and thus enhance cross-area synchrony by phase locking in gamma oscillations[14].

Hypothesis. Layer 3 PCs interconnecting PPC and DLPFC differ in intrinsic excitability but share functional properties relevant for production of local oscillatory synchrony in low (membrane resonance) and high (synaptic inhibition) frequency bands.

Methods. Current- and voltage-clamp recordings are obtained (as in our previous studies[1,14,42,43]) in brain slices from areas 7a and 46 of macaque monkeys that receive cortical injections of Retrobeads (Fig.3.5).

Experiment 3.1. Membrane properties of layer 3 PCs interconnecting areas 7a and 46 are determined using: i) rectangular current steps (Fig.3.2B, 500 ms, amplitude -50 to 1000 pA) and ii) sinusoidal stimulus waveforms (Fig.3.2C, constant amplitude of 50 or 100 pA, frequency decreasing or increasing between 30-1 Hz in 5 s). From protocol i), we measure input resistance, membrane time constant, rheobase, spike voltage threshold and build current-frequency plots to assess intrinsic excitability. From protocol ii), an impedance amplitude profile is obtained to estimate the resonant frequency for each neuron using Fast Fourier Transformation of the voltage response and input current[44,45]. Results are integrated with those of Aim 2. See **Stats/DM Core**.

Expected and alternative outcomes. In i), layer 3 PCs are expected to be less excitable in DLPFC than PPC, because greater dendritic complexity and spine density likely indicate higher levels of excitatory synaptic input and consequently intrinsic excitability is adjusted to be lower. Alternatively, excitability may not differ between regions, or layer 3 PCs in PPC may be more excitable. The former suggests greater diversity of inputs onto DLPFC layer 3 PCs, with no differences in levels of excitatory input, and the latter suggests that PCs in adult primate neocortex do not adjust excitability as a function of mean level of input. In ii), if membrane resonance contributes to synchronization of PPC and DLPFC at low frequencies, then layer 3 PCs interconnecting these areas should show similar resonance. Alternatively, different resonance of PCs interconnecting areas PPC and DLPFC would suggest the contribution of membrane resonance to cross-area synchrony is relatively weak.

Comments. **1)** PCs in areas V1 and 46 of monkey neocortex differ in some physiological properties[7], but membrane resonance has not been studied in PCs of any cortical area in macaques. **2)** If excitability does not differ between PCs in areas 7a and 46, we will apply focal stimulation of excitatory inputs[12] to build input-output curves and compare the levels of excitatory input received by layer 3 PCs in each area.

Experiment 3.2. To compare the properties of synaptic inhibition from PV cells onto layer 3 PCs, IPSCs are recorded in the presence of conotoxin, which blocks GABA release from non-PV cells, and cholecystokinin (**CCK**), which stimulates PV neuron firing, evoking PV cell-mediated IPSCs[46,47]. To test if the profile of IPSC properties differs between layer 3 PCs interconnecting areas 7a and 46, amplitude and rise time are measured for individual IPSCs (Fig.3.4A) and 2D distributions (Fig.3.4B) are compared. To test if DLPFC-to-PPC (and PPC-to-DLPFC) PCs receive common inhibitory input from local PV cells, we estimate the probability of finding synch-IPSCs (Fig.3.4D,E). IPSCs are recorded from PC pairs in areas 7a or 46, and synch-IPSCs are identified (difference in onset across PCs≤125 µs) and their proportions out of total sIPSCs are compared. See **Stats/DM Core** for analyses.

Expected and alternative outcomes*.* Cross-area synchrony in the gamma band (**P4**) may be facilitated if the local properties of oscillatory gamma activity are similar in each area[48]. Because inhibition from PV cells plays a crucial role in gamma oscillation production, similar properties of inhibition in PCs that interconnect areas 7a and 46 would facilitate cross-area phase locking**.** Thus, we expect to find similar IPSC properties and similar proportions of synch-IPSCs in 7a or 46 PC pairs, consistent with a similar local circuit level contribution of PV inputs to gamma synchrony in both regions. Alternatively, different IPSC properties or synch-IPSCs proportions between areas 7a and 46 PCs may be related to the excitation-inhibition balance in each area, indicating synaptic inhibition is less likely to mediate cross-area gamma synchrony via layer 3 PCs.

C*omments.* **1)** Endogenous CCK levels are nearly identical throughout monkey neocortex[49], suggesting that the magnitude of CCK effects does not vary across areas. **2)** Whenever possible, to directly study common inhibition onto PCs we will record simultaneously from PCs and PV neurons[50] in each area, specifically from triplets (PV/PC/PC), in which the PV neuron is identified based on its fast-spiking firing pattern[50]. **3)** If PV-mediated inhibition differs between PPC and DLPFC, we will test the impact on gamma activity in each area using simulations in a network model as in our previous experimental/computational modeling studies[51].

VERTEBRATE ANIMALS

Description of the proposed use of animals. Ten (10), young adult (5-7 years of age), experimentally naïve, male, rhesus (*Macaca mulatta*) macaque monkeys will be used in the proposed studies. Sample size is based on the power calculations shown under **P3** in **Stats/DM Core**. All animals are from a single source and are housed in pairs, or in single cages, in the same social setting at the University of Pittsburgh's Plum Borough Research Center Animal Facility (see **Lewis Resources**). Enrichment for all animals is under the guidance of the University of Pittsburgh's Department of Laboratory Animal Resources and with approval of the IACUC.

Justification for the use of these animals. The purpose of the proposed studies is to advance the understanding of the structure and function of the human neocortex, and nonhuman primates are an obvious choice of experimental subject because of their close phylogenetic relationship to humans, especially in the enormous expansion and specialization of neocortical association regions that are unique to primates. The proposed studies involve important experimental manipulations that are not possible in humans. These studies involve PPC and DLPFC, neocortical areas with major differences between rodents and primates.

Veterinary care of the animals involved. All animals are housed at the University of Pittsburgh's Plum Borough Research Center Animal Facility (PBRC). The PBRC facility comprises eight major areas, including: 1) administrative and infrastructure support, 2) non-human primate (NHP) routine housing and procedural support; 2) a NHP holding and procedure suite, 3) indoor pen housing units for group housing and reproduction studies of NHPs accommodating family groups with a behavioral testing procedure core, 4) a quarantine suite with an intensive care isolation room, 5) large animal surgical, 6) radiological, and 7) necropsy suites, and 8) a cognitive behavioral suite. DLAR staffing at the site includes assigned veterinarians, veterinary technical staff, and husbandry and NHP enrichment support staff. All occupied cage housing areas are outfitted with enrichment televisions or alternate devices.

Surgical procedures and approaches to minimize discomfort. Monkeys are anesthetized with ketamine (25 mg/kg), treated with atropine (0.5mg/kg) and dexamethasone (0.5 mg/kg), intubated, and placed in a stereotaxic apparatus. Anesthesia is maintained throughout the procedure with 1% isoflurane in 28% O_2/air delivered through the endotracheal tube. Using sterile techniques, the skull is exposed and a craniectomy over the right or left DLPFC and the contralateral PPC is performed. The dura is resected over the area of interest, and injections (0.3 µl) of inert Retrobeads are injected in both regions of interest. The exposed areas are covered with Gelfoam, and the skin closed. Following the experiment, animals receive a systemic antibiotic (chloramphenicol 15 mg/kg, 3 times daily) and an analgesic (buprenorphine .01 mg/kg, 2 times daily) for three days. We have substantial previous experience with this procedure, and animals consistently recover very quickly and do not manifest any alterations in behavior.

Euthanasia. Two weeks after surgery, animals are anesthetized with ketamine (25 mg/kg), intubated and ventilated with 28% O_2/air delivered through the endotracheal tube, given pentobarbital sodium (40 mg/kg, IV). When fully anesthetized, the chest is opened, the descending aorta is clamped, the animal is perfused transcardially with cold ACSF, and the brain is removed. These methods are consistent with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association.

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SPECIFIC AIMS

The Center's **Central Hypothesis** states that the cognitive deficits at the core of schizophrenia arise because pathological changes in layer 3 pyramidal cells (**PCs**) interfere with functional connectivity between cortical areas, including dorsolateral prefrontal (**DLPFC**) and posterior parietal (**PPC**) cortices (**Center Plan** Fig.0.2). **P1**&**P2** will test this hypothesis by characterizing the gene expression and morphology of layer 3 PCs in postmortem tissue from individuals with schizophrenia. **P5** will test this hypothesis by characterizing functional connectivity between DLPFC and PPC through in vivo studies of individuals with schizophrenia. The role of **P3**-**4** is to provide a link between these domains of inquiry. The unique role of **P4** is to pose the question whether layer 3 PCs, as studied in **P1**-**2**, play a crucial role in functional connectivity between DLPFC and PPC, as studied in **P5**. This question is important because it concerns a key assumption of the **Central Hypothesis** for which no solid evidence currently exists. Although we know that direct axonal projections between DLPFC and PPC arise predominantly from layer 3 PCs, we do not know that projections from layer 3 PCs mediate functional connectivity as characterized through the coherence and causality measures used in the human EEG, MEG and fMRI studies. Cross-area phase-locking and co-variation of oscillatory activity could be mediated by projection neurons in other layers and might not even depend on direct area-to-area connections. For example, phase-locking and co-variation could arise because two areas receive a common input. Accordingly, **P4** will address the following significant and unanswered question: *Does functional connectivity between DLPFC and PPC, as revealed by coherence and causality analysis, depend on the layer 3 PCs that provide reciprocal projections between these regions?*

To answer this question, we will monitor electrical activity simultaneously in DLPFC and PPC of nonhuman primates using innovative methodology. During each session, an electrode inserted into one of the areas will consist of a linear array of contacts spanning the depth of the cortex from the surface to the white matter. This will allow us to determine not only whether functional connectivity is present but also, critically, whether it is centered in certain layers. We will address this issue by analyzing neuronal spiking activity and local field potentials. The analysis will be based on data collected while monkeys perform tasks requiring working memory and attention. These tasks are an appropriate context in which to assess whether functional connectivity depends on layer 3 PC projections because attention and working memory are known to depend jointly on DLPFC and PPC and to induce coherent DLPFC and PPC oscillatory activity. The tasks will parallel those used in **P5** so as to facilitate comparison between monkey and human results.

We hypothesize that attention and working memory will induce phase-locking between DLPFC and PPC because phase-locking has been observed previously in comparable cognitive contexts[15,44]. We predict that the effect will increase with the level of cognitive demand because greater demand will more strongly recruit processes that require coordinated DLPFC-PPC activation. We predict that the effect will be strongest in layer 3, as compared to other layers, because most PCs projecting between DLPFC and PPC reside in layer 3[3,36,47].

Aim 1. Characterize the laminar organization of functional connectivity related to attention. In monkeys performing a task that requires attending to one item in a multi-item display we will monitor neuronal activity and local field potentials at DLPFC and PPC sites simultaneously. *We predict that 1) DLPFC and PPC will exhibit coherent activity during the period of sustained attention, 2) this effect will increase with the level of demand for top-down control of attention, and 3) it will be most prominent in layer 3.*

Aim 2. Characterize the laminar organization of functional connectivity related to working memory. In monkeys performing a task that requires holding information in working memory, we will record electrical activity using identical methods. *We predict that 1) DLPFC and PPC will exhibit coherent activity during the memory delay, 2) this effect will increase with memory load, and 3) it will be most prominent in layer 3.*

Aim 3. Characterize the dependence of functional connectivity on direct area-to-area connections. We will identify neurons in each area that send axonal projections to the other, using the method of antidromic electrical activation in a fixation task. *We predict that neurons thus identified as projecting between areas will exhibit especially strong signs of functional connectivity in attention and working memory tasks as revealed by coherence and causality analysis.* We will reversibly block white matter connections between prefrontal and parietal cortex. *We predict that this manipulation will attenuate functional connectivity*.

OVERVIEW

DLPFC and PPC neurons carry attention and working memory signals. The dorsolateral prefrontal cortex (**DLPFC**), encompassing area 46 and the adjacent frontal eye field (**FEF**), and the posterior parietal cortex (**PPC**), encompassing area 7a and the adjacent lateral intraparietal area (**LIP**), play a critical role in attention and working memory in macaque monkeys. Neurons in all four areas fire during periods when the monkey is allocating attention to a particular location in visual space[8,15,28,29,62] or is holding information in working memory[18,55]. Although task-related activity varies slightly from area to area in this network[5,13,26,52,62], the overriding point is that neurons in all four areas carry similar signals related to attention and working memory.

DLPFC and PPC are linked by connections originating and terminating in layer 3. DLPFC and PPC are reciprocally connected in the macaque monkey. Each DLPFC area noted above is linked to each PPC area[2,16,36]. Both supragranular (layer 1-3) and infragranular (layer 5-6) pyramidal cells (**PCs**) give rise to axons linking DLPFC and PPC[3,45,47]. However, most projection PCs (around three quarters) are located in layer 3[3,36,47]. Axons originating from these PCs terminate in supragranular and infragranular layers but, in cases where a distinction has been noted, are denser in the supragranular layers[2,36,51]. We conclude that direct reciprocal connections between DLPFC and PPC preferentially originate from and terminate in layer 3.

DLPFC and PPC exhibit functional connectivity. There are various ways in which to assess coordination between DLPFC and PPC. Possible approaches include recording in one region after inactivation of the other[17] and electrically stimulating one region to identify neurons that project to it from the other[23]. These approaches are invasive and are necessarily confined to animal studies. Another approach, capable of being applied non-invasively, and therefore widely used in human studies and recently used with growing frequency in animal studies, is to carry out coherence and causality analysis in the frequency domain[11,19,22,33,38,57]. This approach, when employed in monkeys, involves determining whether oscillatory activity in one area, measured at the level of the local field potential (**LFP**), multi-unit activity (**MUA**) or single-unit activity (**SUA**), is significantly phase-locked to oscillatory activity in the other area. Studies based on this approach have revealed phase-locking in the theta (4-8 Hz), alpha (7-13 Hz), beta (15-30 Hz) and gamma (30-80 Hz) bands, with the particular frequency dependent on the areas and the task. Table 1 summarizes studies using this approach to demonstrate phase-locking between posterior and frontal cortical areas in the macaque monkey. No such study has analyzed functional connectivity by cortical layer as we propose to do.

Posterior Area	Frontal Area	Frequency	Task Context	Source
7a, LIP	46, FEF	15-60 Hz	Visual Search	Buschman & Miller 2007
V4	FEF	30-60 Hz	Visual Attention	Gregoriou et al. 2009
V4	FEF	35-60 Hz	Visual Attention	Gregoriou et al. 2012
7a, LIP	46, FEF	15 Hz	Working Memory	Salazar et al. 2012
V4	46	3-9 Hz	Working Memory	Liebe et al. 2012
7b	MI	22 Hz	Manual Go/NoGo	Brovelli et al. 2004
PRR	PMd	15 Hz	Free Reach	Pesaran et al. 2008

Table 1. PMd=dorsal premotor area; PRR=parietal reach region; MI=primary motor cortex.

Key question: Does functional connectivity depend on projection neurons in layer 3? The **Central Hypothesis** of the Center is that pathology of layer 3 PCs gives rise to abnormal functional connectivity between DLPFC and PPC in schizophrenia and that impaired functional connectivity underlies the attention and working memory deficits central to the disease. To test this hypothesis requires establishing a link between layer 3 PCs (studied post mortem in **P1-2)** and measures of functional connectivity based on coherence and causality analysis (studied in patients in **P5**). Toward this end, we will measure functional connectivity in different layers of monkey cortex. We will ask whether activity in layer 3 of each area is especially strongly phase-locked with activity in the other area, at what frequencies phase-locking occurs and whether phase-locking depends on direct connections originating in layer 3. These results will allow us to relate effects observed at particular frequencies in the human studies of **P5** to particular layers of cortex.

Strategy for answering the question: Record from individual layers with a linear array electrode. To identify cortical layers that participate in functional interactions between DLPFC and PPC, as measured by coherence and causality analysis, we will record from multiple sites in one area with standard microelectrodes, while recording in the other area a linear array electrode with closely spaced contacts yielding laminar resolution. This approach will maximize the yield of functionally connected sites. Linear array electrodes are well established as a means for studying laminar organization in a single cortical area in the monkey[9,10,25,32,37,46,48,50,61]. They have very recently been used to characterize the laminar specificity cross-area interactions between areas V1 and V2[63]. To the best of our knowledge, they have not previously been used as a means for characterizing the laminar specificity of interactions between posterior and frontal cortical areas.

Commercially available multicontact electrodes (e.g., U-Probe, Plexon, Linear Microelectrode Array, Alpha-Omega) allow simultaneous recording of LFP, MUA and SUA at regular intervals across the cortical depth[30]. A typical electrode contains 8 contacts arranged in a linear array along the shaft at intervals of 200 microns[63]. This configuration allows simultaneous recording over the distance of approximately two mm which separates the cortical surface from the white matter in monkeys. To place the electrode at an appropriate depth requires taking note of signal changes that occur while it is advanced. These mark the boundary between the CSF and the cortex and the boundary between gray matter and white matter[46]. Once the electrode is in position, contacts can be roughly assigned to layers on the basis of their distance from the cortical surface. More precise assignment is possible if the LFP response to a simple visual probe such as whole-field flash reveals consistent laminar signatures. The most common signature is an early current sink in layer 4[46,50]. This is particularly useful because it demarcates the supragranular from the infragranular layers. Laminar signatures vary from area to area and accordingly must be worked out for each new area[46].

Most studies of functional connectivity in primate cortex have relied on recording the LFP in at least one of the areas. In principle, recording simultaneously from neurons in areas A and B should suffice. In practice, however, the sampling of electrical activity is so sparse in space (a few neurons) and in time (a handful of action potentials per second per neuron) as to make the detection of coherence difficult. The LFP, which is thought to represent the sum of thousands of postsynaptic currents within a region with a radius of around 250 microns[61], has emerged as a preferred means, in studies including those in Table 1, for characterizing functional connectivity. The virtue of the LFP (spatial summation) is also a potential problem in any experiment that aims to characterize activity with high spatial resolution. The problem, however, is soluble. Placement of contacts at regular intervals along the shaft of a linear array electrode allows filtering out volume-conducted signals common to adjacent contacts. This is accomplished by basing analysis on the difference in voltage between two contacts[50] or on local current source density computed as the second spatial derivative of voltage[46]. Studies based on this approach have revealed clear functional differences across layers in a single cortical column. In response to a brief visual stimulus, the trajectory of voltage over time differs systematically across layers[46]. Oscillatory activity in the alpha band also varies systematically across layers[9,50].

Even if we discover that signs of functional connectivity are particularly prominent in the supragranular cortical layers, this will not, in itself, establish that layer 3 PCs—neurons sending axons directly from DLPFC to PPC or vice versa—are crucial for functional connectivity. For example, phase-locking could arise if two areas had a common input from a third area. To assess the role of layer 3 PCs will require two adjunctive procedures. First, we will identify by antidromic activation[23], neurons that project from each region to the other. This will allow us to determine whether these neurons exhibit especially strong signs of functional connectivity as revealed by coherence and causality analysis. Second, in the concluding phase of study for each monkey, we will measure functional connectivity before and after reversible blockade[1] of white matter connections between DLPFC and PPC. The results will provide a direct test of the premise that functional connectivity between the regions, as revealed by coherence and causality analysis, depends on direct connections between them. They may reveal that attention and working memory performance depend on direct connections.

SIGNIFICANCE

The significance of this project arises from its contribution to the overarching goal of the **Center**. That goal is to test a specific hypothesis concerning the origin of cognitive deficits that constitute the core symptoms of schizophrenia. The hypothesis states that pathology in layer 3 PCs leads to an impairment of functional connectivity between cortical areas which in turns leads to cognitive deficits. **P1**&**P2** will characterize the pathology of layer 3 PCs in schizophrenia through the analysis of postmortem brain tissue. **P5** will characterize abnormalities of functional connectivity in schizophrenia with coherence and causality measures collected during performance of tasks that require attention and working memory. The role of **P3**&**P4** is to provide a bridge between these domains of inquiry. They will use a nonhuman primate model to establish how the electrophysiological activity of layer 3 PCs within a region contributes to functional connectivity between regions as measured with coherence and causality analysis. The use of rhesus macaque monkeys is critical because these studies, being invasive, require an animal model and because rhesus monkeys are closer to humans than any other laboratory animal in their pattern of brain organization. **P3** will use slice recording to characterize the electrophysiological properties of layer 3 PCs that predispose them to oscillate at low or high frequencies. **P4** will use in vivo recording to determine whether layer 3 PCs exhibit cross-area phase coherence at low or high frequencies in tasks requiring attention and working memory. The results will allow us to relate pathology of layer 3 PCs, as revealed by **P1**&**P2**, to disorders of functional connectivity in low or high frequency bands, as revealed by **P5**.

INNOVATION

This project is both conceptually and methodologically innovative. At the conceptual level, this is the first study to pose the question whether measures of functional connectivity based on coherence and causality analysis depend differentially on particular cortical layers. At the methodological level, this is the first study to use linear array electrodes as a means for assessing the contributions of particular layers to functional connectivity.

APPROACH

Attention task.

Rationale. We will collect neural data in the context of a task requiring visual attention. On each trial of this task, the monkey must allocate attention to one item out of several in an array. Because this task requires remembering a cue early in the trial, it may also cast light on neural events related to working memory.

Basic design. Before each trial, a fixation spot appears at the center of the screen. The monkey initiates the trial by fixating this spot and must maintain fixation until the end of the trial. After a period of steady fixation, a foveal color cue appears. The monkey must hold this color in working memory during the ensuing memory period. At the end of the memory period, an array of six colored annuli appears. The monkey must search for and attend to the annulus matching the cue in color so as to detect the brief appearance of a gap during the instruction interval. The monkey must make a saccade to the target at the top, left, bottom or right side of the screen in response to a gap located at the top, left, bottom or right side of the attended annulus. The gaps in the other annuli must be ignored. The sequence of events in a typical trial is shown in Fig.4.1. The insertion of an attention interval before onset of the instruction differentiates this task from the one used in **P5**. This will benefit the experiment by providing a prolonged window of sustained attention during which to carry out coherence and causality analysis. The loss of the ability to measure behavioral reaction times will not matter. Unlike **P5**, **P4** does not need to compare performance by healthy and clinical populations. We will compare search efficiency across conditions simply by measuring the time of onset of neuronal activity reflecting target acquisition.

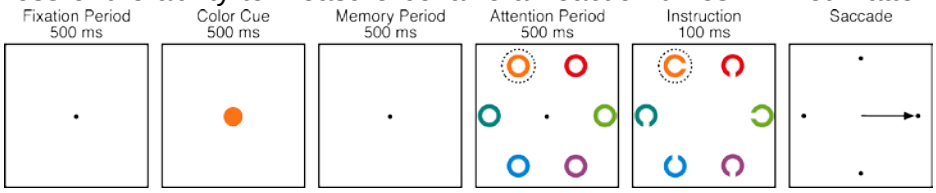

Fig.4.1. Events in a representative trial of the attention task. Dashed circle indicates locus of attention.

Manipulating the demand for executive control of attention. Three blocks of trials, imposed in random order, differ with regard to the difficulty of finding the target and of subsequently maintaining attention on it (Fig.4.2). In the most demanding "flexible" block, the color of the target and the colors of the distractors vary randomly from trial to trial. In the less demanding "habitual" block, the color of the target is fixed across trials. In the least demanding "habitual+popout" block, both the color of the target and the color of the identical distractors are fixed. The rule for response selection (make a saccade to the target on the side corresponding to the gap) is constant.

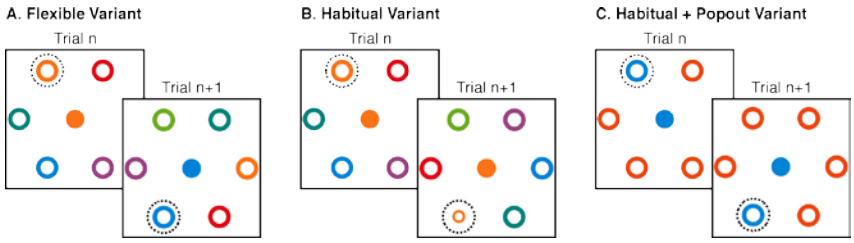

Fig.4.2. The demand for executive control of attention declines across conditions A, B and C. In each panel, the cue (central) and the probes (eccentric) are shown together although they occur in sequence. Each pair of staggered panels depicts two representative trials under a given condition.

Attention hypothesis 1. Functional connectivity will increase in conjunction with attention. We predict that phase coherence between DLPFC and PPC will increase during the attention period if the attended item lies within the receptive fields of neurons at the DLPFC and PPC recording sites. This prediction is based on published observations linking phase coherence to visual search and attention (Table 1). We predict that this effect will decrease across blocks in the order: "flexible">"habitual">"habitual+popout". This prediction is based on the observation that monkeys with lesions of DLPFC are severely impaired at performance of the "flexible" version of a similar task but can perform "habitual" and "popout" versions[43] and on the observation in humans that DLPFC and PPC BOLD activation is stronger under the flexible condition than under the other conditions[41].

Attention hypothesis 2. This phenomenon will be strongest in layer 3. We predict that these effects will be most prominent in layer 3. This prediction is based on reports indicating that the cell bodies of most DLPFC neurons projecting to PPC and of most PPC neurons projecting to DLPFC are located in layer 3[3,36,47].

Attention hypothesis 3. This phenomenon will depend on direct connections. We predict that these effects will be most pronounced in PCs projecting between the two areas and will be reduced or abolished by blocking direct connections between the two areas. These predictions are not tautological. Phase coherence between areas could arise from other mechanisms including common inputs (e.g., from thalamus: see **P2**).

Working memory task.

Rationale. This task will require monkeys to hold the colors of objects at particular locations in working memory during a delay period. It will be used to assess the dependence of DLPFC-PPC phase-locking on working memory. Because it requires the monkey to allocate attention to a single visual hemifield early in the trial, it may also cast light on neural events related to attention. Working memory and attention, although operationally distinguishable and associated with different patterns of cross-area phase coherence (Table 1), are thought to depend on an overlapping set of areas including DLPFC and PPC[4]. The use of a working memory task as well as an attention task will allow us to assess whether the observed effects generalize across multiple cognitive domains.

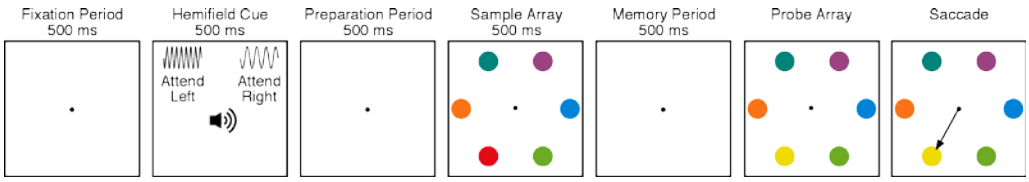

Fig.4.3. Events in a representative trial of the working memory task.

Basic design. Before each trial, a fixation spot appears at the center of the screen. The monkey initiates the trial by fixating this spot and must maintain fixation until the end of the trial. After a period of steady fixation, a high- or low-frequency tone sounds. This instructs the monkey to process information in the left or right hemifield. Then a sample array of colored disks is presented. A delay follows during which the monkey must hold in working memory the colors of the disks in the instructed hemifield. Finally, a probe array appears. On one quarter of trials, the probe array will be identical to the sample array (match condition: withhold response). On another quarter of trials, one item in the probe array will differ in color from the corresponding item in the sample array but the changed item will be in the unattended hemifield (also a match condition: withhold response). On the remaining half of trials, one item in the probe array will differ in color from the corresponding item in the sample array and the changed item will be in the attended hemifield (non-match condition: make a saccade to the changed item). Each correct response, whether making or withholding a saccade, is rewarded with juice. The sequence of events in a typical non-match trial is shown in Fig.4.3.

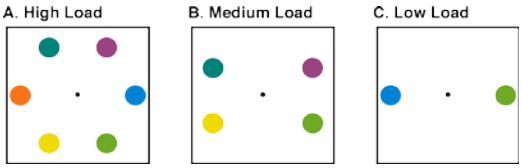

Fig.4.4. The load placed on working memory declines across conditions A, B and C.

Manipulating memory load. From trial to trial, the level of demand placed on working memory varies. The sample array may consist of two, four or six items divided equally between the left and right hemifields (Fig.4.4). The probe array on any given trial has the same number and arrangement of items as the sample array.

Working memory hypothesis 1. Functional connectivity will increase in conjunction with working memory. We predict that phase coherence between DLPFC and PPC cortex will increase during the memory period and that this effect will grow stronger as memory load increases. This prediction is based on previous reports of DLPFC-PPC phase coherence during an object working memory task (Table 1).

Working memory hypothesis 2. This phenomenon will be strongest in layer 3. We predict that these effects will be most prominent in layer 3 because most projection neurons are located in layer 3.

Working memory hypothesis 3. This phenomenon will depend on direct connections. We predict that these effects will be most pronounced in PCs projecting between the two areas and will be reduced or abolished by blocking direct connections between the two areas because it is plausible although neither obligatory nor established that direct connections mediate phase coherence between areas.

Experimental methodology.

Training. We estimate on the basis of past experience with tasks involving this degree of difficulty that training will occupy up to a year for each monkey[6,7].

Chamber placement. Recording chambers 2 cm in diameter will be implanted over DLPFC and PPC of the same hemisphere. The chambers will be oriented so as to allow penetrations orthogonal to the cortical surface in areas 46 and 7a (monkey cohort 1) or orthogonal to the sulcal wall in FEF and LIP (monkey cohort 2). Areas

46 and 7a are homologous to cortex selected for human postmortem studies in **P1**&**P2** and are the focus of in vitro electrophysiology in **P3**. Adjacent sectors of DLPFC and PPC, namely FEF and LIP, are also included in **P4** and **P5** to test the generality of the results obtained in 46 and 7a (see Table 3. Milestones in **Center Plan**).

***Electrode placement*.** On any given day, a linear array electrode will be placed in one of the regions and four separate microelectrodes will be inserted into the other region. The rationale for this approach is that not all pairs of recording sites exhibit signs of functional connectivity. One principle governing the variability is that sites containing neurons with spatially matched response fields tend to be functionally connected[28,29]. We will characterize spatial selectivity at each of the recording sites by monitoring neuronal activity while the monkey performs a memory-guided saccade task. The area in which the linear array electrode is placed will vary from day to day. We will also record the surface potential over each area. In general, local invasive measures are well correlated with the simultaneously recorded surface potential[39,58]. Monitoring the surface potential will, however, facilitate direct comparison to EEG signals recorded at the scalp in **P5**.

***Recording*.** We will record LFP, MUA and SUA activity from all electrodes while the monkey performs attention and working memory tasks. Monkeys typically work for ~1000 trials per day. Five hundred trials per task will form an adequate sample for assessing the impact on functional connectivity of the key task variables. These will be target location (6 levels) and degree of demand for executive control (3 levels) in the case of the attention task. They will be hemifield (2 levels) and load (3 levels) in the case of the working memory task.

***Antidromic activation*.** During blocks of trials requiring the monkey simply to maintain central fixation, we will deliver brief bipolar pulses of current through each of the microelectrodes in one region while recording through the linear array electrode in the other region[23]. By noting responses that occur at short latency and are abolished in a collision test, which constitutes the canonical criterion for antidromic activation, we will identify PCs recorded through the linear array electrode that project monosynaptically to the electrically stimulated site. It is feasible to detect projection neurons in reasonable numbers by this procedure[23]. They typically respond to electrical stimulation with a latency of several ms, reflecting the antidromic axonal conduction time between DLPFC and PPC[23].

***Blockade*.** After data have been collected in each monkey sufficient to allow firm characterization of functional connectivity between DLPFC and PPC, the experiment will conclude with a phase in which we record each day before and after blocking direct connections between DLPFC and PPC. To allow blockade, we will install a third chamber, vertically oriented and positioned under MRI guidance to give access to the second branch of the superior longitudinal fasciculus as it passes through the white matter deep to the precentral gyrus (level 93 in Fig.2 of Thiebaut de Schotten[53]). To induce blockade, we will inject lidocaine through a Hamilton syringe. The effectiveness of the injection will be assessed by measuring the response in one region to electrical stimulation of the other. All aspects of this approach have been demonstrated to be feasible[1].

Data analysis.

***Cross-area phase coherence*.** We will analyze functional connectivity as a function of cortical layer by carrying out independent analyses on data from each linear array contact. The key analyses will focus on LFP-LFP and spike-LFP coherence during periods of sustained attention and working memory. Spike-LFP and LFP-LFP measures generally are concordant[28,29]. However, spike-LFP coherence conveys two kinds of information that LFP-LFP coherence cannot. First, it may reveal an asymmetry whereby spikes in area A are more strongly coherent with LFP oscillations in area B than vice versa[40,56]. On the assumption that spikes in one area induce an LFP response in the other area, this observation is taken to indicate that area A is the primary driver of oscillatory phase-locking. This is a causality measure independent of Granger causality. Second, the spike-LFP approach allows us to ask whether coherence is confined to neurons in a certain category[28]. For the analysis of coherence, we will adopt an approach described and applied in several previous studies[24,28,29,31,40]. As a preprocessing step, applied independently to data from each condition of interest, we will subtract from the response measured on each trial the mean response computed across trials. We will calculate spike-LFP and LFP-LFP coherence by the use of multi-taper methods for spectral estimation. An optimal family of orthogonal tapers given by the discrete prolate spheroid sequences (Slepian functions) will be used. The same number of trials will be used for each condition of interest. This eliminates any possible bias from differing sample sizes. Data from each of the successive 500 ms trial epochs in each task will be included in the analysis.

***Cross-area Granger causality*.** If the activity of two cortical areas is correlated, this could be because they are both under the influence of some third area or because activity in one of them influences activity in the other.

Relations in time can shed light on this issue. If the current state of area A allows predicting subsequent activity in area B, then one can infer provisionally that activity in A causes activity in B. Granger causality analysis in the frequency domain is a systematic method for establishing such relations[11]. According to Granger[27], at a given point in time we can say that one stochastic process (Xt) is "causal" to a second stochastic process (Yt) if the autoregressive predictability of Yt is improved by the inclusion of past values of Xt. To evaluate the relative strengths of influences between the two areas in the two directions (DLPFC to PPC and PPC to DLPFC) we will adopt an approach described and applied in several previous studies[12,19,21,28,29]. This approach is based on the use of multivariate autoregressive time series models for the estimation of spectral quantities. To determine the relative strength of directional influences, we will compute Granger causality values for each task condition within each 500 ms trial epoch. To test the temporal evolution of the directional influences we will carry out analyses in a 150 ms window advanced through the trial in 10 ms steps.

***Statistical analysis*.** To evaluate the statistical significance of coherence or causality between any pair of sites under any given task condition, we will use a permutation procedure based on shuffling data across trials. We will also use a permutation-based approach to evaluate the statistical significance of any difference between task conditions or between cortical layers. We have consulted with colleagues in the **Stats/DM Core** concerning this approach and will continue to receive guidance from them as the experiments unfold.

***Comments*.** We have focused on coherence and causality analysis during periods of sustained attention and working memory because these analyses are most directly relevant to testing the **Central Hypothesis**. However, we note that these represent only a small subset of the analyses that it will be possible to carry out on the very rich data set generated by the proposed experiments. Further avenues for exploration include:

• Layer-specific oscillatory patterns such as, for example, dependence of gamma oscillations on superficial layers and of alpha oscillations on deep layers[14]. Gamma oscillations are thought to depend on layer 3 parvalbumin (**PV**) neurons as characterized in **P1**, **P2** & **P3**.

• Effects involving complex measures of cross-area correlation such as mutual information with task content[44] and cross-frequency interaction including phase-amplitude coupling[54] and causality across frequency bands [59].

• Effects in which causal direction changes. For example, DLPFC might lead PPC under the "flexible" attention condition but PPC might lead DLPFC under the "habitual+popout" condition[15,62].

• A tendency for DLPFC and PPC activation to vary in tandem across identical trials. This is termed noise correlation[20]. It will be a point of contact between this project and fMRI in **P5**.

Potential pitfalls.

In designing this project, we have been guided by considerations related to the needs of the Center. It is important as a means for meeting the goals of the Center to connect two levels of analysis. Level 1: **P1**&**P2** will characterize layer 3 PCs in human postmortem tissue and **P3** will do so in monkey cortical slices. Level 2: **P5** will characterize functional connectivity between DLPFC and PPC by coherence and causality analysis in humans with schizophrenia. **P4** alone has the ability to bridge between these two levels by establishing that layer 3 PCs mediate functional connectivity between DLPFC and PPC. If we show that functional connectivity at certain frequencies or under certain task conditions depends on layer 3, then it will be possible to infer that abnormalities of functional connectivity at those frequencies or under those task conditions in schizophrenia (**P5**) are a product of layer 3 pathology in schizophrenia (**P1**&**P2**). No previous study has posed this question. Consequently there are some unknowns with implications for feasibility. These include the following.

• Will we be able to find sites that are functionally connected? Whether two sites exhibit functional connectivity depends on factors such as receptive field congruence[28,29]. In previous experiments with no targeting criterion other than that recording sites be in interconnected areas of posterior and frontal cortex, the fraction of paired sites exhibiting significant phase locking was 23%[40] and 26%[44]. Thus, with a linear array electrode in one area and four electrodes at different sites in the other area, we expect to be able to acquire at least one coherent pair of recording sites per session. If this is not the case, then we will increase the number of recording sites.

• The attempt to blockade direct connections between DLPFC and PPC may not work. The method has been demonstrated to be feasible only in the anesthetized cat[1]. If it does work, it may interfere with performance of the tasks. This would be of interest in itself but would prevent analyzing cross-area phase coherence in task context. If successful, this manipulation will provide valuable information. If not, we will focus on determining whether projection neurons identified by antidromic activation display particularly strong cross-area coherence.

VERTEBRATE ANIMALS

Description of the proposed use of animals. The experiments in this proposal involve electrophysiological recordings from macaque monkeys (*Macaca mulatta*). Extracellular recordings will be made from individual neurons in the cerebral cortex with the goal of better understanding neural coding and the functional organization of primate and thus of human cerebral cortex. The animal subjects will weigh between 4 and 15 kg, and will be of either sex. Two animals will be in use during each year.

Justification for the use of these animals. In vitro systems or computer models cannot be used to answer the questions posed in this proposal. Macaque monkeys have been selected because their cortical organization is well understood and is an excellent model for human systems controlling attention and working memory. Macaques were selected for study because they embody perceptual and cognitive processes closely related to those in humans and possess the intelligence needed for the performance of demanding behavioral tasks. Macaques learn quickly and will work consistently for several hours during daily experimental sessions. The number of animals that will be used is the lowest feasible number that can yield dependable results.

Veterinary care of the animals involved. Members of the laboratory and a full-time animal care technician with AALAS accreditation perform daily care of the animals under the supervision of the Project Director. An attending veterinarian examines the animals regularly and is available at any time for consultation regarding health problems.

Surgical procedures and approaches to minimize discomfort. Head restraining posts and recording chambers are implanted under general anesthesia with sterile procedures. Discomfort and injury to animals will be limited to that which is unavoidable in the conduct of this research. Analgesic, anesthetic, and tranquilizing drugs are used where indicated and appropriate to minimize discomfort to the animals. All surgeries will be done under general anesthesia and analgesics are provided during recovery from surgery. For recovery surgeries, all instruments, drapes and dressings will be sterilized. All surgical personnel wear sterile gowns, gloves, caps and masks throughout the surgical procedure. Each animal is conditioned to handling and trained to move between its cage and a primate chair guided by a metal pole attached to a collar it wears. The primate chairs are adjustable along several dimensions to allow a comfortable primate sitting posture. During training or recording sessions, the animal's head movement is restricted for monitoring eye position and neurophysiological recording. Head posts allow head stabilization without pressure on the skin. The length of training or recording sessions is controlled by the animal's interest in working, and is typically two to five hours. Each animal remains completely unrestrained in its home cage outside of training and recording sessions. During training and recording the animal's water intake is restricted to motivate them to work for fluid rewards. While water intake is restricted, the animal's hydration is monitored daily by fluid intake, weight, skin turgor and examination of feces. Animals that do not work for sufficient fluid will be given additional water. Operant conditioning will be accomplished by use of positive rewards.

Euthanasia. At the conclusion of the period of physiological experimentation, animal subjects will be euthanized by deeply anesthetizing with barbiturates followed by perfusion to assure the proper fixation necessary for the anatomical studies. This procedure conforms to the guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association.

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SPECIFIC AIMS

Impairments of attention and working memory are core deficits in schizophrenia. The Center's **Central Hypothesis** (Fig. 0.2 **Center Plan**) states that these deficits arise in part from abnormal functional connectivity between dorsolateral prefrontal cortex (**DLPFC**), posterior parietal cortex (**PPC**) and primary visual cortex (**V1**) which in turn results from pathology of layer 3 pyramidal cells (**PCs**) linking these regions. **P1**&**P2** will characterize the molecular and morphological alterations of layer 3 PCs in schizophrenia through postmortem studies. **P3**&**P4** will characterize the functional properties of layer 3 PCs linking DLPFC and PPC in monkeys and will characterize their role in functional connectivity. **P5** will bring this enterprise full circle by characterizing abnormalities of functional connectivity in subjects with schizophrenia. Through the lens of the monkey studies, it will be possible to assess the hypothesis that abnormalities of functional connectivity (**P5**) are a direct consequence of layer 3 pathology in schizophrenia (**P1**&**P2**). The **Central Hypothesis** (Fig. 0.2) holds in addition that pathology of layer 3 PCs increases along a posterior to anterior axis, with deficits minor in V1, intermediate in PPC and large in DLPFC. **P5**, at the level of regional cortical function, and **P1**&**2**, at the levels of gene expression, cellular morphology and local circuitry, will carry out complementary tests of this hypothesis.

The overarching goal of **P5** is to measure cortical activation and functional connectivity in antipsychotic-naïve, first-episode psychosis patients and matched controls performing tasks that require attention and working memory. We will employ a concurrent multimodal imaging approach combining fMRI, for high spatial resolution, with MEG and EEG (**M/EEG**), for high temporal resolution. We will be attentive to patterns of activation throughout the cortex but will focus, in accordance with the Center's goals, on V1, PPC and DLPFC. To test the hypothesis that functional connectivity between PPC and DLPFC is impaired in schizophrenia, we will employ spectral measures of coherence and causality (M/EEG) and analogous measures of slow temporal co-variation (fMRI). To test the hypothesis that impairments increase along a posterior-to-anterior axis, we will measure the strength of activation during task performance in V1, PPC and DLPFC.

We will select for testing patients with a first psychotic break. Because definitive diagnoses cannot be made until 6 months later, we will recruit all identified individuals. Analysis will focus on patients with schizophrenia or schizoaffective disorder, as in **P1**&**P2** (as well as subjects with schizophreniform disorder); for simplicity in presentation, the term schizophrenia is used below to refer to all three DSM diagnoses. The "wide net" recruiting approach has the advantage that it will allow us to perform secondary analyses independent of DSM diagnosis and consistent with the NIMH RDoC initiative (see **Center Plan 1.0**)

Aim 1: To investigate attentional impairments in first-episode schizophrenia. We will assess regional activity and interregional functional connectivity in patients with first-episode schizophrenia and matched controls performing tasks that place a variable demand on attention. *We predict that:*

1) Performance of subjects with schizophrenia will be impaired. The impairment will be most prominent under conditions imposing the greatest demand on attention.

2) Cortical activation associated with attention will be reduced in schizophrenia. The disease effect will increase across areas in the order V1 to PPC to DLPFC.

*3) Functional connectivity between DLPFC and PPC associated with attention will be reduced in schizophrenia. The reduction will be most prominent for measures found in **P4** to depend on layer 3.*

Aim 2: To investigate working memory impairments in first-episode schizophrenia. We will assess regional activity and interregional functional connectivity in patients with first-episode schizophrenia and matched controls performing a task that places a variable load on working memory. *We predict that:*

1) The performance of subjects with schizophrenia will be impaired. The impairment will be most prominent under conditions imposing the greatest load on working memory.

2) Cortical activation associated with working memory will be reduced in schizophrenia. The disease effect will increase across areas in the order V1 to PPC to DLPFC.

*3) Functional connectivity between DLPFC and PPC associated with working memory will be reduced in schizophrenia. The reduction will be most prominent for measures found in **P4** to depend on layer 3.*

Attainment of these aims will provide novel insights into the local and distributed circuit pathophysiology underlying attention and working memory disturbances in schizophrenia. In concert with **P1**-**P4**, the results will help to identify pathophysiologically-based biomarkers to guide novel diagnostic and therapeutic approaches.

OVERVIEW

Cortical networks mediating attention and working memory. Human neuroimaging and nonhuman primate studies have identified a distributed cortical network, engaged during working memory, which includes DLPFC, PPC, and V1[1]. The same network is engaged during covert attention[2-6]. In the context of tasks requiring attention, DLPFC and PPC are thought to be the source of a top-down influence brought to bear on sensory areas such as V1 which results in enhanced sensory activation by attended stimuli[5,7,8]. While DLPFC and PPC contribute jointly to working memory and attention[9], they presumably make specialized contributions. PPC may play a greater role in the bottom-up capture of attention whereas DLPFC may be preferentially involved in its top-down control[10,11]. Cooperation between DLPFC and PPC depends on functional connectivity between them. Functional connectivity is evident in cross-area oscillatory phase coherence. This has been documented in frequency bands including alpha and gamma[12]. Coherence may depend in a frequency-dependent manner on task demands such as the requirement for bottom-up or top-down attention[11,13].

Abnormality of these cortical networks in schizophrenia. The impairment of working memory in schizophrenia is a core deficit more closely correlated than the positive or negative symptoms with prognosis and social functioning (see **Center Plan 2.3**). Schizophrenia also involves an impairment of attention, especially under conditions requiring top-down control[14]. Performance in tasks requiring only bottom-up attention typically is intact[14] but other bottom-up processes, notably early sensory processing, may be affected[15,16].

An extensive and growing literature across multiple neuroimaging modalities has documented abnormalities of cortical function and of functional connectivity in schizophrenia in regions including DLPFC and PPC. EEG and MEG studies have produced evidence for cortical and subcortical abnormalities in the context of tasks requiring attention and working memory[17-23]. Abnormalities of local oscillatory activity have been noted in particular in the gamma and alpha bands[24-31]. Anomalies have also been noted in the magnitude of BOLD activation accompanying the performance of cognitive tasks[32-38]. Impairments have been found throughout PPC[39,40] but it is in the inferior parietal lobe that they are particularly prominent[39,41,42]. The abnormalities associated with schizophrenia extend to interregional functional connectivity. EEG studies have revealed a reduction of anterior-posterior gamma band phase-synchrony associated with Gestalt perception[28,43] as well as identifying alterations of functional connectivity during working memory[44-47]. However, these results all derive from scalp EEG and therefore are limited in defining anatomic sources of the disturbances. On a longer time scale, fMRI studies have revealed that co-variation of activity in DLPFC and PPC is reduced in schizophrenia during the resting state and in the context of cognitive tasks[48-50]. This effect increases with working memory load[51]. and is present specifically in DLPFC and PPC[52]. There is also an impairment at the level of structure, including tracts presumed to connect DLPFC and PPC[53-55] (see **P4**, Aim 3).

Although the existing literature suggests that regional activation and inter-regional interaction are abnormal in the cerebral cortex in schizophrenia, there is not yet any consistent account of the abnormalities, based on testing in contexts analogous to those used for assessing the functions of DLPFC and PPC in nonhuman primates, nor is there evidence for or against the idea that the abnormalities arise from pathophysiology in the circuitry mediated by layer 3 PCs.

Key Questions: 1) Is functional connectivity between DLPFC and PPC impaired in schizophrenia? 2) Do impairments of local activation increase in severity from V1 to PPC to DLPFC? The **Central Hypothesis** holds that impairments of cognitive function in schizophrenia, as revealed by tests of attention and working memory, arise from pathology of layer 3 PCs. The hypothesis suggests two specific predictions with regard to cortical function in schizophrenia. First, functional connectivity between DLPFC and PPC will be impaired because direct connections between the regions derive primarily from layer 3 PCs. Second, local impairments of function will become progressively more severe in the sequence V1 to PPC to DLPFC because pathology in layer 3 PCs becomes progressively more pronounced in this order.

Strategy for answering the questions: Multimodal neuroimaging during cognitive task performance. **P5** will answer these questions by conducting neuroimaging studies in antipsychotic-naïve patients with first episode psychosis and matched controls. For neuroimaging, it will use an innovative multimodal approach combining the high temporal resolution of M/EEG and the high spatial resolution of fMRI. It will collect data from subjects as they perform tasks that require attention and working memory because activation and inter-regional interaction of DLPFC and PPC are most evident in the context of such tasks.

The use of a multimodal approach is critical for thorough assessment of the state of cortical function in schizophrenia. MEG and EEG jointly offer the advantage of high temporal resolution. High temporal resolution, down to the limit of around 1ms, is obligatory for the analysis of functional connectivity by spectral measures of coherence and causality. It also allows the measurement, in the spectral and temporal domains, of the magnitude of event-related responses, as required for assessing the functional status of individual areas. The methods are complementary in that EEG is sensitive to radial (gyral) and tangential (sulcal) voltage signals, but subject to attenuation by skull and scalp tissue, whereas MEG is particularly sensitive to tangential magnetic signals, which are not subject to attenuation. The unique advantage of fMRI, which measures metabolic activity via the blood-oxygen level dependent (BOLD) response, thus indirectly assessing neuronal activity, arises from its high degree of whole-brain spatial resolution[56]. This is required for definitive localization of abnormal levels of activation in schizophrenia, including abnormal levels both of the BOLD signal in the fMRI data set itself, and abnormal levels of electrophysiological signals in the M/EEG data set. fMRI will also contribute to the assessment of functional connectivity by allowing the measurement, at long time-scales, of inter-regional cross-trial co-variation in the BOLD response. There is an obvious high degree of synergy in the simultaneous application to each subject of three methods possessing unique complementary strengths.

The use of specially designed tasks is a second critical feature of the approach adopted in **P5**. The general goal is to determine whether impairments of regional activation and inter-regional interaction are of the sort that would be expected to arise from pathology of layer 3 PCs. Attaining this goal will require collecting data from humans under circumstances in which regional activation and inter-regional interaction are known, from nonhuman primate experiments, to depend on layer 3 PCs. Accordingly, we will use tasks designed in parallel to the tasks used in **P4** to assess the dependence of functional connectivity between DLPFC and PPC on layer 3 PCs. One task will demand selective visual attention under variable reliance on the capacity for top-down control. A second task will require working memory under a variable degree of load.

SIGNIFICANCE

As detailed in the **Center Plan**, schizophrenia remains a costly problem, both emotionally for those afflicted and their family members, and financially, with less than half of those ill able to work even part time for the rest of their lives. The general goal of the Center is to characterize the local and distributed circuit pathophysiology underlying attention and working memory disturbances in schizophrenia. Attainment of this goal will provide the basis for identifying pathophysiologically-based biomarkers to guide novel diagnostic and therapeutic approaches. **P5** will make a unique contribution to this enterprise by assessing the status of regional activation and inter-regional interaction in the cerebral cortex of patients with schizophrenia and matched controls. It will improve our understanding of the neurobiological basis of schizophrenia in several significant ways:

• The multimodal neuroimaging approach will provide a multifaceted characterization of the integrity of individual cortical areas and of their functional interactions.

• The use of tasks comparable to those employed in the nonhuman primate studies of **P4** will allow testing a specific hypothesis to the effect that abnormalities of cortical function in schizophrenia arise from pathology of layer 3 PCs as characterized in **P1**&**P2**.

• By bridging from the systems level to the cellular level, we will identify physiological biomarkers for the pathology of layer 3 PCs. These may in turn prove to be a target for novel therapeutic approaches based on knowledge of molecular, cellular and circuitry pathology (**P1**&**P2**).

INNOVATION

P5 is innovative in that it is designed to test a specific model of functional disturbances in schizophrenia based on pathology of layer 3 PCs. Previous functional imaging studies have tended toward description as distinct from tight theoretical framing at the level of cellular function. A second innovative feature of **P5** is the adoption of an approach based on multimodal neuroimaging. Previous studies have conformed to the single-modality model and thus have lacked the simultaneous lock on spatial and temporal parameters of activation that can be obtained only through the combined use of fMRI and M/EEG. A third innovative feature of **P5** derives from the way in which it is positioned in the Center as a whole. Using behavioral paradigms similar to those employed in the nonhuman primate studies of **P4** will allow us, by comparing our results to those obtained in **P4**, to determine whether functional abnormalities in schizophrenia (**P5**) can be explained by pathology of layer 3 PCs in schizophrenia (**P1**&**P2**). Specific innovative virtues of **P5** include the following:

• This will be the first study to collect not only concurrent MEG and EEG (M/EEG) measures but also fMRI measures from the same cohort of patients with schizophrenia and matched controls.

• This will be the first study to apply these combined measures to the assessment of functional connectivity between DLPFC and PPC in schizophrenia.

• The use of antipsychotic-naive subjects has the strength of addressing potential confounds between effects arising from schizophrenia itself and indirect effects including 1) antipsychotic effects and 2) chronic effects arising from illness, treatment and social milieu.

APPROACH

Rationale. The general goal of **P5** is to measure neural activation and functional connectivity throughout the cerebral cortex in antipsychotic-naïve, first-episode psychosis patients and matched controls performing tasks that require attention and working memory. We will employ a concurrent multimodal imaging approach combining the high temporal resolution of M/EEG and the high spatial resolution fMRI. The specific goal of **P5** is to test the **Central Hypothesis** that pathology of layer 3 PCs gives rise to the abnormalities of cortical function that underlie cognitive impairments in schizophrenia. We will collect data relevant to testing two key predictions that emerge from this hypothesis. *Prediction 1: Functional connectivity between PPC and DLPFC will be impaired in schizophrenia.* To test this prediction, we will employ spectral measures of coherence and causality (M/EEG) and analogous measures of slow temporal co-variation (fMRI). *Prediction 2: Region-specific functional impairments will become more severe along a posterior-to-anterior axis.* To test this prediction, we will measure the strength of activation during task performance in V1, PPC and DLPFC.

Subjects. A later section provides extensive information on subjects. Here, we highlight that first-episode psychosis patients and healthy controls will be recruited by the Psychosis Recruitment and Assessment Core (PRAC) of the Department of Psychiatry, University of Pittsburgh. The PRAC has over two decades of experience in recruiting first-episode patients, drawing from an extensive network that includes outpatient and inpatient clinical facilities, and community referral sources. All subjects will receive diagnostic and clinical rating assessments. All subjects will participate in the collection of M/EEG and fMRI measures during the performance of tasks requiring attention and working memory. We will take a dimensional approach to examining common cross-diagnostic features associated with symptoms that span nosologic categories such as working memory and attention, and symptom dimensions including reality distortion, poverty and disorganization. Subsequent analyses will compare schizophrenia-spectrum and non-schizophrenia psychosis subgroups along these same dimensions. Comparisons and correlations between all measures will be conducted to assess the effect on social and occupational functioning.

Cortical areas. In the service of addressing key Center-relevant issues, we will focus, in our analysis, on activation in three areas: V1, PPC and DLPFC. We will focus on the entirety of PPC, including the intraparietal sulcus, but will confer particular attention on BA40 because it is a documented site of impairment in schizophrenia[39-42], and represents the cytoarchitectonic area examined in the postmortem studies of **P1**&**P2**. We will examine the entirety of DLPFC (including BA9) but will confer particular attention on BA46 and the frontal eye field because these are probable homologues of areas to be studied in the nonhuman primate experiments of **P4**.

Functional measures. Later sections describe in detail the methodology of multimodal neuroimaging. Here we note briefly the overall strategy relating the measures to be collected to testable predictions that arise from the **Central Hypothesis**. To test the prediction that functional connectivity between PPC and DLPFC is impaired in schizophrenia, we will employ spectral measures of coherence and causality (M/EEG) and analogous measures of slow temporal co-variation (fMRI). To test the prediction that impairments increase along a posterior-to-anterior axis, we will measure the strength of activation (fMRI and M/EEG) during task performance in V1, PPC and DLPFC. In general, we expect that measures based on M/EEG and fMRI will be concordant both with regard to measures of regional activation and with regard to measures of cross-regional interaction. We expect the methods to differ primarily in the degree to which they yield temporal or spatial precision.

Aim 1: Attention.

Patients and healthy controls will perform, in separate blocks, three tasks that impose graded degrees of challenge on top-down attention. In order of increasing challenge, these are a visual pop-out task in “habitual” mode, a visual search task in “habitual” mode and a visual search task in “flexible” mode. The trial structure will

be the same regardless of task (Fig. 5.1). After the appearance of a central cross, on which the subject must fixate, a color cue appears briefly at fixation. This instructs the subject that it will be necessary later in the trial to direct attention to a peripheral item of the same color. After a brief delay, a probe array appears, encompassing a target and five distractors at equal eccentricity in a hexagonal arrangement. All six items are annuli. Each annulus has a gap on either its left or right side. The subject has two seconds in which to direct attention to the target, take note of the location of the gap and respond with a left or right button press depending on whether the gap is located on the left or right side of the target. The size of the gap will be adjusted during preliminary testing so as to ensure that performance requires covert attention.

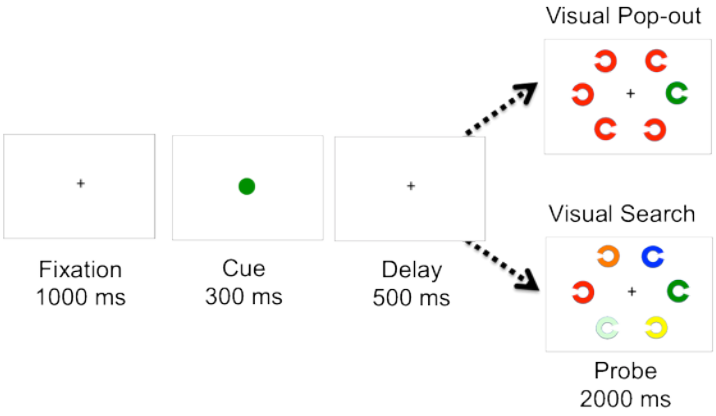

Fig.5.1. Sequence of events in pop-out and search attention tasks.

Each task has distinctive properties that determine the degree of challenge to top-down attention. *Pop-out task in "habitual" mode.* On a given block of trials, the color cue and the target will always be of one color (green in Fig.5.1) and the distractors will all be of the same color, which will be the same from trial to trial (red in Fig.5.1). Thus the target can be selected for attention in a top-down fashion, on the basis of its matching the antecedent cue in color, but top-down selection subject to aid by two bottom-up processes, namely habit (the habit developed across the block of attending to a certain color) and automatic capture of attention by an item salient due to its perceptual oddball status. *Visual search task in "habitual" mode*. In this task, the target will be of the same color across the entire block of trials but the distractors on any given trial will be of multiple colors. Thus the target can be selected for attention in a top-down fashion, on the basis of its matching the antecedent cue in color, but top-down selection is subject to aid by a bottom-up process, namely habit (the habit developed across the block of attending to a certain color). *Visual search task in "flexible" mode*. In this task, both the target and the distractors will vary in color from trial to trial. Thus the target must be selected for attention in a top-down fashion, on the basis of its matching the antecedent cue in color, without any reliance on bottom-up aids. The three tasks will be run in interleaved blocks for a total of 480 trials.

Attention hypothesis 1. *The performance of subjects with schizophrenia will be impaired. The impairment will be most prominent under conditions imposing the greatest demand on attention.*

Attention hypothesis 2. *Cortical activation associated with attention will be reduced in schizophrenia. The disease effect will increase across areas in the order V1 to PPC to DLPFC.*

Attention hypothesis 3. *Functional connectivity between DLPFC & PPC associated with attention will be reduced in schizophrenia. The reduction will be most prominent for measures found in* ***P4*** *to depend on layer 3.*

Comment: Our expectation that performance and cortical function will be most preserved under conditions imposing a low challenge on top-down attention is concordant with the observation that attention to pop-out stimuli is relatively preserved in schizophrenia[57,58]. The observation that bottom-up attention is preserved might be construed as in disagreement with the observation that low-level sensory processing is impaired[15]. However, the detection of pop-out stimuli is easier than the typically fine discriminations required in studies of sensory processing deficits.

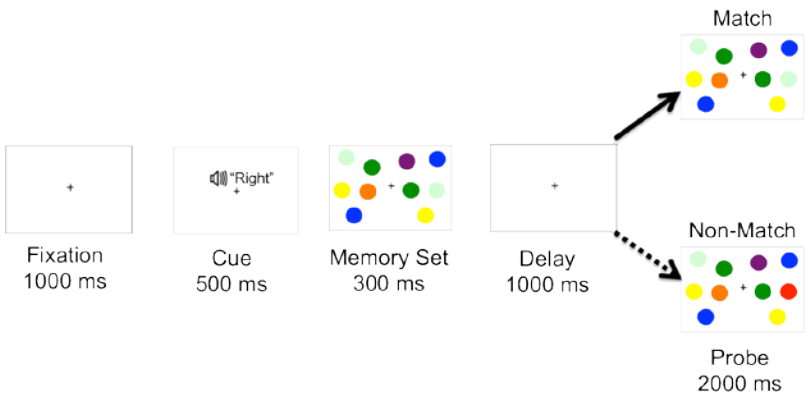

Fig.5.2. Sequence of events in match and non-match working memory trials.

Aim 2: Working memory.

Patients and controls will perform a working memory task requiring retention over a short interval of information concerning the location and color of elements in a multi-item display. The display will consist of a number of items that varies from trial to trial with consequent

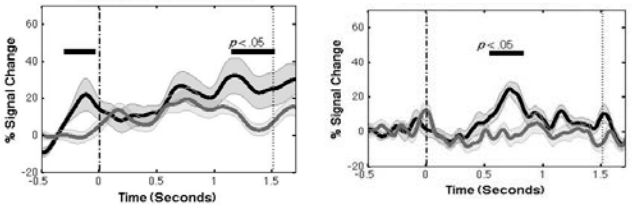

Fig.5.3. Alpha power in FEF (left) and Beta power (right) in) and adolescents (gray line).

variation in the load placed on working memory. Different loads will be imposed on interleaved trials. The sequence of events in a trial is summarized in Fig.5.2. After attainment of fixation on a central cross, subjects are instructed by the pitch of an auditory cue whether to attend to items in the left or right hemifield while processing an upcoming multi-item display. The manipulation of attention allows deconfounding lateralized effects due to mnemonic processing from lateralized effects due to the location of items in the display. A sample array spanning both hemifields is then presented (Memory Set in Fig. 5.2). This consists of a number of colored disks. The subject must hold information about the instructed side of sample array in working memory over an ensuing one-second delay. After the delay a probe array appears. The probe array, with equal frequency, will be identical to the sample array with regard to the attended hemifield (match condition) or will differ from it with regard to the color of one item in the attended hemifield (non-match condition). Subjects are instructed to make a right button press under one condition and a left button press under the other, with the pairing counterbalanced across subjects. In half of the match trials (involving no change in the attended hemifield) a single item will be changed in the non-attended hemi-field to serve as a catch for failure to confine attention to one hemifield as instructed. There will be three load conditions (1, 3 or 5 stimuli in each hemifield), with 120 trials of each load for a total of 360 trials.

Working memory hypothesis 1. *The performance of subjects with schizophrenia will be impaired. The impairment will be most prominent under conditions imposing the greatest load on working memory.*

Working memory hypothesis 2. *Cortical activation associated with working memory will be reduced in schizophrenia. The disease effect will increase across areas in the order V1 to PPC to DLPFC.*

Working memory hypothesis 3. *Functional connectivity between DLPFC and PPC associated with working memory will be reduced in schizophrenia. The reduction will be most prominent for measures found in* ***P4*** *to depend on layer 3.*

Comment: Our expectation that cortical activation will be reduced in subjects with schizophrenia is generally consistent with the existing literature. However, there are reports of increased BOLD activity for patients that are often interpreted as 'inefficient activation' and/or a leftward shift of the inverted-U relationship between brain activation and working memory load in schizophrenia. The parametric design employed in our study, involving three degrees of challenge to working memory, will allow us to examine whether such effects occur.

PRELIMINARY RESULTS

Track record in using the proposed methods. Our group has extensive collective experience in the use of all the neuroimaging and data-analytic methods proposed for use in this project (See Biographical Sketches). This statement applies in particular to spectral power analysis[30,59,60], MEG phase-locking analysis[61,62], fMRI connectivity analysis[63,64] and the use of fMRI and MEG in parallel[65]. We present preliminary data both to establish the plausibility of the guiding hypotheses and to demonstrate the feasibility of the proposed approaches.

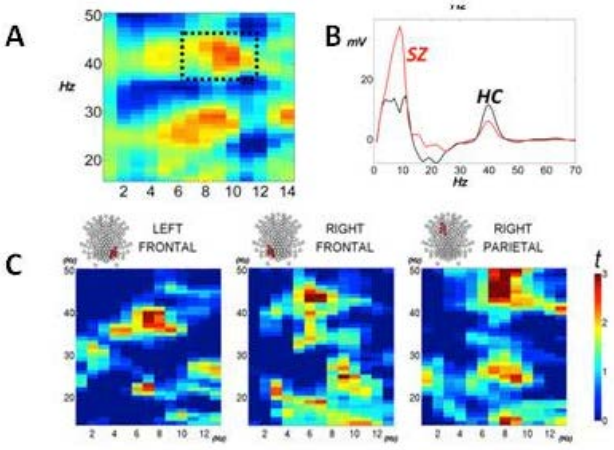

Fig.5.4. Cross-frequency coupling in healthy controls vs. schizophrenia subjects. **A)** T-statistic plot for cross-frequency coupling (CFC) comparisons of controls vs. patients. Note significant group differences (black outline) in alpha (8-10 Hz)-gamma(40Hz) coupling. **B)** Spectral power plot showing higher alpha power in patients and higher gamma power in controls. **C)** T-stat plot of CFC in frontoparietal areas during WM task in healthy controls.

Example: Detecting group differences in oscillations with MEG during cognitive control. In order to demonstrate our ability to use MEG to detect oscillations localized to regions, we present data from a study investigating top-down inhibitory control in adolescents and young adults (Hwang and Luna, in preparation). Although this dataset focused on alpha and beta oscillations, our proposed studies will focus initially on alpha and gamma. We collected MEG data from 17 adolescents (age 14-16) and 20 adult participants (age 20-30), where participants performed an antisaccade

(AS) task that required voluntary response suppression, and a control prosaccade (PS) task. During correct trials, compared to adults, adolescents showed decreased alpha-band power in the oculomotor regions in preparation to inhibit an upcoming reflexive saccade, suggesting immaturities in controlled inhibition of task-inappropriate activity (Fig.5.3). Furthermore, adolescents showed weaker beta-band power in prefrontal cognitive control regions during response preparation, suggesting less robust top-down biasing of sensory and motor processes. *These results demonstrate that the neurophysiological measures and analytic techniques proposed are not only feasible for group comparisons but are informative and yield superior combined temporal and spatial resolution. Of note, we will match groups by age and use age as a regressor to take into consideration possible age effects.*

Example: Detecting cross-frequency coupling disturbances in schizophrenia. The modulation of gamma oscillations by lower frequency rhythms is thought to be an important mechanism underlying cognitive processes including executive functions as well as sensory processing[66-69]. We present a preliminary cross-frequency coupling analysis of published data[60] comparing schizophrenia and healthy controls during an auditory cortical driving paradigm[60,70-73]. Our preliminary data indicate schizophrenia subjects had impaired alpha-gamma coupling (Fig.5.4A). This decreased coupling was found in the context of increased alpha power and decreased gamma power in patients compared to controls, indicating that coupling strength cannot be predicted simply by activity patterns at the respective frequencies (Fig.5.4B). To investigate cross-frequency coupling in the context of executive function tasks that tap frontoparietal circuits relevant to the current project, 10 healthy controls (age 19.6±1.0 years; 3 females) performed a pilot version of the working memory task (Fig.5.4C) involving just the high load (5 items) condition. During the maintenance period, there was robust coupling between high theta/alpha (6-10 Hz) and gamma (30-50 Hz) frequency bands across left and right frontal areas and right parietal areas. *These findings demonstrate the feasibility of evaluating cross-frequency coupling during tasks ranging from the level of basic sensory processing to the level of executive control. They provide preliminary support for decreased alpha-gamma coupling in schizophrenia at the level of scalp-recorded data. We will extend these methods to source-reconstructed space in the current project.*

Example: Detecting stages of working memory processing by use of fMRI. While fMRI does not have the temporal resolution to directly assess information flow in real time, isolating different stages of processing for each task can reveal different aspects of bottom-up and top-down processes. The initial cue stage engages predominantly bottom-up processing of the visual cue. The delay period engages a loop of bottom-up and top-down processing enabling the maintenance of working memory information. The response/retrieval period predominantly engages top-down processing to direct a voluntary response. We present preliminary data from an ongoing project using a Sternberg-type spatial working memory task where the location of serially presented stimuli must be retained in working memory. Catch trials were presented in order to dissociate encoding, maintenance, and response/retrieval phases of the task[74]. Preliminary ROI analyses on 79 healthy adults show that activity in DLPFC and PPC is greatest during the target phase engaging retrieval processes compared to cue and delay periods (Fig.5.5). *These data indicate that a parametric task approach in the fMRI environment identifies distinct regions and distributed systems independently associated with different stages of information processing. A fractionating approach will also allow us to identify specific impairments tied to bottom-up or top-down processing or confined to particular stages in the performance of tasks requiring attention and working memory.*

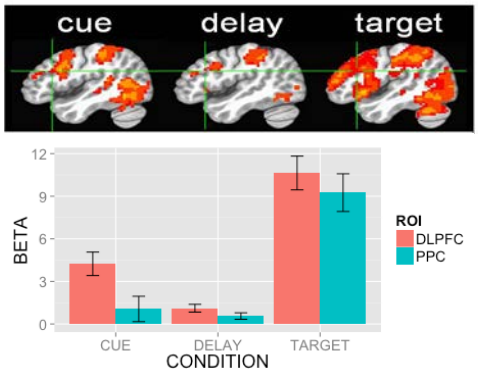

Fig 5.5 ROI analyses by stage of WM.

Example: Applying Granger causality analysis (GCA) to MEG data. In this project we propose to apply GCA to M/EEG data to characterize causal connections and their relative strength in patients during attention and working memory tasks. We present data to demonstrate the utility of this approach (Fig. 5.6). Twelve healthy adults performed an object recognition task for images that were filtered to only contain low spatial frequencies (LSF) or high spatial frequencies (HSF), and intact images (Ghuman et al. in preparation). The direction of information flow between occipital visual areas, orbital frontal cortex, and the fusiform gyrus was examined. Granger causality

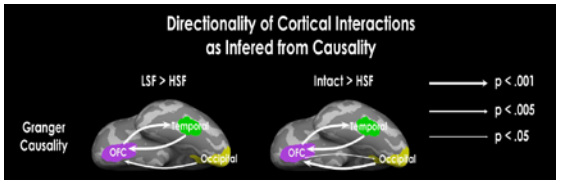

Fig.5.6. Granger causality showing information flowing from early visual cortex to orbital frontal cortex and back to the fusiform gyrus.

analysis was used to compare the direction of information flow. This analysis revealed that the orbital frontal cortex received bottom-up input reliant on LSF from occipital visual areas and projected back to the temporal cortex in a top-down manner. *These results demonstrate our ability to extract directionality and causality at specific cortical frequencies. Granger causality analysis will allow us to assess the integrity of PPC-DLPFC interaction in schizophrenia.*

DATA COLLECTION METHODOLOGY

Concurrent M/EEG. MEG data will be collected on a 306-channel Elektra NeuroMag system in a shielded room at 1 kHz sample rate. Concurrent EOG (eye-blink artifacts), EKG (cardiac artifacts) and 64 channel low-impedence EEG will also be recorded through this system. Head positioning (HPI) coils are also mounted in the EEG head cap to allow registration of the head to the MEG sensors. A Polhemus isotrak system is used to register the HPI coils and EEG sensors to landmarks on the head prior to scanning, which is then used to coregister the sensors to a structural MRI. Ocular, muscle and EKG artifacts will be removed using ICA-based approaches. Event-related activity will be analyzed in the same manner as our preliminary data using a technique we have used in the past[61,65,75]. Briefly, the M/EEG data will be projected onto a grid of dipoles created on each individual's structural MRI (MPRAGE acquired as part of fMRI scan; see below). Source reconstruction on the cortical surface will use the l_2 minimum norm estimate (MNE) inverse solution (implemented in MNE-Python). The activation in response to the stimulus or task will be determined using the MNE and dynamic statistical parametric maps will be generated[76]. These maps correspond to the event-related electro-magnetic fields on the brain and will be used to determine the regions of interest that are selectively activated by the respective task or stimulus.

fMRI. fMRI data will be obtained to assess magnitude of activity at different stages of task processing and to aid spatial localization of M/EEG data. We will also obtain whole brain network information including subcortical and medial wall regions that are not readily accessible with MEG. fMRI data will also be used to construct functional connectivity maps to identify relationships among cortical core region connectivity and the integration of subcortical regions. Given that we will be performing the same tasks with fMRI, we will use fMRI data as weighted maps to constrain source localization of MEG data[76,77]. Image Acquisition: A Siemens 3T Tim Trio research dedicated scanner with echo planar imaging (EPI) capabilities and a CP transmit/receive head coil with integrated mirror for functional MR studies will be used. It has a small fringe field; 5 Gauss at 3.4m (radial) x 4.5m (axial) and a 40 mT/m max. gradient strength per axis (or 69 mT/m effective). The DTI matches the slice definition of the BOLD EPI. Parameters for BOLD acquisition are TR=1.5s, TE=25ms, 79°=flip angle, 29 slices, axial plane AC/PC aligned, 64x64 acquisition matrix, FOV=205x205mm^2, 4mm thickness, 0 gap, yielding 4 x 3.125 x 3.125 cubic mm^3 voxels. Structural images for anatomic localization of the activation will be obtained using an MPRAGE sequence: sagittal plane, TR = 1630, TI=0.8s, TE=2.48ms, 8° flip angle (maximizing gray/white contrast), 256x256x224 acquisition matrixes, FOV=205x205 mm^2, 0.8mm slice thickness, yielding isotropic voxels (0.8x0.8x0.8 mm^3). High-resolution spin echo EPI images parallel to functional studies will be obtained to aid in registering anatomic and functional data (TR=4.5s, TE=68ms, 128x128 acquisition matrix). All task designs will be performed in a mixed block-event related design. The event related design will include incomplete "catch" trials that enable analyses of stages of task processing[74]. Despite the limited temporal resolution of fMRI, activation for different phases of working memory and attention can also be discerned by using designs that dissociate different stages of processing[74]. DLPFC, PPC as well as visual areas have been found to be involved in different phases of working memory including encoding and maintenance[78-81]. The ability to probe the relative contributions of each region of interest to unique phases of attention and working memory can provide a model to identify specific physiologic and cognitive disturbances that may arise from abnormal function of circuits formed by layer 3 PCs in schizophrenia.

DATA ANALYTIC METHODOLOGY

For all measures, we will compare antipsychotic-naïve, first-episode schizophrenia subjects to psychiatrically-well subjects matched for age, sex and IQ. Auxiliary analyses will make comparisons of controls versus all first-episode psychosis subjects, as well as by DSM classifications. Basic analytic approaches are described in the **Stats/DM Core**. To supplement hypothesis testing, corresponding confidence intervals will be provided to support alternative explanations when required. Power considerations for the N=40 subjects/group are also provided in the **Stats/DM Core**.

M/EEG analysis. Scalp analyses will include EEG and MEG[82,83] measures to complement our source space analyses. This will allow basic validation of the signals and allow contact with the extensive scalp recording literature focused on working memory and attention event related potentials. Our primary measures in the

scalp EEG (and analogous components in MEG) will be the P1, N1, and P2 sensory potentials, arising in early visual areas; N2 and P3 for attention and target detection in pop-out versus serial search tasks, and N2pc, an index of the degree to which attention via PFC and PPC top-down activity has modulated sensory processing[84]; and the load-sensitive CDA (contralateral delay activity), during memory maintenance, thought to arise from PPC, and P300 on the visual working memory task[85-87]. These measures are particularly relevant because unique abnormalities in N2pc and CDA have been found in schizophrenia[88]. The latencies for these components identified from the complementary scalp-recorded M\EEG can help to inform identification critical periods for analysis of the source reconstructed oscillatory activity and subsequent measures of connectivity and causality. The respective potentials will be indexed as follows: visual evoked potentials (O1/O2); N2 and N2pc (PO7/8); CDA (P1/2, 3/4, 5/6; PO1/2, 3/4, 5/6); P300 (Fz and Pz). Although Fz is traditionally associated with automatic orienting and the earlier P3a component, and Pz is typically associated with the later "cognitive" P3b component Li et al.[10] presented evidence to suggest that pop-out P300 displayed a peak consistent with a bottom-up parietal terminus, and serial search P300 displayed a later frontal peak. We will measure from both scalp areas to assess timing and amplitude differences and compare them between tasks and groups.

M/EEG source localization. The precise location of the cortical current sources cannot be precisely determined using the measured magnetic fields and currents from outside the head, and therefore is estimated using cortically constrained MNE, described extensively elsewhere[76,89]. Briefly, a linear inverse operator W is applied to the measured signal to calculate the MNE:

$$y(t) = W\, x(t)$$

where x(t) represents the M/EEG channel data at time t and y(t) is the corresponding current projected onto the cortical surface. The expression of W is defined as

$$W = RA^T (ARA^T + \lambda^2 C)^{-1}$$

where C and R are the noise and source covariance matrices, respectively. A is the free source orientation solution of the forward problem calculated using the boundary element method[90,91]. To compensate for the bias towards superficial currents of the MNE a scaling factor (i.e. depth weighting) is applied to A[92]. λ^2 is a regularization parameter[76,93]. Finally, to estimate the time course and statistical significance of the cortical activity, noise normalized values are calculated at each time point and each dipole location[76]. This transforms power values into dynamic statistical parametric values and makes the point spread function of the estimated signal relatively uniform across cortical dipoles[93].

Source reconstructions will be further refined by the addition of fMRI BOLD activations as 'seeds' to constrain the source modeling[76], helping to solve the inverse-solution problem for M/EEG by providing empirically determined source areas within the distributed network.

M/EEG spectral analyses of functional connectivity. M/EEG spectral measures of power, cross-frequency and connectivity and measures will all be based on amplitude and phase estimates derived from the Morlet wavelet transform[94]. The wavelet will be applied at 1 Hz steps from 1-100 Hz across the epoch. Spectral measures will be baseline-corrected using 300 ms of a 600 ms pre-stimulus baseline. Evoked power will index the amplitude of oscillations that are phase-locked to stimulus onset. "Induced" (non-phase locked) power will be derived as total power minus the evoked power. Cross-frequency coupling will be calculated as the mutual information between the lower frequency oscillation phase timeseries and the higher frequency amplitude timeseries. Stimulus-dependent phase locking values (PLVs), a measure of neural synchrony between regions will be determined by analyzing the variability in the phase difference across trials[95]. The PLV is defined as: PLV=|mean(exp(i *(θ_1(t)-(θ_2(t)))|, where θ_1(t) and θ_2(t) are the phases of the signal in region 1 and 2 respectively across trials[95]. Multiple comparisons controlled statistics will be generated using a cluster-level permutation test[96]. Analysis will be done using the MNE™ software package as well as MATLAB and Python using custom-written programs.

To assess the direction of information flow between V1, PPC, and PFC, we will employ Granger causality[97] and its spectral extension[98] to investigate causal influence from one time series to another. Qualitatively, we say that "X" is "Granger causal" of "Y" if combined information from both "X" and "Y" can significantly improve the prediction of the time series "Y" rather than using the information from "Y" alone. In practice, it is common to employ auto-regressive (AR) model to implement Granger causality as in the equation in Fig.5.7 where p is the model order, the A matrices contain the

$$Y(t)=\sum_{j=1}^{p} A_{1,j} Y(t-j)+\sum_{j=1}^{p} A_{2,j} X(t-j)+\varepsilon$$

Fig.5.7 Autoregressive model for derivation of Granger causality.

regression coefficients, and ε is the residual/error term. If this error term is significantly reduced by inclusion of the lagged values of X (i.e., the terms in the second summation above), X is said to "Granger cause" Y. This is done by testing the null hypothesis that A_2=0 using an F-test. We will use the Schwarz's Bayesian Information Criterion to estimate the order of the AR model and stepwise least squares to estimate model parameters[99,100].

fMRI. FSL[101] will be used for preprocessing including, slice timing correction to adjust for interleaved slice acquisition, estimating rotational and translational head motion for correction and reconstruction of images. Data from each run are then scaled by their mean intensity to reflect percent signal change, followed by concatenation across runs. AFNI (Analysis and Visualization of Functional Magnetic Resonance Neuroimaging) software[102] will be used for statistical analysis[102]. Data will be spatially smoothed using a 4mm Full-Width at Half Maximum kernel. Individual data will be analyzed using deconvolution methods available in AFNI[102]. Sine basis functions are used to model the hemodynamic response function. Partial F-statistics for each task regressor and t-scores for various time-delays will be calculated. The overall baseline is calculated as the mean activation for each voxel across all fixation time points. Subjects view the screen via a mirror mounted on a standard radio-frequency (RF) head coil. Our approach will be to study the *combined* effects of bottom-up and top-down processing on working memory, thus we will run voxel-wise mixed factor ANOVAs with condition (Pop out, visual search, working memory), and patient group (schizophrenia and controls) as fixed factors and participant as a random factor, using individual estimated impulse response maps (see **Stats/DM Core**). We will examine mean percent MR signal changes in *a priori* defined regions underlying each task, primarily DLPFC, PPC, and V1. We will also perform analyses on the extent of the attention and working memory circuitry which include additional regions including the anterior cingulate cortex (ACC), precuneus, striatum, and thalamus. ROIs are defined anatomically in each hemisphere on a Talairach-normalized MPRAGE anatomical image of a representative participant to create a mask. This mask will be applied to a mean percent signal change map for each participant. Activation thresholds for statistical maps will be corrected for multiple comparisons based on a Monte Carlo simulation modeling voxel cluster size and individual voxel probability. <u>fMRI Functional Connectivity Analyses</u>: We will employ beta-series functional connectivity measures[103], to assess the relative differences in connectivity at different stages of each task such as encoding, maintenance, and retrieval. This will allow us to assess the strength of connections in the regions of interest for bottom-up and top-down networks as well as strength and number of connections to subcortical regions. Assessing the integrity of corticosubcortical connections would provide an approach for investigating the possible effects of bottom-up and top-down impairments to the full brain. Dynamic Causal Modeling (DCM)[104] will be considered to assess group differences in the number and strength of causal connections arising from each of the identified regions of interest. The **Stats/DM Core** will provide input as to the optimal implementation of DCM in our data.

M/EEG fMRI Integration. Regions of interest (ROI) defined from a main effect of time map will be used as seeds to constrain the source modeling[76] to help constrain the inverse-solution problem in M/EEG. Correlation and regression models will be used to integrate BOLD related activity with M/EEG power for each region of interest. Strength of BOLD functional connectivity will also be associated with frequency information from M/EEG using regression models guided by consultation from the **Stats/DM Core**.

SUBJECTS

Subjects. Our primary aim is to compare 40 first-episode schizophrenia spectrum (schizophrenia, schizophreniform disorder or schizoaffective disorder) subjects to 40 healthy controls matched for parental socio-economic status, gender, age, IQ and handedness. The target age range will be 14-30 years old. Because definitive diagnoses cannot be made at first episode, our recruitment cohort will include subjects with schizophrenia spectrum, affective psychoses or psychotic disorder NOS. As such, the recruitment process will necessarily generate a cohort of non-schizophrenia psychosis subjects who can serve as a psychosis comparator group for secondary analyses. To meet our schizophrenia subject recruitment goals, we will aim to recruit a total of 80 first-episode psychosis patients and ensure that a majority of these subjects will be schizophrenia spectrum patients by requiring that the Criterion A of the DSM criteria for schizophrenia be met at screening (i.e. at least two symptoms, including delusions, hallucinations, disorganized speech, disorganized/catatonic behavior, or negative symptoms, for one month period). This strategy will allow us to acquire the targeted number of antipsychotic-naïve, first-episode schizophrenia patients, with the added benefit of secondary exploratory analyses examining cross-diagnostic dimensions (e.g. working memory performance) in line with the RDoC initiative (see **Center Plan *4.1.iii***).

We expect to have 20 first episode and 10 control subjects tested per year during years 1 to 4, but to ensure that datasets are adequately powered, we have allowed for "makeup" testing in year 5 to ensure all cells are full as proposed. These numbers are consistent with our experience in recruiting for the Department of Psychiatry's long-standing first-episode schizophrenia studies. Over the past 4.5 years, 83 eligible first-episode psychotic individuals have been enrolled in studies in the Department of Psychiatry. The majority (66%) were antipsychotic-naïve at time of consent. Forty-two received a schizophrenia spectrum disorder, with the remaining being psychotic disorder NOS (n=18), delusional disorder (n=2), or a mood disorder with psychosis (n=14). Seven await diagnostic review at the current time. Based on this diagnostic distribution, we will recruit 20 first-episode patients per year, anticipating that 10/year will fall in the schizophrenia spectrum. Although we anticipate high diagnostic stability, we will also conduct follow-up confirmatory diagnostic interviews at 6 months. Over the past 10 years, diagnostic stability has been maintained at approximately 90% with only 10% of subjects moving between schizophrenia spectrum and other diagnoses. The non-schizophrenia spectrum subject data will permit secondary analyses of diagnostic specificity as well as dimensional analyses across all diagnoses. We will also focus recruitment on younger subjects, 14-30 years of age. While we have historically recruited from a broader age range (12-50 years), the vast majority of subjects have fallen in this younger age range. Targeting this narrower range will help to reduce subject heterogeneity, and set the stage for future investigations of developmentally relevant questions, while still being broad enough to accommodate the recruitment of female patient subjects who tend to have a later age of illness onset. Our prior recruitment experience supports the feasibility of this approach.

Inclusion criteria. 1) First-episode psychotic patients: a) ages 14-30 years; b) first episode of a psychotic illness, defined by report of symptoms and/or history of assessments; c) no current antipsychotic treatment and less than 2 months of lifetime antipsychotic treatment or taking medications that interfere with brain function. 2) Healthy comparison subjects: a) ages 14-30 years; b) no lifetime history of Axis I disorder; and c) no first-degree family history of schizophrenia spectrum Axis I disorder or mood disorder with psychotic features.

Exclusion criteria. General (regardless of group): a) DSM-IV mental retardation; b) significant head injury; c) medical illness affecting brain function or structure; d) pregnancy or postpartum (<6 weeks after delivery or miscarriage); e) significant neurologic disorder (e.g seizure disorder); f) inability to provide informed consent; and g) color blindness. For first-episode psychotic patients: a) a psychotic illness with a temporal relation to a substance use disorder; b) co-morbidity for DSM-IV psychoactive substance dependence within the past six month; c) substance abuse - other than cannabis and/or alcohol within the past one month; and d) temporal relation between illness onset and head injury. For healthy comparison subjects: a) treatment with an antipsychotic at any time; b) co-morbidity for DSM-IV psychoactive substance dependence within the past six month or substance abuse within the past one month.

First-episode subject recruitment. First-episode psychotic individuals will be identified through Western Psychiatric Institute and Clinic's inpatient and outpatient services, Allegheny County's re:Solve Crisis Network, UPMC hospitals, and college and university counseling centers throughout western Pennsylvania. Our clinical staff members have worked with these referral sources for up to 20 years. These referral sources have been educated regarding the importance of delaying antipsychotic treatment, when possible, until an evaluation by study staff has been completed. There is an established on-call system, whereby research staff is available for referrals 24/7. Our recruitment core is notified no more than 24 hours after any first episode psychosis individual enters the clinical system.

Healthy control subject recruitment. Healthy control subjects will be recruited from the local Pittsburgh community, with advertisement targeting the students of the University of Pittsburgh, adjacent Carnegie Mellon University, other nearby colleges and Pittsburgh public and private high schools. Control subjects will be matched to patients on parental socio-economic status, gender, age, IQ and handedness. The Office of Clinical Research (OCR) at the University of Pittsburgh also offers a website that lists research studies that are actively seeking volunteers. Flyers and notices are posted on physical and electronic bulletin boards and, if necessary, advertisements will be placed in local newsletters and circulars. We also accept referrals from other research studies and we utilize referral chains, whereby participants are asked to identify other potential participants.

Clinical and diagnostic assessments. In addition to detailed demographic information, we obtain diagnostic information using the Structured Clinical Interview for DSM-IV Disorders (SCID, changing to the DSM5 when available) and childhood disorders using the Kiddie SADS, as well as a measure of general intelligence

(WASI). Linked to the assessment program is a diagnostic review process that utilizes information obtained at baseline and the 6 month follow-up [Diagnostic and Disposition Conferences (DDC)] to ensure that diagnosis is based on both information from structured interviews as well as the consensus review of expert diagnosticians, using the LEAD (Longitudinal, Expert, All Data) standard advocated by Spitzer[105]. Psychopathological ratings are conducted in the psychotic subjects only and include the Positive and Negative Syndrome Scale (PANSS), the Scale for the Assessment of Positive Symptoms (SAPS), the Scale for the Assessment of Negative Symptoms (SANS), the Hamilton Depression Rating Scale (HDRS), the Bech-Rafaelsen Manic Rating Scale, the 7-point Clinical Global Impression (CGI) Scale, the Global Assessment Functioning (GAF), the Global Functioning: Social and Role Scales, the Social Functioning Scale (SFS) and University of California Performance-based Skills Assessment (UPSA-Brief).

HUMAN SUBJECTS

1. Risks to the Subjects

1.a. Human subjects involvement and characteristics. A total of 120 subjects (80 first episode psychosis and 40 healthy controls) 14 to 30 years of age will be tested throughout the 5 years of the project. Our primary aim is to compare 40 first-episode *schizophrenia spectrum* (schizophrenia, schizophreniform disorder and schizoaffective disorder) subjects to 40 matched controls. Because definitive diagnoses cannot be made at first episode, our recruitment cohort will include schizophrenia-psychosis spectrum, affective psychosis and psychotic disorder NOS. As such, the recruitment process will necessarily generate a cohort of non-schizophrenia psychosis subjects who can serve as a psychosis comparator group for secondary analyses. To meet our schizophrenia subject recruitment goals, we will aim to recruit a total of 80 first-episode psychosis patients and ensure that a majority of these subjects will be schizophrenia spectrum patients by requiring that the Criterion A of the DSM criteria for schizophrenia be met at screening (i.e. at least two symptoms, including delusions, hallucinations, disorganized speech, disorganized/catatonic behavior, negative symptoms, for one month period). This strategy will allow us to acquire the targeted number of antipsychotic naïve first episode schizophrenia patients, with the added benefit of secondary exploratory analyses examining cross-diagnostic dimensions (e.g. WM performance).

Subject testing will occur at the University of Pittsburgh School of Medicine. Participants will be screened by interview to ensure study eligibility (see inclusion/exclusion criteria below), including characteristics that would disqualify them from MRI testing. Medical status and history will be obtained by interview. Demographic, diagnostic, clinical, neuropsychological and neuroimaging assessments will be administered as per the protocol. All participants will be in good medical health and will have no known history of head injury or illness with CNS implications. All participants will be free of medications that affect blood flow response or alertness. Smoking and coffee consumption are prohibited within 2 hours of laboratory testing because of established effects on blood oxygen level dependent measures during fMRI. Participants will have at least 20/40 far acuity (either uncorrected or corrected) and no eye movement abnormalities (e.g. strabismus, amblyopia). For fMRI studies, participants will fulfill these additional criteria: no recent antihypertensive agents, no cardiac pacemaker, aneurysm clip, cochlear implants, pregnancy, IUD, shrapnel, history of metal fragments in eyes, neurostimulators, weight of 250 lbs. or more, or claustrophobia. Sedation will not be used.

We expect to have 20 first episode and 10 control subjects tested per years 1 to 4, but to ensure that we will have adequately powered datasets, we have allowed for “makeup” testing in year 5 to ensure all cells are full as proposed. These numbers are consistent with our experience in recruiting for the Department of Psychiatry’s long-standing first episode studies. Since July 2008, 85 eligible first-episode psychotic individuals have been recruited for studies in the Department of Psychiatry. The majority (n=56, 66%) were antipsychotic naïve at time of consent. 44 have received a schizophrenia spectrum disorder, with the remaining being psychotic disorder NOS (n=18), delusional disorder (n=2), or a mood disorder with psychosis (n=14). Seven await diagnostic review at the current time. Based on this diagnostic distribution, we will recruit 20 first-episode patients per year, anticipating that 10/year will fall in the schizophrenia spectrum. Although we anticipate high diagnostic stability, we will also conduct follow-up confirmatory diagnostic interviews at 6 months. Over the past 10 years, diagnostic stability has been maintained at approximately 90% with only 10% of subjects moving between schizophrenia spectrum and other diagnoses. The non-schizophrenia spectrum subject data will permit secondary analyses of diagnostic specificity as well as dimensional analyses across all diagnoses. We will also focus recruitment on younger subjects, 14-30 years of age. While we have historically recruited from a broader age range (12-50 years), the vast majority of subjects have fallen in this younger age range. Targeting this narrower range will help to reduce subject heterogeneity, setting the stage for future

investigations of developmentally relevant questions, while still being broad enough to accommodate the recruitment of female patient subjects who tend to have later age of illness onset. Our prior recruitment experience supports the feasibility of this approach.

Inclusion criteria. 1) First-episode psychotic patients: a) ages 14-30 years, b) first episode of a psychotic illness, defined by report of symptoms and/or history of treatment, and c) no current antipsychotic treatment and less than 2 months of lifetime antipsychotic treatment or taking medications that interfere with brain function. 2) Healthy comparison subjects: a) ages 14-30 years, b) no lifetime history of Axis I disorder, and c) no first-degree family history of schizophrenia spectrum Axis I disorder or mood disorder with psychotic features.

Exclusion criteria. General (regardless of group): a) DSM-IV mental retardation; b) significant head injury; c) medical illness affecting CNS function or structure; d) pregnancy or postpartum (<6 weeks after delivery or miscarriage); e) significant neurologic disorder (e.g., seizure disorder); f) inability to provide informed consent. For first-episode psychotic patients: a) a psychotic illness with a temporal relation to a substance use disorder; b) co-morbidity for DSM-IV psychoactive substance dependence within the past six month; c) substance abuse - other than cannabis and/or alcohol - within the past one month; and d) temporal relation between illness onset and head injury. 2) Healthy comparison subjects: a) treatment with a antipsychotic at any time; b) co-morbidity for DSM-IV psychoactive substance dependence within the past six month or substance abuse within the past one month.

1.b. Sources of materials. Sources of research material will be obtained by subject interview of psychiatric history, neuropsychological testing and neuroimaging data as described in detail in the *Research and Methods* section. The data obtained will be used for research purposes only. Only research staff with a "need to know" will have access to individually identifiable private information about human subjects. A review of existing clinical records is conducted to aid in the diagnostic assessment. All material will be identified by a code number to protect the confidentiality of the subjects. All information obtained from subjects is coded and kept locked in confidential paper and electronic files.

1.c. Potential risks of study and protection of subjects. The anticipated risks of participation are minimal. The risks of paper and pencil tests are minimal and are limited to performance anxiety or fatigue. To minimize these problems, testing will not begin until the subject is comfortable with the office and the tester. The same technician will perform all tests, provide breaks as needed, and adjust the length of the test sessions to each individual. Participants will be provided with frequent positive feedback and each examiner will be attentive to subject fatigue and provide breaks accordingly. There may discomfort in revealing personal information regarding psychiatric and medical status, hence, we will assure participants that personal information is kept in a locked cabinet with no personal identifiers on the forms except for a subject number. All records are subject to standard confidentiality procedures followed at the University of Pittsburgh. Information will not be released from these records to any party except with the written consent of the subject. Subject identity will be concealed in data records and files by use of assigned code numbers. Only those code numbers will appear on any data sheets and documents used for statistical analysis. Research data will be maintained in locked storage cabinets in a locked suite. If any new information becomes available to the investigators during the course of the research which may affect the subject's willingness to participate, each subject will be notified. Additionally, if any new adverse effects of the procedures are demonstrated in the future, every attempt will be made to notify participants about such possible risks.

The fMRI studies involve a 3.0 Tesla MR scanner and the MEG studies involve a Elektra Neuromag 306-channel MEG system, which conform to FDA safety guidelines. The fMRI scanner is identical to clinical scanners with the added features of echo-planar imaging which is FDA approved. Such scanners are available for patient studies at other major medical centers and have been used for a number of years without problems. There are no known biological risks due to exposure to magnetic fields from MRI exams using techniques such as those that will be utilized in this study. There is a potential risk of the main magnetic field attracting ferromagnetic objects toward the magnet. This risk is minimized by careful screening of participants prior to entry into the magnetically shielded room. Participants may experience some discomfort associated with the noise of the fMRI scanning. This is minimized by use of earplugs and sound padding over the ears. Anxiety and discomfort may be experienced from lying in the magnet during the fMRI scan. To address these problems, potential participants will be screened for claustrophobia during recruitment and again prior to the fMRI testing using a mock simulation scanner. The project staff member with whom they have become familiar will accompany each subject to the imaging center. The MR technologist will regularly provide information

about the progress of the examination and there is opportunity for communication during the procedure with the technologist. Sedation will not be used and the subject may abort the examination at anytime. Although there are no known risks during pregnancy associated with MRI scanning, the possibility that risks may be discovered in the future cannot be ruled out, hence we will exclude participation of pregnant female participants. Female participants of child-bearing age and their families will be informed, during initial verbal contact (i.e., during the telephone screen) with the research team, that a urine pregnancy test, conducted at the MR center on the day of scanning, will be a preliminary requirement for participation in MR research. The test will be provided and read by a trained MR registered nurse or physician who has extensive experience with the procedure, and with discussing its importance and possible consequences with young women and their families. Participants will again be reminded that their participation is voluntary and that they may withdraw at any time.

Risks associated with the fMRI procedures will be minimized by: 1) checking participants for possession of ferromagnetic objects prior to entry into the scan room, 2) use of adequate padding to ease discomfort and reduce noise, 3) verification that there are no contraindications to MRI (cardiac pacemaker, aneurysm clip, cochlear implants, pregnancy, IUD, shrapnel, history of metal fragments in eyes, neurostimulators, weight of 250 pounds or more, claustrophobia) and that all participants are medically stable, 4) heart rate and respiration will be monitored during the scanning procedure, 5) patients will be directly observed throughout the procedure, and 6) availability of trained medical personnel at all times. The 3.0 Tesla MR scanner meets FDA parameters for field strength, gradient switching, and RF power disposition for all FDA-approved acquisition schemes including echo-planar imaging. The power disposition parameters are verified on a phantom using the power monitoring system installed on the 3.0 Tesla scanner. All participants will have properly fitted earplugs with a noise reduction rating of 29 dB or greater, as supplied by the MR Research Center.

The Elekta Neuromag® MEG System (Elekta Neuromag Oy, Helsinki, Finland) is a completely non-invasive bioelectromagnetic measurement system for functional brain studies. The sensor system includes 306 MEG-channels and up to 128 EEG-channels, all registering the electromagnetic signatures of the intracranial ionic currents associated with brain function. The MEG-sensor unit in its floor-mounted gantry, the movable subject chair and bed, together with the patient audio-visual monitoring and stimulus delivery systems are contained in a magnetically shielded room MSR (IMEDCO AG, CH – 4614 Hägendorf, Switzerland). A full set of stimulus delivery and subject response equipment is integrated in the system using 16 trigger lines to allow flexible combinations of somatosensory, visual and auditory stimuli. Audiovisual stimuli are delivered using a dedicated PC with the E-prime presentation program [Psychology Software Tools, Inc. (PST), Pittsburgh, PA]. Panasonic PT-D7700 premium projector (Panasonic Corporation of North America, One Panasonic Way, Secaucus, NJ 07094) is used for visual presentations. The study participants will either sit [or lie] in the MSR with the head in a helmet that covers the entire head except the face. Brain magnetic fields will be recorded with the Elekta Neuromag 306-channel MEG system. Visual and two-way audio communication with the participant will be maintained throughout the session. Head position within the sensor array will be determined before the MEG session by registering the position of four indicator coils that are attached to the head of a subject: one high behind each ear, and one on each side of the forehead. For this purpose, a brief weak electrical pulse is sent to the coils. Subjects will perform oculomotor tasks similar to those performed in our behavioral laboratory and fMRI experiments (see above). MEG has no known risks to fetuses, children or adults, and the entire system includes necessary approvals for human use and is 510(k) cleared by the FDA. Occasionally, minor inconveniences may involve boredom and mild discomfort after trying to remain still during data acquisition. There is a risk that a subject may feel claustrophobic while in the MSR. Before the start of the MEG scan subjects will be asked to sit in the MSR and their comfort level will be carefully checked. The MEG technicians are in constant communication with the subject and they can choose at any time during the scan to stop the testing by talking through an auditory communicating device. If this occurs at any time during their participation, the subject will be removed from the MSR immediately.

For the EEG recording concurrently with MEG in the Neuromag system, the scalp must be lightly abraded at each contact point to reduce electrical impedance and acquire signals. We note that this is a standard clinical practice in EEG. There is a possibility of skin irritation from this procedure. This risk will be minimized by skilled technicians using care and avoiding undue pressure. Subjects will be encouraged to tell the technician of any discomfort. If the backup EEG systems are needed, both are high impedance systems that do not require abrading the skin.

2. Adequacy of Protection against Risks

2.a Recruitment and informed consent

First-episode subject recruitment. First-episode psychotic individuals will be identified through Western Psychiatric Institute and Clinic's inpatient and outpatient services, Allegheny county's re:Solve Crisis Network, UPMC hospitals, and College and University counseling centers throughout western Pennsylvania – referral sources who clinical staff have worked with for over 20 years. They have been educated regarding the importance of delaying medication, when possible, until an evaluation by study staff has been completed. There is an established on-call system, whereby staff is available for referrals 24/7. These resources and approaches aid in the recruitment and study of first-episode psychotic subjects. Our recruitment core is notified no more than 24 hours after any first episode psychosis individual enters the clinical system.

Healthy control subject recruitment. Healthy control subjects will be recruited from the local Pittsburgh community, with advertisement targeting the students of the University of Pittsburgh, the University of Pittsburgh Medical Center, nearby Carnegie Mellon University and Pittsburgh public and private schools. Control subjects will be matched to patients on parental socio-economic status, gender, age, IQ and handedness. The Office of Clinical Research (OCR) and the Department of Psychiatry at the University of Pittsburgh also offer websites that lists research studies that are actively seeking volunteers. Flyers and notices are posted on physical and electronic bulletin boards and, if necessary, advertisements will be placed in local newsletters and circulars. We also accept referrals from other research studies and we utilize referral chains, whereby participants are asked to identify other potential participants.

Informed consent. The University of Pittsburgh IRB has granted a waiver for referral sources to share the identity of a first episode psychotic individual with the recruitment core, as the core, as well as the specialized treatment program for first episode psychosis (STEP Clinic), are under Dr. Raymond Cho's direction. In practice, we ask the referring agent to obtain permission before the patient is approached by the core. Once permission is granted, the first episode patient is contacted immediately. Interested controls contact the office by telephone or email. Individuals who contact us by phone are provided with a brief description of the study, including its purpose, research procedures, risks and benefits, time commitment and compensation for study completion (which is included in an IRB-approved screening script). Eligible and interested respondents will then meet in person with the study coordinator to extensively review the consent document. Special attention is paid to assess the ability of subjects (and family members, if applicable) to understand and evaluate the risks associated with the study. Witnessed oral and written consent is obtained by both the participant and for minors, a parent or guardian. Assent for study participation by minors is required.

Participation in this research is completely voluntary. Only subjects who give their written informed consent, including parents for subjects under the age of 18, will participate in these studies. Informed consent is obtained before any research procedures are administered. Subjects may withdraw their consent at any time, without any effect on their clinical care.

2.b. Protections against risk. Please see **1.c** above.

3. Potential Benefits of the Proposed Research to the Participants and Others

There are no direct benefits to the participants except for a sense of satisfaction at contributing to scientific investigations that may promote a better understanding of schizophrenia. Participants and their parents often compliment us regarding the educational experience of participating in our studies. Patients will receive a thorough diagnostic evaluation, which will be available to their physician upon request and patient and healthy control subjects could potentially benefit by the physical exam and any labs that are performed as part of the study. Subjects' participation will contribute to the advancement of our understanding of the impairments in working memory and attention in schizophrenia, without known risk.

Participation in the research study may be of benefit to the participants with psychosis since this information may provide insight into the neurobiology, diagnosis, and possible treatments. Control participants may have various motivations and, hence, varying rewards for participation in the study. Some control participants may participate to learn about imaging research, while others may participate because they know a disabled individual or are committed to community service. Finally, some participants will be primarily motivated by the participant payment.

4. Importance of the Knowledge to be Gained

Knowledge generated from our work may increase our understanding of the pathophysiology of schizophrenia. This may help in establishing future treatments of this debilitating illness. The potential gain in knowledge far outweighs the limited and low risks involved in participation.

5. Data and Safety Monitoring Plan

All records obtained are subject to standard confidentiality procedures followed at the Western Psychiatric Institute and Clinic. Information will not be released from these records to any party, including the subject, except with the written consent of the subject. The screening, diagnostic, and neuropsychological tests are maintained in folders for each subject, stored in locked cabinets within a locked suite. When the data are transferred to data summary sheets, only the subject number appears on the sheet and it is entered into the database. A private data file is used for tracking subject flow and testing on a day-to-day basis; subject names are used in this system. This system is double password protected and is in compliance with IRB regulations. We continuously monitor both our procedures and our data to be sure we are in compliance with confidentiality guidelines. Research staff members are carefully trained about the critical nature of participants' confidentiality and about the procedures for respecting and maintaining it. They are instructed to file data immediately after they have been collected, never to leave unlocked or opened data files unattended, never to discuss participants' behavior outside of the research study, and never to mention the name(s) of participants except to other research staff on this project. They are also instructed on the procedures for assigning ID numbers and for filing data separately from participant-identification information. All staff members complete training modules on human participants' protection and an examination to obtain certification.

Dr. Luna will oversee all data and safety monitoring. She will hold weekly meetings with staff to discuss progress of the research study (data quality, timeliness, and participant recruitment), outcome and adverse event data and changes to the benefit-to-risk ratio that would change the design of the experiment, and issues related to confidentiality ensuring that procedures for obtaining information in a private manner were performed and that documentation was stored in the appropriate locked file cabinets. Dr. Luna will hold separate weekly journal meetings to discuss any new information relevant to this study. Dr. Luna will also be responsible for reporting any serious and unexpected adverse reactions within the time stated in the IRB Policy manual. Any information about the participants' participation in this study will be kept strictly confidential. The participants will be assigned an exclusive number code for all data, and all information in research files will be coded using that number rather than the subject's name. A name log will be kept in a protected database table, only accessible to those with a "need to know". All data will be entered into a secured database. Scientifically trained and properly authorized employees of the FDA and/or UPMC may inspect the relevant records. Dr. Luna will submit a written report indicating the procedures that were performed to ensure confidentiality, any adverse event data, any journal findings, a summary of confidentiality policies, and any new findings pertaining to data and safety monitoring at every renewal period. We are committed to complying with the IRB's policies for the reporting of serious and unexpected adverse events as delineated in IRB manual.

6. Inclusion of Women and Minorities

Females and males will be recruited in roughly equal numbers. In assessing the gender composition of first episode subjects recruited over the past 10 years, however, the distribution is approximately 60% male and 40% female.

The racial composition of first-episode subjects recruited in the same timeframe approximates the demographic composition of the city of Pittsburgh: 51% Caucasian, 43% African American and 6% American Indian, Asian, and Pacific Islander. Although the Hispanic population of Pittsburgh is approximately 1.3%, our sample over the past 10 years included 7% Hispanic. No one will be excluded based on race or ethnicity. Our Targeted Enrollment Report outlines the planned enrollment by gender, ethnicity and race, based on the demographic proportions of Pittsburgh.

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ADMINISTRATIVE ORGANIZATION OF THE CENTER

We believe that the scientific efforts of the investigators in the proposed Silvio O. Conte Center for Translational Mental Health Research are best supported by a simple organizational structure that minimizes the constraints of traditional departmental or disciplinary barriers while maximizing the opportunities for collaborative interactions. With this in mind, the organizational framework for the proposed Center is based primarily on the leadership of faculty from two departments at the University of Pittsburgh (the Department of Psychiatry in the School of Medicine and the Department of Neuroscience in the School of Arts and Sciences) and the Center for the Neural Basis of Cognition (which spans the University of Pittsburgh and Carnegie Mellon University.) Two major administrative/scientific committees, a Steering Committee and a Scientific Coordinating Committee, will support the Center Director in managing the activities of the Center. In addition, the members of an External Scientific Advisory Board will provide consultation and critiques of Center activities.

Center Director

Scientific and administrative leadership of the Center will be provided by the Center Director, David A. Lewis, MD. Dr. Lewis will have responsibility for the overall scientific and fiscal management of the Center, for facilitating the interactions of basic and clinical research investigators engaged in collaborative projects, and for promoting the training and outreach efforts of the Center. Dr. Lewis is the UPMC Professor in Translational Neuroscience, holds appointments as Professor in both the Departments of Psychiatry and Neuroscience, and serves as Chair of the Department of Psychiatry and Medical Director of Western Psychiatric Institute and Clinic. The research proposed in this application represents the principal focus of Dr. Lewis' scientific activities. Accordingly, he will commit 4.2 months of effort to the Center (1.2 months as Center Director, 1.8 months as Project Director of **P3**, and 0.6 months as a Co-Investigator for both **P1&2**). The scientific focus of the Center reflects the substantial influence of Dr. Lewis' research activities on those of his colleagues, as well as the recognition and support of his leadership abilities by the other members of the Steering Committee.

Steering Committee

Dr. Lewis will be assisted in his role as Center Director by the members of a Steering Committee who will provide 1) advice on major programmatic and financial decisions that affect the Center as a whole, 2) assistance in the coordination of Center activities with other components of the Pittsburgh neuroscience community and 3) an interface with the relevant administrative units at the University of Pittsburgh and Carnegie Mellon University. In addition to Dr. Lewis, the members of the Steering Committee include Robert Friedlander, MD, Chair of the Department of Neurological Surgery; David Kupfer, MD, Thomas Detre Professor of Psychiatry; Peter Strick, PhD, Chair of the Department of Neurobiology and Co-Director, Center for the Neural Basis of Cognition (University of Pittsburgh); Alan Sved, PhD, Chair of the Department of Neuroscience; and Michael Tarr, PhD, Co-Director, Center for the Neural Basis of Cognition (Carnegie Mellon University). In addition to providing their considerable scientific and administrative expertise, these individuals will ensure that faculty in their respective areas of responsibility have the time and research facilities necessary for their productive involvement in the Center. Dr. Lewis will meet regularly with all or a subset of the other members of the Steering Committee to review progress and to plan new initiatives. Moreover, they will meet periodically with the Scientific Coordinating Committee to critically evaluate ongoing experiments and progress against milestones (see Table 3 in **Center Plan**). All members of the Steering Committee have confirmed their willingness to serve.

Scientific Coordinating Committee

The Scientific Coordinating Committee consists of the leaders of the proposed research projects (Drs. David Lewis, Etienne Sibille, Robert Sweet, Carl Olson, Beatriz Luna and Dean Salisbury) and the director of the Stats/DM Core (Dr. Allan Sampson). This committee will meet monthly to review the research activities of the Center. Formal presentations by each project will permit the group to critically evaluate the progress of ongoing experiments and to facilitate communication and collaboration across the projects. The major objectives of the Scientific Coordinating Committee are to support and encourage high-quality research within the Center, to keep all research teams fully aware of each other's work, to enhance the flow of information between basic and clinical research teams, to nurture the *esprit de corps* of the Center as a collective entity, and to coordinate training opportunities for pre- and postdoctoral students.

EVALUATION PLAN AND ADVISORY BOARD

An External Scientific Advisory Board (SAB) will review the progress of each project against the Center's milestones (see Table 3 in the **Center Plan**). The SAB will also provide advice regarding administrative decisions, goals in research and training, and the communal resources and needs of the Center. In addition, they will review the results of the separate research projects, advise the investigators regarding the interpretation and application of their findings in designing other studies, and suggest new directions of research. Although SAB members may be consulted individually throughout the year for their input on particular projects, the full SAB will meet annually to review the Center as a whole.

All members of the SAB will be selected in conjunction with NIMH staff for their eminence in areas of research relevant to the Center projects, and for their broad range of experience and mature scientific perspectives. Membership of the SAB will be adjusted as needed to obtain the types of expertise and critical evaluation that are most critical for the activities of the Center as it evolves. Per the Program Announcement instructions, we have not included the names of prospective members of our SAB. The costs associated with convening the SAB will be shared with institutional resources (see Administrative Core budget justification).

ACTIVITIES AND RESOURCES THAT ENHANCE AND EXTEND THE EFFECTIVENESS OF THE CENTER

Schizophrenia as a focus of scientific exchange

Center seminar series. To stimulate the collaborative interactions between basic and clinical scientists in the areas of research represented in this application, we initiated an evening seminar series. These two-hour meetings (which are always followed by a number of spontaneous smaller group discussions lasting 30-45 minutes or more) are attended by all Center investigators and take a variety of formats. For example, for one series, a primary speaker gave a presentation, focused on a single aspect of his research. Following this presentation, a designated discussant provided a 15-20 minute commentary and critique, emphasizing possible links to other areas of research. The remainder of the session was then used for general discussion. At these meetings, when the speaker was a basic neuroscientist, the selected discussant was typically a clinical researcher, and vice versa.

Translational Neuroscience Program seminar series. The Department of Psychiatry has also supported a weekly seminar series specifically devoted to issues involved in translational neuroscience in general, with a specific emphasis on schizophrenia. This series involves both Center investigators and speakers from other departments and institutions.

Website

In order to inform other scientists and the general public about the activities, research findings and resources of the proposed Center, we will establish a dedicated website (www.ctmhr.pitt.edu) that will 1) describe the overall goals and organization of the Center, 2) summarize recent research findings, 3) provide a list of publications, 4) describe new methods and protocols, 5) describe infrastructure resources, 6) announce upcoming conferences and presentations, and 7) include links to related sources of information.

Training and Novel Outreach and Dissemination Programs

Opportunities for young investigators. The proposed Center makes an important contribution to an academic environment committed to providing, at a number of points along the career development "pipeline," outstanding training for the next generation of translational neuroscientists through the following mechanisms: 1) Each of the proposed projects and **Stats/DM Core** involve pre-and/or postdoctoral trainees and/or junior faculty members in translational research experiences designed to enhance their professional development in addition to accomplishing specific experimental goals. 2) A unique training arrangement provided by the Center's **Administrative Core** is the opportunity for trainees at all stages and for basic neuroscientists to learn about schizophrenia and related disorders first-hand by participating in clinical activities (see **Appendix 2**). 3) A number of Center investigators are also involved in other training mechanisms that are linked to the Center. For example, Dr. Robert Sweet directs an NIMH training grant (MH016804) for postdoctoral trainees in transformative discovery in psychiatric disorders, and Dr. Haas directs an NIMH training grant (MH054318) for recruiting medical students to careers in mental health research. 4) As a group, we have a documented track record of recruiting minority and disadvantaged undergraduate students to engage in translational neuroscience research, and we plan to build on this success through our proposed **Undergraduate Summer**

Research Experience. 5) Early stage investigators will be able to develop new projects related to the Center and obtain pilot data for NIH grant applications through our proposed **Pilot and Feasibility Program**.

Outreach to clinicians and the public

Pittsburgh Schizophrenia Conference. The annual Pittsburgh Schizophrenia Conference, now in its 30th year, offers reviews of the latest advances in schizophrenia research by leading international experts. The conference is designed to disseminate the latest research findings to a wide audience of clinicians, researchers, patients and their relatives, and the lay public. Patients and family members are also always on the program to share their experiences and their priorities for future research efforts. The deliberate effort to provide the best scientific evidence has resulted in a regular attendance of between 400-500 persons per year.

Pittsburgh Search for Treatment in Early Psychoses Conference. This biennial conference with a focus on early intervention and prevention in psychotic disorders began in 1998 under the auspices of the Services for Treatment in Early Psychoses (STEP program) directed by Center investigator, Dr. Raymond Cho. This activity is designed to educate patients, families, professionals and mental health students regarding current themes in the detection, prevention and treatment of prodromal and early psychosis. The program includes national-known speakers, presentations by Center faculty, and panel sessions involving family members and patients. Typical attendance is 250-300 participants.

Community outreach. As part of our efforts to educate groups regarding the recognition of prodromal and early psychosis, our investigators and staff speak about mental illness at community groups in the tri-state area (Western PA, Eastern OH, Northern WV) including: Parent-Teacher Associations in area school districts; college counseling centers (e.g., University of Pittsburgh, Carnegie Mellon University, Carlow College, Point Park University, the Art Institute); health fairs (e.g., Center for Minority Health, Washington County Health Fair, Beaver County Health Fair, PITT Health Fair, Carlow College Health Fair, Chatham College Health Fair, Peabody HS Health Fair, Duquesne Minority Health Fair); local partial hospitalization programs (e.g., Mercy Behavioral, Washington Hospital), shelters (e.g., Bethany Shelter) and drop-in programs (e.g., Renaissance Center, Wellspring, New Horizons, People's Oakland, Mon Yough, etc); family groups at local community mental health centers; NAMI conferences and Consumer only groups (e.g., Consumer Conferences: Day of Discovery, Undependence Conference, Recover Conference, Angora Gardens, Families in Need Conference).

Educational outreach. With the assistance of the Pittsburgh Neuroscience Society (the local chapter of the Society for Neuroscience), Center investigators engage in a range of public outreach activities that provide a vehicle for communicating the goals and research findings of the proposed Center and the importance of translational research in schizophrenia. The activities include 1) An annual Pittsburgh Brain Bee competition that tests the neuroscience knowledge of high school students from the Pittsburgh area. 2) A Brain Program that entails visits by members of the Pittsburgh Neuroscience Society to Pittsburgh area middle and high schools to present a series of “stations” about the brain. Center investigators and trainees have developed and staff a station focused on schizophrenia. 3) Neuroscientist-Teacher Partnership Day, which is a day-long event during which middle school teachers from the Pittsburgh area listen to lectures on basic concepts in neuroscience, visit neuroscience labs, and are paired with members of the Pittsburgh neuroscience community to develop teaching tools and hands-on projects to help the teachers bring neuroscience into their class rooms. Center investigators are involved in presenting a lecture on schizophrenia and providing assistance to teachers in developing schizophrenia-related neuroscience projects that can be done in class rooms.

Special projects. To facilitate communication of the status and promise of schizophrenia research to a range of trainees, scientists and the general public, Center investigators also participate in special projects. These include speaking appearances at local and national NAMI conferences, and on televised specials (e.g., Charlie Rose show), and service on national scientific advisory boards (e.g. International Mental Health Research Organization, Brain and Behavior Foundation, SFARI, etc).

DIVERSITY RECRUITMENT PLAN

The Department of Psychiatry remains committed to the recruitment of underrepresented minorities including racial minorities, persons with disabilities, and those of disadvantaged backgrounds. The Department’s overall approach emphasizes “early recruitment” with a strong focus on identifying talented underrepresented minority trainees with an eye toward eventual faculty appointments. This strategy is implemented by 1) assigning senior faculty members to serve on School of Medicine and UPMC committees related to diversity; 2) asking our training directors to actively participate in initiatives that foster a more inclusive environment for

underrepresented minorities within our Department; and 3) dedicating time within the Department's training programs to include minority-relevant content when issues such as launching and maintaining clinical and academic careers, career development and professional enhancement are addressed.

Our strategy of "early recruitment" is implemented in the following ways: 1) Our approach to recruiting undergraduates to consider a career in mental health research is described in the **Undergraduate Summer Research Experience** section below (see also **Appendix 3**). 2) Department of Psychiatry faculty and residents conduct psychiatry sessions for minority high school and college students through the Medical Explorers and the Summer Premedical Academic Enrichment programs. 3) Of the medical students from other institutions who applied for travel grants to do clinical electives in our department, 40% were from underrepresented minority groups. 4) We develop our current minority faculty members by supporting their participation in career development activities, including the **Pilot and Feasibility Program** described below. Consistent with the Department's philosophy of maintaining a strong "internal pipeline" for faculty recruitment, we expect the efforts described above to enhance our recruitment of underrepresented minority scientists and physicians.

PILOT AND FEASIBILITY PROGRAM (Optional funding not requested)

Although no funding is requested for this program, the Center will provide a means for Early Stage Investigator (ESI) investigators to develop preliminary data using support provided by The Pittsburgh Foundation (see Letter of Support). These projects will leverage Center resources, such as research subjects (**P5**) and tissue samples (**P1**&**P2**), and will contribute to the goals of the Center by advancing methods relevant to Center projects and by pursuing promising new leads suggested by findings from Center investigators. Applications, containing biosketch, specific aims (1 page), approach, significance and use of Center resources (3 pages), may be submitted by ESI faculty members. Applications are reviewed for scientific merit by the Scientific Coordinating Committee using the NIH scoring system and recommendations for funding are approved by the Steering Committee. IRB and/or IACUC approvals are required before funds are provided. One example of a potential ESI project is summarized in the following paragraph.

Complex brain disorders, such as schizophrenia, are seen as reflecting circuit-level brain pathology, involving the interactions of multiple brain regions. Of particular interest is how the precise interregional synchrony of these interactions is disturbed in the disorder (see **P4**&**P5**). However, current methods for measuring brain synchrony have two known limitations: 1) Interregional interactions can be modulated by purely local changes in brain activity (termed attenuation of correlation) that can result in false positives; 2) Traditional measures may be relatively insensitive to certain types of group differences in interregional interactions leading to false negatives. To address these limitations, this project will develop a high order autoregressive statistical model that will provide a framework to overcome these two limitations.

UNDERGRADUATE SUMMER RESEARCH EXPERIENCE (Optional funding requested)

Specific Aims

To recruit talented students into graduate training for careers in mental health research, the proposed Undergraduate Research Program will provide early-career, hands-on engagement in cutting-edge translational neuroscience research focused on schizophrenia. Under the mentorship of Center and related neuroscientists at the University of Pittsburgh and Carnegie Mellon University, and through educational, clinical exposure and career counseling experiences, student participants will be exposed to the exciting challenges and opportunities of a career in mental health research. ***The aims of this program are to 1) increase the number of talented students who enter and complete doctoral (MD/PhD and PhD) programs that prepare them for careers in translational mental health research; 2) recruit talented disadvantaged and minority students to pursue careers in mental health research; and 3) provide a foundation for success in the subsequent stages of education, training and career development***. Methods to achieve these aims include 1) early identification and recruitment of promising students; 2) personal mentoring by highly successful clinical and basic neuroscientists; 3) hands-on engagement in an intensive and longitudinal program of research; 4) exposure to the clinical challenges of schizophrenia that motivate translational research; and 5) instruction in the skills for a successful research career.

Program structure

Student recruitment. Fifteen freshman and sophomore students will be recruited/supported each year of the proposed Center funding period. This goal is feasible given that more than 1300 students currently major in neuroscience, chemistry, biological sciences or psychology at Pitt and CMU. Trainees will be selected by the Selection and Advising Committee, comprised of the Program Director, Program Co-Director, a member of the Center training faculty, and the Program Coordinator, based on 1) academic record; 2) personal statement; 3) recommendation letters; and 4) interviews with committee members.

We will specifically encourage members of under-represented minority groups, individuals with disabilities and individuals from disadvantaged backgrounds (i.e., students from low-income families, from rural or inner-city environments, or who are first generation to attend college) to apply. Based on past experience we expect that across years the selected trainees will include approximately 50% women, 25-30% disadvantaged students, 25-30% under-represented minorities, and 5% individuals with disabilities. To achieve these goals, we will: 1) Coordinate with University-wide recruiting functions including (a) Honors College programs that target the recruitment of talented science students from local and rural Pennsylvania high schools; and (b) Office of Diversity Programs, which recruit under-represented high school seniors and college freshmen to strengthen their academic skills and focus their interest on a medical career. 2) Identify promising students through the career counseling staff in the Office of Student Affairs. 3) Advertise our program to the >40 student organizations that promote the multi-ethnic life of students at Pitt and CMU, including Pitt's Pre-medical Organization for Minority Students (POMS). 4) Advertise our program at nearby universities, including Carlow and Duquesne Universities, and Point Park College, which have traditionally had higher proportions of female, minority and disadvantaged students.

Personal mentoring. The probability of recruiting outstanding talent into mental health research careers is substantially increased when seasoned neuroscientists inspire young trainees, work with them on a jointly-chosen research project, and serve as role models. With the guidance of the Selection and Advising Committee, program applicants select their top three research mentors from among the Conte Center-funded and affiliated training faculty. Students are matched to an investigator/mentor based on their interests, faculty availability and project feasibility. Due to the multiple collaborations that exist among Center investigators, students will be exposed to, and may work in, the laboratories of their mentor's collaborators. Mentoring begins with a full-time 10 week research experience during the summer following the freshman or sophomore year. Interested students will be encouraged (and supported with institutional funds) to continue their research project during the school year and the following summer. Mentors will 1) work directly with their trainees in all phases of the research project, 2) have regular one-on-one meetings with them to discuss project details and career development, 3) provide a brief reports of student progress at the midpoint and end of the summer term, and at the end of the academic term, research experiences and 4) facilitate the interactions of the trainee with other members of the mentor's lab.

Research activities. We believe students are inspired to pursue a research career by the intellectual challenges encountered in their projects, the quality of their research experience, the performance expectation as junior partners in a specific project, and the success and associated emotional tone of their research efforts. To provide this type of inspiration, students will work with one or more Center scientists on a project designed to enable the student to 1) participate in the process of translating an idea into an experiment, 2) learn the skills to execute the experiment, 3) analyze data and interpret findings, 4) consider the findings in relation to published work and 5) give a formal presentation of their work to Center faculty and other trainees, and at a national research meeting. Students will begin their research activities during the summer following the freshman or sophomore year, working 40 hours per week. With approval from the mentor and Program Director, students will continue to work 10-15 hours per week in the lab during the following school year, with the option of using their research experience as the basis for an undergraduate honors thesis.

Clinical exposure activities. We believe that the motivation to pursue a career in mental health research, and in translational research in schizophrenia in particular, is greatly enhanced by a first-hand understanding of the real-life challenges that accompany the illness. To build this understanding, trainees will participate in 7 different types of clinical activities under the direction of Dr. Debra Montrose. These activities (each lasting 2-4 hours) provide first-hand exposure to the different illness phases of schizophrenia, the challenges of managing schizophrenia in different living environments, the diagnosis of schizophrenia, and the lifetime outcomes associated with the illness (see **Appendix 2**).

Career development activities. Each summer, weekly training sessions, tailored for undergraduate students, will provide the fellows opportunities to discuss research career development with program leadership and

selected faculty. Two meetings will be devoted to discussion of ethical issues in research, including research integrity and maintaining lab notebooks. Two additional sessions will involve discussion of trajectories and resources for careers in biomedical research, giving the students an opportunity to interact with individuals at different training and career stages. Students will also participate in a weekly journal club. At these meetings, two students will each present on a journal article related to the research they are conducting in their mentors' labs, and lead a discussion on the strengths and weaknesses of the paper. Journal club discussions will be facilitated by Program Co-Director Dr. John Enwright. In addition, students will be invited to have one-to-one meetings with the Program Director to discuss career interests and goals, and pathways for reaching these goals.

Feasibility of proposed program

Our ability to successfully conduct the proposed program is demonstrated by our past success in conducting similar programs funded by the NIMH and Commonwealth of Pennsylvania. For example, over a four year period, we selected 37 students from a total of 121 applications. With an average GPA of 3.7, the group included 33% under-represented minority, 35% disadvantaged and 51% female students.

COST SHARING AND INSTITUTIONAL SUPPORT

The University of Pittsburgh and the Department of Psychiatry have covered the costs of the method development and pilot studies described in the individual projects and provided dedicated funds for required instrumentation. For the proposed studies, they will provide funds for maintenance of all equipment to be used. In addition, the Department of Psychiatry will cover 1) salary support as needed for the postdoctoral scholar/associates in **P2**&**P5**; 2) travel and honorarium costs for the External Scientific Advisory Board meetings; 3) all costs associated with the annual trips of the Center Director and selected Center Scientists to the NIMH to discuss progress and achievements toward the scientific goals of the project as well as education, outreach, and diversity recruitment efforts as indicated in the Program Announcement; 4) salary costs for Summer Undergraduate Research Program Co-Director, John F. Enwright, PhD and 5) stipends of up to $1,000 for each of the Fall and Spring 14-week academic terms to enable the Summer Undergraduate Research Program trainees to continue their research projects during the school year. In addition, The Pittsburgh Foundation has generously agreed to provide full support (up to $150,000 annually) for the Pilot and Feasibility Program for Early Stage Investigators (see Letter of Support).

RESOURCE SHARING PLAN

All resources generated by Center investigations (e.g., data sets acquired through Center studies, detailed protocols for new methods, software and other research tools developed by Center investigators) will be announced on the Center website (www.ctmhr.pitt.edu) and made available per NIH policy. According to NIH guidelines, the data to be obtained from the proposed experiments in the experimental animals (**P3**&**P4**) do not represent unique data, and the publication of the final data will constitute an acceptable mechanism for sharing data. Second, findings from the proposed studies in postmortem human brains (**P1**&**P2**) represent the type of data that need to be independently replicated by other investigators in other subject cohorts. Following such studies, the conduct of meta-analyses may be informative to the field. Thus, under the auspices of the principal investigators, the data spreadsheets containing the raw quantitative data for each subject (without identifiers) will be made available following the acceptance for publication of the final data set. The existence of these data sets and the mechanism to access them will be posted on the Center website. In addition, as described in the database management section of the **Stats/DM Core**, a gene/miRNA database will be established to bridge the human data from **P1** and monkey data from **P3**, and made available. Whole brain data from the MEG/EEG and fMRI studies of **P5** will be made available after publication of the results from the a priori regions of interest analyses that directly test the project's main hypotheses. The available data will include information generated from brain regions that were not the focus of the initial published analyses. Investigators interested in analyzing the data are asked to submit a summary of the proposed analyses, the hypotheses to be tested and the desired data format. The proposal will be reviewed for scientific merit and feasibility by the Scientific Coordinating and Steering Committees. The requesting investigator is provided feedback on the proposed study including costs associated with formatting the data into the requested configuration. Subsequently, an amendment will be submitted to the University of Pittsburgh Medical Center Institutional Review board for approval of the data transfer. An identical approach is used for all investigators, independent of whether they are members of the proposed Center, a member of a different program at the University of Pittsburgh, or from a different institution.

RATIONALE

The Statistics and Data Management Core (Stats/DM Core) will 1) provide statistical support to investigators conducting Center research studies and 2) maintain the databases required by projects. Core and Project investigators collaborate on experimental design, statistical modeling and analyses, and statistical graphics and presentations in Projects 1-5. These capabilities include design support ranging from broad conceptual support to detailed power calculations; analytic support ranging from advice on appropriate statistical models to conducting complex statistical analyses; and presentation support ranging from graphical advice to co-authoring papers and abstracts. In addition, the Stats/DM Core will develop new statistical methodology to enhance the research conducted in the Center, and provide training opportunities for graduate students in statistics and all levels of trainees in neuroscience and psychiatric research. The Stats/DM Core data manager will work with the Department of Psychiatry's Office of Academic Computing and Center investigators to develop databases that facilitate the integration of results across projects (e.g., organized and retrievable databases for mRNA, miRNA, functional annotation and regulatory network data from **P1**&**P3**).

The statistical support provided for Center investigators will be established jointly by the investigator, the Stats/DM Core statistician and the Stats/DM Core Director (Dr. Allan Sampson), and will depend on the experimental complexity of the project, the project's similarity to previous projects, and the statistical experience of the investigator. In addition, the Stats/DM Core has the capabilities to develop innovative statistical methodology to advance the Center's research when current methods are not fully effective. The Stats/DM Core's development of such methodology would not only enhance the Center's research but also benefit the broader neuroscience community when encountering similar statistical issues.

PERSONNEL

Allan Sampson, PhD (Core Director), George Tseng, PhD (Core Co-Investigator) and Kehui Chen, PhD (Core Co-Investigator) will be the primary University of Pittsburgh statistical collaborators for the Center's investigators. Dr. Sampson's primary academic appointment is Professor, Department of Statistics, with a secondary appointment in the Department of Biostatistics; Dr. Tseng's primary appointment is Associate Professor, Department of Biostatistics with secondary appointments in the Departments of Human Genetics and Computational Biology; and Dr. Chen holds a joint appointment as Assistant Professor, Departments of Statistics and Psychiatry. Drs. Sampson, Tseng and Chen will actively consult with Center Investigators, and for selected studies provide more extensive statistical design and methodological support, including supervising the study's data analysis. Dr. Sampson is an internationally recognized statistical methodologist who has extensive experience in applications to neuroscience and psychiatry. Dr. Tseng is a nationally recognized expert in statistical approaches for genomics and bioinformatics. Dr. Chen brings her expertise in functional data analysis, high-dimensional data analysis and applications of statistics to neuroscience. The members of the Stats/DM Core collectively bring a broad range of established statistical expertise and experience to the designs and analyses of the Center's studies.

The University of Pittsburgh's Department of Statistics and Department of Psychiatry have a successful relationship of longstanding duration. In addition to Dr. Chen, two other faculty members in the Department of Statistics also have joint appointments in the Department of Psychiatry. The Department of Biostatistics has also extensively collaborated with the Department of Psychiatry on multiple research programs. Satish Iyengar, PhD and Lisa Weissfeld, PhD will serve as University Consultants in the Stats/DM Core (see Letters of Support). Dr. Iyengar brings a broad range of expertise in neural modeling and also meta-analytic studies in psychiatry. Dr. Weissfeld brings general expertise in imaging modeling and analysis and neuroimaging, in particular.

One to two senior statistics graduate student researchers (GSRs) from the Department of Statistics will devote a combined total of 12.0 calendar months to the project. Under the supervision of Drs. Sampson, Chen and Tseng, the GSR(s) will assist with designing studies, computerized data analyses and preparing manuscripts. As required, the GSRs will also be involved in developing any needed new statistical methodology. During peak workloads for the Center, the Statistical Consulting Center (a student training center) of the Department of Statistics and the Biostatistics Consulting Service (a student training service) will both be available. When projects could benefit from access to additional statistical expertise from the greater Pittsburgh statistical community, including other departments at the University of Pittsburgh and Carnegie-Mellon University, the Stats/DM Core will serve as a referral and coordination resource for Center investigators.

HISTORY OF RESEARCH INTERACTIONS WITH CENTER INVESTIGATORS, CAPABILITIES AND TRAINING

Dr. Sampson (Core Director) has an extensive history of joint publications with a number of the investigators in the proposed Center (>30 publications, with 90% of them including a Department of Statistics GSR as co-author; see Dr. Sampson's biosketch). A number of Statistics GSRs are currently working on studies with some of the proposed Center Investigators under the supervision of Drs. Sampson and Chen. Dr. Tseng also has a number of publications with proposed Center investigators; many have involved and continue to involve Department of Biostatistics GSRs. All of the GSRs have substantively contributed to these investigators' research. They also wrote doctoral dissertations in statistics and in genomics which were motivated by and applicable to the scientific research of these investigators and also applicable for several of the Center's proposed projects.

Previous GSR dissertations were based on collaborative work with some of the Center investigators. The following are some examples of this research. Multivariate methodology was used to analyze studies in which neuronal measures arise from two different populations of neurons in the target region which are not physically distinguishable[1,2]. A novel statistical mixtures model was developed for such repeated measures and used to estimate the diagnostic neuronal differences for each of the different populations of neurons with an application to schizophrenia research[3]. Progress toward the longer-term goal of clustering subjects with schizophrenia on the basis of postmortem brain tissue studies was made with the development of methods for structured multivariate data that arise naturally in this setting[4,5]. Adaptive stereological designs were introduced[6] for postmortem tissue studies that control Type I error and typically maintain power with a reduction in expected use of subjects. Novel methodology was obtained[7] to adjust for matching and covariates in linear discriminant analysis. Building on genomic meta-analysis methods of Tseng[8,9], a novel model with variable selection and meta-analysis was developed[10] to combine multiple transcriptomic studies in major depressive disorder with a case-control paired design having weak signal and large numbers of confounding co-factors.

The Center's projects provide multiple statistical research opportunities for GSRs. Innovative methods to identify and validate possible cluster types and their relative frequencies common to multiple DSM diagnoses would be beneficial for Center's projects, as well as other settings. These methods may depend on whether considering paired post-mortem tissue data, in vivo human performance data or imaging data. With multimodal imaging data, opportunities and challenges are presented for developing suitable machine learning and functional data analysis methods to integrate various measures of functional impairment with cognitive deficits in subjects. For postmortem tissue data, the RDoC paradigm raises sampling issues when constructing matched multiple disorder designs that provide low-variance unbiased estimators of "clinic level" population characteristics. Similar, but more complex concerns occur for mRNA studies. We expect that the interplay of the Center project results and RDoC concerns will identify further statistical research opportunities.

DESIGN AND STATISTICAL METHODOLOGY

Details concerning design and statistical analysis approaches for each Project follow. Each statistical presentation reflects the primary themes of the Center concerning relationships of neuronal properties among brain regions as impacted by diagnosis in **P1, P2** and **P5**, with related issues in **P3** and **P4**. The analytics vary in complexity depending on the nature of the Project so that statistical detail in each subsection varies. For some projects (e.g., **P1**, **P2** and **P5**) there may be interest to identify whether subpopulations of subjects are present within or across DSM diagnoses. See Volk et al[11] for an example of a clustering/subpopulation approach in schizophrenia. When germane, relationships of results across projects will be examined (e.g., **P1**&**P2, P1**&**P3**, or **P4**&**P5**). Individual study design considerations are typically based on pilot or previous related data, with power computed using conservative statistical models. Our philosophical approach to statistical analyses is hierarchical where all analyses start with both numerical summaries and graphical displays. Modeling begins with the most parsimonious models, and then, as appropriate, models are expanded to include more subtle explanatory effects. Throughout, modeling diagnostics are used for validation and also to inform data transformations when necessary. One key statistical principle adhered to throughout is evaluating experimental results for robustness, which involves examining alternative models or analysis methods and demonstrating that they produce results similar to the primary analysis. In each subsection, we highlight the main statistical models and hypotheses. For all hypotheses, p-values will be accompanied by suitable confidence intervals to quantify the estimated effect and its variability. To be conservative, all alternative hypotheses and confidence intervals are expressed as 2-sided even when the scientific hypothesis is one sided.

Project 1-Sibille

CEL files from mRNA and miRNA arrays will be processed using the manufacturer's Expression Console package and nCounter® Analysis System packages. Extensive controls will ensure the quality of retained scans and the absence of potential batch effects. For each given gene or miRNA, the difference of expression intensities between the schizophrenia subject and paired control will be modeled with brain region as a factor, other confounding variables as covariates and accounting for the 3 brain regions measured within subject. We test the null hypothesis that no brain region has a significant diagnosis effect versus at least one brain region has a significant diagnosis effect. Since the number of potential confounders (expected at 10) can be relatively large relative to the sample size (n = 50 subject pairs), we will adopt a variable selection scheme that we previously developed[10] where we search through all possibilities of no more than 2-3 confounding variables and choose the model with the greatest Bayesian Information Criterion (BIC). Due to the model selection bias, the p-values will be re-assessed via conventional genome-wide permutation analysis where expression values of cases and controls are randomly permuted. Finally, the false discovery rate will be controlled by the Benjamini-Hochberg procedure at 5%. Our previous studies have shown the feasibility and improved accuracy of this strategy in detecting candidate markers in major depressive disorder[10] and for a currently in progress schizophrenia project.

In the noted model, candidate markers are detected if any brain region shows a significant difference from the other regions. As a result, genes with different regional patterns of expression can be discovered in an unsupervised manner. To obtain gene modules of major distinct patterns, post hoc cluster analysis is used with the K-means algorithm (with Mahalanobis distance instead of Euclidean distance to account for repeated measure dependence). The Gap statistics[12] are applied to estimate the number of gene modules in the detected markers. Modules with disease differences, region differences and disease by region changes (see Aim 1, **P1**) will be investigated. The analysis will be performed in PCs (Aim 1) and PV cells (Aim 2) separately and an in-house meta-analysis method using adaptive weighting[13] will be applied to investigate the commonality and differences between cell types. As an alternative approach to the unsupervised method, we will also investigate potential gene markers highly correlated ($|r|>0.6$) with a variable of interest (e.g. somal size) that passes the decision-tree-type hypothesis tests described under **P2** below and perform related functional annotation. This will elucidate pathways potentially related to the phenotypic variables.

Gene set enrichment analysis[14] will be applied to each gene module matching our central hypothesis to identify associated biological pathway annotation. For each identified miRNA in the gene modules, we will apply a new ComiR system[15] that integrates and improves existing miRNA target gene prediction databases (PITA, miRanda, mirSVR and TargetScan). The predicted targets of each miRNA will be verified from the data using this system with functional annotation to understand their potential functional roles in mediating the central hypothesis.

Gene coexpression network analysis will be performed as described in Aim 3, **P1** to identify alterations in measures of network conservation (e.g. connectivity and clustering coefficients in Aim 3, **P1**), information transfer efficiency, off-hub functional players and potential leads to therapeutic targets. In addition to the approaches proposed in **P1**, other bioinformatics approaches and packages, such as Ingenuity Pathway Analysis (IPA), MetaCore, Weighted Gene Co-Expression Network Analysis (WGCNA), Cytoscape, Connectivity Map, Bayesian networks and graphical models, will also be tested and compared. The integrative analysis will generate enhanced knowledge about subcellular location, functional gene family, association with drugs, pathways and known connections to diseases. The supported visualization and network graphs also enhance further mechanism understanding and hypothesis generation.

We used the "sizepower" package in Bioconductor to calculate statistical power of the mRNA expression experiment. Assuming that 10,000 genes are tested simultaneously after filtering, at most an average of 25 false positives are generated, the estimated statistical power for n=50 in each group is >95% at 50% log2-scale group differences (~41% fold change) and the within-group standard deviation of log-scaled expression is 0.5. If the group difference is smaller (40% log2-scale; 32% fold change), the power is 90%. If the within-group stdev increases to 0.7, the power reduces slightly to 79%.

Project 2-Sweet

The structure of the modeling and testing for layer 3 of Aim 1 are essentially the same for Experiments 1.1 and 1.2. The first goal is to assess the diagnosis difference in layer 3 for a particular cellular measure (e.g., somal size), between all schizophrenia and schizoaffective subjects and controls collectively across the 3 regions, as

well as individually within region controlling the family-wise type 1 error within measure. The second level of analysis deals with regional effects and assesses diagnosis differences in layer 3 inter-regionally from V1 to PPC to DLPFC, controlling family-wise error within measure. The statistical models take into account subject pairing, region being a within-subject effect and repeated cellular measures within subjects. In addition, the possible inclusion of the covariates freezer storage time and pH is examined for each variable. To assess diagnosis effect in layer 3 across regions, a suitable mixed model initially tests for layer 3 diagnosis effect = 0 across all 3 regions for the measure versus at least one region's effect ≠ 0. This omnibus test is followed by diagnosis tests within each separate region where each test is at the .05/3 level to control type 1 error. For the inter-regional diagnosis differences in layer 3, we initially test at the .05-level that the diagnosis effect for V1 = the diagnosis effect for DLPFC, versus V1 ≠ DLPFC. With the expectation this is significant, we then step-down and separately test DLPFC = PPC and PPC = V1, each at .05/2 level. This combination of step-down and Bonferroni testing controls the overall type 1 error for the 3 inter-regional tests at .05 and allows for detecting potential alternative explanations. The power for the omnibus layer 3 test of diagnostic effect across all three regions is based on previously obtained dendritic spine density effect sizes[16] of 0.93 in DLPFC and 0.51 in V1 obtained from 15 control subjects and 15 subjects with schizophrenia and an estimated inter-regional correlation, 0.5, for within-subject dendritic spine density[16]. Hotelling's T^2 -test power calculations assumed PPC effect size, 0.72 was half-way between that of V1 and DLPFC. With 50 subject pairs, power is >95% for the omnibus test for dendritic spine density. If the effect sizes were reduced by 50%, 50 pairs still produces 80% power. Individual region diagnosis powers adjusting for the multiple comparisons are for DLPFC, >95%; for V1, 86%; and for PPC, >95%. To detect the difference between the diagnosis effect for DLPFC versus the diagnosis effect for V1, 50 pairs provide 89% power. V1 versus PPC and PPC versus DLPFC, power (adjusting for multiple comparisons) substantially varies depending on assumed effect size for PPC. At .72, the power for the PPC to DLPFC comparison with 50 pairs is 25%; however, if PPC effect were smaller (e.g., 0.6), the power would increase to approximately 60%, although the power for the V1 to PPC comparison would decrease. Power confirmation is based on layer 3 DLPFC somal volume effect size[17] from 28 subject pairs where we assumed proportionally reduced effect sizes in PPC and V1. This yielded 78% power for the omnibus test for somal volume with 50 pairs and 60% power to compare V1 diagnosis effect to DLPFC effect. Layer 5 measurements will be examined and estimated in an exploratory manner parallel to layer 3 analyses. Without focal hypotheses, in place of formal testing, layer 5 results are given as appropriate confidence intervals for diagnosis effect to assess the range of population diagnosis effects consistent with the observed data. Supplementary analyses will examine certain measure effects only for the control subjects.

The Aim 3 testing structure (e.g., SYP levels in VGlut1 expressing excitatory boutons) parallels the testing structure for Aim 1. For Aim 2, Cox1, GAD67 and PV layer 3 measures' testing structure is the also the same as that in Aim 1. Aim 4's confound assessments are handled by standard statistical tests. The relative difference between the control subject and schizophrenia subject in a pair is computed and the pair-wise relative differences are compared between, for example, subjects whose deaths were by suicide versus those whose were not[18,19]. To examine whether inter-regional diagnosis differences can be attributable to a confound, essentially the same approach is employed with the ratio within pair across regions of the relative diagnosis differences being compared between confound levels. To examine if any findings differ between subjects with schizophrenia and those having schizoaffective disorder, similar approaches to the preceding will be used and additionally supported by auxiliary factorial models. The monkey testing in Aim 4 is analogous to Aim 1 testing except using antipsychotic exposure versus sham effects instead of diagnosis effects[20].

A secondary model to assess robustness of any testing results from Aims 1-3 will ignore subject pairing and instead use the pairing variables as additional covariates: age, PMI and gender[18,19,21].

Project 3-Lewis

The statistical approach to Aim 1 mirrors the approach employed in **P1.** Detection of differences in markers across regions will be similarly performed with follow-up functional annotation and network analysis. Since the monkeys are genetically and environmentally better controlled, we expect that the biological variations will be significantly smaller than the human study in **P1** and n=10 will be sufficient. Particularly, if the within-group standard deviation reduces to 0.3 in the power calculation in **P1**, the statistical power will remain at 96.6% at n=10.

For both Aims 2 and 3, 10 monkeys each will provide approximately 10-20 brain slices with approximately 50% of the slices from each region, PPC or DLPFC. From each region's total of 50 to 100 slices, a single PC connecting to the other region is chosen. The same PCs will be used in both Aims 2 and 3, although for

methodological reasons some of the PCs in Aim 3 may not be usable for the Sholl analysis of Aim 2. Aim 2 examines several aspects of neuronal morphology: Dendritic spine density and complexity measures based on dendritic tree reconstruction. These two measures are compared between layer 3 PCs in PPC that project to DLPFC and layer 3 PCs in DLPFC that project to PPC. To examine the extent of the selected dendritic tree, Sholl analysis provides for each neuron histograms of the numbers of crossings of the Sholl rings by dendritic branches from the basal/apical dendrites at each radial distance from the soma. Previous related morphological research suggests that the neurons within a monkey can be viewed as independent, so that comparisons of dendritic spine density between the PPC and DLPFC regions can be done by a standard independent two sample tests, e.g., t- or Wilcoxon tests. To obtain 80% power for spine density differences between regions assuming moderate inter-regional effect sizes =.5 requires 65 neurons/region. An average of 13 slices/monkey will provide the necessary 65 neurons, with this number of slices being experimentally quite achievable. Because the apical and basal dendrite histograms are computed for each neuron, comparing PPC to DLPFC can be more complex. A basic approach useful for descriptive purposes collapses all of the individual neuron histograms into 4 histograms: Apical and basal for PPC and apical and basal for DLPFC. Chi-squared testing can then test inter-regional differences. To handle the individual neuron histograms, exploratory models will be used to fit a parametric distribution to each type of dendritic tree for each neuron e.g., a 3-parameter gamma distribution or a mixture of 2 gamma's (if the Fig 3.3C 's bimodality holds more generally). Each neuron's apical and basal fitted parameters can be compared across regions.

In Aim 3 Experiment 3.1, from each slice in a region, a single PC that projects to the other region is chosen. For each neuron, the resonant frequency will be obtained and will be statistically modeled by independent samples models comparing PCs in PPC projecting to DLPFC versus those in DLPFC projecting to PPC. Previous related monkey brain electrophysiological research indicates that measurements on neurons between slices and within a slice can be viewed as independent of each other. For Experiment 3.2, a pair of PCs will be selected from each slice and the IPSCs will be recorded from each PC in the presence of both conotoxin and CCK for a fixed time period. The number of IPSCs, n_s, that are experimentally determined to be "synchronous" across PCs is measured as are the total numbers, n_1 and n_2, respectively, of IPSCs recorded from PC1 and PC2 in the same time window. For each slice the two resulting proportions are obtained: $p_1 = n_s/n_1$ and $p_2 = n_s/n_2$. These proportions are treated as repeated proportions from each of the slices in PPC and from each of the slices in DLPFC and are dependent due to the synchrony aspect. To compare proportions of synch-IPSCs in DLPFC to PPC, we employ a generalized mixed model with a logistic link function and region as the main effect. To compare the joint distributions of rise time and amplitude between the two regions on the PCs selected from each region, we compare mean log (rise time) and mean log (amplitude) simultaneously between regions using Hotelling's independent samples T^2-test, with suitable supporting graphical displays.

Project 4-Olson

This project will mainly study the involvement of layer 3 neurons in functional connectivity between prefrontal and parietal cortex in macaque monkeys. Aim 1 conducts analysis under attention tasks and Aim 2 studies connectivity related to working memory tasks. The statistical modeling approaches for Aims 1-2 are essentially the same. For a carefully computed connectivity measure (coherence or causality) between cross-area pairs of sites, we will first test the statistical significance of these functional connectivity measures by a permutation test based on shuffling trials[22] as described in the methods section of **P4**. Functional connectivity is predicted to increase in tasks with high demands in comparison to tasks with low demands, and is predicted to be more prominent in supragranular layers than in infragranular layers. These can be primarily assessed by paired-t tests. As a secondary approach, we will also employ a suitable mixed model analysis, accounting for correlated observations, to test the effects of task demands and layers together, as well as their interactions. For the first experiment of Aim 3, we will employ the chi-squared test of association to characterize the dependence of functional connectivity on direct neuron projections. The second experiment involves a comparison of functional connectivity measured before and after blockade of inter-regional connections. Similar measures and models as in Aims 1-2 will be used with an additional blockade effect, and the blockade effect will be tested under different scenarios. Family-wise type 1 error rates will be controlled when conducting multiple tests. Power was based on paired-t tests. With 200 recording sites (more than 200 will be recorded), power = 80% to detect a small effect size (d= 0.2).

Project 5-Luna and Salisbury

For Aim 1 H1, we will test if the performance, measured by percent correct and reaction time, is impaired in schizophrenia subjects compared to control subjects for each task, and also step-down test the impairment in the visual search flexible task versus pop-out task, and then visual search flexible versus habitual. For Aim 1 H2, different measures of brain activity, including frequency specific oscillations and BOLD activity from fMRI data, will be quantified (transformed as necessary). For each task, the diagnosis effect for a given measure will be generically denoted in each brain region as, β_1, β_2, β_3 for V1, PPC, and DLPFC, respectively. Taking into account the repeated measurement structures within a subject, we will employ a suitable mixed model analysis, where we initially test H_0: $\beta_1 = \beta_2 = \beta_3 = 0$ versus H_A: there is at least one $\beta_i \neq 0$. This omnibus test is followed by tests (at $\alpha/3$ level) for each separate region. To facilitate comparisons of impairment across regions, regional measures will first be meaningfully standardized or ranked. We will first compare in the ascending stream V1 versus DLPFC, and if this is significant, we will step-down to test individually (at $\alpha/2$ level) V1=PPC and PPC=DLPFC (see Sec 4.3 for analogous multiple testing details). For Aim 1 H3, for a connectivity measure, $m(R_i,R_j)$ between region R_i and R_j, such as PLV or Granger causality, similar tests to Aim 1 H2 will be adopted to test the diagnostic impairment in m(V1,PPC), in m(PPC,DLPFC), and in the difference between m(V1,PPC) and m(PPC,DLPFC). The actual model used in each analysis will reflect the hypothesis under consideration and include other covariates; however, approximating two-sample multivariate tests are used to provide conservative power calculations. We report the power for Aim 1 H2 in detail as an example and note that powers for the other tests were analogously computed and found sufficient. Assuming small (0.2), medium (0.5) and large effect (0.8) sizes for regions V1, PPC and DLPFC, respectively, and within-subject cross-regional correlation = 0.5, we found with sample size N=40/group, the two-sample Hotelling's T-squared .05 level test has 90% power to detect the overall difference between the group means of the 3 regions. When comparing regions V1 and DLPFC, with an effect size 0.6, a two sample t-test gives 75% power. The analysis for Aim 2 is analogously parallel to that for Aim 1.

In the above, we focus on schizophrenia, schizoaffective and schizophreniform subjects. As a supplementary analysis, we will study all recruited medication naïve psychosis patients and make comparisons among subgroups relative to control subjects. As the project proceeds**,** we will also employ various cutting-edge methodologies and develop suitable tools specifically for this project to visualize and explore the data. For example, we will study the association between brain functional deficits and cognitive impairments and try to build feasible prediction models and conduct cluster analyses. For the integrity of fMRI data and EEG/MEG data, we will model association between BOLD activity and frequency band specific oscillations using suitable regression models[23,24]. We will also try functional regression methods[25] to study how the BOLD connectivity relates to the neuron synchrony in a continuous frequency spectrum and its complex dynamics.

DATABASE PROCEDURES

For the human studies, Center Investigators can access the existing databases for the Department of Psychiatry Psychosis Recruitment and Assessment Core (**P5**) and the Human Brain Tissue Donation Program (**P1**&**P2**). Both of these are managed by the Department's Office of Academic Computing (see **Stats/DM Resources**). In addition to the participant demographic and clinical data, the following information will be maintained for **P5**: MEG/EEG oscillation power and phase values and measures of fMRI BOLD activation data from the regions of interest, and related task performance data. These databases are designed to support the linkage of the multi-investigator, multi-project databases of the Department of Psychiatry Psychosis Recruitment and Assessment Core and ensure ready access for all investigators in the Center. In addition, they are compatible with many statistical software packages.

A gene/miRNA database will be established to bridge the **P1** (human data) and **P3** (monkey data). Homolog/ortholog gene annotations will be obtained from the Affymetrix NetAffx Analysis center. Probe sequences will be blast across species for accurate annotation if needed. All information will be organized in an extensible and retrievable MySQL database with patient information and inference results (e.g., p-values, expression patterns, etc.) functional annotations and graphical presentations, linked across gene/miRNA expression species, brain regions and (PC/PV) cell types. The database will allow investigators across all projects to cross-reference, interpret and integrate results.

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The Pittsburgh Neuroscience Community

One of the hallmarks of the neuroscience community at the University of Pittsburgh and the adjacent Carnegie Mellon University is the extensive interaction among basic and clinical investigators. Listed below is a selection of organizational units which exemplify and promote such interactions, and that help create a rich and stimulating intellectual environment for a Conte Center for Translational Mental Health Research.

Department of Psychiatry

Western Psychiatric Institute and Clinic (WPIC) houses the Department of Psychiatry (chaired by **Dr. David A. Lewis, Center and P3 Principal Investigator**) of the University of Pittsburgh School of Medicine. As one of the nation's leading university-based psychiatric hospitals, WPIC offers the most advanced diagnosis and treatment regimens to patients with virtually every type of psychiatric condition. WPIC now has 310 inpatient psychiatric beds and offers 78 ambulatory programs. These facilities include a dedicated inpatient unit for individuals with psychotic conditions and the outpatient Comprehensive Recovery Services Division which oversees Services for the Treatment of Early Psychosis Program (STEP). The STEP clinic, directed by **Dr. Raymond Cho** (**P5 Co-Investigator**), provides clinical care for many of the subjects to be studied in **P5**. In addition to a multitude of clinical and teaching activities, more than 100 full-time faculty members of the Department of Psychiatry are principal investigators with their own research grants. An additional 30 serve as principal investigators with subawards from other departments or institutions. WPIC is committed to providing its basic and clinical scientists with the facilities and resources they need to support their investigations. For example, in concert with the Department of Radiology, a world-class Functional Imaging Center was developed to support a range of neuroimaging procedures, all of which are being utilized in the study of schizophrenia. In addition, research conducted at WPIC is strategically integrated with patient care activities, training and regional consultation.

The Department of Psychiatry and Western Psychiatric Institute and Clinic (WPIC) have created a specialized Residency Research Track (RRT) for the purpose of facilitating the career development of psychiatry residents into faculty positions with expertise in a broad range of investigative strategies and tools that will advance our ability to diagnose, treat and ultimately prevent psychiatric disorders. The mission of the RRT emphasizes six critical components for developing a research career: 1) cultivating a passion for mental health research; 2) connecting with the best mentors; 3) participating in ongoing studies and constructing an exciting research project; 4) creating time for research; 5) obtaining consultation for career development; and 6) continuing training in grant and manuscript writing. The RRT has had great success in producing promising research faculty, including former RRT resident, **David Volk, MD, PhD** (**P1 Co-Investigator**) and Co-Director of the RRT. In addition, several RRT residents currently participate in projects conducted by investigators of the proposed Center.

Department of Neuroscience

The Department of Neuroscience had its origins in the Psychobiology Program of the Department of Psychology. In 1986, a separate Department of Behavioral Neuroscience was established under the leadership of Edward M. Stricker, PhD, and in 1994, the unit was renamed the Department of Neuroscience to better reflect the diversity of its membership. The department (currently chaired by **Alan Sved, PhD, Steering Committee member**) has 18 faculty members with primary tenure-stream appointments and approximately 20 additional faculty members with a primary departmental appointment outside the tenure stream or with a secondary appointment (whose primary appointments are elsewhere on campus or at neighboring Carnegie Mellon University). In addition, the department has >15 postdoctoral fellows, 14 PhD research staff and 35 graduate students, in addition to numerous research staff members with undergraduate degrees. Departmental facilities include an extensive microscopic facility (including electron and confocal microscopes), an image analysis facility, a complete vivarium for small laboratory animals staffed with certified animal care workers, and machine and electronics shops staffed by experienced staff. The department provides a local area computer network which links departmental investigators with other Center participants. The department has a considerable undergraduate enrollment (with more than 400 students as majors) which includes the largest percentage of honors students of any department in the College. Many of these undergraduates participate in research projects conducted by investigators of the proposed Center and have been supported by the Department of Psychiatry's externally-funded programs for undergraduate summer research (see Section 6 of **Administrative Core-A**).

Department of Neurological Surgery

The Department of Neurological Surgery (chaired by **Robert Friedlander, MD, Steering Committee member**) at the University of Pittsburgh was founded more than 75 years ago with a strong commitment to patient care, education and research. Today, the department is the largest neurosurgical academic provider in the United States performing more than 11,000 procedures annually. The goal of the Department is to improve the care and treatment of patients with neurosurgical disease. This goal is being achieved partly though the conduct of state-of-the-art basic and translational research. The department, with more than 40 faculty members and investigators, seeks to be at the forefront of this endeavor; and numerous advances have already been achieved, resulting in research translated into practice. Annually, the department has been highly ranked in National Institutes of Health funding, a direct result of the success and quality of its research and development. In the past fiscal year, departmental faculty and residents were involved in more than 75 research projects having an annual budget award of almost $6.5 million.

Facilities include the most technologically-advanced equipment (e.g., modern surgical microscopes, advanced image-guided brain and spine navigational tools, state-of-the-art monitoring techniques), which improves patient outcomes by reducing operative complications. Over 25 years ago, the first Gamma Knife unit in North America was installed at the University of Pittsburgh's Center for Image-Guided Neurosurgery. This center is now one of the world's pre-eminent locations for stereotactic treatment and education. In the last 10 years, the Center for Cranial Base Surgery has made major advancements in developing the Expanded Endonasal Approach, a unique minimally invasive method of removing deep-seated brain tumors, affording patients a quick return to normal life. The department's faculty performs unique, federally funded research into methods for improving clinical outcomes; continually developing new tools and techniques to improve the status of patients with potentially life-threatening illnesses. It provides the most advanced training to future neurosurgeons from the United States and abroad. The department also houses a Brain Trauma Research Center (BTRC), Neurotrauma Clinical Trials Center and the Safar Center for Resuscitation Research.

A recent study published in the *Journal of Neurosurgery* showed that the department ranked first in academic output in top-tier specialty journals among all departments of neurosurgery across the United States and Canada. Another *Journal of Neurosurgery* article showed that it ranked as the most productive residency program in the nation in terms of graduates remaining in and contributing to academic neurosurgery. Still another article, published in *informaHealthcare*, showed that the department's stereotactic research effort was the most productive in the world.

The commitment of the Department of Neurological Surgery to research is further evident from their investment in establishing the MEG facility and the recruitment of **Dr. Avniel Ghuman**, **P5 Co-Investigator**, to direct that facility.

Department of Neurobiology

The Department of Neurobiology (chaired by **Peter Strick**, **PhD**, **Steering Committee member**) emerged in 1993 from the reorganization of two basic science departments in the medical school, Neurobiology, Anatomy and Cell Science and Physiology. At present there are 20 tenured or tenure stream and 7 non-tenure stream research faculty. In addition, the Department serves host to 24 secondary or adjunct faculty members holding primary appointments in other departments within the University of Pittsburgh. The Department of Neurobiology is housed in the Biomedical Science Tower 3 (BST3) and on the 14th floor of the Biomedical Science Tower (BST), in close proximity to the Department of Psychiatry basic laboratories on the 16th floor of the BST. Departmental facilities include image analysis and light microscopy (which are augmented by the Structural Imaging Center that contains EM, SEM, and confocal imaging facilities), analytical biochemistry and molecular biology equipment, tissue culture, small animal embryo injections, darkrooms, x-ray and autoradiography rooms, and department server with computer network. The department also administers the Rodent Behavior Analysis Core (RBAC), a core facility at the University of Pittsburgh Schools of the Health Sciences. The purpose of the RBAC is to facilitate characterization of the behavioral phenotype of rat and mouse models studied by investigators throughout the University of Pittsburgh and its affiliated centers, institutes and hospitals.

Center for Neuroscience at the University of Pittsburgh

Many trainees working in laboratories of faculty in all of the above departments are members of the Neuroscience Graduate Training Program that is administered through the Center for Neuroscience (CNUP), a campus-wide organization that promotes research and graduate training activities in neuroscience. The

University of Pittsburgh established the CNUP in 1984 to link traditional administrative units with the goal of promoting research and education in the neurosciences. The CNUP strives to encourage and coordinate research and academic programs in neuroscience, develop and coordinate academic training programs in neuroscience at the undergraduate, doctoral and postdoctoral levels, foster collaborative research between and among basic and clinical neuroscientists, and provide a resource to the local community on neuroscience-related issues. **Steering Committee members, Drs. Peter Strick and Alan Sved** are the present Co-Directors of the CNUP. The three examples below are among the many CNUP activities that would facilitate the goals of the proposed Center:

Seminars and Workshops. The CNUP organizes a Distinguished Scientist Series, workshops and an annual retreat for trainees and faculty. In addition, a major symposium is organized at least every other year. During these events, time is specifically allotted for speakers to meet alone with trainees both in groups and individually. These programs bring a large number of distinguished neuroscientists to Pittsburgh, a number of who have research interests related to those of Center investigators. The CNUP also sponsors monthly dinners at which graduate students present research seminars. This activity facilitates student interactions and helps prepare students for their thesis defense and other professional presentations. The CNUP's Clinical Neuroscience Committee invites distinguished visitors to present seminars on issues related to the neurobiology of disease.

Publicity and Coordination of Events. CNUP publishes an electronic biweekly newsletter, "The Neurotransmitter." It lists event and news items, including seminars, new courses, awards, and grant opportunities.

Visiting Scholars Program. CNUP faculty members may invite a faculty collaborator from outside the University to visit for a period of one to six months. The CNUP provides stipends to partially offset living expenses and research costs.

Center for the Neural Basis of Cognition

The Center for the Neural Basis of Cognition (CNBC) was established in January 1994 as a joint venture of Carnegie Mellon University and the University of Pittsburgh. The Center was initiated by a major gift from the R. K. Mellon Foundation, and is currently directed by **Drs. Peter Strick and Michael Tarr, Steering Committee members.** The following scientists of the proposed Center are members of the CNBC: **Drs. Raymond Cho, Carol Colby, Avniel Ghuman, David Lewis, Beatriz Luna, Carl Olson and Etienne Sibille.** The CNBC builds on the established strengths of Carnegie Mellon University in cognitive science and computer science and those of the University of Pittsburgh in basic and clinical neuroscience to produce a coordinated research and educational program dedicated to the study of the neural basis of cognitive processes, including learning and memory, language and thought, perception, attention, and planning; to the study of the development of the neural substrate of these processes; to the study of disorders of these processes and their underlying neuropathology; and to the promotion of applications of the results of these studies to artificial intelligence, technology and medicine. The CNBC synthesizes the disciplines of basic and clinical neuroscience, cognitive psychology and computer science, combining neurobiological, behavioral, computational and brain imaging methods.

A principal objective of the CNBC is to foster new collaborations between investigators at the University of Pittsburgh and Carnegie Mellon University in order to address basic and applied questions about all aspects of higher brain functions. The long term goals of the CNBC are to (a) establish organizational mechanisms for inter-university collaboration; (b) create a nucleus of scientists who have research interests in the neural basis of cognition; (c) train future scientists who will work in this area; (d) foster new interdisciplinary research projects; (e) disseminate knowledge across disciplines and beyond the two universities; and (f) promote the development and commercialization of technologies spawned by CNBC investigators.

The Graduate Training Program of the Center for the Neural Basis of Cognition offers exciting interdisciplinary training opportunities in conjunction with many affiliated PhD granting programs and departments. This program allows students to work with mentors outside their home discipline to develop core competencies in another field that complements the student's main research activities.

The Multimodal Neuroimaging Training Program (MNTP) at the University of Pittsburgh, in collaboration with Carnegie Mellon University, and with funding from the National Institutes of Health (NIH) (grants R90DA023420 and T90DA022761) was developed so that neuroscience students and investigators can learn about and gain experience in the rapidly advancing in vivo imaging fields. The MNTP teaches underlying

principles of these neuroimaging methods, as well as their inherent limitations, applications and modeling for integrative research. Methodologies include structural magnetic resonance imaging (MRI), functional MRI, positron emission tomography, magnetoencephalography and optical imaging. To fulfill these aims, MNTP has developed two programs: 1) The Graduate Training Program in Neuroimaging, administered through the CNBC Graduate Program and 2) The MNTP Summer Workshop, hosted by the CNBC, and coordinated by Seong-Gi Kim (University of Pittsburgh) and William Eddy (Carnegie Mellon University).

The CNBC also provides a very important world-class computational modeling facility. The computational modeling facility houses a 15-processor IBM SP supercomputer capable of delivering 1.5 gigaflops of computing power, either to one or many simulations at a time. The CNBC facility provides several neural modeling tools, including NEURON, GENESIS and other, higher-level network modeling environments. Desktop workstations are available for visualization and interactive modeling use.

Clinical and Translational Science Institute

The University of Pittsburgh was among 12 institutions nationwide to receive an inaugural NIH grant to develop and advance clinical and translational science as a distinct discipline. This grant was used to establish the Clinical and Translational Science Institute (CTSI). The CTSI serves as the integrative academic home for clinical and translational scientists across the University's six health sciences schools, Carnegie Mellon University, the University of Pittsburgh Medical Center (UPMC) and the region. Steven E. Reis, MD, Associate Vice Chancellor for Clinical Research, Health Sciences, serves as the Director of the Institute. CTSI has one central focus: to facilitate the translation of biomedical research advances into clinical and public health practice and policy—bridging from laboratory bench to patient bedside to community-based practice. To do so, CTSI develops, nurtures and supports a cadre of clinical and translational scientists by building on the University's existing clinical research training programs (Roadmap K12, K30) to establish a comprehensive program with activities ranging from early research exposure for high school students to advanced doctoral programs. CTSI, through its Research Facilities and Clinical Resources (CRRF) core, supports multiple research centers and networks devoted to advancing clinical and translational research. Researchers can utilize the Clinical and Translational Research Centers (CTRCs) and Research Networks listed below for the provision of facilities, staff, equipment, laboratory testing and other research resources in both inpatient and outpatient settings.

- Children's Hospital of UPMC Clinical and Translational Research Center (P-CTRC)
- Magee-Womens Hospital of UPMC Clinical and Translational Research Center (MWH-CTRC)
- UPMC Montefiore Clinical and Translational Research Center (MUH-CTRC)
- Neuroscience Clinical and Translational Research Center (N-CTRC)
- Physical Therapy Clinical and Translational Research Center (PT-CTRC)
- Pediatric PittNet (PittNet)
- University of Pittsburgh Cancer Institute Clinical and Translational Research Center (UPCI-CTRC)
- Vascular Clinical and Translational Research Center (V-CTRC)
- Multidisciplinary Acute Care Research Organization (MACRO)

In addition, CTSI's Translational Technologies and Resources Core (TTRC) provides centralized resources for the integration of state-of-the-art technologies needed to conduct translational and clinical research. Core facilities are available to provide advanced or specialized instrumentation, expertise in the use of such instrumentation, and access to relevant informatics capabilities to maximize efficiency. CTSI offers researchers the services of the following specialized Core Laboratories: Genomics and Proteomics Core Laboratories (GPCL); Peptide Synthesis Core Laboratory; Health Sciences Tissue Bank Small Molecule Biomarker Core; Stem Cell Core; Division of Laboratory Animal Resources and Molecular Systems and Modeling (MSM) Core.

The resources of the CTSI will be useful to the proposed Center in several ways. For example, the UPMC-wide patient Research Participant Registry will increase our capacity to recruit individuals with a first-episode of psychosis from all UPMC facilities (including 19 hospitals and 350 outpatient practice locations/clinics) into studies. The Community PARTners Core of the CTSI supports community based participatory research to foster collaboration between the community and clinical researchers.

Magnetic Resonance (MR) Research Center

The fMRI studies proposed in **P5** will be conducted at the University of Pittsburgh Medical Center (UPMC)'s state-of-the-art MR Research Center on the 8th floor of Presbyterian University Hospital, located one block

from WPIC and from the laboratories of **Dr. Beatriz Luna (P5 Co-Project Director)**. This facility has dedicated space for imaging systems, support laboratories and office space. The MR Center's Research Program includes 11 academic and 12 support staff. The Program is built around development and application of acquisition and image reconstruction schemes primarily for research as well as clinical MRI studies. The center has access to medical support facilities and staff. All scanners are equipped with MRI-compatible cardiac EKG equipment and blood oxygen monitors. The scanners are operated by registered MR technologists. Patient screening and set up is performed by PALS-certified registered nursing personnel.

The Center also houses a MRI scanner simulator that can be used to acclimatize children and claustrophobic subjects to the MRI scanning environment. The acoustic environment is reproduced using a high fidelity audio system that plays back an exact copy of the particular scanning protocol or a shortened version of it as the need arises. A head movement tracker allows subjects to be trained not to move their heads while being scanned. This laboratory is in a separate room that can be darkened as needed. The physiological (heart rate, arterial blood oxygenation, blood pressure) parameters and behavioral responses (finger switches) are recorded on a control system in the simulator just as they are with the real scanners.

Instrumentation. The center has several whole-body human imaging/spectroscopy systems including two at field strengths of 3T. The two identical 3.0T Tim systems (Siemens Tim Trio) from Siemens Medical Systems are equipped with 32 independent receive channels. The scanners have the full complement of product coils provided by Siemens (which includes a 12 channel head coil for each scanner). Both scanners operate under version VB13 of the scanner software which provides multi-nuclear capabilities not previously available under the Tim platform. The gradient coil set achieves 40 mT/m maximum amplitude and a slew rate of 200 mT/m/sec. The combination of the array coils, and multiple RF receivers enables the use of parallel imaging techniques, reducing the total scan time by up to 40% for various imaging protocols. Both Trio scanners are fitted with a MRI-compatible eye tracking monitoring and recording system (ASL, Bedford, Massachusetts) as well as with a MR compatible physiological monitor (MEDRAD, Indianola, PA). The magnet rooms are magnetically, acoustically and RF shielded. All systems are interfaced to a high-speed (Gigabit) local area network for data transfer to workstations for analysis. All conventional and echo planar MR imaging and MR angiographic functions (both phase contrast and time-of-flight methods) are supported. Quality assurance procedures include daily signal stability scans for echo planar imaging (1% maximum RMS over a continuous 30 minute acquisition with a 64x64 matrix size) and daily signal-to-noise measurements with the standard RF head coil. The MR Research Center has maintenance agreements with the scanner manufacturers that guarantee service in less than 12 hours whenever daily stability scans fail to meet the required specifications.

Computer laboratory**.** Three independent, but interconnected, LAN's support the operation of the MR Center: a research network, an administrative network and a scanner network. The computational power for the research network is supplied by a 8 CPU Silicon Graphics (SGI) Power Challenge L server, a 32 CPU Linux server and a Linux cluster (4x2.0GHz dual-core opterons). The SGI and Linux servers have 2GB and 4GB of RAM, respectively. Together they provide over 12TB of online data storage. Access to the servers is performed through multiple Linux PC workstations. All computers on the research network are interconnected by a 1Gb/s LAN. Commercial software for image analysis (AVS, SAGE, IDL, Analyze, MATLAB) as well as freeware packages (FSL, SPM) complement the development of customized software for individual projects. The scanner network operates on a combination of thin-wire Ethernet and 1Gb/s switched router. This network provides low latency access to all computers supporting the different subsystems for 3.0T scanners and is connected through a router to the research and administrative networks.

Center for Advanced Brain Magnetic Source Imaging

The M/EEG studies proposed in **P5** will be conducted in the Center for Advanced Brain Magnetic Source Imaging (CABMSI) at UPMC Presbyterian Hospital, which is immediately adjacent to WPIC and near the laboratories of **Drs. Cho, Ghuman and Salisbury**. The Elekta Neuromag® Vectorview (Helsinki, Finland) housed in this facility is a completely non-invasive bioelectromagnetic measurement system for functional brain studies. The M/EEG suite includes dedicated rooms for acquisition and analysis, setup facilities which allow for subject preparation, including EEG setup and behavioral training facilities. The facility houses a cutting-edge Elekta-Neuromag Vector view 306 MEG system. MEG signals are recorded from the entire head using a Neuromag Vectorview system (Helsinki, Finland). The sensor system includes 306 MEG-channels and up to 128 EEG-channels, all registering the electromagnetic signatures of the intracranial ionic currents associated with brain function. The MEG-sensor unit in its floor-mounted gantry, the movable subject chair and bed, together with the patient audio-visual monitoring and stimulus delivery systems are contained in a

magnetization-shielded room (IMEDCO AG, CH – 4614 Hägendorf, Switzerland). The integrated EEG-system consists of 124 single-ended channels and four differential channels, enabling recordings of EEG, EOG, EMG and ECG signals. The maximal acquisition sampling rate is 5 kHz. The entire system includes necessary approvals for human use and is 510(k) cleared by the FDA.

EEG will be recorded simultaneously on the NeuroMag system above. If subjects cannot tolerate that environment, the following systems (housed in electrically- and acoustically-shielded chambers) are available for alternate EEG recording. *BioSemi System*: Two identical 72 channel high impedance EEG systems, using custom designed BioSemi Active2 biopotential amplifiers, which run on 2 dedicated Intel Core2 2.4 Ghz, 2 G RAM, 160 G HD computers. Presentations stimulus delivery software runs on 2 dedicated stimulus delivery Intel Quad 4 3.0 Ghz, 16 G RAM, 500 G HD computers with 4G video cards. *ElectroGeodesics System*: A 128 channel Geodesic Sensor Net (EGI systems, Eugene OR). Eprime (PST, Pittsburgh PA) software is used for stimulus presentation and response recording. A precision timing device tethers the two computers that house the Eprime and EEG recording software (Netstation, EGI Systems), respectively, to ensure precision locking of EEG data to stimulus/response events.

Pittsburgh Supercomputing Center

The Pittsburgh Supercomputing Center provides an integrated array of high performance computing and communications products and related services, including supercomputing-class hardware, software, mass storage facilities, consulting, visualization services and training. Its computational supercomputing-class resources include the following machines:

- Backlight is an SGI UV shared memory system with 4096 cores and 32 Tbytes of shared memory).
- Sherlock is a YarcData uRiKA™ (Universal RDF Integration Knowledge Appliance) data appliance with PSC enhancements. It enables large-scale, rapid graph analytics through massive multithreading, a shared address space, sophisticated memory optimizations, a productive user environment, and support for heterogeneous applications.
- Anton is a special purpose supercomputer for molecular dynamics (MD) simulations, designed and constructed by D. E. Shaw Research (DESRES). In colllaboration with DESRES, the National Resource for Biomedical Supercomputing at PSC is hosting an Anton machine for general availability to the national biomedical community.
- Salk is an SGI Altix SMP machine with 144 cores and 288 Gbytes of shared memory, dedicated to biomedical research.
- Warhol is an HP c3000 cluster machine with 64 cores and 128 Gbytes of memory.
- Axon is a 32-node cluster with a total of 256 cores. Most nodes contain 8 Gbytes of memory; 4 nodes have 16 Gbytes.
- BioU is a 3-node computational cluster containing 16 cores and 128 Gbytes of memory per node. BioU is available to researchers conducting biomedical research.
- The Data Supercell, PSC's archival system, is a low cost, high bandwidth, high capacity and high reliability data management system.

Linux front end pscuxa is used to access the supercomputing-class machines and for mail. pscuxa is a 1.6GHz dual-CPU Opteron system running Red Hat Enterprise Linux (resembling other Unix systems) and has 2GB of memory and 80GB of mirrored local disk space.

Center for Biologic Imaging at the University of Pittsburgh

The Center for Biologic Imaging (CBI) provides centralized imaging services including light fluorescent microscopy, confocal laser scanning, electron microscopy, advanced computer aided morphometry and image analysis. The Center has 17 confocal microscopes, three SEM & TEM, 6 Reichert Ultracut (R,S,E Ultramicrotomes 1 with cryokits), 3 Microm cryostats, 17 light microscopes, flat-bed and slide scanners, 58 Pentium-based PCs (all with MetaMorph and Photoshop, other software packages including Imaris, Amira, Image Pro), four large network servers with distributed online storage of 150 terabytes and Tektronix phaser 860 color printers. Miscellaneous equipment includes multiple CCD cameras for light microscopes, multiple (15) live cell chambers and syringe pumps for media perfusion, miscellaneous small light (inverted phase)

microscopes, PCR machine, assorted protein and nucleic acid gell running apparati, tiissue culture hoods (2), 3 x C02 tissue culture incubators, water baths, 5 table top microfuges, Beckman Allegra tissue ulture refrigerated centrifuge(6K), clinical centrifuge, pH meters, digital balances(2), refrigerated high speed (26k x g) microfuge, Beckman Airfuge with EM90 and A100 rotors, access to departmental cold room, dark room, x-ray developer, preperative centrifuges, ultracentrifuges and rotors. CBI is located in S233 of the Biomedical Science Tower.