Elevated Viral Transcription Factor Levels in Cortical Blood Vessels in Schizophrenia

by

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Context and Rationale: Schizophrenia is a pervasive and devastating mental disorder, but the current available treatments do not alleviate the most debilitating feature of the disease: the cognitive deficits, specifically impairments in working memory. Through a translational approach, which involves tracing the disorder from its etiologic components to its clinical manifestations, novel and effective treatment strategies can be developed. Although the etiology of schizophrenia is extremely complex, a predominant risk factor is immune overactivation early in life, especially in-utero, that persists into adulthood. Additionally, the core pathologic source of the working memory impairments can be traced to dysfunctional GABA circuitry in the dorsolateral prefrontal cortex (DLPFC). In a previous study using a relatively small sample of schizophrenia subjects, mRNA levels of IFITM, a broad-spectrum viral restriction factor, has been shown to be markedly elevated in the DLPFC. This localized elevation, when considered with IFITM's role as marker of immune activity, suggests that IFITM may be related to both the GABA circuitry deficiency and the residual immune dysfunction characteristic of schizophrenia.

Study Objectives: To further evaluate IFITM's status in the DLPFC in schizophrenia, the present study had four aims: (1) replicate the finding of higher levels of IFITM mRNA in the prefrontal cortex in a larger cohort of schizophrenia subjects; (2) evaluate whether the IFITM elevation in schizophrenia is attributable to factors that are associated with, but not central to, the disorder; (3) determine the cell types that overexpress IFITM mRNA in schizophrenia; and (4) investigate whether higher IFITM mRNA levels are correlated with abnormal GABA-related marker mRNAs in the same schizophrenia subjects.

Methods: We used quantitative PCR (qPCR) and in situ hybridization (ISH) with film and grain counting analyses to quantify IFITM mRNA levels in postmortem samples of prefrontal cortex area 9 of 57 schizophrenia subjects and 57 comparison subjects. We then compared the findings to previously reported mRNA expressions of GABA-related markers in the same cohort of schizophrenia subjects. Transcript levels for IFITM mRNA were also assessed by qPCR in the prefrontal cortex of monkeys chronically exposed to antipsychotic medications.

Results: In schizophrenia subjects, IFITM mRNA levels were markedly higher in both qPCR and ISH analyses. The finding could not be attributed to infection- or inflammation-related cause of death, psychotropic medication use at time of death, or nicotine use at time of death. IFITM mRNA levels were not altered in the prefrontal cortex of antipsychotic-exposed monkeys. ISH analyses revealed that IFITM mRNA levels were predominantly elevated in cortical blood vessels in schizophrenia. Furthermore, higher IFITM mRNA levels measured by qPCR are associated with deficits in prefrontal GABA markers in schizophrenia.

Conclusions: The localization of IFITM to blood vessels in the prefrontal cortex suggests that the viral restriction factor is involved in immunoprotection provided by the blood-brain barrier, which has also been shown to be disrupted in schizophrenia. Considering that indicators of neuroinflammation are a pervasive finding in schizophrenia and that alterations in both GABA-related markers and IFITM have been associated with inflammatory processes, the correlation between IFITM mRNA overexpression in blood vessels and deficits in GABA-related markers may be explained by an common upstream insult, such as maternal immune activation mediated by inflammatory cytokine elevation or chronic neuroinflammation.

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1.0 INTRODUCTION

Schizophrenia is a devastating mental disorder that affects approximately 1% of the global population (McGrath et al. 2008). The disease leads to debilitating deficits in interpersonal and intellectual abilities that often prevent its sufferers from maintaining relationships with friends and family. Possibly because of the destructive effects that the disorder has on one's ability to function, 20% of individuals with schizophrenia are homeless (Folsom et al. 2005). Onset of the disorder typically occurs during late adolescence or early adulthood, and suicide is disturbingly common –it is estimated that between 5% (Palmer et al. 2005) and 10% (Tsuang 1978) of individuals with schizophrenia ultimately take their own lives. Indeed, suicide is one of the leading causes of death for schizophrenia.

While it is a tremendously complex disorder, schizophrenia can be broken down into three broad categories of symptoms: positive, negative, and cognitive. The positive symptoms are marked by *additions* to a person's behavior and experience and include delusions, fixed, false, internally validated beliefs that have little or no basis in reality and cannot be eradicated by appeals to logic; visual or (more commonly) auditory hallucinations, in which the individual perceives objects or voices that are not actually present; and thought disorders, which render the patient unable to think clearly enough to produce coherent speech. Conversely, the negative symptoms are named as such because they are the *absence* of parts of a person's behavior and experience. For example, social withdrawal, lack of personal hygiene, and amotivation are all

considered negative symptoms. The cognitive deficits include deficiencies in general processing speed and problem solving, impairments in the processing of social information, inability to select and maintain appropriate attentional foci, as well as difficulty with verbal and visual learning (Green 2006). Of particular note are the deficits in working memory, which normally allows individuals to use temporary information related to a stimulus (like a phone number in a phonebook) to guide behavior (like dialing the number) before it is forgotten.

All of the medications currently available to treat schizophrenia act primarily by attenuating the positive symptoms of the disease. Despite this predominance of positive symptom-oriented therapy in the treatment of schizophrenia, it has been well documented that there is only a minimal relationship between psychotic symptoms and either social or occupational functioning (Green 1996). Instead, these outcome measures have been shown to be strongly associated with cognitive deficits Indeed, deficits in cognition have been shown to be the best predictors of long-term outcome for individuals with schizophrenia (Elvevåg and Goldberg 2000). Consequently, in order to develop more effective treatments and preventative strategies for the disorder, one must first understand the underlying processes, such as genetic and environmental risk factors as well as developmental phenomena, that culminate in the debilitating cognitive deficits characteristic of the disorder.

1.1 TRANSLATIONAL APPROACH TO SCHIZOPHRENIA

In the April 2005 issue of *Nature Reviews Neuroscience*, Lewis et al presented a framework through which these processes may be elucidated (Figure 1)(Lewis et al. 2005). This framework is structured around the assumption that the clinical phenotype known as schizophrenia can

ultimately be traced back to some combination of genetic abnormalities and environmental insults, which initiate a series of pathological phenomena that eventually result in the clinical phenotype. These genetic and environmental causes comprise the etiology of the disorder, and lead to pathogenic changes in normal brain mechanisms. The disease mechanisms result in anatomic or molecular alterations (pathological entity), which upset normal brain functioning (pathophysiology). In turn, the abnormal brain function produces the clinical syndrome characteristic of the disorder. Importantly, there are two intervention points (prevention and treatment) along this cascade that provide an opportunity to eliminate, or at least attenuate, the clinical syndrome. That is, by preventing the pathogenic mechanisms from altering brain structure or by treating the pathophysiologic activity directly, the disease symptoms can be effectively mitigated.

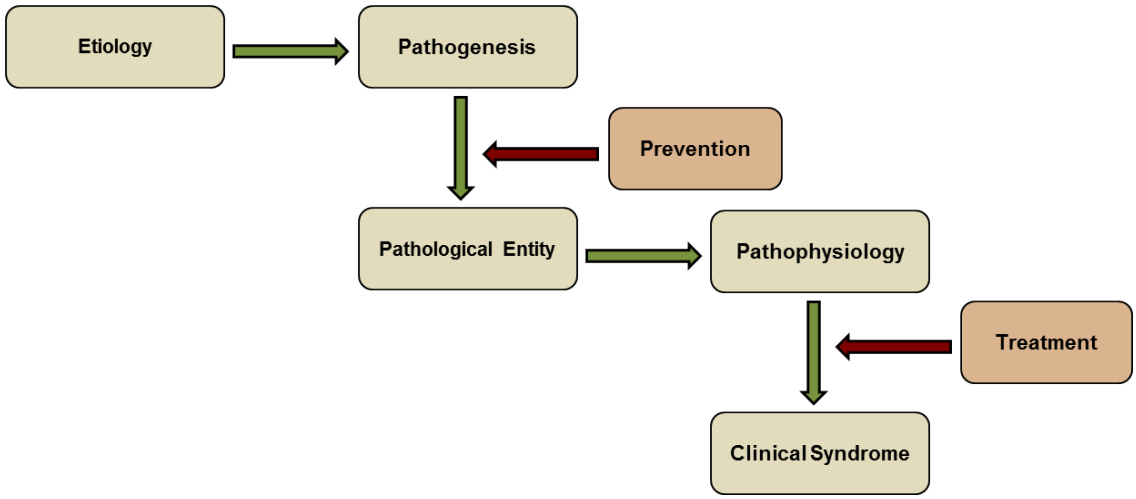

Figure 1. Translational neuroscience approach to disease (modified from (Lewis et al. 2005))

1.1.1 Pathophysiology

Impairments in working memory are commonly reported in schizophrenia (Lee and Park 2005; Forbes et al. 2009) and have been implicated as a core neurocognitive feature (Goldman-Rakic 1994) of the disorder. Working memory is subserved by the circuitry of the dorsolateral prefrontal cortex (DLPFC) (Petrides 2000), so one would expect there to be abnormal functioning of the DLPFC in schizophrenia during working memory tasks. Indeed, functional neuroimaging studies have demonstrated profound alterations in DLPFC activity during working memory tasks (Minzenberg et al. 2009). For example, studies examining fMRI data during the N-back working memory task have shown that individuals with schizophrenia have a limited working memory capacity and abnormal DLPFC functioning (Callicott et al. 2003;Callicott et al. 2000). Rather than being consistently hypo- or hyperfrontal, these abnormalities were characterized by differences in activity that varied by location within the region, suggesting poor modulation of neural activity in response to a changing cognitive load. That is, the working memory deficits seen in the disorder may be due in part to dysfunctional information handling and processing within the DLPFC.

The abnormal neural activity suggested by fMRI studies can be investigated by examining the EEGs of patients with schizophrenia during working memory tasks. Indeed, a strong body of research has shown that DLPFC neural firing patterns in the gamma frequency range (30-80 Hz), which are known to be associated with top-down processes such as working memory (Howard et al. 2003), are abnormal in schizophrenia (Minzenberg et al. 2010;Cho et al. 2006).In normal individuals, the amplitude (or power) of gamma band oscillations is positively correlated with working memory load (Howard et al. 2003). However, this increase in prefrontal gamma power is diminished in schizophrenia (Minzenberg et al. 2010;Cho et al. 2006). Gamma

oscillations have been shown to be associated with fMRI signal change (Logothetis et al. 2001;Mukamel et al. 2005), so it is likely that the impaired modulation of gamma activity seen in EEG studies is involved in the formation of the deficits found in neuroimaging studies. Therefore, given that the gamma power deficits are correlated with symptom severity (Cho et al. 2006), they may represent a pathophysiologic mechanism through which the working memory deficits emerge.

1.1.2 Pathological Entity

The origin of prefrontal gamma oscillations is not fully understood, but it is likely that they emerge from rhythmic firing of GABA-releasing inhibitory interneurons onto pyramidal cells, which are the major excitatory neurons in the cortex. The rhythmic inhibition results in the synchronous firing of millions of pyramidal cells in gamma frequency range. If prefrontal GABA neurotransmission is reduced, then gamma oscillations become disrupted (Lodge et al. 2009) and cognitive function is impaired (Enomoto et al. 2011;Gruber et al. 2010;Paine et al. 2011;Sawaguchi et al. 1989). It has been shown that interneurons expressing parvalbumin (PV), a calcium-binding protein, play a particularly important role in this process. Specifically, inhibition of PV interneurons has been shown to suppress gamma oscillations in mice, while stimulation increases gamma oscillations (Sohal et al. 2009).

In schizophrenia, there are severe alterations in both PV interneurons and GABA neurotransmission in general. For example, a number of studies examining post-mortem human DLPFC tissue have found reductions in mRNA levels of GAD_{67}, a GABA synthesizing enzyme, in schizophrenia (Akbarian et al. 1995;Volk et al. 2000). In PV interneurons specifically, the

GAD_{67} deficits appear to be especially severe – in one study, 50% of PV interneurons were found to contain undetectable levels of GAD_{67} mRNA in the schizophrenia subjects (Hashimoto et al. 2003). Without adequate levels of the GABA synthesizing enzyme, PV interneurons are unable to adequately inhibit their pyramidal cell targets, which may prevent the synchronous firing required to create gamma oscillations. Further support for this explanation comes from the finding that mRNA levels of the α1 subunit of the GABA receptor, which is the postsynaptic target for PV interneurons onto pyramidal cells, have been shown to be reduced in schizophrenia (Glausier and Lewis 2011).Taken together, these findings suggest that dysfunctional GABA-mediated inhibitory circuitry in the DLPFC, especially as it relates to PV interneurons, is one of the pathological entities that results in a pathophysiological reduction in gamma oscillations, which then contributes to the clinical syndrome of working memory dysfunction. Figure 2 superimposes these conclusions onto the Lewis et al framework.

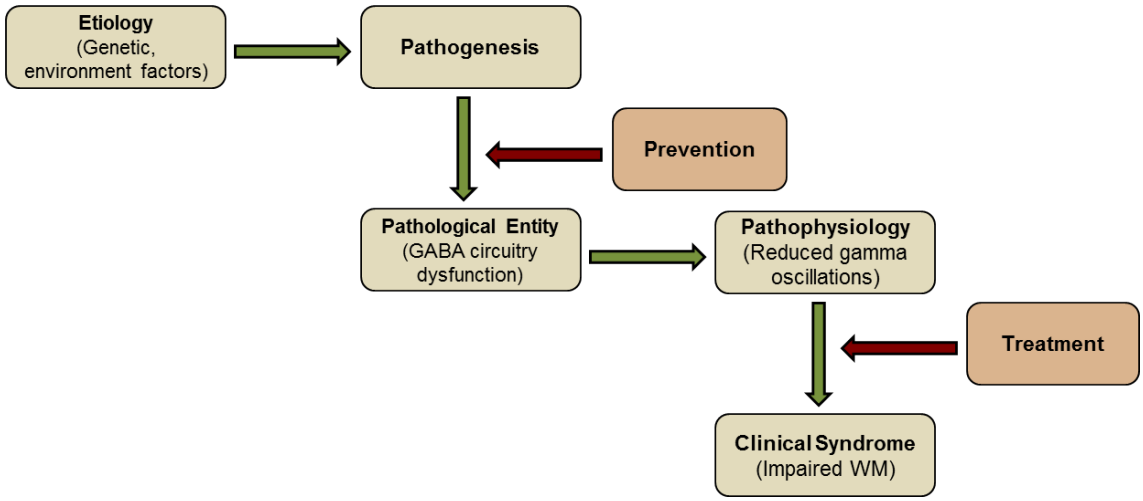

Figure 2. Translational neuroscience approach to schizophrenia (modified from (Lewis et al. 2005))

1.1.3 Uncovering Etiology

While little is known about the pathogenetic processes that produce GABA circuitry dysfunction in schizophrenia, a number of converging lines of research have shed light on the disorder's etiology. Most importantly, it is clear that schizophrenia emerges from both genetic and environmental factors. Indeed, the heritability for the disorder is approximately 0.81, meaning that about 81% of the variability between individuals in the disorder is due to genetic factors (Sullivan et al. 2003).Identifying the specific genes responsible for the disorder is tremendously complicated; however, evidence suggests that the disorder is polygenic, potentially involving thousands of SNPs (Purcell et al. 2009).

Regardless of the precise nature of the genetic risk factors, schizophrenia appears to fit a diathesis-stress model, in which individuals possess a certain degree of genetic predisposition that makes them more vulnerable to developing the disease when exposed to certain environmental stressors. Indeed, while genetics play a crucial role in the disease, it has been shown that the concordance rate for identical twins is only about 50%, implying that environmental factors heavily influence whether an individual actually develops the disorder (Tsuang 2000). Importantly, many of these environmental stressors tend to occur during periods of high neurodevelopmental activity. For example, heavy cannabis use during adolescence and childhood trauma are both associated with higher risk for developing schizophrenia. Furthermore, individuals with schizophrenia are more likely to have suffered from pre- or perinatal complications, such as fetal hypoxia and prenatal exposure to infection or toxins (Brown 2011).

1.2 NEURODEVELOPMENT AND IMMUNE DYSFUNCTION

The timing of these environmental risk factors suggests that there is a core neurodevelopmental component to schizophrenia. Indeed, the cognitive deficits that become so pronounced later in the illness are actually present in a less severe form in individuals prior to the onset of the disease. In addition, the cognitive problems worsen as the individual traverses adolescence into adulthood, even if schizophrenia onset does not occur until later in life (Reichenberg et al. 2010). These findings suggest that in individuals who ultimately develop the disorder, there is an offset in normal neurodevelopment that prevents proper brain maturation, possibly resulting in the dysfunctional prefrontal inhibitory circuitry characteristic of the disorder.

Interestingly, developmental delays have even been reported before age one in individuals who will eventually development schizophrenia (Sorensen et al. 2010), suggesting that the neurodevelopmental trajectory may begin to go off track very early in life. Given that Brown et al estimated that up to 30% of schizophrenia cases may be attributable to maternal infection (Brown and Derkits 2010), prenatal exposure to infection may represent an environmental insult that may be involved in this neurodevelopmental offset. Indeed, in one study 20% of fetuses who were exposed to the rubella virus during the first trimester of pregnancy went on to develop schizophrenia (Brown et al. 2001), although it should be noted that later studies have shown that the type and timing of infection in-utero are not the critical factors that affect schizophrenia risk (Brown and Derkits 2010).

One potential explanation for the association between maternal infection and schizophrenia is that, when the mother becomes infected with a pathogen, the fetal brain experiences an elevation in cytokines (Meyer et al. 2006). Because PV interneurons express cytokine receptors (Wang et al. 2011;Sanchez-Alcaniz et al. 2011;Meechan et al. 2012), perhaps

altered fetal cytokine levels in response to maternal immune activation (MIA) could disrupt normal neuronal migration of PV neurons, and therefore play a role in the inhibitory circuit abnormalities discussed above.

1.3 IFITM IN SCHIZOPHRENIA

Because it is impossible to directly look at the brains of schizophrenia patients in-utero, which would be the ideal way to investigate the role of maternal immune activation in the disease's etiopathogenesis, more indirect (but converging) approaches must be employed. For example, studying adults with schizophrenia can provide indications of insults that happened much earlier in life. Indeed, altered serum cytokines is a well-documented finding in adults with schizophrenia (Erbagci et al. 2001;Di Nicola et al. 2012;Drzyzga et al. 2006), and one postmortem study found elevated mRNA levels of cytokines in the DLPFC of individuals with the disorder (Fillman et al. 2012). Given that maternal immune activation has been shown to elevate levels of cytokines in the fetal brain (Meyer et al. 2006;Oskvig et al. 2012), and that maternal immune activation is relatively common in schizophrenia (Brown and Derkits 2010), these findings suggest there may be a residual immune activation that begins in-utero and persists into adulthood. Consistent with this interpretation, a microarray study of DLPFC tissue in a relatively small number of schizophrenia subjects found elevations in mRNA levels of interferon-induced transmembrane protein (IFITM) (Arion et al. 2007), which is a viral restriction factor that impedes viral entry into cells and inhibits viral replication (Diamond and Farzan 2013). IFITM has been shown to be involved in cellular resistance to a wide range of viruses (including influenza, West Nile, dengue, and HIV) (Brass et al. 2009;Huang et al.

2011;Lu et al. 2011), so it likely plays an important role in broad-spectrum antiviral activity (Diamond and Farzan 2013). The expression of IFITM mRNA in schizophrenia subjects in the Arion et al study was remarkably high – almost an 80% difference between schizophrenia and controls. Such an elevation is rare in postmortem studies of schizophrenia, so considering its potential involvement in an immune-related pathogenic mechanism, it warrants further investigation.

The marked elevation of IFITM mRNA levels in the DLPFC of schizophrenia raises several important questions which are addressed in the present study. First, how common is higher IFITM mRNA expression in schizophrenia? Second, is higher IFITM mRNA expression an integral part of the disease process, or is it either a consequence of disease-related factors, such as antipsychotics and smoking, or a confound of immune/inflammation-related cause of death? Third, as little is known about IFITM function in the brain, which cell types normally express IFITM and do these cells express higher IFITM mRNA levels in schizophrenia? Fourth, is there a relationship between elevated IFITM mRNA levels and deficits in GABA neuron-related markers in the disorder? Addressing these questions will allow for further investigation into IFITM as a potential player in the pathogenesis of schizophrenia.

2.0 METHODS

From these questions, we derived four study aims:

1. Replicate the finding of higher levels of IFITM mRNA in the prefrontal cortex in a larger, new cohort of schizophrenia subjects.
2. Evaluate whether elevated IFITM mRNA levels in schizophrenia is related to disease severity or attributable to factors such as antipsychotic use, smoking or late-life immune events.
3. Determine the cell-types that overexpress IFITM mRNA in schizophrenia.
4. Investigate whether higher IFITM mRNA levels are related to abnormalities in expression of GABA-related markers in the same schizophrenia subjects.

To address each of these aims, we used postmortem brain tissue of adult individuals with and without schizophrenia. Specifically, we studied IFITM mRNA expression in area 9 of the PFC using two methods: quantitative PCR (qPCR) and in situ hybridization. qPCR was employed to determine the differential mRNA expression of IFITM in DLPFC gray matter homogenates of schizophrenia subjects and unafflicted controls. In situ hybridization was used to confirm the qPCR findings and to determine which cells in the DLPFC express higher levels of IFITM mRNA in schizophrenia. We also used qPCR to quantify IFITM mRNA levels in the prefrontal cortex of antipsychotic-exposed monkeys.

2.1 HUMAN SUBJECTS

With consent from next-of-kin, the brain specimens for the study were obtained during routine autopsies conducted at the Allegheny County Medical Examiner's Office. For each schizophrenia subject, an independent committee of experienced research clinicians made consensus DSMIV (American Psychiatric Association 1994) diagnoses using structured interviews with family members and review of medical records (Volk et al. 2010).For the healthy comparison subjects, the absence of a psychiatric diagnosis was confirmed in a similar manner. To control for experimental variance, subjects with schizophrenia or schizoaffective disorder (n=57) were matched individually to one healthy comparison subject for sex and as closely as possible for age, as previously described (Volk et al. 2010;Volk et al. 2011;Curley et al. 2011), and samples from subjects in a pair were processed together throughout all stages of the study. Of the fifty-seven subject pairs, fourteen had previously been studied for IFITM mRNA levels by microarray in the Arion et al study (Arion et al. 2007). As shown in Table 1, the mean age, postmortem interval, freezer storage time, brain pH, and RNA integrity number (RIN; Agilent Bioanalyzer) did not differ between subject groups. Additionally, each subject had a RIN $\geq$ 7.0. All procedures for the study were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board.

Table 1. Control and Schizophrenia Subject Characteristics

	Controls		Schizophrenia	
	Number	**Percent**	**Number**	**Percent**
Gender	15F / 42M	26.3F / 73.7M	15F / 42M	26.3F / 73.7M
Race	47W / 10B	82.5W / 17.5B	43W / 14B	75.4W / 24.6B
	Mean	**SD**	**Mean**	**SD**
Age (years)	48.1	13.9	46.9	12.9
PMI[a]	18.2	5.4	18.9	8.5
ST qPCR[b]	113	50	109	54
ST ISH[b]	92	34	81	25
RIN[c]	8.2	0.6	8.1	0.6
Brain pH	6.7	0.3	6.6	0.3

[a] PMI, postmortem interval (hours); [b] ST, storage time (months) at -80C; [c] RIN, RNA integrity number; F, female; M, male; W, white; B, black

2.2 TISSUE PREPARATION

Frozen tissue blocks containing the middle portion of the right superior frontal sulcus were confirmed to contain prefrontal cortical area 9 using Nissl-stained, cryostat tissue sections for each subject (Volk et al. 2000). The gray-white matter boundary of prefrontal area 9 in the tissue block from each subject was carefully scored with a scalpel blade where the gray matter had uniform thickness and the gray-white matter boundary was easily delineated. The scored gray matter region of the tissue block was then digitally photographed, and the number of tissue sections (40 µm) required to collect ~30 mm^3 of gray matter was determined for each subject. The calculated number of required tissue sections for each subject was then cut by cryostat, and gray matter was separately collected into a tube containing TRIzol reagent in a manner that

ensured minimal white matter contamination and excellent RNA preservation(Hashimoto et al. 2008).

2.3 QUANTITATIVE PCR

2.3.1 Overview

As indicated above, qPCR can be used to quantify mRNA levels for whole-tissue homogenates. In general terms, qPCR is carried out in the following procedure. First, for each subject, RNA is isolated from homogenized gray matter via a series of purification reactions. cDNA is then synthesized from the tissue mRNA via a reverse transcriptase. Using primers specific for the target cDNA sequence, which corresponds to IFITM in this study, the target cDNA sequence is then amplified exponentially. As the cDNA is amplified, a fluorescent binding agent (SYBR Green) intercalates to the amplified, double stranded cDNA. The fluorescence of the sample can then be measured. In this study, the relative mRNA amount in a sample is determined by the number of PCR cycles it takes for the fluorescence to reach a standardized detection threshold relative to three normalizer genes. Figure 3 shows this process pictorially.

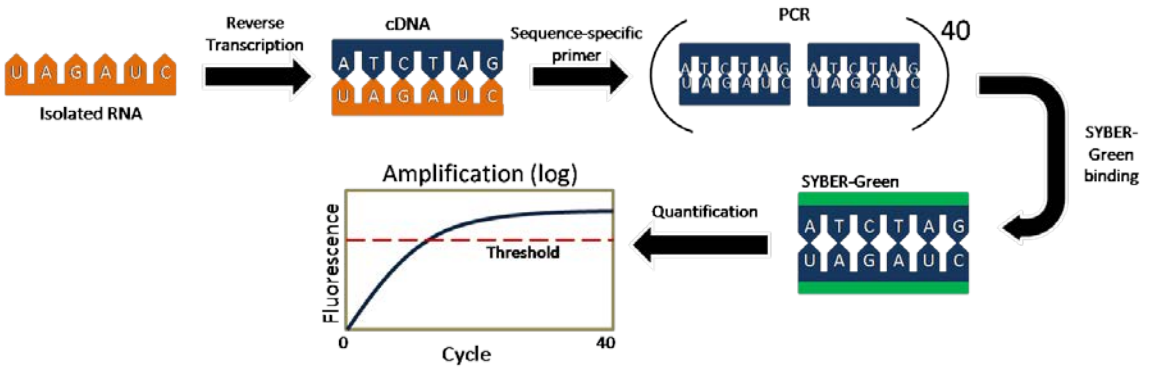

Figure 3. qPCR Overview

2.3.2 Specific Procedure

cDNA was synthesized via standardized dilutions of total RNA (10 ng/µl) for each subject using the standardized amounts of cortical gray matter. There are three relevant variants of IFITM mRNA (IFITM1, IFITM2, IFITM3; IFITM4 is a pseudogene and IFITM5 is only found in osteoblasts (Diamond and Farzan 2013). One primer set was designed to quantify IFITM1 mRNA by targeting a unique region not found in the two other reported IFITM variants in humans (IFITM2 and IFITM3). However, due to the very high sequence homology between IFITM2 and IFITM3 in humans, a second primer set was designed to quantify IFITM2 and IFITM3 mRNAs by targeting a region that is identical between IFITM2 and IFITM3 (termed IFITM2/3; Table 2). All primer pairs demonstrated high amplification efficiency (>96%) across a wide range of cDNA dilutions and specific single products in dissociation curve analysis.

Table 2. qPCR Primer Design

Gene	Species	Accession #	Amplicon Size (bp)	Position	Forward Primer (F) Reverse Primer (R)
Interferon-induced transmembrane protein 1 (IFITM1)	H[†]	NM_003641	82	576-657	(F) CAACCTTTGCACTCCACTGT (R) GTATCTAGGGGCAGGACCAA
IFITM2/3*	H	NM_006435 (IFITM2) NM_021034 (IFITM3)	59	441-499 (IFITM2) 459-517 (IFITM3)	(F) CTGCTCATCATCATCCCAGT (R) TGATGCCTCCTGATCTATC
Beta actin	H	NM_001101	101	1146-1246	(F) GATGTGGATCAGCAAGCA (R) AGAAAGGGTGTAACGCAACTA
Cyclophilin	H	NM_021130	126	159-284	(F) GCAGACAAGGTCCCAAAG (R) GAAGTCACCACCCTGACAAC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	H	NM_002046	87	556-642	(F) TGCACCACCAACTGCTTAGC (R) GGCATGGACTGTGGTCATGAG
IFITM1/3[#]	M[†]	XM_001085444 (IFITM1) XM_001106166 (IFITM3)	106	323-428	(F) AGTCCAGGGACAGGAAGATG (R) GTCATGAGGATGCCCAAAAT
Beta actin	M	NM_001033084	101	1087-1187	(F) GATGTGGATCAGCAAGCA (R)AGAAAGGGTGTAACGCAACTA
Cyclophilin	M	NM_001032809	126	76-201	(F) GCAGACAAGGTTCCAAAG (R) GAAGTCACCACCCTGACAC
GAPDH	M	XM_001105471	93	527-619	(F) TGCACCACCAACTGCTTAGC (R) AGTGATGGCGTGGACTGTG

* Due to the very high sequence homology between IFITM2 and IFITM3 in humans, this primer set was designed to target an mRNA region that is identical between IFITM2 and IFITM3 (termed IFITM2/3).

This primer set was designed to target an mRNA region that is identical between all known IFITM variants in monkeys, IFITM1 and IFITM3 (termed IFITM1/3). No IFITM2 mRNA sequence for monkeys was available in the NCBI data base at the time of primer design.

[†] Abbreviations: H, human; M, Macaca mulatta

Quantitative PCR was performed using the comparative cycle threshold (CT) method with Power SYBR Green dye and the StepOnePlus Real-Time PCR System (Applied Biosystems). cDNA samples from both subjects in the same pair were processed together on a single quantitative PCR plate, and a different plate was utilized for each of the 57 subject pairs. Four replicate measures were performed for each transcript for each subject with a detection threshold for each gene applied consistently for all subjects. The mean coefficient of variance (±SD) of the replicate measures were IFITM1 0.036 (±0.018) and IFITM2/3 0.038 (±0.026). Control studies in which the cDNA template was not included in the qPCR reaction resulted in a complete lack of amplification. Based on their stable relative expression level between schizophrenia and comparison subjects (Hashimoto et al. 2008), three reference genes (beta actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were used to normalize target mRNA levels. The difference in CT (dCT) for each target transcript was calculated by subtracting the geometric mean CT for the three reference genes from the CT of the target transcript (mean of four replicate measures). Because dCT represents the log2-transformed expression ratio of each target transcript to the reference genes, the relative level of the target transcript for each subject is reported as 2^{-dCT} (Vandesompele et al. 2002;Hashimoto et al. 2008;Volk et al. 2010;Volk et al. 2011).

2.4 IN SITU HYBRIDIZATION

2.4.1 Overview

In situ hybridization (ISH) is used in this study to determine which cells in the DLPFC express the highest levels of IFITM mRNA, and to evaluate whether the elevation seen in the overall tissue (via qPCR) is also present in these cells. The ISH was carried out by adding a radioactive riboprobe specific for IFITM to intact sections of DLPFC tissue. The probe bound to IFITM mRNA, such that the areas of the tissue that exhibited the most radioactivity corresponded with the cell types that have the highest levels of IFITM mRNA. We then quantified the relative difference in radioactivity between individuals with schizophrenia and control subjects. We used two methods to quantify mRNA expression: film analysis and grain counting. For the film analysis, each slide of tissue processed by in situ hybridization was exposed to a sheet of film for a standardized length of time along with radioactive standards, and optical density measures were taken of the gray and white matter from the film. For the grain counting analysis, tissue sections were dipped in a nuclear emulsion. Over a period over 10 days, the radioactive particles from the bound riboprobe hit the emulsion, creating a superimposed autoradiograph of silver grains over the slide. To identify cell bodies, tissue sections were then counterstained for Nissl substance. We then examined each slide under a microscope in dark field, for the visualization and quantification of silver grain density which is related to the amount of IFITM mRNA expression over cell bodies.

2.4.2 Specific Procedure

A 266–base pair fragment corresponding to bases 145-410 of the human *IFITM2* gene (NM_006435), a homologous region in all three human IFITM variants, was PCR-amplified, and nucleotide sequencing confirmed 100% homology for the amplified fragment to the reported sequence. Sense and antisense ^{35}S-labeled riboprobes were generated by in vitro transcription. In the hybridization procedure, 3 sections separated by at least 320 μm were chosen for each subject, and sections with the same rostral-caudal level were paired. One pair of sections from each subject pair was processed side by side in an in situ hybridization run. Following fixation with 4% paraformaldehyde in 0.1M phosphate-buffered saline, sections were hybridized with ^{35}S–labeled riboprobes in a standard hybridization buffer at 56°C for 16 hours. Sections were subsequently washed in a solution containing 50% formamide at 63°C, treated with RNase A at 37°C, and washed in 0.1×SSC (150 mM sodium chloride, 15mM sodium citrate) at 66°C. Sections were then dehydrated through a graded ethanol series, air-dried, and exposed to BioMax MR film (Eastman Kodak, Rochester, New York) for 43 hours. Sections were then coated with NTB emulsion (Eastman Kodak), exposed for 10 days at 4°C, developed with D-19 developer (Eastman Kodak), and counterstained with cresyl violet (Volk et al. 2000;Hashimoto et al. 2003;Morris et al. 2008).

Levels of IFITM mRNA expression were quantified in film autoradiograms using a Microcomputer Imaging Device system (Imaging Research Inc, London, Ontario, Canada) without knowledge of diagnosis or subject number by random coding. Images of the tissue sections were also captured and superimposed onto the autoradiographic images to identify the gray matter/white matter border. Gray matter optical density was measured by drawing contours of the full thickness of the cortex exclusively in the zones where the cortex was cut perpendicular

to the pial surface. The mean (SD) total area of gray matter sampled in each subject was 76 (28) mm^2 for control subjects and 72 (40) mm^2 for subjects with schizophrenia. White matter optical density was determining by outlining the gray matter/white matter border and including all white matter on the section. The mean total areas of the sampled total white matter per subject were 243 (113) mm^2 for control subjects and 223 (99) mm^2 for subjects with schizophrenia. Optical density is expressed as nCi/g of tissue by reference to radioactive carbon 14 standards (American Radiolabeled Chemicals, St Louis) exposed on the same film.

IFITM mRNA expression was then evaluated at the cellular level using the MCID system coupled to a microscope equipped with a motor-driven stage. Silver grains generated by the ^{35}S-labeled riboprobe in emulsion-dipped sections were counted over traced outlines of blood vessels, identified as distinct clusters of packed cells with at least two elongated nuclei and the occasional presence of a lumen or branching point, in two 500 µm-wide gray matter traverses in each of three sections/subject. The mean (SD) number of blood vessels sampled in each subject was 70 (10) for control subjects and 73 (10) mm^2 for subjects with schizophrenia.

2.5 ANTIPSYCHOTIC-EXPOSED MONKEYS

Young adult, male, long-tailed monkeys (*Macacafascicularis*) received oral doses of haloperidol, olanzapine or placebo (n=6 monkeys per group) twice daily for 17–27 months(Dorph-Petersen et al. 2005).One monkey from each of the three groups were euthanized together on the same day, and the six triads were euthanized on separate days. RNA was isolated from prefrontal cortical area 9, and qPCR was conducted for the same three reference genes and IFITM (Table 2) with all monkeys from a triad processed together on the same plate. The expression levels of the

reference genes (beta actin, cyclophilin A, and GAPDH) did not differ between haloperidol-exposed, olanzapine-exposed, and placebo-exposed monkeys ($F_{(2,10)} \leq 1.3$, $p \geq 0.32$).All animal studies followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.6 STATISTICAL ANALYSIS

The ANCOVA model we report includes mRNA level (i.e. normalized expression level, optical density, or grain density) as the dependent variable, diagnostic group as the main effect, subject pair as a blocking factor, and postmortem interval, brain pH, RIN, and freezer storage time as covariates. Subject pairing may be considered an attempt to account for the parallel processing of tissue samples from a pair and to balance diagnostic groups for sex and age, and not a true statistical paired design. Therefore, a second ANCOVA model without subject pair as a blocking factor and including sex and age at death as covariates was also used, and both models produced similar results. Subsequent analyses of differences in mRNA levels between schizophrenia subjects grouped by predictors and indicators of disease severity, psychotropic medications, nicotine use, and cause of death were conducted using the unpaired ANCOVA models with $\alpha = .05$.For the antipsychotic-exposed monkey study, an ANOVA model with mRNA level as the dependent variable, treatment group as the main effect, and triad as a blocking factor was employed.

3.0 RESULTS

3.1 AIM 1: QPCR AND AUTORADIOGRAPH ANALYSIS OF IFITM MRNA

qPCR analysis revealed that mean mRNA levels in schizophrenia subjects were markedly higher for IFITM1 (+72%; $F_{(1,53)}$=20.0, p<.0001) and IFITM 2/3 (+116%; $F_{(1,53)}$=26.8, p<.0001) relative to healthy comparison subjects (Figure 4). This finding is consistent with the elevation in IFITM mRNA levels reported in schizophrenia in the 2007 Arion et al. study, which used fourteen of the 57 schizophrenia subjects in the present study (Arion et al. 2007). In the newly studied 43 schizophrenia subjects alone, we also found higher mRNA levels for IFITM1 (+76%; $F_{(1,39)}$=13.9, p=.001) and IFITM 2/3 (+118%; $F_{(1,39)}$=16.6, p<.001). Since mRNA levels for IFITM1 and IFITM2/3 are highly correlated across all subjects (r=0.74, p<.0001) and the different IFITM variants have similar roles as viral restriction factors (Brass et al. 2009;Huang et al. 2011;Lu et al. 2011), we focused on IFITM2/3 mRNA levels for subsequent data analyses due to the more robust elevation seen in this variant.

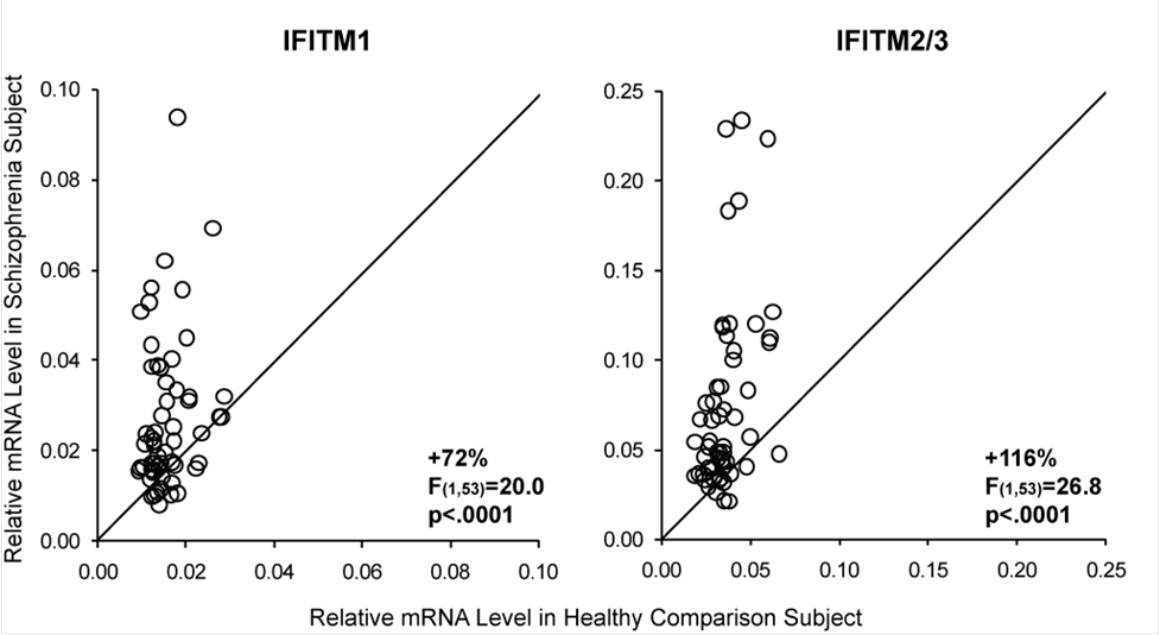

Figure 4. Quantitative PCR analysis of IFITM mRNA levels in the prefrontal cortex in schizophrenia.
mRNA levels for schizophrenia subjects relative to matched healthy comparison subjects in a pair are indicated by open circles. Data points to the left of the unity line indicate higher mRNA levels in the schizophrenia subject relative to the healthy comparison subject and vice versa. Mean mRNA levels (± standard deviation) in schizophrenia subjects were statistically significantly higher for IFITM1 (0.027 ± 0.017) and IFITM2/3 (0.078 ± 0.058) relative to healthy comparison subjects (IFITM1: 0.016 ± 0.005; IFITM2/3: 0.036 ± 0.011).

We further validated this finding using in situ hybridization in 21 (out of 57) of the subject pairs that had higher IFITM mRNA expression in the schizophrenia subject by qPCR and had a sufficient number of available tissue sections. To our knowledge, in situ hybridization analysis of IFITM mRNA expression has not been previously reported in human PFC. Optical density analysis of film autoradiographs revealed that mean IFITM mRNA levels were higher in the gray matter (+110%; $F_{(1,17)}$=16.0, p=.001) and white matter (+85%; $F_{(1,17)}$=9.6, p=.006) in schizophrenia (Figure 5). Furthermore, gray matter IFITM mRNA levels quantified by in situ hybridization and by qPCR were correlated in the same subjects (r=.54, p=.01), which indicates

the reliability of the mRNA quantification techniques and the reproducibility of the results. Interestingly, pseudocolor film autoradiographs from schizophrenia subjects (Figure 5B) revealed small structures with intense signal that were linear, round, and/or had branch points, which is consistent with labeling of blood vessels.

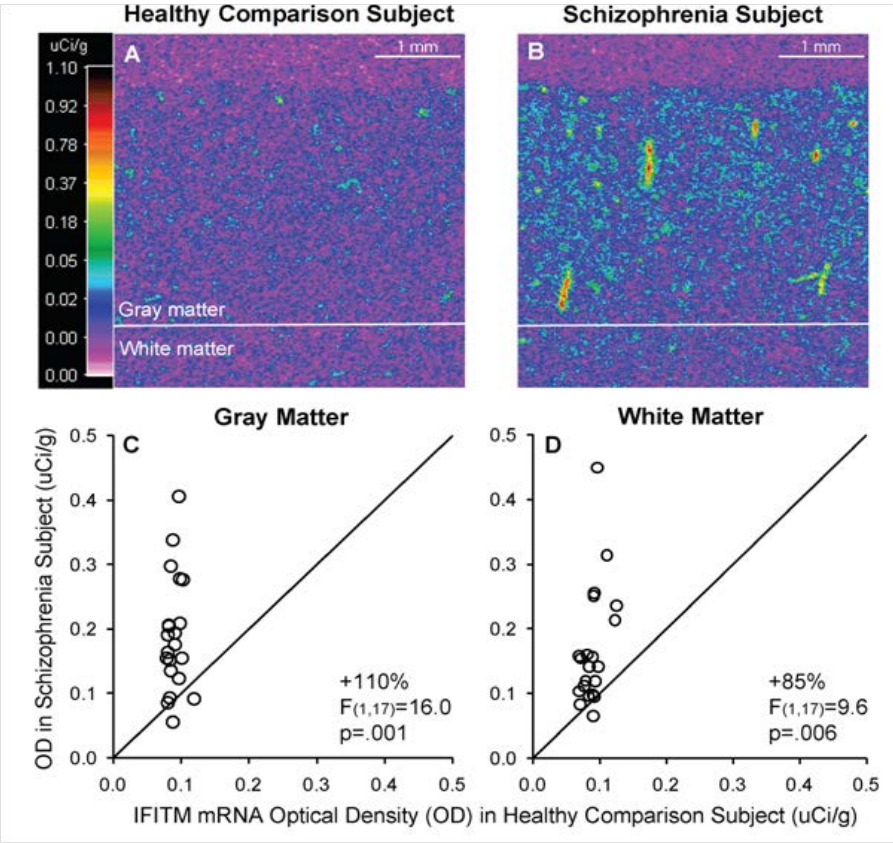

Figure 5. Autoradiograph analysis of in situ hybridization for IFITM mRNA in the prefrontal cortex of schizophrenia and healthy comparison subjects.
Pseudocolored film autoradiographs of tissue sections from a matched pair of healthy comparison **(A)** and schizophrenia **(B)** subjects processed by in situ hybridization with an ^{35}S-riboprobe for IFITM mRNA are shown. Solid white line indicates the gray matter/white matter border. While the healthy subject has relatively low levels (colder colors; scale) of IFITM mRNA throughout the gray and white matter, the schizophrenia subject has distinct small structures with intense signal (warmer colors) that are linear, round, and/or have branch points, which is consistent with the anatomical appearance of blood vessels. Mean IFITM mRNA levels in the gray matter **(C)** and white matter **(D)** of prefrontal cortical area 9 for schizophrenia subjects relative to matched healthy comparison subjects in a pair are indicated by open circles. Data points to the left of the unity line indicate higher mRNA levels in the schizophrenia subject relative to the healthy comparison subject and vice versa. Mean IFITM mRNA levels are markedly higher in the gray matter (+110%) and white matter (+85%) in schizophrenia subjects.

3.2 AIM 2: IFITM AND DISEASE-ASSOCIATED FACTORS

Among schizophrenia subjects, IFITM mRNA levels did not differ as a function of factors that predict a more severe course of illness (male sex, a diagnosis of schizophrenia rather than schizoaffective disorder, first-degree relative with schizophrenia, early age at illness onset [≤18 years of age]) or measures of illness severity (suicide, no history of marriage, low socioeconomic status as measured by the Hollingshead Index of Social Position, living dependently at the time of death) (all $F<.33$, $p>.56$; Figure 6A). We also found no relationship between use of antipsychotic, antidepressant, benzodiazepine, or nicotine at time of death and IFITM mRNA levels in schizophrenia subjects (all $F<1.2$, $p>0.28$; Figure 6B). IFITM mRNA levels also did not differ in the PFC of monkeys chronically exposed to antipsychotics (haloperidol and olanzapine) or placebo (Figure 7; $F_{(2,10)}=1.3$, $p=.31$). These data suggest that elevated IFITM mRNA levels in schizophrenia are not associated with disease severity and are not attributable to the effects of psychotropic medication.

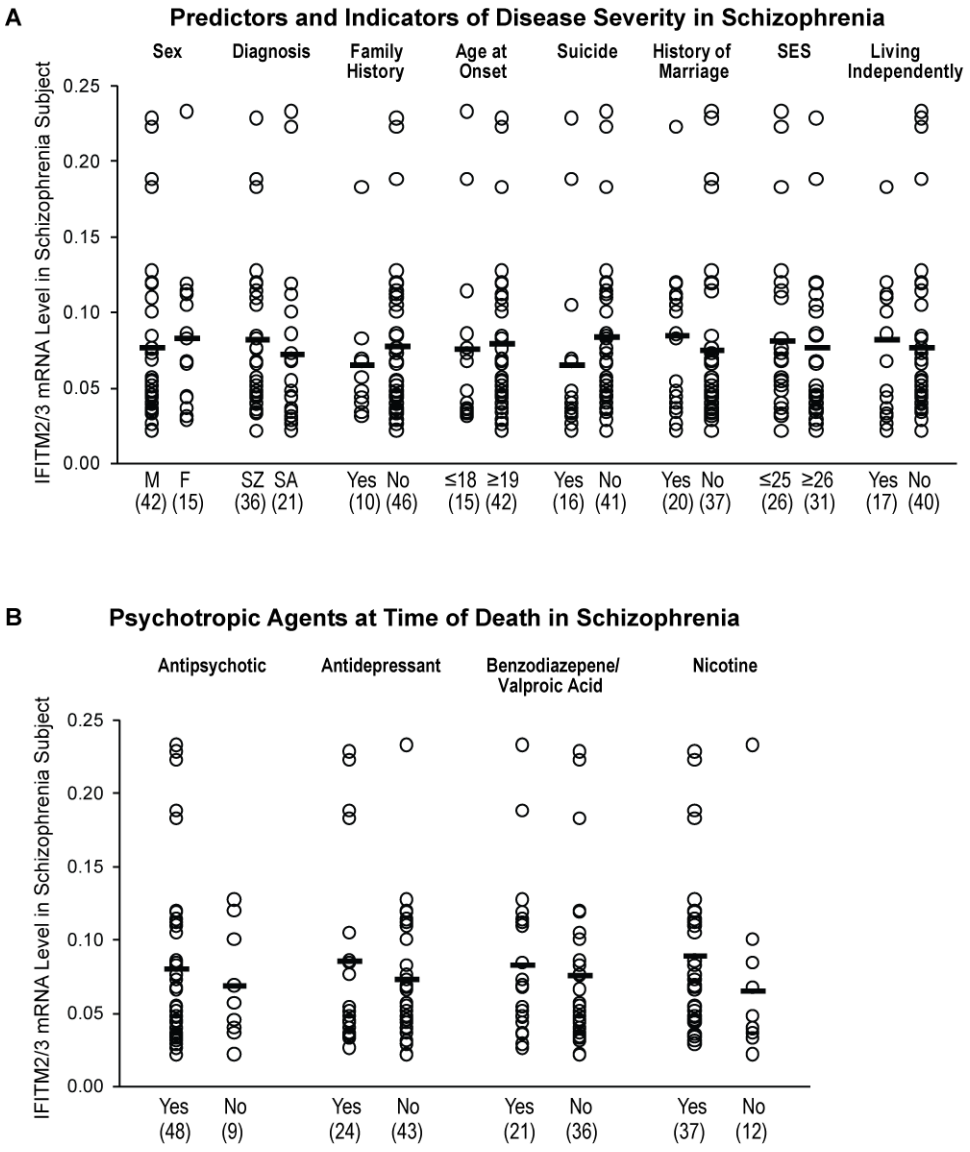

Figure 6. Relationship between disease severity and psychotropic medications and IFITM mRNA levels in schizophrenia.

A. Schizophrenia subjects were divided into groups based on 1) factors predictive of disease severity including sex, diagnosis of schizophrenia (SZ) or schizoaffective disorder (SA), family history of a first degree relative with schizophrenia, and age at onset of schizophrenia in years; and 2) factors that measure impairment in functioning including suicide as cause of death, history of marriage, highest achieved socioeconomic status (SES) as indicated by Hollingshead Index of Social Position, and living independently at time of death. Horizontal black line indicates mean IFITM mRNA level for each subject group. Information on family history was not available for one schizophrenia subject. IFITM mRNA levels did not differ as a function of any of these factors (all $F<.33$, $p>.56$).

B. Schizophrenia subjects were also divided into groups based on use of antipsychotic, antidepressant, or benzodiazepine or valproic acid medications or use of nicotine at time of death. Information on nicotine use was not available for eight schizophrenia subjects. IFITM mRNA levels did not differ as a function of any of these psychoactive substances (all $F<1.2$, $p>0.28$).

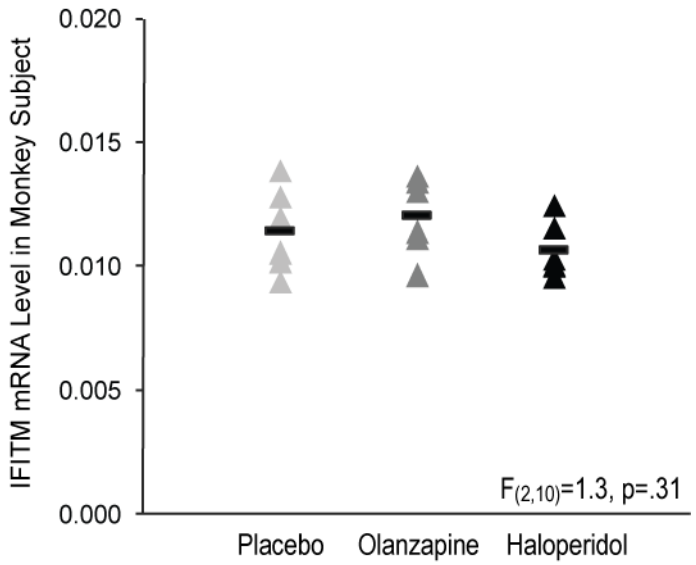

Figure 7. IFITM mRNA levels in prefrontal area 9 of antipsychotic-exposed monkeys. qPCR analysis revealed no statistically significant differences in IFITM mRNA levels (mean ± standard deviation) in monkeys chronically exposed to either olanzapine (0.0120 ± 0.0016; dark gray triangles) or haloperidol (0.0107 ± 0.0011; black triangles) compared to placebo (0.0115 ± 0.0017; light gray triangles). Mean values are shown as horizontal black bars.

We next sought to determine whether higher IFITM mRNA levels in schizophrenia may be related to infection/inflammation-related cause of death (See Appendix A). Brain tissue from our cohort of human subjects is acquired from autopsies conducted at the Allegheny County Medical Examiner's Office. Not surprisingly, the most common cause of these initially unexplained deaths for both schizophrenia (24/57 subjects) and healthy comparison subjects (43/57) is cardiopulmonary-related illness (e.g., atherosclerotic coronary vascular disease, cardiomyopathy, pulmonary embolism;) while only five schizophrenia and one healthy comparison subject had infection/inflammation-related cause of death (i.e. peritonitis, myocarditis, pneumonia, and anaphylaxis). (Sixteen schizophrenia subjects but no healthy comparison subjects died by suicide.) Only including subjects with cardiopulmonary-related cause of death revealed that IFITM mRNA levels were still much higher in schizophrenia

subjects (+77%; 0.061± 0.030; $F_{(1,59)}$=25.9, p<.0001) relative to comparison subjects (0.034 ± 0.010), suggesting that higher IFITM mRNA levels in schizophrenia cannot be explained by infection/inflammation-related cause of death.

3.3 AIM 3: CELL SPECIFICITY OF IFITM MRNA VIA GRAIN COUNTING

As discussed above, qualitative assessment of the film autoradiographs suggested that IFITM expression was particular strong in cortical blood vessels. Consequently, we exposed the same tissue sections from the in-situ hybridization to nuclear emulsion and then developed and stained the sections for Nissl substance to conduct a grain counting analysis. Dense silver grain clusters indicating IFITM mRNA expression were present over distinct clusters of packed cells with elongated nuclei that often contained a lumen or branching point, and these cell clusters had an either linear or round orientation, consistent with longitudinal or transverse profiles of blood vessels, respectively (Figure 8). Grain clusters were also consistently observed over pia mater (not shown). In contrast, qualitative inspection of all schizophrenia and healthy comparison subjects revealed an absence of distinct grain clusters over neurons or glia. In schizophrenia subjects, the mean grain density over blood vessels was higher in schizophrenia subjects (+64%; $F_{(1,17)}$=18.7, p<.001; Figure 8C) relative to comparison subjects and was highly correlated with gray matter optical density measures (r=.74, p<.0001). However, we were not able to quantify grain density over pia mater due to the limited presence of pia mater in the tissue sections.

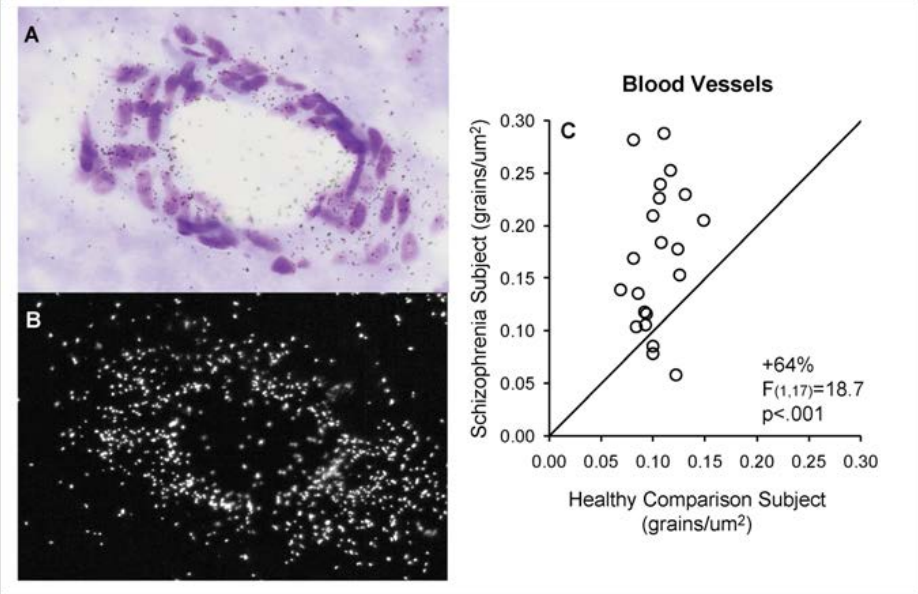

Figure 8. Grain counting analysis of in situ hybridization for IFITM mRNA in the prefrontal cortex of schizophrenia and healthy comparison subjects.
The tissue sections used for the autoradiograph analysis were exposed to nuclear emulsion, developed, and Nissl stained. A representative brightfield image **(A)** from the same tissue section shown in frame B illustrates a cluster of packed cells with elongated nuclei comprising a round structure with a lumen that is consistent with the transverse profile of a blood vessel. The darkfield image **(B)** reveals the accumulation of silver grains representing IFITM mRNA labeling over the blood vessel. Mean grain density per profile area (μm^2) of blood vessel was also higher in schizophrenia subjects (+64%) relative to healthy comparison subjects **(C)**.

3.4 AIM 4: IFITM MRNA LEVELS AND GABA MARKERS

As discussed above, disturbances in PFC inhibitory neurons have been commonly reported in schizophrenia (Akbarian et al. 1995;Volk et al. 2000;Hashimoto et al. 2003;Glausier and Lewis 2011). In a 2012 study of 42 of the schizophrenia subjects included in the present study, Volk et al reported lower mRNA levels for the GABA synthesizing enzyme GAD67, neuronal subpopulation markers parvalbumin and somatostatin, and the GABA neuron-specific

transcription factor Lhx6 (Volk et al. 2012). Interestingly, in schizophrenia subjects, IFITM mRNA levels were inversely correlated with mRNA levels for GAD67 ($r=-.38$, $p=.01$), somatostatin ($r=-.56$, $p<.0001$), Lhx6 ($r=-.42$, $p=.005$), but did not reach significance for parvalbumin ($r=-.24$, $p=.13$). In contrast, in healthy subjects, IFITM mRNA levels were not correlated with GAD67, parvalbumin, or Lhx6 (for all, $r<-.23$, $p>.15$), but were inversely correlated with somatostatin ($r=-.51$, $p=.001$) (Figure 9A). Furthermore, the 2012 Volk et al study we also identified a "low-GABA-marker" subset of schizophrenia subjects (n=20) that had the most severe deficits in GAD67, parvalbumin, somatostatin, and Lhx6 mRNA levels (Volk et al. 2012). Interestingly, differences in mRNA levels were found for IFITM mRNA ($F_{(2,76)}=15.6$, $p<.0001$) among the low-GABA-marker schizophrenia subjects (n=20) relative to all other schizophrenia (n=22) and healthy (n=42) subjects (Figure 9B). IFITM mRNA levels were even higher in the low-GABA-marker schizophrenia subjects relative to other schizophrenia subjects (+61%, $p=.001$) and to healthy comparison subjects (+148%, $p<.0001$). The finding that schizophrenia subjects with higher IFITM mRNA levels in endothelial cells also have greater disturbances in cortical GABA neurons suggests that these cell-type distinct pathological disturbances may be influenced by a shared upstream insult.

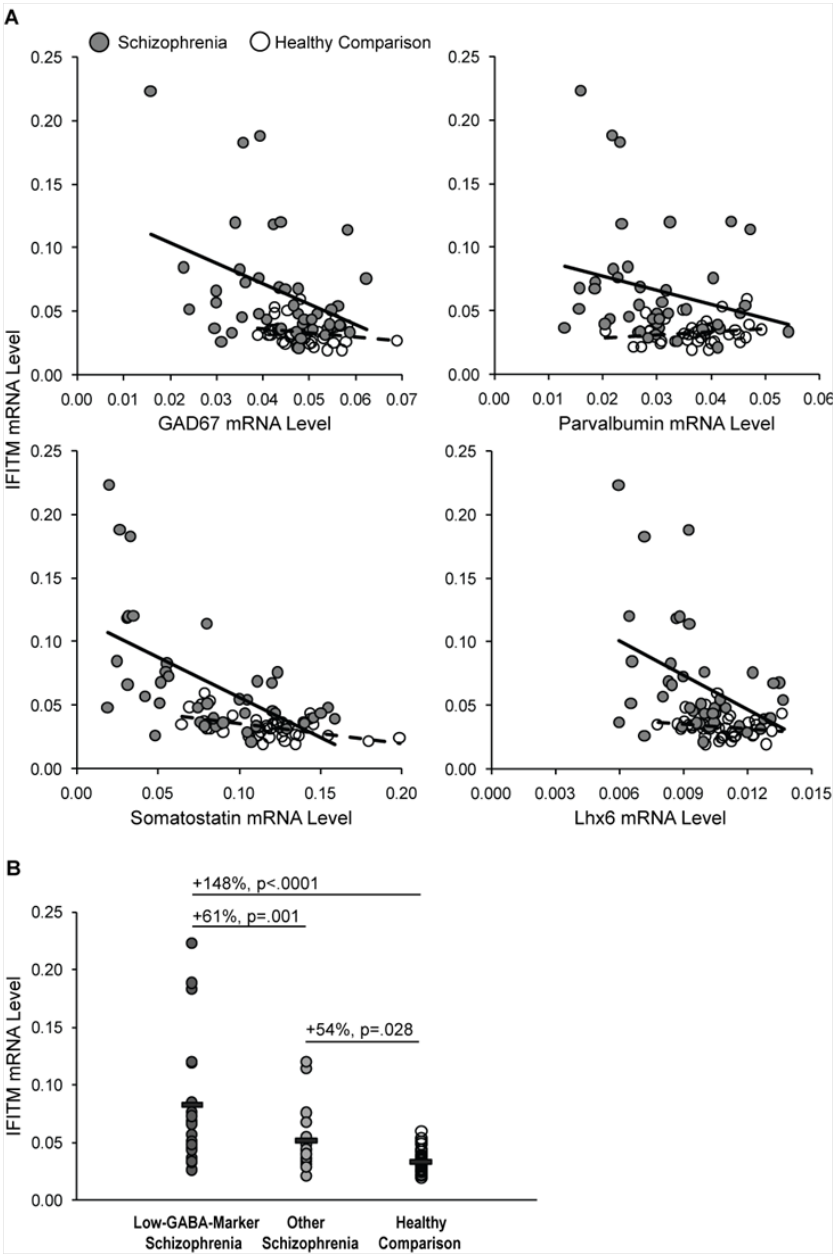

Figure 9. Relationship between IFITM mRNA levels and GABA neuron-related markers in schizophrenia.

A. Quantitative PCR analysis revealed that in schizophrenia subjects (grey circles; black linear regression lines, n=42 subjects), IFITM mRNA levels were inversely correlated with mRNA levels for GAD67 (r=-.38, p=.01), somatostatin (r=-.56, p<.0001), Lhx6 (r=-.42, p=.005), and almost for parvalbumin (r=-.24, p=.13). In contrast, in healthy subjects (open circles, dashed linear regression lines, n=42 subjects), IFITM mRNA levels were not correlated with GAD67, parvalbumin, or Lhx6 (for all, r<-.23, p>.15), but were inversely correlated with somatostatin (r=-.51, p=.001). **B.** IFITM mRNA levels were even higher in the low-GABA-marker schizophrenia subjects (dark grey circles; n=20) relative to other schizophrenia subjects (light grey circles; n=22) and to healthy comparison subjects (open circles; n=42). IFITM mRNA levels were also higher in the schizophrenia subjects not included in the low-GABA-marker subset relative to healthy comparison subjects.

4.0 CHAPTER 4: DISCUSSION

4.1 SUMMARY OF FINDINGS

In this study, we found marked elevations in IFITM mRNA levels in the prefrontal cortex in schizophrenia which is consistent with the findings of prior studies (Arion et al. 2007;Saetre et al. 2007) and suggests that higher IFITM mRNA levels are a common feature of schizophrenia. Specifically, via qPCR analysis, there was a 72% elevation for IFITM1 mRNA and a 116% elevation for IFITM2/3 mRNA expression suggesting elevated expression of all known isoforms of IFITM mRNA in the disorder.

Further evaluation using film autoradiographs revealed intense signal over small structures with features characteristic of blood vessels (i.e. linear, round, branching structures). Furthermore, film analysis demonstrated the absence of a detectable laminar pattern for IFITM mRNA labeling (data not shown) and similar levels of IFITM mRNA expression in the gray and white matter, which is also consistent with the presence of IFITM mRNA expression in blood vessels. Using a film analysis, we found a 110% elevation in optical density for IFITM mRNA expression in gray matter in schizophrenia and an 85% elevation in white matter. Analysis of Nissl-stained, emulsion dipped tissue sections finally confirmed that IFITM mRNA is predominantly expressed in anatomical structures consistent with blood vessels. Grain counting analysis revealed a 64% increase in grain density over gray matter blood vessels in schizophrenia

subjects relative to control subjects. Large, concentrated grain clusters were also visible over pia mater, although the lack of consistently available pia for analysis across subjects prevented quantitative study.

Interestingly, we also found that in schizophrenia subjects, IFITM mRNA levels quantified by qPCR were negatively correlated with the mRNA levels of GABA-related markers detected via the same method. Specifically, there were statistically significant, negative correlations between IFITM and GAD67, somatostatin, and Lhx6 mRNA, as well as a trend association between IFITM and parvalbumin in schizophrenia subjects. Moreover, when grouped as high- and low-GABA-marker expressers, the low-GABA-marker schizophrenia subjects had even higher levels of IFITM expression. In contrast, in control subjects, there were no statistically significant correlations between IFITM and GAD67, parvalbumin, or Lhx6 (although SST was negatively correlated). This finding suggests that the IFITM elevation and GABA-related marker deficits may share the same upstream result.

4.2 POTENTIAL CONFOUNDS

We next sought to further establish the validity of these findings by determining whether higher IFITM mRNA levels were related to potential confounding factors. We found no relationship between psychotropic medication or nicotine use and IFITM mRNA levels at time of death in schizophrenia subjects. Similarly, monkeys chronically exposed to antipsychotics or placebo did not differ in IFITM mRNA levels.

Because of IFITM's role in immunoprotection, the elevation in postmortem IFITM mRNA levels in schizophrenia subjects might have been related to a late-life immune event, instead of a core feature of the disease. However, this is highly unlikely because the vast majority of control subjects and schizophrenia subjects died of cardiopulmonary events, trauma, or suicide. Indeed, less that 6% of the causes of deaths for the cohort in the study were related to infection or inflammation. Furthermore, higher IFITM mRNA levels were present in the vast majority of schizophrenia subjects (i.e. higher in 50 of the 57 schizophrenia subjects relative to matched healthy comparison subjects), suggesting that elevated IFITM mRNA in schizophrenia is unlikely to be explained by undetected late-life immune events that may have occurred at a higher frequency in schizophrenia than in comparison subjects. These data suggest that higher IFITM levels in schizophrenia cannot be explained by a late-life immune event, such as infection or inflammation.

A final piece of evidence for the validity of the study findings is that there was a strong association between the qPCR and in situ hybridization data which utilize completely different methods of mRNA quantification. Indeed, there was a high, positive correlation between IFITM mRNA levels found via qPCR and optical densities found via in situ hybridization for. This suggests that the finding of elevated IFITM mRNA in schizophrenia is both robust and reproducible across different techniques.

4.3 POTENTIAL UNDERLYING MECHANISMS

The present study clearly illustrates that there is an elevation in IFITM mRNA levels in the DLPFC of individuals with schizophrenia that is especially profound in gray matter blood vessels. Recent studies have found that IFITM proteins act to restrict viral replication early in the viral life cycle by preventing the virus from fusing with the cell membrane (Feeley et al. 2011;Huang et al. 2011). This early restriction allows IFITM to have antiviral effects against a wide range of viruses that involve viral fusion, including influenza, filoviruses, flaviviruses, and coronaviruses. IFITM isoforms1-3, which were the variants studied here, are ubiquitous in the body and are present at basal levels in cells, even in the absence of interferon induction (Tanaka et al. 2005). This may help explain why there were detectable levels of IFITM mRNA in all subjects, including controls, even though the degree of expression was different between groups. Given its broad-spectrum antiviral function and its potential role in immune cell signaling, IFITM overexpression in schizophrenia may be an indicator of a state of heightened immune activity(Diamond and Farzan 2013). From this limited knowledge of IFITM function, we now discuss three pathogenetic mechanisms that may contribute to overexpression of IFITM mRNA in cortical blood vessels in schizophrenia. Namely, the elevation in IFITM mRNA levels could be involved in or a consequence of a(n):

1. Early immune insult such as maternal immune activation
2. Chronic state of neuroinflammation
3. Disruption in the microvasculature of the blood-brain barrier

4.3.1 Maternal Immune Activation

As discussed above, maternal immune activation (MIA) has been reported to increase the risk of schizophrenia in offspring (Brown and Derkits 2010). For example, the presence of antigens to influenza or antibodies to toxoplasmosis in maternal serum during pregnancy not only predicts development of schizophrenia in the offspring, but is actually associated with worse performance on cognitive tasks even among schizophrenia patients (Brown et al. 2005). Furthermore, in studies that injected pregnant mice with immune-activating agents such as lipopolysaccharide or poly I:C, offspring have been shown to exhibit deficits in working memory (Oskvig et al. 2012;Meyer et al. 2008;Meyer et al. 2006;Dickerson et al. 2010). MIA in mice has also been shown to cause reductions in cortical parvalbumin immunoreactivity and abnormalities in PFC neural firing patterns associated with parvalbumin neurons that subserve working memory function in offspring (Dickerson et al. 2010;Meyer et al. 2008). Taken together, these findings indicate that MIA may be an integral part of the development of the PFC dysfunction characteristic of the cognitive deficits in schizophrenia.

Because increased schizophrenia risk has been associated with MIA due to viral (Brown et al. 2000), parasitic (Brown et al. 2005), or bacterial (Sorensen et al. 2009) pathogens, the phenomenon does not appear to be pathogen-specific. In other words, the increased risk is not directly caused by any given pathogen in-utero, but rather by a generalized immunological mechanism initiated by the mother's immune system. A prominent theory in the literature is that these elevations in fetal cytokines, which at normal levels facilitate a number of crucial neurodevelopmental processes such as neural induction, neurogenesis, neuron differentiation, and neural migration (Deverman and Patterson 2009), is a primary mediator of the disruptions in neurodevelopment that ultimately put the fetus at risk for developing schizophrenia later in life

(Gilmore and Jarskog 1997;Patterson 2009). Consistent with this theory, studies in which cytokines were injected directly into newborn rodent pups found that the cytokines disrupted neurodevelopment and led to cognitive and behavioral deficits similar to those seen in schizophrenia (Nawa and Takei 2006).

An important implication of the cytokine-mediated offset in neurodevelopment is that the MIA-related elevation in cytokines early in life may persist into adulthood as result of permanently altered gene regulation. Indeed, maternal immune activation can induce long-lasting epigenetic changes at gene promoters in offspring (Tang et al. 2013), although it is unclear if MIA specifically induces epigenetic changes at the promoters for IFITM or GABA-related markers. Furthermore, MIA has been shown to lower mRNA levels for GAD67 and vesicular GABA transporter in the prefrontal cortex of adult mice (Richetto et al. 2013), similar to that seen in schizophrenia (Volk et al. 2012;Hoftman et al. 2013). MIA also raises levels of pro-inflammatory cytokines in splenocytes of adult rats (Basta-Kaim et al. 2012), including cytokines (e.g, IL-6) known to induce IFITM expression (Bailey et al. 2012). In addition, mice that were exposed to poly:IC early in development (but after birth) exhibited higher IFITM3 expression in astrocytes that was shown to contribute to impairment in neurite development via neuron cell culture. This developmental impairment was not seen in neuron cell cultures with IFITM3 knockout astrocytes, suggesting that IFITM may play a mechanistic role in the immune-related neurodevelopmental offset hypothesized to be involved in schizophrenia (Ibi et al. 2013). Notably, however, IFITM mRNA elevations in astrocytes were not found in the present study.

Taken together, these findings indicate that, while further proof-of-principle testing is needed, an early immune insult such as MIA may contribute to long-lasting elevations in IFITM mRNA levels and deficits in GABA-related mRNAs in adult brain through multiple mechanisms

of disrupting early fetal development by elevating cytokine levels in fetal brain (Meyer et al. 2006) and through enduring epigenetic modifications at gene promoter regions (Tang et al. 2013). One way to further investigate this hypothesis would be create a mouse model of MIA via poly I:C injection and then measure prefrontal IFITM mRNA expression in the offspring with the prediction that there will be higher IFITM mRNA levels in the offspring whose mothers were exposed to Poly I:C than those who were not.

4.3.2 Chronic Neuroinflammation

Regardless of its origin, abnormal immune activity is a remarkably conserved finding in schizophrenia. Studies examining serum and cerebrospinal fluid have found consistently abnormal cytokine levels in schizophrenia, although there are many contradictory findings in terms of which specific cytokines are over- or underexpressed in the disease (Di Nicola et al. 2012;Drzyzga et al. 2006;Miller et al. 2011;Potvin et al. 2008). Importantly, however, cytokines involved in inflammatory processes (e.g, IL-6 and IL-1β) are among the most commonly found to be abnormal, suggesting that there is an inflammatory component to schizophrenia. Indeed, PET imaging has revealed that schizophrenia patients injected with a radioactive tag for neuroinflammation (PK-11195) exhibit higher binding than do healthy comparisons (Doorduin et al. 2009;van Berckel et al. 2008). Furthermore, microglia, which heavily contribute to inflammatory processes in the brain, may be present in higher density in area 9 in schizophrenia (Radewicz et al. 2000). More support for the presence of a neuroinflammatory component to the disorder has come from genome-wide association studies (GWAS), which have found that schizophrenia is highly associated with gene variants in the MHC region (Ripke et al. 2011;Irish

Schizophrenia Genomics Consortium and the Wellcome Trust Case Control Consortium 2 ;Jia et al. 2010). These gene variants tend to correspond to players in inflammatory signaling pathways, such as TGFβ and TNF (Jia et al. 2010).

Interestingly, the 2012 Fillman et al study found both elevated pro-inflammatory cytokines (IL-6, IL-1β, IL-8) and deficient GABA markers (somatostatin, parvalbumin, GAD_{67}) in a subset of schizophrenia subjects. In the same cohort, the study also found higher mRNA expression of IFITM1 mRNA in the DLPFC in schizophrenia. IFITM proteins have be shown to be associated with a variety of inflammatory diseases, including ulcerative colitis and inflammatory bowel disease, so it is plausible for IFITM to be involved in inflammatory processes in the brain (Seo et al. 2010;Wu et al. 2007). Indeed, the 2007 Arion et al study demonstrated a strong association between IFITM and immune-related transcripts such as SERPINA3, which has been linked to inflammatory processes (Horvath et al. 2005;Arion et al. 2007). Moreover, elevated SERPINA3, HLA-A, and IFITM mRNA expression were found to co-occur in the PFC in schizophrenia in another microarray study (Saetre et al. 2007). These findings are especially interesting in light of the present study's demonstrations that GABA marker mRNA deficits are correlated with IFITM mRNA elevation and that the IFITM elevation is particularly pronounced in blood vessels, which are intricately involved in neuroinflammation. Taken together, these findings suggest that neuroinflammation may be related to both GABA circuitry dysfunction (e.g. perhaps parvalbumin neurons are particularly susceptible to the deleterious effects of chronic neuroinflammation) and IFITM mRNA overexpression in the DLPFC in schizophrenia, although they do not elucidate the directionality the relationships.

4.3.3 Blood Brain Barrier Abnormality

Because the present study shows that IFITM mRNA is present in high levels specifically in DLPFC blood vessels, it is plausible that there is disrupted microvasculature within the region. If so, then elevated IFITM mRNA levels in schizophrenia in endothelial cells could be indicative of a dysfunctional blood-brain barrier (BBB) in the disorder.

Indeed, CSF albumin protein levels, which are a broad indicator of BBB function and permeability, are elevated in schizophrenia (Kirch et al. 1992;Kirch et al. 1985;Muller et al. 1999). Additionally, a study looking at mRNA expression of vascular endothelial growth factor (VEGF), an angiogenetic factor involved in the regulation of cerebral blood flow, found markedly decreased mRNA levels in the DLPFC in schizophrenia (Fulzele and Pillai 2009).

The status of BBB function can also be assessed by investigating the function of astrocytes, which are heavily involved in the development and maintenance of the BBB. Specifically, astrocytic endfeet envelop the outer surface of the CNS blood vessel endothelium, allowing for induction of new vessels and vessel branches (Janzer and Raff 1987). Additionally, the anatomical proximity of astrocytes and endothelia allows for both short- and long-term modulation of the permeability of the tight junctions that form the BBB (Abbott 2002). Therefore, if markers of astrocyte function are shown to be abnormal in schizophrenia, then BBB function can also be assumed to be abnormal. Indeed, a number of studies have investigated glial acidic fibrillary protein (GFAP), which is involved in astrocyte cytoskeleton integrity (Harrison and Mobley 1992), and overall glial cell density. In both cases, there has not been an overall difference in schizophrenia, although both GFAP expression and glial cell density might be down in cortical layer 5 and there may be lower GFAP expression in the anterior cingulate cortex (Cotter et al. 2001;Rajkowksa et al. 2002).

Furthermore, in schizophrenia it has been well-established that S100B, a calcium-binding protein that is integrally important to and predominantly expressed by astrocytes, is elevated in the serum of individuals with schizophrenia (Rothermundt et al. 2009). There is also evidence that S100B is elevated in both the CSF and post-mortem cortical brain tissue, especially in the DLPFC (Rothermundt et al. 2009;Steiner et al. 2006;Steiner et al. 2008). Interestingly, there are positive associations between elevated S100B serum levels and negative symptom severity in schizophrenia (Rothermundt et al. 2004;Pedersen et al. 2008). S100B has also been implicated in the regulation of inflammatory processes and other immune functions (Donato 2003), so elevated S100B in schizophrenia lends support to the hypothesis that dysfunctional BBB is involved in the immunological abnormalities found in the disorder. In fact, it has been proposed that the cerebral microvasculature is the primary site of neuroinflammation in the disease (Hanson and Gottesman 2005). One way to test the status of IFITM in BBB dysfunction in schizophrenia would be to investigate the expression of BBB-related markers such as S100B and VEGF in the same schizophrenia subjects who have elevated IFITM mRNA levels with the prediction that alterations in these BBB-related markers are greatest in the same schizophrenia subjects.

Taken together, these findings suggest that the agents involved in BBB formation and maintenance are distinctly abnormal. Because neurodevelopment and cerebral microvasculature function are closely interdependent (Saunders et al. 2008), abnormalities in the cells that form the BBB, such as increased IFITM expression in the DLPFC, may be related to the abnormalities in neurodevelopment that ultimately result in core pathologic findings seen in schizophrenia, such as GABA circuitry dysfunction.

4.3.4 An Integrative Interpretation

The three interpretations discussed here (MIA, chronic neuroinflammation, and BBB disruption) are by no means mutually exclusive, and in fact may all be involved in the blood vessel-specific overexpression of DLPFC IFITM mRNA found in schizophrenia in the present study. For example, maternal immune activation may serve as an etiologic source which, when combined with genetic predisposition and other environmental factors, results in chronic elevation of cytokines that cause persistent neuroinflammation. This elevation in cytokines and resulting neuroinflammation may contribute directly to GABA circuitry dysfunction and ultimately to the cognitive deficits seen in schizophrenia. At the same time, the inflammation may also disrupt the BBB such that a recursive increase in inflammation occurs, further disrupting normal neurodevelopment (Hanson and Gottesman 2005). The association of IFITM overexpression with blood vessels and markers of both GABA circuitry dysfunction and neuroinflammation in the DLPFC suggests that IFITM plays an integral role in these phenomena.

4.4 CLINICAL IMPLICATIONS

If the GABA-marker deficits and IFITM overexpression in the DLPFC are downstream consequences of MIA, then preventative prenatal care may reduce the risk of schizophrenia development in at-risk offspring. For example, prevention of the maternal infection in the first place (via vaccinations or personal health practice) or rapid treatment of maternal infection if it occurs may help mitigate the deleterious effects of MIA (Brown and Derkits 2010).

Independent of its relationship to MIA, the chronic state of neuroinflammation seen in schizophrenia could be targeted by immune-modulating agents like non-steroidal anti-inflammatory drugs (NSAIDs). By reducing neuroinflammation, such drugs could alleviate some of the circuitry dysfunction seen in the disorder. Indeed, the COX-2 inhibitor celecoxib has been shown to improve both positive and negative symptoms, as measured by the Positive and Negative Symptom Scale (PANSS), more than antipsychotic treatment alone (Akhondzadeh et al. 2007;Muller et al. 2010).

A third clinical approach would be to target the pathway leading to IFITM synthesis directly, thereby preventing its overexpression. IFITM expression is a downstream result of INFα/β receptor binding and subsequent activation of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins. The JAK-STAT signaling pathway results in the translocation of a transcription factor complex into the nucleus of the cell, where IFITM mRNA is transcribed. Ruxolitinib is an example of a drug that interferes with this process by inhibiting JAK. Although FDA approved for the treatment of myelofibrosis, Ruxolitinib could a potentially lower IFITM expression (Deisseroth et al. 2012). In fact, the JAK-STAT pathway is involved in the signal transduction for broad range of pro-inflammatory cytokines, so inhibition the pathway could conceivably attenuate the effects of the cytokine overexpression seen in the disorder (Pesu et al. 2008).

APPENDIX

DEMOGRAPHIC, POSTMORTEM, AND CLINICAL CHARACTERISTICS OF HUMAN SUBJECTS USED IN THIS STUDY

Table 1. Control subject characteristics

Pair	Case	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
1	592	M/B	41	22.1	182		9.0	6.7	ASCVD
2	567	F/W	46	15.0	186		8.9	6.7	Mitral valve prolapse
3	516	M/B	20	14.0	194		8.4	6.9	Homicide by gun shot
4	630	M/W	65	21.2	176		9.0	7.0	ASCVD
5	604	M/W	39	19.3	180		8.6	7.1	Hypoplastic coronary artery
6	546	F/W	37	23.5	190		8.6	6.7	ASCVD
7	551	M/W	61	16.4	189		8.3	6.6	Cardiac tamponade
8	685	M/W	56	14.5	169		8.1	6.6	Hypoplastic coronary artery
9	681	M/W	51	11.6	170		8.9	7.2	Hypertrophic cardio-myopathy
10	806	M/W	57	24.0	149		7.8	6.9	Pulmonary embolism
11	822	M/B	28	25.3	146		8.5	7.0	ASCVD
12	727	M/B	19	7.0	163		9.2	7.2	Trauma
13	871	M/W	28	16.5	135		8.5	7.1	Trauma

Table 1. Control subject characteristics

Pair	Case	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
14	575	F/B	55	11.3	185		9.6	6.8	ASCVD
15	700	M/W	42	26.1	167		8.7	7.0	ASCVD
16	988	M/W	82	22.5	114		8.4	6.2	Trauma
17	686	F/W	52	22.6	169		8.5	7.0	ASCVD
18	634	M/W	52	16.2	176		8.5	7.0	ASCVD
19	852	M/W	54	8.0	139		9.1	6.8	Cardiac tamponade
20	987	F/W	65	21.5	114		9.1	6.8	ASCVD
21	818	F/W	67	24.0	147		8.4	7.1	Anaphylactic reaction
22	857	M/W	48	16.6	138		8.9	6.7	ASCVD
23	739	M/W	40	15.8	162		8.4	6.9	ASCVD
24	1047	M/W	43	13.8	105	116	9.0	6.6	ASCVD
25	1086	M/W	51	24.2	99	110	8.1	6.8	ASCVD
26	1092	F/B	40	16.6	99		8.0	6.8	Mitral Valve Prolapse
27	10005	M/W	42	23.5	86	97	7.4	6.7	Trauma
28	1336	M/W	65	18.4	64	75	8.0	6.8	Cardiac Tamponade
29	1122	M/W	55	15.4	95	106	7.9	6.7	Cardiac Tamponade
30	1284	M/W	55	6.4	74		8.7	6.8	ASCVD
31	1191	M/B	59	19.4	88	99	8.4	6.2	ASCVD
32	970	M/W	42	25.9	117		7.2	6.4	ASCVD
33	10003	M/W	49	21.2	88		8.4	6.5	Trauma
34	1247	F/W	58	22.7	80	91	8.4	6.4	ASCVD
35	1324	M/W	43	22.3	67	78	7.3	7	Aortic Dissection
36	1099	F/W	24	9.1	98	109	8.6	6.5	Cardiomyopathy
37	1307	M/B	32	4.8	70		7.6	6.7	ASCVD

Table 1. Control subject characteristics

Pair	Case	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
38	1391	F/W	51	7.8	56		7.1	6.6	ASCVD
39	1282	F/W	39	24.5	75		7.5	6.8	ASCVD
40	1159	M/W	51	16.7	92	103	7.6	6.5	ASCVD
41	1326	M/W	58	16.4	66		8.0	6.7	ASCVD
42	902	M/W	60	23.6	131	142	7.7	6.7	ASCVD
43	1374	M/W	43	21.7	64	69	7.2	6.6	ASCVD
44	1555	M/W	17	15.1	29		7.9	6.9	Blunt force trauma to head, thorax, abdomen
45	1268	M/B	49	19.9	82	87	7.9	7.1	ASCVD
46	1466	F/B	64	20.0	47	52	8.8	6.7	Blunt force trauma to trunk
47	1518	M/W	50	20.7	36	41	7.7	6.4	ASCVD
48	1386	M/W	46	21.2	61		8.3	6.7	ASCVD
49	1472	M/W	61	23.8	46	51	8.0	6.5	Pulmonary infarction
50	1026	M/W	59	20.0	113	118	7.4	6.3	ASCVD
51	694	M/W	38	20.7	174	179	7.7	7.0	Subarachnoid hemorrhage
52	1350	M/W	21	24.2	67		7.3	6.4	Trauma
53	1201	F/W	52	16.4	92	97	8.1	6.2	ASCVD
54	1524	M/W	66	9.4	34		8.1	6.4	Small intestinal ischemic infarction
55	1270	F/W	73	19.7	81	86	7.7	6.7	Trauma
56	1372	M/W	37	20.5	65		9.0	6.6	Asphyxiation
57	1543	F/W	45	17.9	31	36	7.4	6.8	Subarachnoid hemorrhage
		Mean	**48.1**	**18.2**	**112.5**	**92.5**	**8.2**	**6.7**	
		SD	**13.9**	**5.4**	**49.9**	**33.7**	**0.6**	**0.3**	

[a] PMI, postmortem interval (hours); [b] Storage time (months) at -80C; Other abbreviations: ASCVD, arteriosclerotic cardiovascular disease; RIN, RNA integrity number

Table 2a. Schizophrenia subject characteristics (Part 1)

Pair	Case	DSM IV diagnosis	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
1	533	Chronic undifferentiated schizophrenia	M/W	40	29.1	192		8.4	6.8	Accidental Asphyxiation
2	537	Schizoaffective disorder	F/W	37	14.5	191		8.6	6.7	Suicide by hanging
3	547	Schizoaffective disorder	M/B	27	16.5	190		7.4	7.0	Heat Stroke
4	566	Chronic undifferentiated schizophrenia; AAR	M/W	63	18.3	187		8.0	6.8	ASCVD
5	581	Chronic paranoid schizophrenia; ADC; OAC	M/W	46	28.1	184		7.9	7.2	Accidental combined drug overdose
6	587	Chronic undifferentiated schizophrenia; AAR	F/B	38	17.8	183		9.0	7.0	Myocardial hypertrophy
7	625	Chronic disorganized schizophrenia; AAC	M/B	49	23.5	177		7.6	7.3	ASCVD
8	622	Chronic undifferentiated schizophrenia	M/W	58	18.9	177		7.4	6.8	Right MCA infarction
9	640	Chronic paranoid schizophrenia	M/W	49	5.2	175		8.4	6.9	Pulmonary embolism
10	665	Chronic paranoid schizophrenia; ADC	M/B	59	28.1	173		9.2	6.9	Intestinal hemorrhage
11	787	Schizoaffective disorder; ODC	M/B	27	19.2	152		8.4	6.7	Suicide by gun shot
12	829	Schizoaffective disorder; ADC; OAR	M/W	25	5.0	144		9.3	6.8	Suicide by salicylate overdose
13	878	Disorganized schizophrenia; ADC	M/W	33	10.8	135		8.9	6.7	Myocardial fibrosis
14	517	Disorganized schizophrenia; ADC	F/W	48	3.7	194		9.3	6.7	Intracerebral hemorrhage
15	539	Schizoaffective disorder; ADR	M/W	50	40.5	191		8.1	7.1	Suicide by combined drug overdose
16	621	Chronic undifferentiated schizophrenia	M/W	83	16.0	178		8.7	7.3	Accidental asphyxiation
17	656	Schizoaffective disorder; ADC	F/B	47	20.1	174		9.2	7.3	Suicide by gun shot
18	722	Chronic undifferentiated schizophrenia; ODR; OAR	M/B	45	9.1	164		9.2	6.7	Upper GI bleeding

Table 2a. Schizophrenia subject characteristics (Part 1)

Pair	Case	DSM IV diagnosis	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
19	781	Schizoaffective disorder; ADR	M/B	52	8.0	154		7.7	6.7	Peritonitis
20	802	Schizoaffective disorder; ADC; ODR	F/W	63	29.0	150		9.2	6.4	Right ventricular dysplasia
21	917	Chronic undifferentiated schizophrenia	F/W	71	23.8	127		7.0	6.8	ASCVD
22	930	Disorganized schizophrenia; ADR; OAR	M/W	47	15.3	124		8.2	6.2	ASCVD
23	933	Disorganized schizophrenia	M/W	44	8.3	123		8.1	5.9	Myocarditis
24	1209	Schizoaffective disorder	M/W	35	9.1	86	97	8.7	6.5	Suicide by diphenhydramine overdose
25	10025	Disorganized schizophrenia; OAR	M/B	52	27.1	78	89	7.8	6.7	ASCVD
26	1178	Schizoaffective disorder	F/B	37	18.9	90		8.4	6.1	Pulmonary embolism
27	1256	Undifferentiated schizophrenia	M/W	34	27.4	78	89	7.9	6.4	Suicide by hanging
28	1173	Disorganized schizophrenia; ADR	M/W	62	22.9	90	101	7.7	6.4	ASCVD
29	1105	Schizoaffective disorder	M/W	53	7.9	97	108	8.9	6.2	ASCVD
30	1188	Undifferentiated schizophrenia; AAR; OAR	M/W	58	7.7	89		8.4	6.2	ASCVD
31	1263	Undifferentiated schizophrenia; ADR	M/W	62	22.7	78	89	8.5	7.1	Accidental asphyxiation
32	1222	Undifferentiated schizophrenia; AAC	M/W	32	30.8	84		7.5	6.4	Suicide by combined drug overdose
33	1088	Undifferentiated schizophrenia; ADC; OAC	M/W	49	21.5	99		8.1	6.5	Accidental combined drug overdose
34	1240	Undifferentiated schizophrenia; ADR	F/B	50	22.9	81	92	7.7	6.3	ASCVD
35	10020	Paranoid schizophrenia; AAC; OAC	M/W	38	28.8	80	91	7.4	6.6	Suicide by salicylate overdose
36	10023	Disorganized schizophrenia	F/B	25	20.1	79	90	7.4	6.7	Suicide by drowning

Table 2a. Schizophrenia subject characteristics (Part 1)

Pair	Case	DSM IV diagnosis	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
37	10024	Paranoid schizophrenia	M/B	37	6.0	79		7.5	6.1	ASCVD
38	1189	Schizoaffective disorder; AAR	F/W	47	14.4	89		8.3	6.4	Suicide by combined drug overdose
39	1211	Schizoaffective disorder	F/W	41	20.1	86		7.8	6.3	Sudden unexplained death
40	1296	Undifferentiated schizophrenia	M/W	48	7.8	72	83	7.3	6.5	Pneumonia
41	1314	Undifferentiated schizophrenia	M/W	50	11.0	69		7.2	6.2	ASCVD
42	1361	Schizoaffective disorder; ODC	M/W	63	23.2	62	73	7.7	6.4	Cardiomyopathy
43	904	Schizoaffective disorder	M/W	33	28.0	136	141	7.1	6.2	Broncho-pneumonia
44	1649	Undifferentiated schizophrenia	M/B	17	21.8	15		8.1	6.9	Suicide by hanging
45	1230	Undifferentiated schizophrenia	M/W	50	16.9	87	92	8.2	6.6	Suicide by doxepin overdose
46	1341	Schizoaffective disorder; ODC	F/W	44	24.5	68	73	8.8	6.6	Trauma
47	1367	Schizoaffective disorder; ADC; ODR	M/W	47	28.9	66	71	7.2	6.6	Combined drug overdose
48	1420	Schizoaffective disorder; AAR; ODC; OAR	M/W	47	23.4	54		8.2	6.7	Suicide by jump
49	1453	Paranoid schizophrenia; ADR	M/W	62	11.1	49	54	8.2	6.4	Trauma
50	1454	Paranoid schizophrenia; AAR; ODC	M/W	59	24.1	48	53	7.6	6.1	Trauma
51	1455	Paranoid schizophrenia; AAR; OAC	M/W	42	8.2	48	53	7.7	6.2	Peritonitis
52	1474	Schizoaffective disorder; ADR	M/W	37	39.9	46		7.0	6.7	Suicide by hanging
53	1506	Schizoaffective disorder; ADC	F/W	47	14.1	40	45	7.5	6.6	Combined drug overdose
54	1542	Paranoid schizophrenia	M/W	65	17.4	31		7.8	6.7	Combined drug overdose
55	1579	Schizoaffective disorder; ADR; ODC	F/W	69	16.1	25	30	7.7	6.7	ASCVD

Table 2a. Schizophrenia subject characteristics (Part 1)

Pair	Case	DSM IV diagnosis	Sex/ Race	Age	PMI[a]	Storage Time qPCR[b]	Storage Time In Situ	RIN	pH	Cause of Death
56	1581	Paranoid schizophrenia; ODC; OAC	M/W	32	18.4	24		9.0	6.8	ASCVD
57	10026	Undifferentiated schizophrenia	F/W	46	23.8	83	88	7.6	6.6	Suicide by thermal injuries
			Mean	**46.9**	**18.9**	**109.2**	**81.1**	**8.1**	**6.6**	
			SD	**12.9**	**8.5**	**53.7**	**24.7**	**0.6**	**0.3**	

[a] PMI, postmortem interval (hours); [b] Storage time (months) at -80C;Other abbreviations: ASCVD, arteriosclerotic cardiovascular disease; MCA, middle cerebral artery; ADC, alcohol dependence, current at time of death; ADR, alcohol dependence, in remission at time of death; AAC, alcohol abuse, current at time of death; AAR, alcohol abuse, in remission at time of death; ODC, other substance dependence, current at time of death; ODR, other substance dependence, in remission at time of death; OAC, other substance abuse, current at time of death; OAR, other substance abuse, in remission at time of death; U, unknown.

Table 2b. Schizophrenia subject characteristics (Part 2)

Pair	Case	History of Cannabis Use	APD ATOD	APD ATOD	BDZ/ VPA ATOD	Family History[c]	Age at Onset of Illness	History of Marriage	Hollingshead Two Factor ISP	Independent Living ATOD
1	533	None	Y	N	N	N	25	N	20	N
2	537	None	N	N	N	N	29	Y	20	Y
3	547	None	Y	Y	Y	N	18	N	17	N
4	566	None	Y	Y	Y	N	43	Y	16	N
5	581	Use	Y	N	Y	Y	16	Y	19	Y
6	587	Use	Y	N	Y	N	18	N	17	N
7	625	None	Y	Y	N	Y	35	Y	34	N
8	622	None	N	N	N	N	42	N	27	N
9	640	None	Y	Y	N	N	21	N	63	N
10	665	None	Y	Y	N	Y	27	N	16	Y

Table 2b. Schizophrenia subject characteristics (Part 2)

Pair	Case	History of Cannabis Use	APD ATOD	APD ATOD	BDZ/ VPA ATOD	Family History[c]	Age at Onset of Illness	History of Marriage	Hollingshead Two Factor ISP	Independent Living ATOD
11	787	Dependent	Y	N	N	N	24	N	30	N
12	829	Abuse in remission	N	N	Y	Y	20	N	22	N
13	878	Use	Y	Y	Y	N	16	N	57	N
14	517	None	Y	N	N	N	28	Y	53	N
15	539	None	Y	Y	Y	N	19	Y	45	Y
16	621	None	N	N	N	Y	28	N	8	N
17	656	None	Y	N	N	Y	17	N	19	Y
18	722	Use	Y	N	N	N	26	N	24	N
19	781	None	Y	Y	N	N	37	Y	24	N
20	802	None	Y	N	Y	N	20	N	42	Y
21	917	None	Y	N	N	Y	24	Y	19	N
22	930	Abuse	Y	N	Y	N	19	N	17	N
23	933	None	Y	Y	Y	N	22	N	27	N
24	1209	None	Y	N	N	N	21	Y	35	Y
25	10025	Abuse in remission	N	N	N	N	22	N	27	N
26	1178	None	Y	N	Y	N	26	N	40	N
27	1256	None	Y	N	N	N	28	N	36	N
28	1173	None	Y	N	N	N	29	Y	16	Y
29	1105	None	Y	N	N	N	48	N	24	N
30	1188	Abuse in remission	Y	N	N	Y	25	N	45	N
31	1263	Use	Y	Y	N	N	21	N	11	N
32	1222	Use	Y	Y	N	N	16	N	14	Y
33	1088	Abuse	Y	Y	N	N	25	Y	45	N
34	1240	None	Y	N	N	N	25	N	32	N
35	10020	None	Y	Y	Y	N	18	N	27	N

Table 2b. Schizophrenia subject characteristics (Part 2)

Pair	Case	History of Cannabis Use	APD ATOD	APD ATOD	BDZ/ VPA ATOD	Family History[c]	Age at Onset of Illness	History of Marriage	Hollingshead Two Factor ISP	Independent Living ATOD
36	10023	None	Y	N	Y	Y	15	N	17	Y
37	10024	None	N	N	N	N	20	Y	27	N
38	1189	None	Y	Y	Y	N	43	N	66	Y
39	1211	Use	Y	Y	N	N	29	Y	30	N
40	1296	None	Y	Y	N	N	13	N	14	N
41	1314	None	Y	Y	N	N	17	N	19	N
42	1361	Use	Y	N	Y	N	16	N	17	N
43	904		Y	N	Y	N	25	N	30	N
44	1649		Y	Y	N	Y	15	N	11	N
45	1230		Y	Y	N	N	22	N	30	N
46	1341		Y	N	Y	U	14	N	11	N
47	1367		N	N	N	N	23	Y	50	Y
48	1420		Y	Y	N	N	18	N	30	N
49	1453		N	N	Y	N	30	N	19	N
50	1454		Y	Y	N	N	26	Y	32	Y
51	1455		Y	N	Y	N	23	Y	14	Y
52	1474		N	N	N	N	35	Y	35	Y
53	1506		Y	Y	N	N	18	Y	42	Y
54	1542		Y	N	Y	N	25	N	25	N
55	1579		Y	N	Y	N	25	Y	32	Y
56	1581		Y	Y	N	N	29	N	37	N
57	10026		Y	Y	N	N	22	Y	30	N

[c] First degree relative with schizophrenia; Abbreviations: ATOD, at time of death; APD, antipsychotic drug; ADD, antidepressant drug; BDZ, benzodiazepine; VPA, valproic acid; ISP, index of social position; Y, yes; N, no.

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