



Extracellular Matrix Abnormalities in Major Psychoses: CS-6 Expression in Human Dermal Fibroblasts

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Extracellular Matrix Abnormalities in Major Psychoses: CS-6 Expression
in Human Dermal Fibroblasts

Janey Ronxhi

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Abstract

Emerging research on the extracellular matrix (ECM) has identified chondroitin sulfate proteoglycans (CSPGs) as one of the key contributors to abnormalities observed in major psychoses. This study aimed to explore expression of a specific form of sulfation, CS-6 (CS56), on chondroitin sulfate chains in dermal fibroblasts as an approach in understanding the role that ECM, and more specifically CSPGs, play in Schizophrenia and Bipolar Disorder. In this study, 39 human dermal fibroblast cell lines from normal controls, schizophrenic, and bipolar subjects were used. Cell lysates and conditioned media were collected at 24 and 72 hours to assess expression as well as secretion. Immunocytochemistry staining using anti-chondroitin sulfate anti-body CS-56 revealed that human dermal fibroblasts abundantly express CS56. Western Blot analyses showed no significant differences in CS-6 expression amongst the groups. Notably, significant changes were instead detected in olfactory mucosa cell lines from subjects with major psychoses, consistent with findings in the central nervous system. Together, these findings provide important clues on CSPG abnormalities in Schizophrenia and Bipolar Disorder, suggesting dysregulation exclusively in the nervous system.

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Chapter I

Introduction

The Research Problem

Schizophrenia and Bipolar Disorder are debilitating chronic disorders of the brain that affect 1.1% and 5.5% of the global population, respectively (NIMH, 2015). The two disorders share many similarities and overlaps in numerous domains, such as genetic, clinical, pharmacological, molecular, and pathophysiological aspects; therefore, it is important to study Schizophrenia and Bipolar Disorder concurrently. Bipolar Disorder and Schizophrenia share the most common genetic variations, with a 15% overlap on heritability (Lee *et al.*, 2013). The two disorders also exhibit some resemblance in their neurotransmitter dysfunction (Möller, 2003), and share many clinical characteristics (i.e. positive and negative symptoms) (Strakowski, 2003). Studies that highlight differences between the two are also necessary to identify molecular mechanisms or biological markers that could lead to better diagnoses and treatment.

Several recent studies have reported changes within the extracellular matrix in patients with Schizophrenia and Bipolar Disorder (Lubbers *et al.*, 2014; Mauney *et al.*, 2013, Enwright *et al.*, 2012). Growing evidence from our laboratory also supports these findings by noting abnormalities in Chondroitin Sulfate Proteoglycans (CSPGs) within the medial temporal lobe of patients with Schizophrenia and Bipolar Disorder (Pantazopoulos *et al.*, 2010; Pantazopoulos *et al.*, 2015). CSPGs, an important element of the extracellular matrix (ECM), play key roles in neural development and regeneration, axon guidance, as well as synaptic plasticity (Dityatev *et al.*, 2003; Berretta, 2012;

Pantazopoulos *et al.*, 2013; Avram *et al.*, 2014). These functions of CSPGs may have implications for both Schizophrenia and Bipolar Disorder, which are generally considered as neurodevelopmental disorders (Harrison, 2007; Bavamian *et al.*, 2015); thereby, understanding the role that CSPGs play in these two disorders can provide insight into their mechanisms.

As a major component of the ECM, CSPGs are not only expressed in the brain, but are richly represented throughout the whole organism even in non-neural samples such as cartilage and skin (Matigian *et al.*, 2008; Tsuneki *et al.*, 2015). Therefore, peripheral tissue, such as dermal fibroblasts can be used to test whether expression abnormalities of CSPGs detected in the Central Nervous System (CNS) (Pantazopoulos *et al.*, 2010) can also be detected peripherally. Dermal fibroblasts have been widely utilized to study pathophysiological mechanisms in a number of neuropsychiatric disorders, including Schizophrenia (Miyamae *et al.*, 1998; Matigian *et al.*, 2008; Wang *et al.*, 2010), with the advantage being that they are easy to grow and maintain, thus allowing for specific group comparisons (Schizophrenic vs. BD vs. Control). We propose to examine the expression of CSPGs using Anti-Chondroitin Sulfate antibody [CS56] in human dermal fibroblasts from subjects with Schizophrenia and Bipolar Disorder. More specifically, we want to assess not only intracellular expression of CSPGs but also secretion. Western Blotting will be used to assess and quantify expression. Immunocytochemistry (ICC) will also be used to validate findings using microscopy. We expect to see affected CSPG levels in patients suffering from Schizophrenia and BD, when compared to the controls. More specifically, we anticipate

observing an altered CSPG expression in subjects with Schizophrenia and to a lesser degree in Bipolar Disorder. Preliminary results from our group support this hypothesis.

If our hypothesis is confirmed, and altered levels of CSPG are noted in our experimental groups, it will highlight the important role that ECM plays in the pathophysiology of these disorders in addition to having potential non-invasive applications. These applications can in turn lead to a more accurate diagnosis, understanding of pathophysiology and its links to genetic vulnerabilities, and potential targeted therapies. Depending on the degree of observed differences between the groups, these findings also have the potential to facilitate a disease progression model by correlating changes in CSPG levels to disease progression that may also be applied to other neurodevelopmental disorders.

Definition of Terms

“Extra-cellular Matrix (ECM)”: a complex meshwork of proteins and polysaccharides that contribute to the structure and function of a tissue.

“Chondroitin Sulfate Proteoglycans (CSPGs)”: extracellular matrix components containing two structural parts: a protein core and glycosaminoglycan (GAG) side chains.

“Perineuronal Nets (PNNs)”: extracellular matrix structures consisting of groups of CSPGs that enwrap neurons in the brain.

“Human Dermal Fibroblasts”: cells from the dermis layer of the skin

“Concordance rate”: a statistical index that researchers use to determine the relative influence of nature vs. nurture

“CS-6”: a chondroitin sulfate chain highly expressed in the CSPG aggrecan

“Central Nervous System (CNS)”: brain and spinal cord, the complex of nerve tissues that controls the activities of the body

“Matrix metalloproteinases (MMPs)”: calcium-dependent zinc-containing endopeptidases

“Pleomorphic”: able to assume different forms

“Proteolysis”: breakdown of proteins into smaller polypeptides or amino acids

“GABAergic interneurons”: inhibitory neurons of the nervous system that play a vital role in neural circuitry and activity, named due to their release of the neurotransmitter gamma-aminobutyric acid (GABA)

“Chondroitin sulfotransferases”: Enzymes belonging to the family of transferases which transfer sulfur-containing groups

“Parvalbumin”: calcium-binding albumin protein

“Epigenetic”: relating to or arising from nongenetic influences on gene expression

“Endophenotype”: a genetic epidemiology term used to separate behavioral symptoms into more stable phenotypes with a clear genetic connection

“Calretinin”: calcium-binding protein involved in calcium signaling

“Dentate gyrus” part of the hippocampus and/or hippocampal formation, thought to contribute to the formation of new episodic memories & the spontaneous exploration of novel environments

Current knowledge on the pathophysiology of Schizophrenia and Bipolar Disorder

Schizophrenia is a multi-faceted neurological disorder that affects an individual's mental and emotional health. Symptoms of Schizophrenia include hallucinations, delusions, being emotionally dull, and loss of interest in daily tasks as well as lack of awareness (NAMI, 2016). Multiple interweaving potential causes such as genetics, environment, altered neurotransmitters, and substance abuse add to the complexity of this disorder. Twin and family studies point to intricate interactions between genetic and environmental factors. For instance, the risk for developing Schizophrenia increases 8 to 12-folds in first degree relatives, whereas twin studies show that the concordance rate for Schizophrenia is much higher in monozygotic twins (47%–56%) when compared to the dizygotic twins (12%–16%) highlighting the importance of genetic predisposition in Schizophrenia (Ivleva *et al.*, 2010). More recently, a study found altered mRNA expression of MMPs and ADAMTs in Schizophrenia (Pietersen *et al.*, 2014) further emphasizing genetic components of this disorder. Notably ADAMTSL3 affects cell-matrix interaction, ECM assembly, and CSPG proteolysis (Berretta, 2012). As previously discussed, CSPGs affect neural connectivity, synaptic maturation, and neural activity directly, highlighting the neurodevelopmental aspects of this disorder. Studies have reported that ECM and CSPGs regulate neural migration, axon growth, and synaptic maturation during development, aided by CSPG-enriched PNNs to complete and refine neural development in the postnatal period, and later in adulthood, contribute in synaptic stability and neural firing properties (Pantazopoulos *et al.*, 2015; Miranova *et al.*, 2013).

In addition, numerous environmental factors have also been connected to Schizophrenia including stressful events during prenatal and postnatal development,

malnutrition, substance and childhood abuse, as well as vitamin D deficiency (Mortensen *et al.*, 1999; Brown, 2011; Meyer *et al.*, 2010; McGrath *et al.*, 2010; and Ayhun *et al.*, 2015). More interestingly, recent studies are also looking at the gene/environment interplay. Environmental contribution to psychosis is evidenced by studies that assess the effect of drug abuse and the risk for psychosis. For example, studies that followed cannabis users, reported an elevated risk for Schizophrenia in users when compared to the controls (Arseneault *et al.*, 2002; Zammit *et al.*, 2002; Dean *et al.*, 2005).

Similar to Schizophrenia, Bipolar Disorder (BD) has also a multifactorial etiology. The disorder is characterized by dramatic shifts in a person's moods, excessive excitement or irritability, and depression—often accompanied by thoughts of suicide (NAMI, 2016). An interplay between genetic and environmental factors contribute to its causation. BD is also considered to be a pleomorphic disorder due to its clinical progression: some patients experience a worse clinical course from the get-go, while others have multiple relapses but with good inter-episodic functional recovery (Lopes *et al.*, 2012), making it difficult to diagnoses and even more so to treat. Twin and family studies report that first degree relatives of BD patients have a 4-24% elevated risk of developing BD (Ivleva *et al.*, 2008). More recently, genome-wide studies have identified genes/loci significantly associated with BD, including ODZ4, CACNA1C, ANK3, NCAN, etc., however each of these genes individually contribute only <1% towards the overall disease risk (Fullerton *et al.*, 2015), implying that there is a multivariate subset of genes that cumulatively contributes to the disorder.

Based on the current knowledge on the pathophysiology of Schizophrenia and BD, and the ever-growing evidence of the important role that ECM plays in these

disorders, the proposed study hopes to investigate and confirm that the altered expression of CSPGs could be a tool to distinguish between these two overlapping disorders and aid the efforts for better treatment.

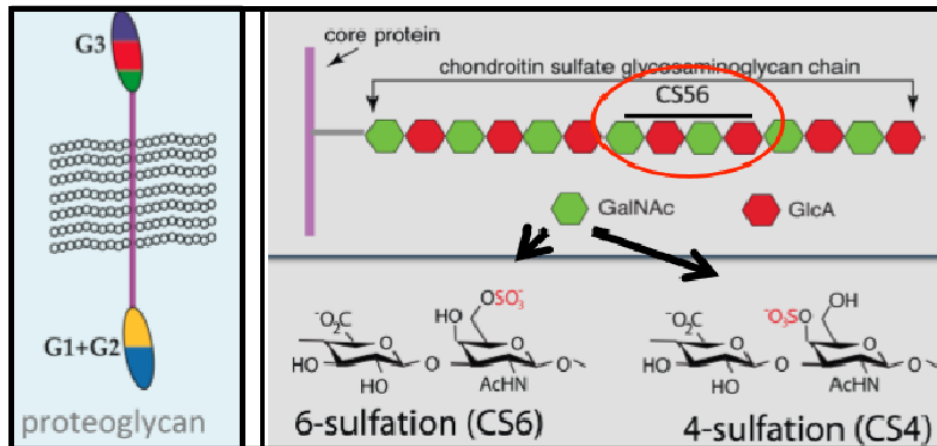
The Role of ECM

Cells need a physical surface where they can grow and develop to form organs within our bodies. This physical surface, has come to be known as the Extracellular Matrix (ECM)—an intricate meshwork of proteins and polysaccharides secreted by the cells that contribute and support their biochemical function (Dityatev *et al.*, 2010). In the body, interactions between the ECM and the cells determine the cells' polarity and function, and also can provide important signals for survival, proliferation, and migration (Lodish *et al.*, 2008). In the brain, ECM also plays a role in glial and neuron organization (Miranova *et al.*, 2013), as well as synaptic activity (Frischknecht *et al.*, 2012); but unlike in other organs, neural ECM is responsible for maintaining and adapting to a very complex environment where neurons, glial cells, and blood vessels cumulatively undergo constant modification in response to stimuli.

The neural ECM consists of a number of key players including: hyaluronan, glycosaminoglycans, proteoglycans, glycoproteins, and chondroitin sulfate proteoglycans (CSPGs) (Dauth et al. 2016). Chondroitin sulfate proteoglycans (CSPGs) are composed of a core protein and side glycosaminoglycans (GAG) sugar chains covalently bonded. These GAG chains are modified by chondroitin sulfotransferases generating several

sulfation patterns which play a key role in defining CSPG function (Pantazopoulos *et al.*, 2015; Boyer-Boiteau *et al.*, 2016).

Figure #1: Structure of CSPGs and its sulfation patterns. (Figure modified from Boyer-Boiteau *et al.*, 2016)



Recent studies have highlighted the important role that ECM plays in neural development and plasticity. For instance, tenascins, an ECM protein, has been reported to inhibit neurite outgrowth (Gilbert *et al.*, 2005) as well as to play a role in neural cellular compartment (Theocharidis *et al.*, 2014). Other studies have focused on the role of perineuronal nets (PNNs). PNNs are specialized extracellular matrix net-like structures that surround mostly the GABAergic, parvalbumin- (PV-) containing, fast-spiking interneurons (Berretta *et al.*, 2015; Slaker *et al.* 2016). Studies have associated PNNs with synaptic function and stability (Andrews *et al.*, 2012; Berretta *et al.*, 2015), and have also reported on the importance of CSPGs and the role that they play in the formation and maintenance of neural networks and neural development (Suttkus, 2014; Ida *et al.*, 2006; Zaremba *et al.*, 1990) highlighting them as a major component of ECM. Mental disorders, therefore, can result from abnormalities in the structure of ECM and the

expression of CSPGs, justifying more research to fully understand the role that ECM, and more specifically CSPGs play in these disorders.

Implications of ECM/CSPGs in Schizophrenia and BD

Indeed, recent studies have highlighted ECM components as key players to development and progression of mental disorders (Pantazopoulos *et al.*, 2015; Woo, 2013; Dityatev *et al.*, 2010). Research into the neurobiological disorders such as Schizophrenia and Bipolar Disorder have directly implicated CSPGs as key contributors that regulate synaptic function and plasticity (Berrretta *et al.*, 2015). For instance, our laboratory has observed a significant reduction in the number of PNNs in the amygdala and the entorhinal cortex of postmortem brains of schizophrenic patients (Pantazopoulos *et al.*, 2010), in addition to abnormal CSPG expression in the olfactory epithelium of schizophrenic patients (Pantazopoulos *et al.* 2013) suggesting impaired ECM and PNNs in subject with Schizophrenia can interfere with normal neurotransmission and GABAergic interneurons.

Genome-wide association studies on CSPGs

Genome-wide association studies have also linked different ECM components to Schizophrenia and Bipolar Disorder. For instance, a recent genome-wide association study linked neurocan to Schizophrenia (Muhleisen *et al.*, 2012). Neurocan is a member of the CSPG family, implicated in neurodevelopment and highly expressed throughout many regions of the brain (Avram *et al.*, 2014). In 2015, Dannlowski *et al.*, explored the association of NCAN with limbic gray matter alterations. The study found that NCAN affects the structure of the gray matter regardless of diagnosis, suggesting that epigenetic

or genetic factors combined with altered NCAN expression may lead to disorder onset (Dannlowski *et al.*, 2015). However, the authors noted that these findings were contradictory to those by Schultz *et al.*, 2014, in which a correlation was observed between modified cortical folding and neurocan risk alleles only in Schizophrenic patients. Further studies exploring the role of CSPGs in brain development are necessary to elucidate these effects on Schizophrenia or other major psychoses, such as BD.

Genome-wide studies have also found similar links in subjects suffering from Bipolar Disorder. For instance, a recent study has identified a link between a genetic variation of the CSPG neurocan and Bipolar Disorder (Cichon *et al.*, 2011), however, our laboratory did not detect changes of PNNs labeled with the lectin Wisteria floribunda agglutinin (WFA) in the entorhinal cortex or amygdala of bipolar subjects (Pantazopoulos *et al.*, 2010) pointing that further research is needed in this area as well.

Examining another component of ECM, Reelin, a large ECM glycoprotein, has been implicated both in Bipolar Disorder and Schizophrenia. In a postmortem study, Fatemi *et al.*, found decreased levels of Reelin expression in the hippocampus of subjects with Schizophrenia and Bipolar Disorder (Fatemi *et al.*, 2000). In Schizophrenia, reduced Reelin levels have been linked to the abnormal GABA neuro-transmission (Lubbers *et al.*, 2014). The current knowledge supports the hypothesis that altered CSPG levels contribute to Schizophrenia and Bipolar Disorder, but further clarification is needed in order to understand the mechanism and how they contribute to the different observed phenotypes. Furthermore, the recent genetic data highlights the possibility that the altered expressions observed in the brain might also be observed remotely (i.e. in the

dermal fibroblasts of patients), and it is precisely this hypothesis that the proposed study seeks to elucidate.

Post mortem studies together with animal models have greatly contributed towards our current knowledge of CSPGs in regards to brain development and how changes within the brain play a role in disease outcome. Recent studies looking at endophenotypes have attempted to find a communality between findings in animal models and those in the postmortem human brain with regards to Schizophrenia and Bipolar Disorder. Walton *et al.*, sought to provide translational validity between immature Dentate Gyrus (iDGs) in mice and phenotypical characteristics in humans such as hyperactivity, working memory, and antisocial behavior. The study found significant upregulation of Calretinin in the Dentate Gyrus of patients with Schizophrenia and Bipolar Disorder matching their findings in mice (Walton *et al.*, 2012). To further explore abnormal neurodevelopment and its implications in major psychoses, Dauth *et al.*, (2016) sought to explore the role and distribution of ECM proteins and ECM structures within the different regions of the brain. Focusing on three major CSPGs (aggrecan, brevican, and tenascin-R) the authors looked at different distribution patterns as well as signal intensity to determine whether specific CSPGs reside within specific brain regions of the rat adult brain, and therefore play unique roles. The study confirmed that ECM proteins have different functions within different regions of the brain, for example Aggrecan was more expressed in PNNs, when compared to brevican and tenascin-r. This study further highlighted the complexity of ECM and ECM proteins within the rat adult brain, and the need to fully understand the specific function of CSPGs in order to identify abnormalities.

In the human brain, Pantazopoulos et al. (2010), conducted a postmortem case-control study to explore CSPG related abnormalities in the amygdala and entorhinal cortex of subjects with Schizophrenia. The study found marked differences in the brains of schizophrenic patients when compared to the controls and bipolar patients, such as increases in CSPG positive glial cells in the amygdala and entorhinal cortex and decreased PNNs, pointing to the pathophysiological distinctions between the two disorders. Although the post-mortem studies capture alterations within the brain from subjects with these disorders, they do not allow for dynamic studies of cellular mechanisms, and are restricted in interpretation in that ECM alterations may have increased sensitivity to proteolysis or have been affected by epigenetic factors such as drug treatment (Matuszko *et al.*, 2017).

Alternative method to post mortem studies: accessibility and consistency of cell culture

Currently, a number of methods have been developed and are being utilized to evaluate ECM's structural and functional roles. For instance, microscopic methods (atomic force microscopy; scanning electron microscopy, confocal reflection microscopy; auto florescence lifetime microscopy, etc.) provide insight into the structure of the ECM proteins and their expression (Zeug *et al.*, 2014). However, most of these microscopic methods are limited in that they cannot probe living unlabeled tissue, especially that of the human brain. Therefore, research depends on post-mortem samples or biopsies from patients. The post-mortem samples are limited in that although they capture changes in the brain, do not allow for dynamic studies of cellular mechanisms and their results are

not easily interpreted in the context of clinical symptoms. On the other hand, biopsies from psychiatric patients are extremely difficult to obtain and justify (Kalman *et al.*, 2016). Furthermore, obtained neural tissues are difficult to maintain functional for long periods of time.

Animal studies are also being utilized to model and aid the understanding of brain diseases (Weinberger *et al.*, 2000; Dauth *et al.*, 2016). However, the challenge with this method is translation and validation of findings, especially since psychiatric disorders are thought to be an exclusively human condition (Nestler *et al.*, 2010). The need for patient-derived bio specimens that can be probed to better understand psychiatric disorders has been in part solved through cell-culture studies due to many advantages associated with this model. For instance, genetic studies suggest that disorders like Schizophrenia and BD have high heritability, and understandably, the genetic makeup should be conserved in the patients' cell cultures (Brennand *et al.*, 2012). Another advantage is that since Schizophrenia and BD are considered diseases of the whole body as opposed to diseases of the brain alone, thus, many of these diseases' dysregulations (i.e. immune, metabolic, etc.) should be detected in peripheral tissue as well (Penninx *et al.*, 2013; Kalman *et al.*, 2016). As a result, many cell-culture models have been utilized to date including leukocytes, olfactory epithelium, iPSCs, human dermal fibroblasts, etc. Amongst these, human dermal fibroblasts stand out for their many advantages including conserved cellular and molecular functions, genetic stability, and ease of culture (Kalman *et al.*, 2016). It, however, as with other models, have some drawbacks (See Table # 1 below). In the proposed study, we plan to control for the shortcomings associated with this model by carefully pairing cells lines based on the donor's age, sex, and condition, in addition to

only using human dermal fibroblasts between passages 5 and 15, which have been found to produce the most consistent and reproducible data (Akin *et al.*, 2004, Fournier *et al.*, 2014).

Table # 1: Advantages and limitations of Human dermal fibroblasts (HDF) cultures (based on Kalman *et al.*, 2016)

Advantages: - Easy to obtain, transport, culture, and store - High sample volume - Feasibility of well-matched case—control and within subject control studies - Primary cell cultures with functional and genetic homogeneity (better signal/noise ratio) - Express many of the same receptors and signaling pathways as neurons - Synthesize and respond to multiple auto-, para-, and endocrine factors - Model amenable to stimulation and genetic manipulation - Lose epigenetic modifications and in vivo effects after 5 passages - Show natural in vivo and in vitro aging
Limitation: - Cultures are a mixture of keratinocytes/fibroblasts in early passages (before P5) and proliferating/post-mitotic cells in late passages (after P20) - Proliferation rate, confluent growing and cell senescent might bias inter-and intragroup variability - Susceptible to environmental influences - Cultured cells grow on artificial surfaces - Lack the variety of physiological, in vivo signals - The age of the donor might affect cell viability - HDFs differ from neurons in multiple relevant features, including synaptic formation, division, ion channel expression, excitability, and glial interaction

In order to investigate CSPG protein expression levels in dermal fibroblasts, two methods can be used: Immunocytochemistry (ICC) and Western Blotting (WB). Though both methods detect proteins of interest in the sample, Western Blotting provides the advantage of not only measuring expression but also secretion of potential CSPGs of

interest into the conditioned media. ICC, by contrast, cannot achieve this, but coupled with WBs can further our understanding with regards to regional localization of CSPGs within the cell. Using both methods Harris *et al.* (2009) for example has observed a decrease in both extracellular CSPGs and the association of CSPGs into PNN in tissue adjacent to the injury core, as CSPGs exist both in the extracellular space and are also loosely bound to membranes (Harris *et al.*, 2009). If the hypotheses of this proposed study is confirmed via these proposed methods, and our findings match the results obtained from the olfactory epithelium and post-mortem brain tissue studies, in vitro investigation of human dermal fibroblasts can emerge as a promising minimally invasive tool to track the course of CSPG abnormalities in patients with Schizophrenia and Bipolar Disorder, as well as aid in our understanding of the clinical disease stages.

Chapter II

Materials and Methods

The purpose of this study was to examine extracellular abnormalities in human dermal fibroblasts from subjects with Schizophrenia and Bipolar Disorder. An established cohort of 39-human dermal fibroblast cell lines from normal controls, schizophrenic, and bipolar subjects was used. Groups of 3 (controls, schizophrenic, and bipolar) were matched to each other for age and gender. I was kept blinded to this information and diagnosis in order to avoid biased results. Cell lysates and conditioned media were collected at 24 hours and 72 hours in order to assess not only expression of but also excretion of affected CSPGs.

Cell Culture

Cryopreserved human dermal fibroblasts were thawed into T-75 flasks (75 cm²) containing 10 mL of medium and incubated at 37 degrees C, 5% CO₂. Cells were grown in Gibco's Minimum Essential Media (MEM) (Lifetechnologies, Cat#11095-080) supplemented with 15% FBS (HyClone, Cat #SH30910.03), 1% Glutamax (Gibco, Cat#35050-061), and 1 % Penicillin/Streptomycin (Gibco, Cat#15140-122). Medium was filter sterilized and stored at 4°C to be used up to 1 week. Medium was changed every other day. After the cells reached 80%-85% confluency, they were sub-cultured by splitting 1:3. One vial was frozen back (~ .4 million cells), whereas the remaining cells were seeded into two flasks and allowed to reach 70% confluency prior to switching to serum free Fibroblast Basal Medium (ATTC Primary Cell Solutions, Cat#PCS-201-030) in preparation of cell lysate and conditioned media collection.

Cell Lysate Preparation

Cell lysis was performed on harvested cells grown to ~ 90% confluency between passages 5 and 15 (similar passages for triplicates). First, conditioned media was collected and labeled accordingly, then cells were washed twice with PBS to remove media. 400 ul of ice-cold RIPA lysis buffer (50mM Tris-HCL pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.5 % Nadeoxycholate; 0.1% SDS; EDTA; Halt protease inhibitor cocktail) will be added to each flask and incubated on ice for approximately 30 minutes. Using a cell scraper, cells were then gently scraped towards the bottom of the flask. Cell lysates will be transferred to Eppendorf tubes and sonicated 4 times 3sec pulses @ amplitude 30 while still on ice. The lysates were spun at 13,000 rpm, 4 °C for 20 minutes to remove cell debris. Supernatant were transferred to new Eppendorf tubes, aliquoted, and stored at -80°C.

Protein Quantification

Protein quantification was done using the DC Protein Microplate MicroAssay Kit (Bio-rad, Cat#5000112) for all cell lysate samples. The protocol provided with the kit was followed. Briefly, after preparation of the working reagent, standards, and sample dilutions, 20 ul of sample or standard was added to a dry microtiter plate, followed by 10 ul of working reagent A, and 80 ul of provided reagent B. After a 15-minute incubation, absorbance was read at 750 nm. Calculations will be done based on the obtained standard curve, and values will be expressed as protein concentration in mg/mL.

Western Blotting

Total protein extracts of each sample (20 µg) were separated by SDS/PAGE electrophoresis (30 V for 90 min.) Using 3-8% NuPAGE Tris-Acetate Mini Gels (Lifetechnology). Proteins were transferred electrophoretically onto FL PVDF membranes (Millipore) using CXELL II Blot Module by Invitrogen transfer system. Membranes were blocked by incubation for 1-hour in Odyssey blocking buffer diluted 1 to 1 w/0.01 PBS with 1% Tween 20. After washing, membranes were incubated with primary antibodies: Monoclonal Anti-Chondroitin Sulfate antibody produced in mouse (Sigma, Cat# C8030) and Anti-VCP antibody (Abcam, Cat#ab111740) at 4°C overnight. The membranes were washed four times by gentle agitation in PBS containing 0.1% Tween 20. Secondary antibodies (Lycor 680RD donkey anti-rabbit and 800CW donkey anti-mouse) were used to detect protein of interest and our loading control, dilution 1:20K in Odyssey blocking buffer diluted 1 to 1 w/0.01M PBS with 0.1% Tween 20 and 0.01 SDS. Membranes were incubated for 1 hour at room temperature. Visualization was done using Odyssey CLx (LI-COR). The intensity of the protein bands on films was quantified using Image Studio Lite (LI-COR) imaging software version 5.2. Lysates were normalized with loading control VCP, while Conditioned media was normalized using total volume and total protein.

Immunocytochemistry (ICC)

Immunocytochemistry was performed to access expression and distribution of chondroitin sulfate proteoglycan (CSPG) in human dermal fibroblasts. Cells were grown on sterile coverslips following cell culture procedures as describe above. The cells were

fixed with 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature, and blocked with 2% Bovine albumin serum (BSA). Cells were incubated in primary antibodies (i.e. CS56, 1:1000 ul) for 24 hrs at 4 degrees C; followed by a 2-hours incubation in biotinylated secondary antibody (i.e. 1:500 goat anti-rabbit, Vector Labs BA-1000) where they were kept protected from light; and then incubated with DAPI for 10-20 mins protected from light. Solutions for all steps will be prepared in phosphate buffer/saline with 0.5% Triton X (PBS-TX). Cells on coverslips were then mounted on gelatin-coated glass slides for quantitative analysis blinded to diagnosis, and stored at -20 until visualization.

Table 2: Experimental Approach

	Logic	Experiment	Control and Why
1	Grow cell lines to similar passage(s) / confluency.	Cell culturing of matched groups, and collection of lysates.	- Cell density, to ensure similar growth environment between cell lines. - Media components and % of FBS.
2	Examine Expression of CSPGs in Controls, Schizophrenic, and Bipolar Patients	Western Blot to measure and quantify proteins – probing antibody CS-56	- Loading control Valosin-containing protein (VCP) measured in the same lane of the gel for each sample to ensure consistency of expression relative to the amount of sample loaded for each sample. - 1 lane with pooled samples to normalize between gels. - 1 lane incubated with IgG Ms/Rb to identify non-specific bands.
3	Confirm that the CSPG(s) of interest are expressed in Fibroblasts.	Immunocytochemistry (ICC) – probing with CS-56	- No primary antibody control, in order to show that labeling is due only to primary and secondary binding. - Isotope control, to ensure that what appears to be specific staining was not caused by non-specific interactions of immunoglobulin molecules with the sample.

Research Limitations

One of the limitations with this model is that the human dermal fibroblast (HDFs), maintained in an artificial environment, can behave differently from their counterparts in vivo. Studies have shown that some cells, including HDFs, are capable of altering their in vitro microenvironment by producing proteases, glycopeptides, and even ECM-proteins (Fullar *et al.*, 2015). Additionally, HDFs differ from neurons in a number of features such as division, synaptic formation, ion channel expression, excitability and neurotransmitters (Auburger *et al.*, 2012; Connolly, GP., 1998). Cumulatively, these limitations can lead to results that might not be fully transferable. Therefore, expecting our findings to correlate with findings or processes in the brain/neurons can be a challenge especially when dealing with such complex disorders. However, even if discrepancies are detected between results in HDFs and CNS, differences between diagnosis groups would provide further evidence for a disruption of CSPG in Schizophrenia and Bipolar Disorder patients and prompt further studies to better understand these discrepancies and, if possible, reconcile them so that these assays may be used in vivo patients.

Although the Western Blot procedure is fairly simple, several problems can arise causing unexpected results such as: unpredicted bands, weak signal, and/or high background observed. These limitations can be minimized through optimization prior to beginning the runs. Additionally, it is important to standardize the loading and transfer rates amongst samples of separate lanes to allow for a more precise comparison of protein levels.

Lastly, a potential weakness is that there might be some variability between subjects. However, n=15 for each of the subgroups (controls, schizophrenic, and bipolar)

is an adequate number that should give us sufficient power to detect the expected changes. Additionally, our findings will be confirmed by ICC. Stepwise analysis of covariance (ANCOVA) will be employed to test for statistical significance of differences for the main outcome variables and the potential effects of confound variables including age, gender, pharmacological treatment, and substance abuse. Data will be analyzed using JMP SAS version number 13.1.0.

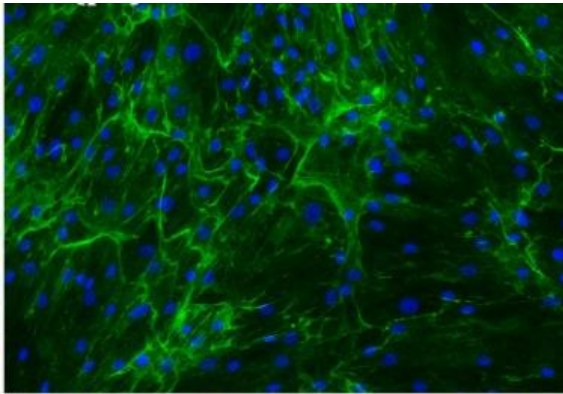
Chapter III

Results

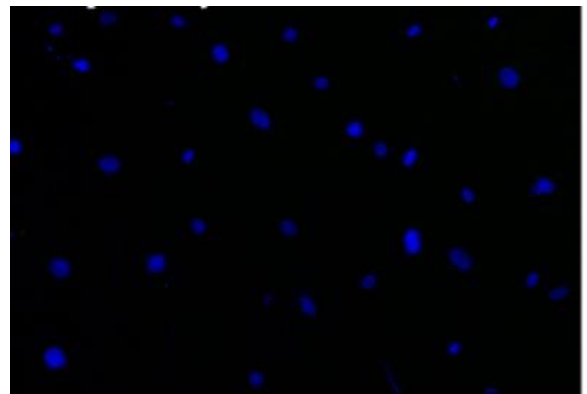
Immunocytochemistry (ICC)

Immunocytochemistry staining using the anti-chondroitin sulfate antibody CS-56 revealed that human dermal fibroblasts abundantly express CS6.

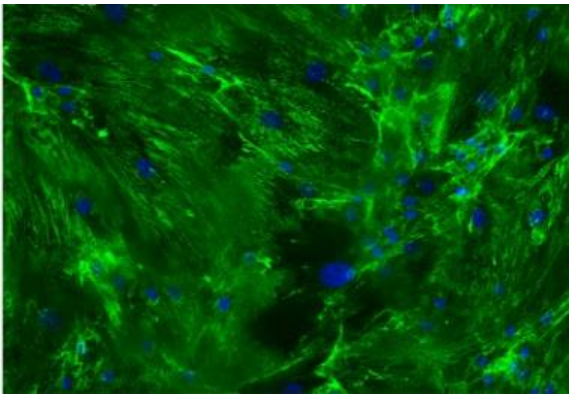
Figure#2: CS6 (CS56) expression in Human Dermal Fibroblasts



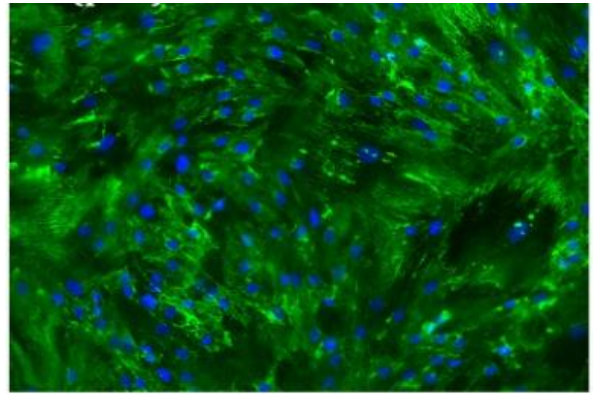
Control



No Primary Antibody



Schizophrenia



Bipolar Disorder

Proliferating Human dermal fibroblasts express CS6 (CS56, green), nucleus stained with DAPI (blue). Representative images from human dermal fibroblasts cultured cells from Normal Controls, Schizophrenia, and Bipolar groups.

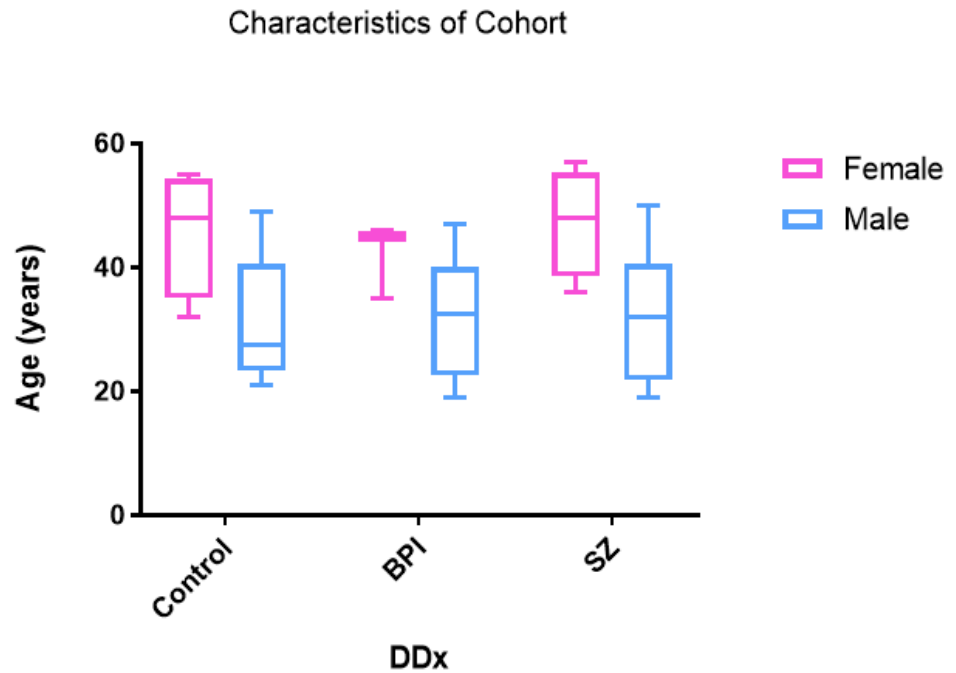
Differences in Age, Sex, and Race between Controls and the Experimental groups

There are no significant differences between the controls and the experimental groups with respect to age, sex and race as seen on the table and figure below.

Table#3: Descriptive Characteristics of the Human Dermal Fibroblast Cohort

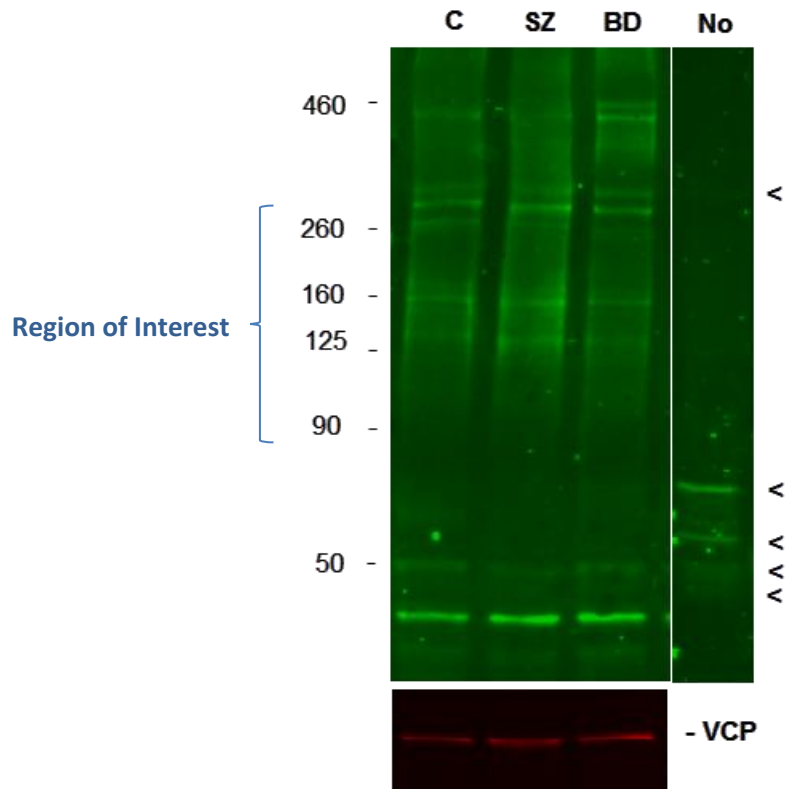
DDx	Sex	Age Mean (SD)	Race
Normal Controls (n=12)	Male: 8	31.5 (10.33)	White = 8
	Female: 4	45.75 (10.27)	White = 4
Bipolar Disorder (n=13)	Male: 10	31.7 (9.69)	White = 10
	Female: 3	42 (6.08)	White = 3
Schizophrenia (n=14)	Male: 10	32.4 (10.27)	White = 10
	Female: 4	42.25 (8.77)	White = 4

Figure#3: Characteristics of Cohort



Western Blotting

Figure#4: Western Blotting: CS56



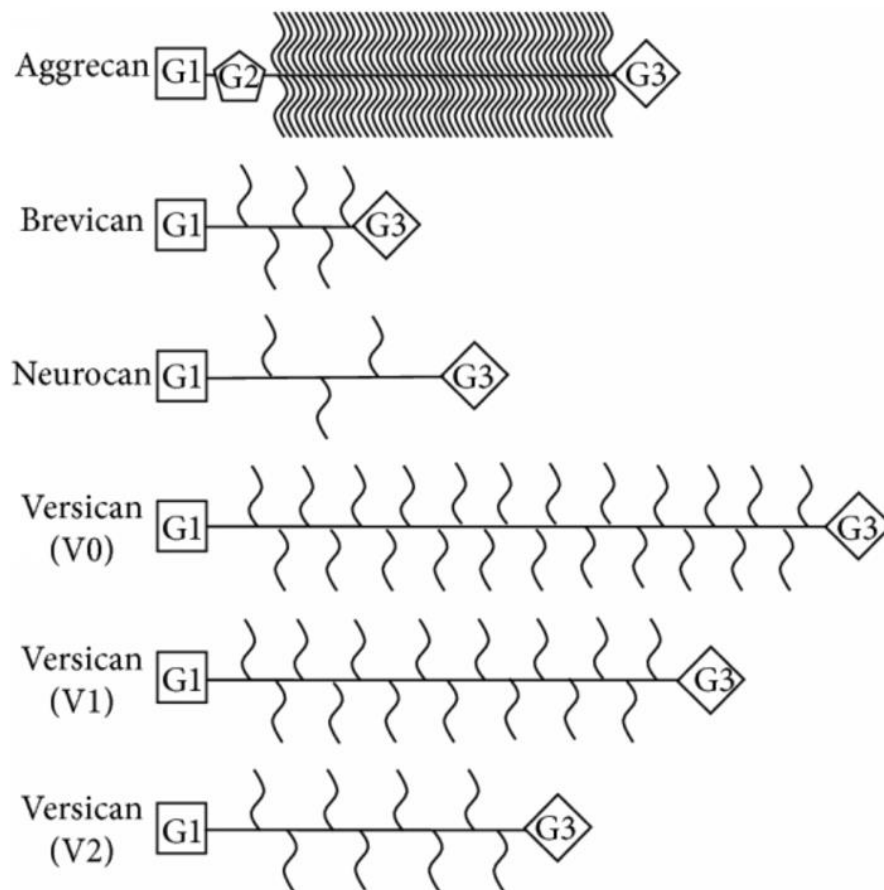
Representative western blot showing CS56 (CS6) expression in human dermal fibroblasts cell lysates. Black arrows indicate non-specific bands.

Table #4: CSPG Splice Variants of Interest

CSPG	Splice Variant	MW (kDa)
Aggrecan	ACAN-201	261.3
	ACAN-202	255.6
	ACAN-008	257.4
	ACAN-004	250.4
Brevican*	BCAN-001	99.1
Neurocan*	NCAN-001	143.1
Phosphacan	PTPRZ1-001	254.6
	PTPRZ1-002	162.6
Versican	VCAN-002	176.8
	VCAN-003	135.7

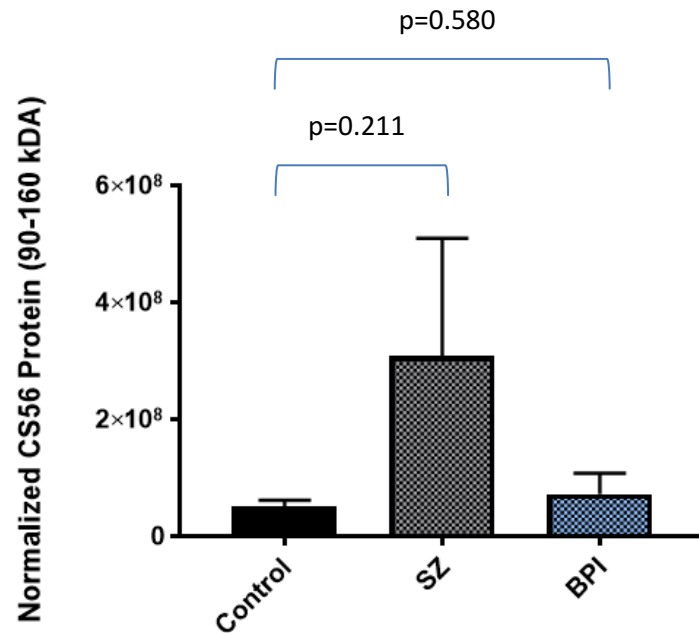
Modified table from Boyer-Boiteau *et al.*, 2016 with information from the Human Protein Atlas (<http://www.proteinatlas.org>) [*] denotes CSPGs that are highly specific to the brain.

Figure#5: Diagram of Individual proteoglycan molecules (adapted from Siebert *et al.*, 2014)



Aggrecan, brevican, neurocan, and the three isotypes of versican. The length of the core protein and the varying number of GAG side chains attached to the central domain are major determinants of the biological activity of an individual proteoglycan (Siebert *et al.*, 2014).

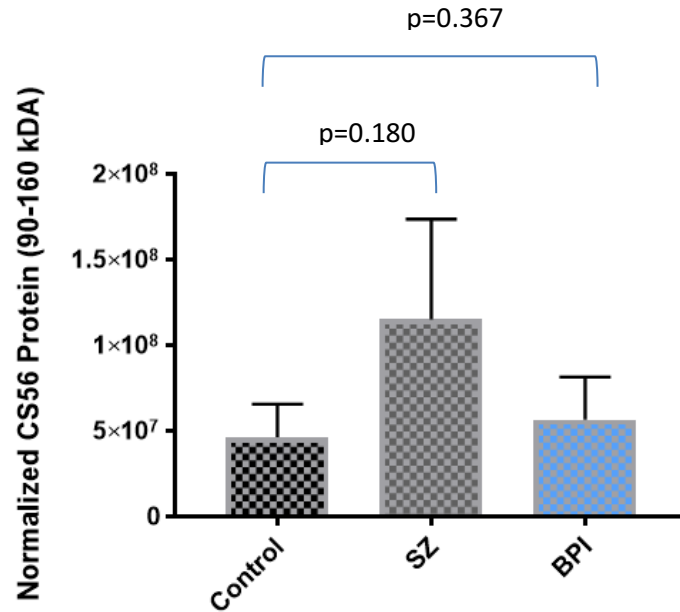
Figure#6: CS6 expression in Day 1 Lysates



CS56 expression in Day 1 of cell lysates from normal controls, subject with Schizophrenia, and from subject with Bipolar Disorder 1. No significant differences were observed between the groups: Controls vs. schizophrenic ($p=0.211$), and controls vs. PBI group ($p=0.580$).

Should be noted that individual bands corresponding to specific CSPG splice variants were also analyzed (data not shown), no significant difference was found between the control and experimental groups for Day 1 Lysates, as well as conditioned medium.

Figure#7: CS6 expression in Day 3 Lysates



CS56 expression in Day 3 of cell lysates from normal controls, subject with Schizophrenia, and from subject with Bipolar Disorder 1. No significant differences were observed between the groups: Controls vs. schizophrenic ($p=0.180$), and controls vs. BPI group ($p=0.367$).

Should be noted that individual bands corresponding to specific CSPG splice variants were also analyzed (data not shown), no significant difference was found between the control and experimental groups for Day 3 Lysates, as well as conditioned medium.

Chapter IV

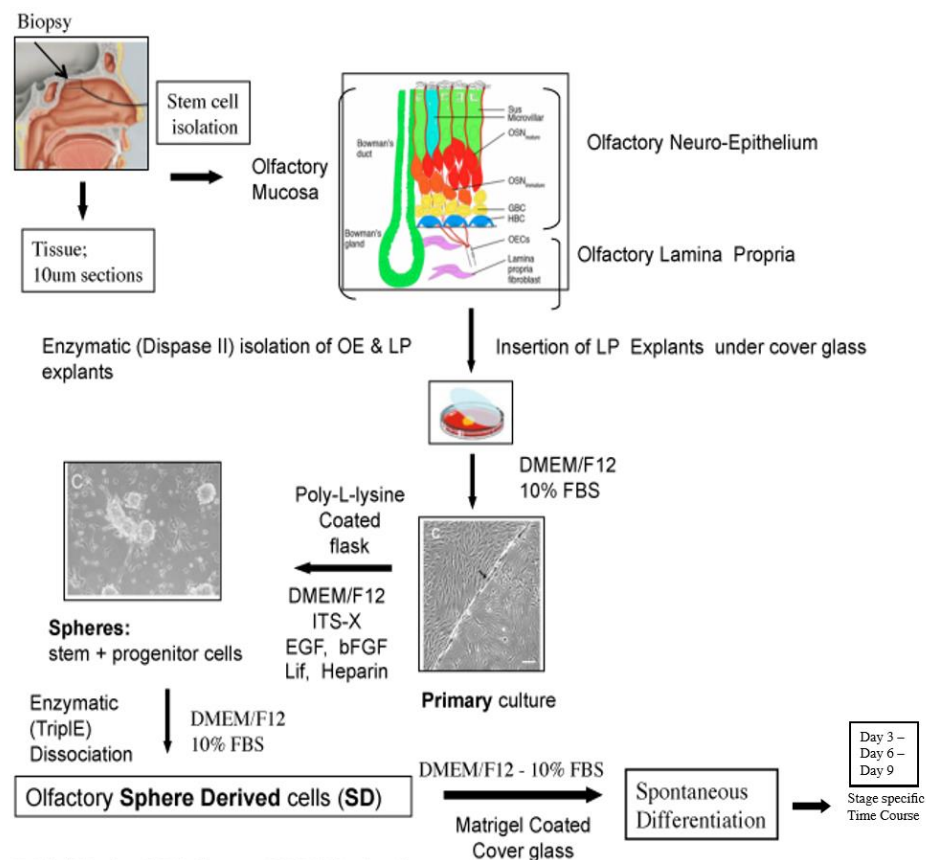
Discussion

In the recent years, there has been an increase in research focusing on ECM and its role in neurological disorders (Pantazopoulos *et al.*, 2015; Lubbers *et al.*, 2014; Mauney *et al.*, 2013). These studies show that observing the changes within the cellular microenvironment more closely can lead to better understanding of the mechanisms and pathophysiology of these disorders. Yet, most studies are conducted either in animal models or from postmortem tissue emphasizing the need for less invasive measures when investigating these disorders. To address this, the goal of this study was to examine the expression of CSPGs using Anti-Chondroitin Sulfate antibody [CS56] in human dermal fibroblasts from subjects with Schizophrenia and Bipolar Disorder. More specifically, we wanted to assess not only intracellular expression of CSPGs but also secretion in hopes of identifying dermal fibroblasts as a potential neuropathological tool. To our knowledge, this approach has not yet been explored by any other group.

Our data indicates that human dermal fibroblasts express and secrete CSPGs resembling the chondroitin sulfation pattern of CS6/CS56 for both day 1 and day 3 in the 90-160 kDa region of CSPG splice variants. However, our western blot analysis showed that there were no significant differences in the levels of expression between the experimental groups (bipolar and Schizophrenia) and the controls at these time points. These findings did not change even when controlling for antipsychotic medications such as Chlorpromazine (CPZ) or Lithium, and do not align with our laboratory's results of the olfactory mucosa (OM) cells which highlighted significant differences between expression of CS6/CS56 amongst controls, bipolar, and schizophrenic subjects as seen in

Figure#9 (Boyer-Boiteau *et al.*, 2016). Olfactory Mucosa cell cultures were established using an adapted protocol for the isolation of human nasal olfactory stem/progenitor cells. For these studies, spheres were dissociated, seeded as monolayer, and harvested at day 3, 6, and 9 timepoints (Figure #8)

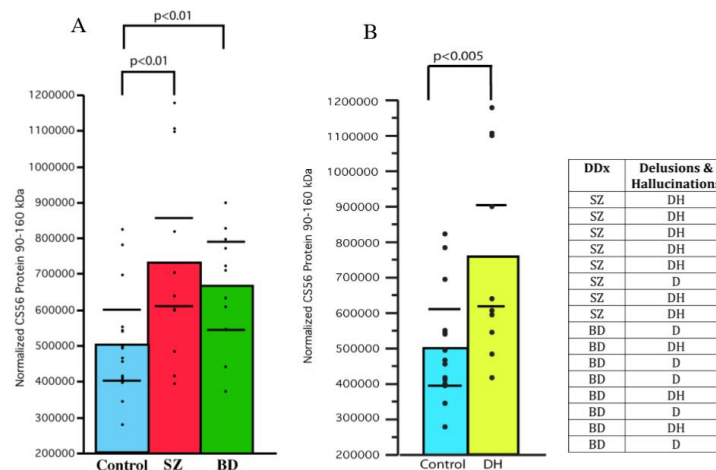
Figure # 8: Establishment of Olfactory Mucosa Cell Culture
(Figure from Boyer-Boiteau *et al.*, 2016)



In the Olfactory Mucosa, both subjects with Schizophrenia and subjects with Bipolar Disorder showed an increase in 90-160 kDa CS6 (CS56) when compared to normal controls ($p < 0.01$). Schizophrenic and Bipolar Disorder subjects presenting both

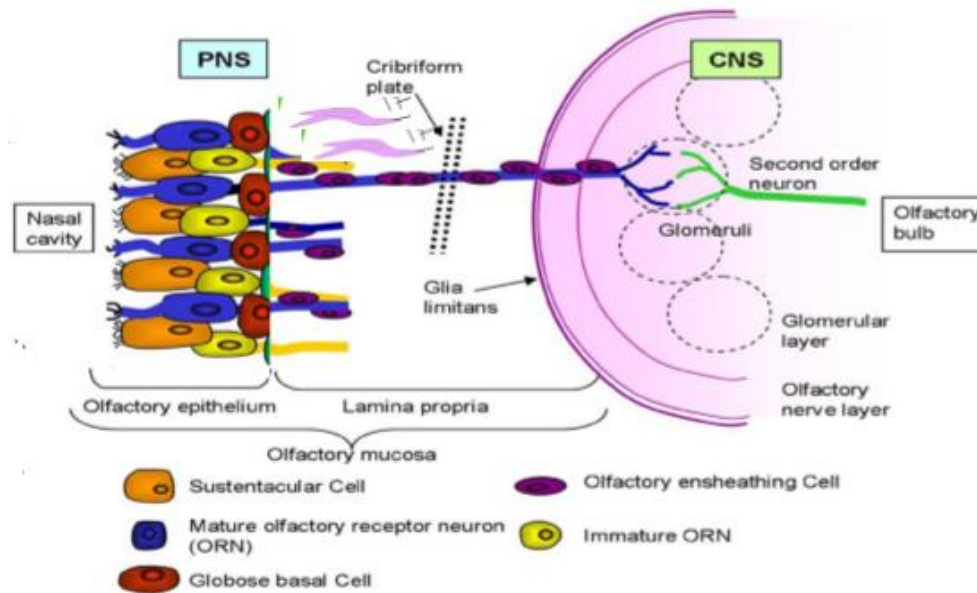
delusions and hallucinations showed strongly significant increase with respect to controls ($p<0.005$) as seen in Figure #9. Previous research has linked olfactory deficiencies to Schizophrenia and Bipolar Disorder (Moberg *et al.*, 1997; Turetsky *et al.*, 2009). In the olfactory system, neuron differentiation and axon outgrowth are retained throughout life, where maturing OE olfactory receptor neurons (ORN) send their axons into the subjacent lamina propria (LP) as seen in Figure#10. In the LP they form axon bundles and find their way to odor-specific olfactory glomeruli in the olfactory bulb (OB), making this system relevant to pathophysiology of these neurodevelopmental disorders (Boyer-Boiteau *et al.*, 2016). The authors suggest that the observed abnormalities in CSPG expression between the groups could lead to miswiring between the Olfactory Bulb and the Olfactory Epithelium, subsequently leading to the observed olfactory deficits.

Figure # 9: CS56 protein expression in day 6 of OM cell lysates



- CS56 protein expression in day 6 of OM spontaneous differentiating cell lysates from normal control, subjects with SZ and subjects with BD.
- SZ and BD subjects presenting with both delusions and hallucinations showed strongly significant increased with respect to controls. (Figure from Boyer-Boiteau *et al.*, 2016.)

Figure # 10: Diagram of the Olfactory System (from Boyer-Boiteau *et al.*, 2016)



In another study by our group on chondroitin-6-sulfate abnormalities in these disorders, differences were also observed within the amygdala of schizophrenic and bipolar patients when compared to the controls (Pantazopoulos, H., 2015). Interestingly, the levels of expression within the amygdala differed from those observed in the OM cells study.

One plausible explanation for this discrepancy in results between human dermal fibroblasts, OM cells, and the amygdala findings, is that perhaps these expression changes are exclusive to neuronal tissue. A key distinction between the models is that the Olfactory Mucosa contains both neurons and glial cells (Borgmann-Winter *et al.*, 2015) and as such, is considered neuronal tissue, whereas fibroblasts are derived from peripheral dermal tissue. This rationale can further be supported by brain specific CSPGs

such as Brevican and Neurocan, markedly linked to astrocytes (Pantazopoulos *et al.*, 2008) and both linked to Bipolar Disorder and Schizophrenia (Muhleisen *et al.*, 2014; Shah *et al.* 2013). Yet, despite the many similarities between astrocytes and dermal fibroblasts, for example transforming $\beta 1$ (TGF- $\beta 1$) which stimulates CSPG expression both in astrocytes and skin fibroblasts (Smith *et al.*, 2005; Westergren-Thorson, *et al.*, 1992), the differences observed further highlight the potential hypothesis that perhaps the observed altered CSPG expression is specific to the brain.

Another reasoning could be that changes in CSPG expression in fibroblasts between groups could be lower than in neuronal tissue, and as such, could be outside the detection levels of the western blot. Although western blots are still held as the golden standard for assessing protein expression, some limitations in terms of sensitivity still remain as seen in genomic studies which highlight discrepancies between gene expression and protein expression levels *in vivo*. (Kendrick, N., 2014). Additionally, our data (Figure # 5 & 6) show that there may be a difference between controls and Schizophrenia, even though not significant, the trend is in the same direction as that observed in the Olfactory Epithelium studies (Figure # 8). Therefore, it is plausible to further postulate that this study did not have enough power to detect a significant difference between and amongst the groups.

Furthermore, a common caveat to *in vitro* studies with human dermal fibroblasts is the biased effects that proliferation rate, confluent growing, and cell senescence might have on inter-and intragroup variability (Kalman *et al.*, 2016). To minimize these effects in our fibroblasts cultures, we controlled for plating density and passage number. However, changes in growth rate were observed between cell lines in culture, likely due

to decreased cell adherence that was reported in Schizophrenia (Mahadik *et al.*, 1994) which could potentially have skewed our observations.

Lastly, despite the many efforts to match our groups for confounding variables such as age, gender, and race, the sample size remained small (n=12 controls, n=13 BD, and n=14 SZ), and might not have had the necessary power to account for unknown genetic and epigenetic factors that could play a role within the cohort to detect the expected changes.

Implications and Future Direction

Although there are many similarities between neuronal cells (i.e. astrocytes) and human dermal fibroblasts, the model did not detect CS-6(CS56) altered expression in our cohort. Regardless, human dermal fibroblasts have been used effectively in studies of psychiatric disorders (Cattane *et al.*, 2015; Kalman *et al.*, 2016), and thus the model deserves special consideration. Future research focusing on CSPG expression in fibroblasts should consider a larger cohort, in combination with genetic and epigenetic information which in turn could have an impact on the trends of expression between and amongst the experimental groups. Another possible approach is to explore altered gene expression in the cohort through rt-qPCR, focusing on specific CSPGs of interest, and later confirming these findings through either ICC or Western Blotting.

Conclusion

Cumulatively, these results show that CSPG abnormalities in major psychoses might be exclusive to the nervous system, supported by the altered expressions of CS-6 (CS56) observed in amygdala and the OE cells, but not in the human dermal fibroblasts.

These results further highlight the intricacy of the ECM and the many roles that CSPGs play within our bodies. Understanding the role that CSPGs play in these disorders within the different regions of the brain, could lead to a better understanding of the mechanisms that are involved. Furthermore, the data suggest that the OM cells could serve an efficient model in exploring ECM abnormalities in the CNS. This in turn could lead to better treatments, therapeutics, and diagnostic tools for both Schizophrenia and Bipolar Disorder.

References

- Andrews, E.M., Richards, R.J., Yin, F.Q., Viapiano, M.S., Jakeman, L.B. (2011). Altercations in chondroitin sulfate proteoglycan expression occur both at and far from the site of spinal contusion injury. *Experimental Neurology*, 235 (1). 174-187.
- Arseneault, L., Cannon, M., Poulton, R., Murray, R., Caspi, A., Moffitt, T.E. (2002). Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. *British Medical Journal*, 325 (7374). 1212-1213.
- Akin, D., Manier, D.H., Sanders-Bush, E., Shelton, R.C. (2004). Decreased Serotonin 5-HT_{2A} Receptor-Stimulated Phosphoinositide Signaling in Fibroblasts from Melancholic Depressed Patients. *Neuropsychopharmacology*, 29. 2081-2087.
- Avram, S., Shaposhnikov, S., Buiu, C., Mernea, M. (2014). Chondroitin Sulfate Proteoglycans: Structure-Function Relationship with Implication in Neural Development and Brain Disorders. *BioMed Research International*, 2014 1-11.
- Alonso, L., Fuchs, E. (2003). Stem cells of the skin epithelium. *Proceedings of the National Academy of Sciences*, 100 (1). 11830-11835.
- Ayhan, Y., McFarland, R., Pletnikov, M. (2016). Animal models of gene-environment interaction in Schizophrenia: A dimensional perspective. *Progress in Neurobiology*, 136. 1-27.
- Berretta, S. (2011). Extracellular matrix abnormalities in Schizophrenia. *Neuropharmacology*, 62. 1584-1597.
- Bavamian, S., Mellios, N., Lalonde, J., Fass, D.M., Wang, J., Sheridan, D., Madison, J.M., Zhou, F., Rueckert, E.H., Barker, D., Perlis, R.H., Sur, M., Haggarty, S.J. (2015). Noncoding RNAs connect genetic risk factors to the neurodevelopmental basis of bipolar disorder. *Molecular Psychiatry*, 20. 573-584.
- Berretta, S., Pantazopoulos, H., Markota, M., Brown, C., Batzianouli, E. (2015). Losing the sugar coating: Potential impact of perineuronal net abnormalities on interneurons in Schizophrenia. *Schizophrenia Research*, 167. 18-27.
- Berretta, S., Heckers, S., Benes, F.M. (2015). Searching human brain for mechanisms of psychiatric disorders. Implications for studies on Schizophrenia. *Schizophrenia Research*, 167. 91-97.
- Berretta, S. (2012). Extracellular matrix abnormalities in schizophrenia. *Neuropharmacology*, 62. 1584-1597.

- Biernaskie, J.A., McKenzie, I.A., Toma, J.G., Miller, F.D. (2006). Isolation of skin-derived precursors (SKPs) and differentiation and enrichment of their Schwann cell progeny. *Nature Protocols*, 1 (6). 2803-2812.
- Bitanhirwe, B.K., Woo, T.W. (2014) Perineuronal nets and Schizophrenia: The importance of neuronal coatings. *Neuroscience and Biobehavioral Reviews*, 45. 85-99.
- Brennan, K.J., Simone, A., Tran, N., Gage, F.H. (2012) Modeling psychiatric disorders at the cellular and network levels. *Molecular Psychiatry*, 17. 1239-1253.
- Borgmann-Winter, K., Willard, S.L., Sinclair, D., Mirza, N., Turetsky, B., Berretta, S., Hahn, C.G. (2015) Translational potential of olfactory mucosa for the study of neuropsychiatric illness. *Translational Psychiatry*, 527. 1-12.
- Boyer-Boiteau, A. (2016, November). An in vitro model to study the role of the extracellular matrix in neurodevelopmental abnormalities in schizophrenia. Poster session presented at the meeting of Society for Neuroscience, San Diego, CA.
- Brown, A.S. (2011). Exposure to prenatal infection and risk of schizophrenia. *Frontiers in Psychiatry*, 2. 1-5.
- Chen, L., Wu, Y., Lee, V., Kiani, C., Adams, M.E., Yao, Y., Yang, B.B. (2002). The Folded Modules of Aggrecan G3 Domain Exert Two Separable Functions in Glycosaminoglycan Modification and Product Secretion. *The Journal of Biological Chemistry*, 277 (4). 2657-2665.
- Cattane, N., Minelli, A., Milanesi, E., Maj, C. Bignotti, S. (2015) Altered gene expression in schizophrenia: findings from transcriptional signatures in fibroblasts and blood. *Plos One*, 10 (2).
- Cichon, S., Muhleisen, T.W., Degenhardt, F.A., Mattheisen, M., Miro, X. et al. (2011) Genome-wide association study identifies genetic variation in neurocan as a susceptibility factor for bipolar disorder. *American Journal of Human Genetics*, 88 (3). 372-381.
- Dauth, S., Grevesse, T., Pantazopoulos, H., Campbell, P.H., Maoz, B.M., Berretta, S., Parker, K.K. (2016). Extracellular Matrix Protein Expression Is Brain Region Dependent. *The Journal of Comparative Neurology*, 524. 1309-1336.
- Dean, K., Murray, R. (2005). Environmental risk factors for psychosis. *Dialogues in Clinical Neuroscience*, 7. 69-80.
- Dityatev, A., Schachner, M. (2003). Extracellular Matrix Molecules and Synaptic Plasticity. *Nature Reviews*, 4. 456-468.

- Dityatev, A., Schachner, M., Sonderegger, P. (2010). The dual role of the extracellular matrix in synaptic plasticity and homeostasis. *Nature Reviews*, 11(11). 735-46.
- Driskell, R., Lichtenberger, B., Hoste, E., Kretzschmar, K., Simons, B., Charalambous, M., Ferron, S., Herault, Y., Pavlovic, G., Ferguson-Smith, A., Watt, F. (2013). Distinct fibroblast lineages determine dermal architecture in skin development and repair. (2013). *Nature*, 504 (12783). 277-281.
- Dannlowski, U., Kugel, H., Grotegerd, D., Redlick, R., Suchy, J., Opel, N., Suslow, T., Konrad, C., Ohrmann, P., Bauer, J., Kircher, T., Krug, A., Jansen, A., Baune, B.T., Heindel, W., Domschke, K., Forstner, A.J., Nöthen, M.M., Treutlein, J., Arolt, V., Hohoff, C., Rietschel, M., Witt, S.H. (2015). NCAN Cross-Disorder Risk Variant Is Associated With Limbic Gray Matter Deficits in Healthy Subjects and Major Depression. *Neuro-psychopharmacology*, 40. 2510-2516.
- Enwright, J.F.I, Arion, D., Fish, K., Lewis, D. (2012). Decreased perineuronal net labeling and altered parvalbumin immunoreactivity in the dorsal lateral prefrontal cortex of subjects with Schizophrenia. *Society for Neuroscience*.
- Fatemi, S.H., Earle, J.A., McMenomy, T. (2000). Reduction in Reelin immunoreactivity in hippocampus of subjects with Schizophrenia, Bipolar Disorder, and major depression. *Molecular Psychiatry*, 5. 654-663.
- Fernandes, K.J., McKenzie, I.A., Mill, P., Smith, K.M., Akhavan, M., Barnabé-Heider, F., Biernaskie, J., Junek, A., Kobayashi, N.R., Toma, J.G., Kaplan, D.R., Labosky, P.A., Rafuse, V., Hui, C.C., Miller, F.D. (2004). A dermal niche for multipotent adult skin-derived precursor cells. *Nature Cell Biology*, 6 (11). 1082-1093.
- Fernandes, K.J., Toma, J.G, Miller, F.D. (2007). Multipotent skin-derived precursors: adult neural crest-related precursors with therapeutic potential. *Philosophical Transactions of The Royal Society B*, 363. 185-198.
- Fournier, P.E., Dubourg, G., Raoult, D. (2010). Clinical detection and characterization of bacterial pathogens in the genomics era. *Genome Medicine*, 6. 114.
- Frischknecht, R., Gundelfinger, E.D. (2012). The brain's extracellular matrix and its role in synaptic plasticity. *Advances in Experimental Medicine and Biology*, 970. 153-171.
- Fullár, A., Dudár, J., Oláh, L., Hollósi, P., Papp, Z., Sobel, G., Karászi, K., Paki, S., Baghy, K., Kovalszky, I. (2015). Remodeling of extracellular matrix by normal and tumor-associated fibroblasts promotes cervical cancer progression. *BioMed Central Cancer*, 15. 256-272.

- Fullerton, J.M., Koller, D.L., Edenberg, H.J., Foroud, T., Liu, H., Glowinski, A.L., McInnis, M.G., Wilcox, H.C., Frankland, A., Roberts, G., Schofield, P.R., Mitchell, P.B., Nurnberg, J.I. (2015). Assessment of first and second degree relatives of individuals with bipolar disorder shows increased genetic risk scores in both affected relatives and young At-Risk Individuals. *American Journal of Medical Genetics*, 168 (7). 617-629.
- Gilbert, R.J., McKeon, R.J., Darr, A., Calabro, A., Hascall, V.C., Bellamkonda, R.V. (2005). CS-4,6 is differentially upregulated in glial scar and is a potent inhibitor of neurite extension. *Molecular Cell Neuroscience*, 29. 545-558.
- Hallifax, D., Houston, J. (2012). Evaluation of Hepatic Clearance Prediction Using *In Vitro* Data: Emphasis on Fraction Unbound in Plasma and Drug Ionisation Using a Database of 107 Drugs. *Journal of Pharmaceutical Sciences*, 101 (8), 2645-2652.
- Harris, N.G. Carmichael, S.T., Hovda, D.A., Sutton, R.L. (2009). Traumatic brain injury results in disparate regions of chondroitin sulfate proteoglycan expression that are temporally limited. *Journal of Neuroscience Research*, 87. 2937-2950.
- Harrison, P.J. (2007). Schizophrenia susceptibility genes and their neurodevelopmental implications: focus on neuregulin 1. *Novartis Foundation Symposium*, 288. 246-255.
- Ida, M. Shuo, T., Hirano, K., Tokita, Y., Nakanishi, K., Matsui, F., Aono, S. (2006). Identification and functions of chondroitin sulfate in the milieu of neural stem cells. *Journal of Biological Chemistry*, 281 (9). 5982-5991.
- Ivleva, E., Thaker, G., Tamminga, C.A. (2008). Comparing Genes and Phenomenology in the Major Psychoses: Schizophrenia and Bipolar I Disorder. *Schizophrenia Bulletin*, 34 (4). 734-742.
- Ivleva, E., Morris, D.W., Moates, A.F., Suppes, T., Thaker, G.K., Tamminga, C.A. (2010). Genetics and Intermediate Phenotypes of the Schizophrenia-Bipolar Disorder Boundary. *Neuroscience & Biobehavioral Reviews*, 34. 897-921.
- Kálmán, S., Garbett, K.A., Janka, Z., Mirnics, K. (2016). Human Dermal Fibroblasts in Psychiatry Research. *Neuroscience*, 320. 105-121.
- Kendrick, N. (2014). A gene's mRNA level does not usually predict its protein level. www.kendricklabs.com/WP1_mRNAsVsProtein-New2014.pdf
- Lander, C., Kind, P., Maleski, M., Hockfield, S. (1996). A Family of Activity-Dependent Neuronal Cell-Surface Chondroitin Sulfate Proteoglycans in Cat Visual Cortex. *The Journal of Neuroscience*, 17 (6). 1928-1939.

- Lee, S.H., Ripke, S. et al. (2013). Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nature Genetics*, 45(9). 984-994.
- Little, C.B., Meeker, C.T., Hembry, R.M., Sims, N.A., Lawlor, K.E., Golub, S.B., Last, K., Fosang, A.J. (2005). Metalloproteinases Are Not Essential for Aggrecan Turnover during Normal Skeletal Growth and Development. *Molecular and Cellular Biology*, 25 (8). 3388-3399.
- Lodish, H., Berk, A., Kaiser, C.A. et al. (2008) Molecular Cell Biology. Sixth Edition, 820-839.
- Lopes, R., Fernandes, L. (2012). Bipolar Disorder: Clinical Perspectives and Implications with Cognitive Dysfunction and Dementia. *Depression Research and Treatment*, 2012. 1-11.
- Lubbers, B., Smit, A., Spijkerm, S., van der Oever, M. (2014). Neural ECM in addiction, Schizophrenia, and mood disorder. *Progress in Brain Research*, 214 (12). 263-271. [12 is the chapter]
- Luo, W., Guo, C., Zheng, J., Chen, T.L., Wang, P.Y., Vertel, B.M., Tanzer, M.L. (2000). Aggrecan from start to finish. *Journal of Bone and Mineral Metabolism*, 18. 51-56.
- Ma, H., Zhang, Z., Tong, T. (2012). The Effects of Epidermal Growth Factor on Gene Expression in Human Fibroblasts. *In Vitro Cellular & Developmental Biology. Animal*, 38 (8). 481-486.
- Mahadik, S.P., Mukherjee, S., Laev, H., Reddy, R., Schnur, D.B. (1991). Abnormal Growth of Skin Fibroblasts From Schizophrenic Patients. *Psychiatric Research*, 37. 309-320.
- Mahadik, S.P., Mukherjee, S. (1996). Cultured skin fibroblasts as a cell model for investigating Schizophrenia. *Journal of Psychiatric Research*, 30 (6). 421-439.
- Mahmood, T., Yang, P.C. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences*, 4 (9). 429-434.
- Matigian, N.A., McCurdy, R.D., Féron, F., Perry, C., Smith, H. Filippich, C., McLean, D., McGrath, J., Mackay-Sim, A., Mowry, B., Hayward, N.K. (2008). Fibroblast and Lymphoblast Gene Expression Profiles in SchizophreniaL Are Non-Neural Cells Informative? *PLoS One*, 3 (6). 1-9.
- Matuszko, G., Curreli, S., Kaushik, R., Becker, A., Dityatev, A. (2017). Extracellular matrix alterations in the ketamine model of Schizophrenia. *Neuroscience*, 350. 13-22.

- Mauney, S.A., Athanas, K.M., Pantazopoulos, H., Shaskan, N., Passeri, E., Berretta, S., Woo, T.W. (2013). *Biological Psychiatry*, 74. 427-435.
- McGrath, J.J., Burne, T.H., Féron, F., Mackay-Sim, A., Eyles, D.W. (2010). Developmental Vitamin D Deficiency and Risk of Schizophrenia: A 10-Year Update. *Schizophrenia Bulletin*, 36 (6): 1073-1078.
- Meyer, U., Spoerri, E., Yee, B.K., Schwarz, M.J., Feldon, J. (2010). Evaluating Early Preventative Antipsychotic and Antidepressant Drug Treatment in an Infection-Based neurodevelopmental Mouse Model of Schizophrenia. *Schizophrenia Bulletin*, 36 (3): 607-623.
- Miranova, Y.A., Giger, R.J. (2013). Where No Synapses Go: Gatekeepers of Circuit Remodeling and Synaptic Strength. *Trends in Neuroscience*, 36 (6). 363-373.
- Miyamae, Y., Nakamura, Y., Kashiwagi, Y., Tanaka, T., Kudo, T., Takeda, M. (1998). Altered adhesion efficiency and fibronectin content in fibroblasts from schizophrenic patients. *Psychiatry and Clinical Neurosciences*, 52. 345-352.
- Moberg P, et al. Olfactory identification deficits in Schizophrenia: correlation with duration of illness. *Am J Psychiatry* 1997 154(7): 1016-8.
- Möller, H.J. (2003). Bipolar Disorder and Schizophrenia: Distinct Illnesses or a Continuum? *Journal of Clinical Psychiatry*, 64. (suppl 6). 23-27.
- Muhleisen, T.W, Mattheisen, M., Strohmaier, J., Degenhardt, F., Priebe, L. et al. (2012). Association between Schizophrenia and common variation in neurocan (NCAN), a genetic risk factor for Bipolar Disorder. *Schizophrenia Research*, 138 (1), 69-73.
- Muhleisen T. W., Leber M., Schulze T. G., Strohmaier J., Degenhardt F., Treutlein J. (2014). Genome-wide association study reveals two new risk loci for bipolar disorder. *Nature Communication*, 5. 3339.
- < <https://www.nami.org/>>. (2016). National Alliance on Mental Illness. Available: <https://www.nami.org/Learn-More/Mental-Health-Conditions/Schizophrenia>
- Kendrick, N. (2014). A gene's mRNA level does not usually predict its protein level. Available: http://www.kendricklabs.com/WP1_mRNAsVsProtein_KendrickLabs.pdf
- < <https://www.nimh.nih.gov/index.shtml>>. (2015). National Institute of Mental Health. Available: <https://www.nimh.nih.gov/health/topics/Schizophrenia/index.shtml>

- Nestler, E.J. Hyman, S.E.. (2010) Animal models of neuropsychiatric disorders. *Nature Neuroscience*, 13(10). 1161-1169.
- Olde-Loohuis, N.F., Nadif Kasri, N., Glennon, J.C., van Bokhoven, H., Hébert, S., Kaplan, B., Martens, G.J., Schrafi, A. (2016). The Schizophrenia risk gene *MIR137* acts as a hippocampal gene network node orchestrating the expression of genes relevant to nervous system development and function. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. 1-10.
- Pantazopoulos, H., Woo, T.W., Lim, M.P., Lange, N., Berretta, S. (2010). Extracellular Matrix-Glial Abnormalities in the Amygdala and Entorhinal Cortex of Subjects Diagnosed with Schizophrenia. *Archives of General Psychiatry*, 67 (2). 155-166.
- Pantazopoulos, H., Boyer-Boiteau, A., Holbrook, E., Jang, W., Chang-Gyu, H., Arnold, S., Berretta, S. (2013). Proteoglycan abnormalities in olfactory epithelium tissue from subjects diagnosed with Schizophrenia. *Schizophrenia Research*, 150. 366-372.
- Pantazopoulos, H., Berretta, S. (2015). In Sickness and in Health: Perineuronal Nets and Synaptic Plasticity in Psychiatric Disorders. *Neural Plasticity*.
- Penninx, M.L., Milaneschi, Y., Lamers, F., Vogelzangs, N. (2013). Understanding the somatic consequences of depression: biological mechanisms and the role of depression symptom profile. *BMC Medical*, 11. 129
- Pietersen, C.Y., Mauney, S.A., Kim, S.S., Lim, M.P., Rooney, R.J., Goldstein, J.M., Petrysehn, T.L., Seidman, L.J., Shenton, M.E., McCarley, R.W., Soontag, K.C., Wood, T.W. (2014). Molecular Profiles of Pyramidal Neurons in the Superior Temporal Cortex in Schizophrenia. *Journal of Neurogenetics*, 28 (1-2). 53-69.
- Rajasekaran, A., Venkatasubramanian, G., Berk, M., Debnath, M. (2015). Mitochondrial dysfunction in Schizophrenia: Pathways, mechanisms and implications. *Neuroscience and Biobehavioral Reviews*, 48. 10-21.
- Rittié, L., Fisher, G.J. (2005). Isolation and Culture of Skin Fibroblasts. *Methods in Molecular Medicine: Fibrosis Research: Methods and Protocols*, 117. 83-98.
- Rutka, J.T., Apodaca, G., Stern, R., Rosenblum, M. (1988). The extracellular matrix of the central and peripheral nervous systems: structure and function. *Journal of Neurosurgery*, 69. 155-170.
- Schultz, C., Engelhardt, M. (2014). Anatomy of the hippocampal formation. *Frontiers in Neurology and Neuroscience*, 34. 6-17.

- Smith, G.M., Strunz, C. (2005). Growth Factor and Cytokine Regulation of Chondroitin Sulfate Proteoglycans by Astrocytes. *GLIA*, 52. 209-218.
- Suttkus, A., Morawski, M., Arendt, T. (2014). Protective Properties of Neural Extracellular Matrix. *Molecular Neurobiology*.
- Strakowski, S.M. (2003). Clinical update in Bipolar Disorders: second generation antipsychotics in the maintenance therapy of Bipolar Disorder. Available: <http://www.medscape.org/viewprogram/2496>
- Shah, A., Lodge, D.J. (2013). A loss of hippocampal perineuronal nets produces deficits in dopamine system function: relevance to the positive symptoms of schizophrenia. *Translational Psychiatry*, 215. 1-7.
- Slaker, M., Blacktop, J.M., Sorg, B.A. (2016) Caught in the Net: Perineuronal Nets and Addiction. *Neuroplasticity*. 1-9.
- Takashima, A. (1998). Establishment of Fibroblast Cultures. *Current Protocols in Cell Biology*, 2.11-2.1.12
- Theocharidis, U., Long, K., Faissner, A. (2014). Regulations of the neural stem cell compartment by extracellular matrix constituents. *Progress in Brain Research*, 214. 3-28.
- Toma, J.G., McKenzie, I.A., Bagli, D., Miller, F.D. (2005). Isolation and Characterization of Multipotent Skin-Derived Precursors from Human Skin. *Stem Cells*, 23. 727-737.
- Tsuneki, M., Madri, J.A. (2015). CD44 influences fibroblast behaviors via modulation of cell-cell and cell-matrix interactions, affecting Survivin and Hippo pathways. *Journal of Cellular Physiology*. 1-44.
- Turetsky B, et al. (2009) An odor-specific threshold deficit implicates abnormal intracellular cyclic AMP signaling in Schizophrenia. *Am J Psychiatry*, 66(2). 226-33.
- Verneti, L.A., Senutovitch, N., Boltz, R., DeBiasio, R., Shun, T.Y., Gough, A., Taylor, D.L. (2015) A human liver microphysiology platform for investigating physiology, drug safety, and disease models. *Experimental Biology and Medicine*, 0. 1-14.
- Vertel, B.M. (1995). The ins and outs of aggrecan. *Trends in Cell Biology*, 5. 458-464.
- Walton, N.M., Zhou, Y., Kogan, J.H., Shin, R., Webster, M., Gross, A.K., Heusner, C.L., Chen, Q., Miyake, S., Tajinda, K., Tamura, K. Miyakawa, T., Matsumoto, M.

- (2012). Detection of an immature dentate gyrus feature in human Schizophrenia/bipolar patients. *Translational Psychiatry*, 2, e135. 1-6.
- Wang, L., Lockstone, H.E., Guest, P.C., Palotás, A., Pietsch, S., Schwarz, E., Rahmoune, H., Harris, L.W., Ma, D., Bahn, S. (2010). Expression Profiling of Fibroblasts Identifies Cell Cycle Abnormalities in Schizophrenia. *Journal of Proteome Research*, 9. 521-527.
- Weinberger, D.R., Al-Amin, H.A., Lipska, B.K. (2000) Exaggerated MK-801-induced motor hyperactivity in rats with the neonatal lesion of the ventral hippocampus. *Behavioral Pharmacology*, 11. 269-278.
- Westergren-Thorsson, G., Schmidtchen, A., Sarnstrand, B., Fransson, L.A., and Malmstrom, A. (1992) Transforming growth factor-beta induces selective increase of proteoglycan production and changes in the copolymeric structure of dermatan sulphate in human skin fibroblasts. *European Journal of Biochemistry*, 205. 277-286.
- Woo, T.U.W. (2014). Neurobiology of Schizophrenia Onset. *Current Topics on Behavioral Neurosciences*, 16. 267-295.
- Yamane, J., Ihn, H., Asano, Y., Jinnin, M., Tamaki, K. (2003). Antagonistic Effects of TNF- α on TGF- β signaling through Down-Regulation of TGF- β Receptor Type II in Human Dermal Fibroblasts. *The Journal of Immunology*, 171. 3855-3862.
- Zaremba, S., Guimaraes, A., Kalb, R.G., Hockfield, S. (1989). Characterization of an Activity-Dependent, Neuronal Surface Proteoglycan Identified with Monoclonal Antibody Cat-301. *Neuron*, 2. 1207-1219.
- Zaremba, S., Naegele, J.R., Barnstable, C.J., Hockfield, S. (1990). Neuronal Subsets Express Multiple High-Molecular-Weight Cell-Surface Glycoconjugates Defined by Monoclonal Antibodies Cat-301 and VC1.1. *The Journal of Neuroscience*, 10 (9). 2985-2995.
- Zammit, S., Allebeck P., Andreasson, S., Lundberg, I., Lewis, G. (2002). Self-reported cannabis use as a risk factor for schizophrenia in Swedish conscripts of 1969; historical cohort study. *British Medical Journal*, 325 (7374). 1199.
- Zeug, A. Strawarski, M., Bieganska, K., Korotchenko, S., Wlodarczyk, J., Dityatev, A. (2014). Current microscopic methods for the neural ECM analysis. *Progress Brain Research*, 214, 287-312.