Novel CS-6 Cluster Microenvironments: The Effect of Fear Conditioning Influencing the Number, Morphology, and Synaptic Activation of CS-6 Clusters

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Novel CS-6 Cluster Microenvironments: The Effect of Fear Conditioning Influencing the Number, Morphology, and Synaptic Activation of CS-6 Clusters

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Abstract

Worldwide, over 50 million suffer with schizophrenia. The deficits associated with schizophrenia are severe, persistent, and largely complex; these effects cause an individual to endure severe cognitive impairments and impede their ability to properly function in daily tasks. These deficits range from emotional, cognitive, memory (verbal and non-verbal), behavior, perception (including hallucinations and deviations to reality), movement disorders, and various other impairments. Schizophrenia is undoubtedly a convoluted and multifaceted disorder in that it has been amalgamated with other disorders and has presentation of various symptoms (e.g., egodisturbances, passivity phenomena, auditory hallucinations, and delusions) (Northoff, 2015).

Given the biological complexity, elucidating the fundamental aspects of schizophrenia along with dissecting the underlying mechanisms is of great importance. In uncovering this, the brain’s extracellular matrix (ECM), is an essential constituent of the brain that initiates chemical and mechanical signals, orchestrates tissue and cellular organization and functions (Naba, et al., 2016). In addition to providing biochemical cues, ECMs also empower signaling cascades that dictate cell survival, cell propagation, stem cell states, and differentiation (Campbell & Humphries, 2011; Rozario & DeSimone 2010; Wickström, et al., 2011). Increasing evidence has suggested that the brain ECM is implicated in the pathophysiology of several brain disorders and critically involved in the regulation of synaptic plasticity (Dzyubenko, et al., 2016; Pantazopoulos & Berretta, 2016). Of specific interest, schizophrenia has been notably linked through studies in genetics, animal models, and human postmortem studies in the pathophysiology of schizophrenia (Pantazopoulos, et al., 2013; Berretta, 2012; Pantazopoulos, et al., 2010; Mauney, et al., 2013; Buxbaum, et al., 2008; Muhleisen, et al., 2012; So, et al., 2010).
Throughout the developmental stages, the brains ECM, including one of its primary components, chondroitin sulfate proteoglycans (CSPGs), play critical roles in synaptogenesis, neuronal migration and connectivity, synaptic development, and axonal outgrowth (Frischknecht & Gundelfinger, 2012; Bandtlow & Zimmerman, 2000; Curran & D’Arcangelo, 1998; Zimmerman, et al., 2008; Dityatev & Schachner, 2006; Dityatev, et al., 2010).

Interestingly, developing data and research from our group and others have shown that these very CSPGs may play a central role in the pathophysiology of psychiatric disorders, specifically schizophrenia (Pantazopoulos & Berretta, 2016). Moreover, our group has revealed that systematized perisynaptic ECM structures enriched in CSPGs, mainly perineuronal nets (PNNs), have diminished counts in various regions throughout the brains of people with schizophrenia and bipolar disorder (Pantazopoulos, et al., 2010; Pantazopoulos, et al., 2015). Within this context, recent findings have pointed to CS-6 clusters, a novel form of ECM/CSPG aggregate, to be markedly reduced within the amygdala of individuals with schizophrenia (Pantazopoulos, 2015). Accounting for the fact that these CS-6 clusters play a critical role in synaptic plastic mechanisms, our aim is to test our hypothesis using a fear conditioning paradigm.

Our main goal of the proposed studies is to ultimately investigate and provide evidence that these CS-6 cluster microenvironments have a linkage to experience-induced active sites of structural synaptic plasticity which contribute to dendritic spine pathology in schizophrenia. Our findings will shed light on the precise nature of CS-6 clusters, a unique ECM structure abundant throughout the cortical and subcortical brain regions. Additionally, we expect that an affirmative finding will illuminate an unknown mechanism which potentially plays a major role in regulating synaptic plasticity within segregated microenvironments. Ultimately, these results will allow a
tool to analyze how experience affects groups of adjacent synapses and in process reveal compelling insight on the prospective contribution of CS-6 cluster density/volume to the pathophysiology of several brain disorders, including schizophrenia and bipolar disorder.
The author’s academic interests have been greatly inspired by his passion in understanding, advancing, and positively impacting the field of medicine and medical research. The author has extensive studies in biomedical sciences as well as psychology and subsequently developed a profound desire to continue his academic endeavors and ultimately utilize the knowledge, skills, and experience acquired to further the field of medical research. Impactful moments, such as having family members who have suffered from various ailments and diseases, being involved in clinical research and settings, assisting in underserved populations, and various other experiences have collectively helped inspire and influence the author’s motivation in uncovering the fundamental aspects and mechanisms in biomedical sciences. It is the author’s hope to use this drive and enthusiasm towards furthering himself and in turn progressing the field of medicine.
Acknowledgments

I extend my deepest gratitude to my mentor and thesis director, Dr. Sabina Berretta. Through her generous guidance, frequent encouragement, inspiration, and confidence in my capabilities, she has given me an opportunity to become a refined student and researcher; ultimately allowing me to utilize my skills, knowledge, and experience to further myself in my ambitions. I would like to thank Dr. Gabriele Chelini for providing an enormous source of direction and guidance along with his constant encouragement and advice. Many thanks to Dr. James Morris for his continued support and assistance, the excellent feedback, mentorship, and motivation. I would like to show my appreciation to Chuck Houston for his kindness and generosity in always advising me throughout my degree and throughout the thesis. Lastly, and most importantly, I would like to thank my family and friends for their everlasting love and support.
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Chapter 1
Introduction

Background of Schizophrenia and Bipolar Disorder

The name Schizophrenia originates and was coined in 1910, which is derived from the Greek words, ‘schizo’ (split) and ‘phren’ (mind) (Burton, 2012). Consequently, this has led to a common misconception of the disorder regarding it as a “split personality” disorder, which is an apparent misapprehension. Schizophrenia is a severe, complex, and disabling brain disorder which is commonly characterized by an individual exhibiting abnormal social behavior and at times an inability to distinguish reality. Table 1 lists the most common symptoms associated with schizophrenia (Picchioni, et al., 2007).

<table>
<thead>
<tr>
<th>Most Common symptoms for Schizophrenia</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lack of insight</td>
<td>97%</td>
</tr>
<tr>
<td>Auditory hallucinations</td>
<td>74%</td>
</tr>
<tr>
<td>Ideas of reference</td>
<td>70%</td>
</tr>
<tr>
<td>Delusions of reference</td>
<td>67%</td>
</tr>
<tr>
<td>Suspiciousness</td>
<td>66%</td>
</tr>
<tr>
<td>Flatness of affect</td>
<td>66%</td>
</tr>
<tr>
<td>Delusional mood</td>
<td>64%</td>
</tr>
<tr>
<td>Delusions of persecution</td>
<td>64%</td>
</tr>
<tr>
<td>Thought alienation</td>
<td>52%</td>
</tr>
<tr>
<td>Thoughts spoken aloud</td>
<td>50%</td>
</tr>
</tbody>
</table>

Table 1
Despite only affecting approximately 1.1% of the population, Schizophrenia has a tremendous economic and social impact worldwide. Schizophrenia has an economic burden of over $60 billion USD per year (Marcus & Olfson, 2008). The severe disorder is commonly diagnosed between the ages of 16 and 30 (NIH, 2016).

A variety of research has laid groundwork on possible causes of schizophrenia which ranges from environmental factors to genetic factors to the various imbalances/abnormalities in neurological complexes and neurotransmission. Despite the extensive investigation over the past several decades, the precise cause of schizophrenia remains elusive. However, it is commonly accepted that the different phenotypes of schizophrenia ascend from multiple factors, particularly environmental and genetic dynamics (Crismon et al., 2014; Siever & Davis, 2004). While other probabilistic causes are more concrete, such as environmental risk factors in childhood, including abuse, neglect, substance abuse, relocation into stressful urban environments, etcetera, there remains scarce evidence supporting protective factors that subsequently and successively reduces the risk of schizophrenia (Heins et al., 1995; Veling et al., 2008).

Intervention, whether by pharmacological or other means, has always been of high interest in research. Specific intervention in the prenatal period has great potential in inhibiting developmental neuropathology which later exacerbates into a severe and chronic mental illnesses such as schizophrenia. One of the proven methods where intervention within the prenatal period has led to the alleviation of a developmental defect is that of the administration of folate to reduce midline structure abnormalities, such as spina bifida, and notably reducing cleft palate and other profound developmental issues (Wilcox et al., 2007; MRC, Lancet, 1991).
Another complex and convoluted neuropsychiatric disorder of interest here is Bipolar Disorder (BD). Bipolar I disorder initiates around the age of 18 and bipolar II disorder typically initiates at an age of 22 (Hirschfield, et al., 2006; Weissman, et al., 1988), also having a considerable heritability risk associated. Bipolar I disorder (BDI) is outlined with having at least one episode of mania. Bipolar II disorder (BDII) on the other hand is outlined by at least one episode of depression and one episode of hypomania (McCormick, et al., 2015).

Bipolar disorder’s effects are widespread—in addition to affecting the individual’s mental health, physical health is also effected. Interpersonal relationships, educational, and professional domains are all impacted (Valente and Kennedy, 2010).

Bipolar disorder is a multidimensional disorder in which patients experience persistent episodes of symptoms. These symptoms are characterized by depressive or manic episodes, which are intermixed by periods of euthymia, or relatively normal mood (see Figure 1).

Figure 1-The various ranges in moods associated with bipolar disorder (Vieta and Goikolea, 2005).
Unfortunately, per various surveys conducted on bipolar patients, more than 50 percent did not seek out treatment for five years, and these same patients received incorrect diagnoses. It wasn’t until an average span of eight years that they would receive their first treatments (Hirschfield, 2001; Lish, et al., 1994). Misdiagnosis of bipolar disorders include being diagnosed as having unipolar disorder, forms of passing psychosis, response to stress/adjustment disorder, and lastly, psychoactive substance abuse (Kessing, 2005). Adding to overall economic costs are misdiagnosis, mistreatment, and undertreatments (Hilty, et al., 2006). The possibility of accurate diagnosis for bipolar disorder in its premature stages could help mitigate the continuing detrimental effects of the illness and the economic impact associated with the disease.

Just as schizophrenia is economically impactful, so too is bipolar disorder as it is a major public health issue with a lifetime prevalence ranging at nearly 4 percent and a range of 1.5 to 6.0 percent in the United States (Kessler, et al., 2005; Ghaemi, 2001). In addition to these concerns, bipolar disorder has a high incidence of mortality with nearly one in four patients attempting suicide, and nearly half of those attempts are fulfilled (Prien and Potter, 1990). Likewise, to issues surrounding schizophrenia, inability to recognize and structure treatment services results in high levels of confinement and imprisoning. Despite advances in pharmacologic alternatives and opportunities, pharmacotherapy, psychotherapy, and other options, gaps persist in the field.
Chondroitin Sulfate Proteoglycans (CSPGs)

Proteoglycans are significant components of the extracellular matrix (ECM) and cell surfaces. The range and variety of functions span from synaptic regulatory functions, regulation of cell adhesion, neurite outgrowth, incursions of tumor cells, assemblers of ECM, and regulators and cofactors of growth factors (Ruoslathi, 1989; Ruoslathi and Yamaguchi, 1991). Interestingly, schizophrenia, a disease with a strong neurodevelopmental factor, shares many of the same properties interconnected to CSPGs and has a direct applicability to the pathophysiology of schizophrenia (SZ) (Harrison, 2007). Recent studies and research has shown major CSPG expression irregularities being discovered in this disease (Pantazopoulos et al., 2010; Enwright et al., 2012; Mauney, et al., 2013; Buxbaum et al., 2008). Precisely, CSPG-enriched PNNs had markedly diminished levels in various regions of the brain, commonly in connection with a noticeable increase of CSPG-positive glial cells (Pantazopoulos, et al., 2010; Enwright, et al., 2012; Mauney, et al., 2013). The significance of these neurobiological irregularities has been hypothesized to be linked in the disruption of neurodevelopmental processes (Berretta, 2012).

Intriguingly, emerging data and research from our group and others have shown that these very CSPGs may play a significant role in the pathophysiology of psychiatric disorders, specifically schizophrenia (Pantazopoulos & Berretta, 2016). Moreover, our group has revealed that systematized perisynaptic ECM structures enriched in CSPGs, mainly perineuronal nets (PNNs), have diminished counts in various regions throughout the brains of people with schizophrenia and bipolar disorder (Pantazopoulos, et al., 2010; Pantazopoulos, et al., 2015) (See Graph 1 Below). Additionally, widespread CS-6 clusters have been observed in the brain across various species (See Figure 2 below) (Pantazopoulos, et al., 2015).
Graph 1-Previous data showing CS-6 clusters and PNNs decreased in people with SZ and BD (Pantazopoulos, et al., 2010; Pantazopoulos, et al., 2015)

Figure 2-Representation of CS-6 clusters and their widespread prominence throughout various animal species.
The importance of these discoveries is based on the increasing amounts of evidence which points to the involvement and function of the ECM/CSPGs in the regulation of synaptic functions and plasticity (Dzyubenko, E., et al., 2016; Pantazopoulos and Berretta, 2016). Perisynaptic CSPG aggregates have been shown to regulate dendritic spine motility and synaptic connectivity in neurons (Orlando, et al., 2012). The effect of postnatal ECM maturation is that it activates the closure of critical periods of plasticity, ultimately leading to the overall regulation of the local circuitry in a mature plasticity state (Pizzorusso, et al., 2002).

CSPGs consist of a core protein and containing a variation of sequence and/or number of chondroitin sulfate sugar chains (CS chains). More specifically, these consist of repeating pairs of N-acetylgalactosamine and glucuronic acid. Recent findings from our research team and others have shown a novel form of an ECM/CSPG aggregate. These structures are referred to as “CS-6 clusters” (see Figures 3A and 3B) due to their predominant sulfation patterns in position 6 of the CSPG chondroitin sulfate (CS) chains. Furthermore, our team has shown that these CS-6 clusters are markedly reduced in the amygdala of patients with schizophrenia and bipolar disorder (Pantazopoulos, et al., 2015). In following the same trend and pattern as PNNs, CS-6 clusters are well signified in cortical and subcortical regions in healthy human, non-human primates and rodents, reaffirming and supporting the notion that they possibly serve an extensive role in neural functioning and properties.
Despite the elusiveness surrounding the role and nature of these structures, various data and research points to the role in regulatory functions in excitatory transmission. Moreover, based on the developing research, data and existing knowledge on the function of CSPGs in synaptic plasticity and based on the morphological observations, our research has allowed us to hypothesize that these CS-6 clusters may be characteristic of synaptic microdomains that are directly involved in the synaptic structural remodeling.

Fig. 3. A) CS-6 cluster in the human amygdala. B) We postulate that CS-6 clusters may be formed in an activity-dependent manner, as part of synaptic plastic mechanisms. In response to experience, dendritic spines on a small set of dendrites (in red) may incorporate CS-6/CSPGs (blue) secreted from the end-feet of groups of surrounding astrocytes (yellow), thus forming a CS-6 cluster (light blue) and inducing spine remodeling.
Definition of Terms

“Amygdala”: derived from the Greek word “almond” due to its almond like shape, this mass of gray matter is located within the cerebral hemisphere. It is integrally involved in the processing of emotions, memory, decision making, motivations, controlling autonomic responses (fear and arousal), and other functions. It has been widely associated with neuropsychiatric disorders.

“Anhedonia”: an inability to experience pleasure. A common symptom of individuals with schizophrenia and other mental disorders.

“Cerebrospinal Fluid (CSF)”: an uncolored and clear fluid found in the brain, specifically in the region of the Choroid Plexus (ChP). The CSF assists the brain by acting as a buffer or cushion from impending impacts and circulating essential nutrients and removing waste products that may exist.

“Central Nervous System (CNS)”: part of the nervous system which comprises of the brain and spinal cord. Functions in the integration and processing of information, controls the overall activities of the body.

“Chondroitinase ABC (chABC)”: a bacterial lyase that is capable of degrading hyaluronan glycosaminoglycans (GAGs), dermatan sulfates, and chondroitin sulfates. The therapeutic
potential of chondroitinase ABC has allowed researchers to strongly consider it as a way to repair and promote the central nervous system and peripheral nervous system due to its capability of degrading chondroitin sulfate glycosaminoglycans.

“Chondroitin Sulfate Proteoglycans (CSPGs)”: a diverse and extensive family of extracellular matrix (ECM) molecules which consist of chondroitin sulfate side chains and a protein core. Involved in numerous cell functions and processes, including but not limited to: cell maturation, cell migration, cell adhesion, receptor binding, and interactions with ECM components. Major CSPGs include: Aggrecan (CSPG1), Versican (CSPG2), Neurocan (CSPG3), Brevican (CSPG7) Phosphacan, and others.

“Dorsolateral Prefrontal Cortex (DLPFC)”: located anterior to the precentral sulcus in the middle and inferior frontal gyrus, corresponding to Brodmann areas 46 and 9 (Pierrot-Deseilligny, et al., 2004). A region of the frontal lobes most commonly known for its involvement in executive tasks such as selective attention and working memory.

“Entorhinal Cortex (ECx)” : located in the rostral end of the temporal lobe, or within the medial portion of the temporal lobe, serves as the primary interface between the hippocampus and the neocortical regions. This interface is involved in critical functions of episodic memory (previously stored spatial and temporal information), memory consolidation, and memory development.

“Euthymia”: in medicine, defined as a normal, tranquil, mood or mental state.
“Extracellular Matrix (ECM)”: serves critical utilities in structural and functional responses to cells within their environments (i.e., developmental interactions, cell differentiation and maturations, cell survival, cell relocation, tissue homeostasis, etc.). The constituents of the ECM interact amongst one another and with embedded proteins and surface proteins. An adult brain tissue is composed of lecticans, which are proteoglycans that are composed of hyaluronan and a lectin domain.

“Gamma-Aminobutyric acid (GABA)”: the primary inhibitory neurotransmitter in the human cortex.

“GABAergic neurons”: a primary inhibitory neurotransmitter which is essential in the maintenance of neurological action.

“Glycoproteins”: macromolecules that have carbohydrates which are covalently linked to a glycosylated N-linked asparagine polypeptide chain or O-linked serine-threonine polypeptide chain.

“Glycosaminoglycans (GAGs)”: extensive unbranched linear polysaccharides which are comprised of a repetitive disaccharide units (consists of N-acetylgalactosamine and galactose). The most common GAGs throughout the central nervous system are chondroitin sulfate, hyaluronan,
“Lateral Nucleus of the Amygdala (LN)”: functions as the sensory interface of the amygdala; a region which receives information from external sources and direct auditory sensory inputs from the cortex and thalamus.

“Lectican”: a family of proteoglycans which are integral constituents to the extracellular matrix. They network with tenascin-R and hyaluronic acid to form a tertiary complex. These are further broken down into four members: Aggrecan, Brevican, Neurocan, Versican.

“Long-term depression (LTD)”: occurring primarily within regions of the central nervous system (CNS), this is a process in which there is a decrease in postsynaptic strength. More specifically, it is an activity-dependent diminution in the effectiveness of neuronal synapses post long-patterned stimulation.

“Long-term potentiation (LTP)”: occurring primarily within the regions of the central nervous system (CNS), and opposite to the effect of LTD, LTP is a process in which there is an overall increase in the postsynaptic strength.

“Nerves”: a collection of fibers that is responsible for the interaction of sending and receiving messages between the brain and the body (sent via chemical and electrical within the cells).

“Oligodendrocyte”: mainly developed during embryogenesis and postnatal stages, a type of neuroglia that provides essential insulation and support to axons within the central nervous system (brain and spinal cord), through their composition of myelin sheaths.
“Perineuronal Nets (PNNs)”: specialized extracellular matrix structures essential in the regulation of the adult brain, including but not limited to, controlling the GABAergic neuron functions and connectivity, overall synaptic functions and plasticity, preserving connections, neuroprotection, and ensuring postnatal development via chondroitin sulfate proteoglycans (CSPG).

“Peripheral Nervous System (PNS)”: part of the nervous system which connects throughout the various limbs and organs of the human body, primarily consisting of ganglia and nerves that are outside of the brain and spinal cord—which are part of the Central Nervous System (CNS).
Background of ECM

One of the most integral components of the central nervous system is the extracellular matrix (ECM). Despite having once been thought to simply be a spectator between cells, discoveries have signified the crucial roles of the ECM. The ECM surrounds the cell and provides a critical physical scaffolding for the cellular components. Furthermore, it is responsible for biochemical signaling, moderating and orchestrating modifications in neural structure and function post injury and/or disease, modifications in reaction to experience induced events, and overall alterations in neuronal activity. Uncovering the processes and molecules involved in the organization of the neural ECM allows further discovery into the underlying functions of the ECM and how the dysregulation of the functions is involved in neuropathologies.

Nearly 20% of the brain’s volume is extracellular space, in which a network of accumulated interacting molecules known as extracellular matrix (ECM) and dispersed within the ECM are various soluble factors (Syková and Nicholson, 2008). These components are consisted of microglias, neurons, astrocytes, oligodendrocytes and other neural and non-neural cells. The vast molecules within the ECM along with the various proteases, carefully cleave matrix protein substrates. Various studies have provided strong evidence showing that disruption of the matrix via proteolytic cleavage may have a potential linkage in plasticity (Gottschall, et al., 2005).

Proteoglycans are vital constituents of the extracellular matrix (ECM) and cell surfaces, especially those throughout the brain. These molecules consist of long, unbranched sugar chains, sulfated polysaccharides (glycosaminoglycans; GAGs) composed of disaccharide unit
repetitions, which are attached to a protein core. In the CNS, they are a significant component of the ECM and the cell surface. Post CNS injury, CSPG expression in the lesioned region is impacted tremendously, rising in the region near the lesion, and ultimately resulting in the overall inhibition of axon regrowth and brain repair (Properzi and Fawcett, 2004).

The multitude of functions range from synaptic regulatory functions, neurite outgrowth, regulation of cell adhesion, incursions of tumor cells, assemblers of ECM, and regulators and cofactors of growth factors (Ruoslathi, 1989; Ruoslathi and Yamaguchi, 1991). One of the main components of the ECM, chondroitin sulfate proteoglycans (CSPGs), is vital in its function in participating in synaptic regulatory functions. (Dzyubenko, E., et al., 2016).
CSPGs and Lecticans

Of the various CSPG molecules expressed in the CNS, one of the significant components in all proteoglycans, and specifically lectican groups, is the consistency of a glycosaminoglycan moiety and a core protein construct. Furthermore, the lectican family includes Brevican, Neurocan, Versican (V0, V1, and V2), and Aggrecan (Figure 4).

![Diagram of proteoglycan molecules](image)

Figure 4. Graphic representation of proteoglycan molecules (i.e. Aggrecan, Brevican, Neurocan, Versican) (Siebert, et al., 2014).

These members consist of the core protein which has a N-terminal with a G1-domain and a C-terminal with a G3 domain. The significance of the main domain is its capability in binding the chondroitin sulfate glycosaminoglycan side chains (CS-GAG) (Bandtlow & Zimmerman, 2000;
Viapiano and Matthews, 2006; Galtrey & Fawcett, 2007). Accounting for the fact these are critical structural components of ECM, the multitude of functions and expressions of lecticans has been analyzed and researched in neural development and maturity along with diseased states within the CNS. Numerous studies have affirmed the involvement of these molecules within the developing CNS, playing a pivotal role in neurite extensions and initiating and promoting signaling for guidance and restriction of cell motility (Hartmann & Maurer, 2001).

A module of the ECM collection within the brain, the composition of chondroitin sulfate proteoglycans, lecticans, function in the impediment of neurite outgrowth, modification of dendritic spine shape, propagate closure of critical period plasticity, and inhibit functional recovery post-injury as the main component of glial scar. Although usage of chondroitinase-ABC results in the deletion of chondroitin sulfate chains from lecticans and consequently improves plasticity, the process of proteolytic cleavage of lectican may alter the conformation of the matrix aggregate and thus moderate neural plasticity (Howell and Gottschall, 2012).

Intriguingly, CSPGs are vital constituents of perineuronal nets (PNNs), which play a critical part in the closure of the childhood critical period, thereby inhibiting experience based neural plasticity in the adult CNS. The reactivation or restoration of critical period like synaptic plasticity in the adult CNS is achievable after degradation (digestion) of the PNNs through enzymatic removal of the chondroitin sulfate (CS) chains (Dityatev & Schachner, 2003). The PNNs are remarkable in affecting neural plasticity and their biophysical properties of the ECM and that affects the overall plasticity. Consequently, enzymatic digestion of the CSPGs considerably increases spine motility, promotes synaptogenesis, and reinstates juvenile forms of synaptic plasticity (Pizzorusso, et al., 2002; Orlando, et al., 2012; de Vivo, et al., 2013).
During early development, these CSPGs are associated but not densely aggregated to the hyaluronic scaffold and compose an ECM with large hydrates CPSG expression from a group of lectican, receptor protein tyrosine phosphatase (RPTP-beta) neuroglycan-C, to adult molecules (Aggrecan, V2, versican and the nature of the matrix would change owing to differences, aggregate around cells and neurites and limit their ability for spatial relocation. Similarly, changes in the structures and expression patterns of the CSPGs in neuropathologies might correspond to changes in the properties and functions of the neural matrix in the diseased CNS.

The ECM within the CNS is engaged in coordinating and modifying alterations in neural functions and structures as a reaction to experience, during disease, post-injury, and due to changes in neuronal activity. Structural neuronal modifications develop during adulthood, affecting functional plasticity, most visibly noted through enlargement of dendritic spines after Hebbian plasticity (Howell and Gottschall, 2012). Changes in plasticity throughout the nervous system are evident in preservation of shape and size of dendritic spines that regulate the overall formation of memory (Kitanishi et al., 2009; Kozorovitskiy et al., 2005; Kasai et al., 2010; Yang et al., 2009), developmental formation of circuitry connectivity (Carulli et al., 2005; Schwartz and Domowicz, 2004; Maeda et al., 2010), axonal outgrowth post CNS disease or injury states (Fawcett, 2009; Thon et al., 2000; Mayer et al., 2005; Busch and Silver, 2007; Schauwecker and McNeill, 1996; Yuan et al., 2002; Deller et al., 2000; Deller, et al., 2006), glial involvement in synaptic plasticity, and experience-dependent closure of developmental critical periods (Pizzorusso et al., 2006; Pizzorusso, et al., 2002). The modifications in dendritic spine structure and their relation to memory are of great interest because it occurs in reaction to long-term
potentiation (a depression state) and the stabilization of these modified spines is related to enduring memories (Yang, et al., 2008; Yang, et al., 2009).

Synaptic Plasticity

Synaptic plasticity has been thought to play a critical role in memory and learning functions. The original theory which proposed that alterations at synapses within the brain are the basis of memory and learning was validated by Donald Hebb in his influential work, *The Organization of Behavior: A Neuropsychological Theory* (Hebb, 1949). One of the most significant aspects of the theory is the discovery within the hippocampus of synaptic long-term depression (LTD) and synaptic long-term potentiation (LTP) (Malenka and Nicoll, 1999; Bear and Abraham, 1996; Bliss and Lomo, 1973; Dudek and Bear, 1992; Lynch et al., 1977). Since the discovery of LTD and LTP, various research has and analysis has reinforced the role of synaptic plasticity in memory and learning. Alterations have been observed in synaptic plasticity depending on the different types of memory and specific neural circuitry including the amygdala and hippocampus (Sigurdsson et al., 2007; Martin et al., 2000). Other factors, such as experiential aspects (i.e. acute or chronic forms of stress), have resounding results in altering synaptic plasticity in various brain regions (Kim et al., 2006).
Fear Conditioning

Fear conditioning is a method of associative learning through which subjects initiate defense or fear related reactions to a neutral conditioned stimulus (CS) that is corresponding with an aversive unconditioned stimulus (US). In an effort to understand animal learning, whether it is related to memory, behavioral tendencies, neurodevelopmental, or biochemically, researchers employ fear conditioning to help elucidate underlying mechanisms. Associative learning is an adaptive process that enables an organism to learn to anticipate events (Curzon, et al., 2009). The primary brain areas that have been discovered to be involved in fear conditioning include the amygdala, frontal cortex, cingulate cortex, and hippocampus. In regards specifically to the amygdala, studies have shown that the basolateral amygdala complex is a significant region for fear conditioning (Curzon, et al., 2009).

The overall process and pairing allows the CS to obtain the capability to elicit autonomic, endocrine, and behavioral responses that are normally manifested in the existence of danger (Smith et al., 1980; Blanchard and Blanchard, 1969; Fanselow and Bolles, 1980). The well-defined stimuli associated with fear conditioning, its ability to be instantly acquired and persist, its universality in animals, and sharing of many similar neural circuits in various mammals has allowed fear conditioning to become a significant and appropriate behavioral model for studying and analyzing the underlying neurobiological mechanisms of memory and learning (LeDoux, 2000; Maren, 1999; Rogan, et al., 2001; Davis and Lee, 1998).
Chapter II

Research Method

The tissues that were utilized throughout this research were all obtained from rodent mouse models, which were categorized into two groups of wild type mice—four conditioned mice and four home-caged littermates. The conditioned mice were ultimately used in a fear conditioning paradigm. Our model for our studies was based on the hypothesis that CS-6 clusters may be formed in response to experience. This is validated through observations in human and rodent tissues indicating distinct CS-6 cluster morphologies to phases of CS-6 cluster formation (See Figure 5).

Rosette

Diffuse

*Fig. 5 “Rosette” CS-6 clusters show distinct ‘process-like’ patterns, ultrastructurally corresponding to CS-/CSPG labeling in spines and astrocyte end-feet along dendrites. “Diffuse” CS-6 clusters are more amorphous and correspond to CS-6/CSPG labeling predominantly in dendritic spines. (Human thalamus)*
Model and Tissue Preparation

To test this hypothesis, we delineated the mice models into four groups of the home-caged littermates and four groups of the conditioned mice, the latter of which were accordingly exposed to a single session of auditory fear conditioning. Auditory fear conditioning, used as a model of behavioral training, was the comparative procedure that was used for analysis amongst the two groups. The experimental group was conditioned and they had one day of habituation. Approximately 24 hours later, they had conditioning which consisted of 10-tone shock pairs. This was followed with sacrificing at 4 hours post-conditioning. Next, after the animals were left alone, paraformaldehyde (PFA) was used to fix the tissue. The tissue was switched from PFA to Cryoprotectant solution. The Cryoprotectant solution that was used was PB with 0.1% sodium azide (0.1%) (commonly used to inhibit fungus growth from the sugar solutions) plus 20% glycerol.

Cryoprotectant solution remained on the tissue for a few days, where thereafter it was cut on a microtome. On the microtome, the tissues were specifically cut into 30-micron thick tissue sections. These were further divided into 24 wells, cut sequentially; this was done in order to have all of the wells account for the appropriate full brain representation.

Succeeding the sequence of methods, which included habituation and conditioning of the mice, the brain was harvested, stained accordingly using a CS-56 antibody which would mark for the CS-6 clusters. CS-56 is a mouse monoclonal IgM that is created by the using of vertebral membranes derived from chicken gizzard fibroblasts as the source of an immunogen.
(Pantazopoulos, et al., 2015). CS-56 has been described to label CS-6 in brain tissue (Miyata, et al., 2012). Accordingly, the CS-56 antibody enables the marking of the CS-6 clusters, allowing for a visual analysis of the clusters of interest, both rosette and diffuse types to be counted (See Figure 6 below for image representation summary of sequence).

![Image 1](image1.png)

**Figure 6-** Representation of sequence in experimental procedures.

Tissue categorization, analysis, and counting

Following these steps, an analysis into the total numbers of clusters would be accounted for. It is noteworthy that identification of CS-6 clusters using a strict and regimented outline be established. As shown in Figure 5, an image of the human thalamus indicates the appropriate classifications of CS-6 clusters as categorized into two distinct patterns. One category of CS-6 clusters is “rosette”, which show more outward-like and “process-like” patterns. These are ultrastructurally similar in their patterns to the CS/CSPG labeling in spines and astrocyte end-
feet. Opposing to the pattern of the rosette is the CS-6 pattern labeled as “diffuse”. These CS-6 clusters are less outward-like, tend to be more amorphous in their patterns, and these correspond to CS-6/CSPG labeling principally in dendritic spines. Through analysis, there were variations in size and density of the above stated noted patterns, but altogether, overall consistencies and matches were observed to the underlying criteria.

Total numbers of rosette and diffuse CS-6 clusters were accounted for in the hippocampus, amygdala, and auditory cortex. These were computed for in both groups, the conditioned and control group of mice.

Quantification procedures for CS-6 cluster identification

The equipment being used to account for these CS-6 clusters in the above-mentioned regions of the brain is stereology-based microscopy. The software being utilize within the microscopy is Bioquant NOVA/NT Image Analysis System. The counting and analysis was performed in a blind manner with the counting performed without knowledge of the diagnosis or conditions of the samples. Parameters within the microscopy analysis portion of the study included 10X, 25X and 40X magnification settings for all tissue observations, tracing, counting, and overall analysis of CS-6 clusters within the brain regions of interest. The outlining of the tissues was used in conjunction with an anatomical atlas, Interactive Atlas Viewer program, which provided an outline and guide of the anatomical structures for all the specific brain layers, the regions of interest, and all their relative subdivisions.
Brain regions and subdivision categorization

Brain regions and subdivisions of the amygdala and hippocampus were matched, traced, and outlined accordingly. The auditory cortex was also matched, traced, and outlined in accordance to the various layers represented in the anatomical outline. The hippocampus was subdivided as such: dentate gyrus (DG), Cornu Ammonis (CA), areas—CA1, CA2, CA3. The amygdala was divided regionally as such: Bas, BL, BM, Cort, Med, Cent. Lastly, the layers for the auditory cortex included and were denoted as such, L1, L2, L3, L4, L5, and L6. In tracing and outlining the auditory cortex layers, we designated layers L2 and L3 collectively into one category denoted as L2/L3 for the purposes of tracing and counting. Full sub-regions were counted and multiplied for thickness and the counting frame in order to account for the total area (multiplied by 30 microns and 24 wells).

A t-test is commonly employed in order to assess whether there is a significant difference between one set of data from one group versus another set of data from another group. Here, the t-test was performed to determine if there was a significant difference in the number of CS-6 clusters in the regions of the groups being analyzed and then compared accordingly. In a similar manner, in order to calculate the effect size, a g-test was used to measure for the overall effect size.
Chapter III

Results

Observations, experimental procedures, and data analysis were all based on the premise of quantification of CS-6 clusters in the brain regions, amygdala, hippocampus, and auditory cortex.

**Graphs 2 A&B- Quantification and Representation of Clusters in the Hippocampal Regions**
CS-6 clusters in the Hippocampus

In order to properly visualize CS-6 clusters, the staining of CS-56 was used. This allowed the CS-6 clusters, in both rosette and diffuse forms to be viewable. After the tissue was stained and the CS-6 clusters were viewable through microscopy, quantification revealed that there was statistical significance. Statistical significance was verified through t-test, which verified the significance between conditioned and home-caged littermates ($p < 0.05$). Additionally, g-test, which measures for effect size was applicable within the data.

In the dentate gyrus (DG) region of the hippocampus observations and analysis revealed that in comparison to the controls there was an overall decrease in the counts of rosette clusters in the fear conditioned mice ($p < 0.05$; $g > 1$). Conversely, observations and analysis in the CA3 region of the hippocampus revealed there was an increase in the number of rosette clusters. With respect to the diffuse clusters, our results indicated an overall increase in the number of clusters in the conditioned mice within the hippocampal region CA2 ($p < 0.05$; $g > 1$).
Graphs 3 A&B-Quantification and Representation of Clusters in the Amygdala Regions

CS-6 clusters in the Amygdala

In order to properly visualize CS-6 clusters, the staining of CS-56 was used. This allowed the CS-6 clusters, in both rosette and diffuse forms to be viewable. After the tissue was stained and the CS-6 clusters were viewable through microscopy, quantification revealed that there was an effect size between conditioned and home-caged littermates (g>1).

In the basolateral (BL) region of the amygdala observations and analysis revealed that in comparison to the controls there was an overall increase in the counts of rosette clusters in the fear conditioned mice (g>1). Similarly, observations and analysis in the BL region of the
amygdala for the diffuse clusters, our results indicated an overall increase in the number of clusters in the conditioned mice (g>1).

\[ \text{Auditory Cortex} \quad * = p < 0.05 \quad \# = g > 1 \]

Graphs 4 A&B- Quantification and Representation of Clusters in the Auditory Cortex Layers

CS-6 clusters in the Auditory Cortex

In order to properly visualize CS-6 clusters, the staining of CS-56 was used. This allowed the CS-6 clusters, in both rosette and diffuse forms to be viewable. After the tissue was stained
and the CS-6 clusters were viewable through microscopy, quantification revealed that there was an effect size between conditioned and home-caged littermates (g>1).

In the L2/3 region of the auditory cortex observations and analysis revealed that in comparison to the controls there was an overall decrease in the counts of rosette clusters in the fear conditioned mice (g>1). Similarly, observations and analysis in the L4 region of the auditory cortex for the rosette clusters, results indicated overall decrease in the number of clusters in the conditioned mice (g>1). This pattern was similar in the L2/3 region of the auditory cortex for the diffused clusters; signifying an overall decrease in the rosette clusters in the conditioned mice (g>1). However, in the L4 region of the auditory cortex for the diffused clusters, results indicated the conditioned mice exhibited an overall increase in the clusters versus the control mice.
Chapter IV

Discussion

Worldwide, over 50 million suffer with schizophrenia. The deficits associated with schizophrenia are severe, persistent, and largely complex; these effects cause an individual to endure severe cognitive impairments and impede their ability to properly function in daily tasks. Despite only affecting approximately 1.1% of the population, schizophrenia has a tremendous economic and social impact worldwide. Schizophrenia has an economic burden of over $60 billion USD per year (Marcus & Olfson, 2008). The severe disorder is commonly diagnosed between the ages of 16 and 30 (NIH, 2016).

Another complex and convoluted neuropsychiatric disorder of interest here is Bipolar Disorder (BD). Bipolar I disorder initiates around the age of 18 and bipolar II disorder typically initiates at an age of 22 (Hirschfield, et al., 2006; Weissman, et al., 1988), also having a considerable heritability risk associated. Bipolar I disorder (BDI) is outlined with having at least one episode of mania. Bipolar II disorder (BDII) on the other hand is outlined by at least one episode of depression and one episode of hypomania (McCormick, et al., 2015).

Bipolar disorder’s effects are widespread—in addition to affecting the individual’s mental health, physical health is also effected. Interpersonal relationships, educational, and professional domains are all impacted (Valente and Kennedy, 2010).

Given the biological complexity, elucidating the fundamental aspects of schizophrenia along with dissecting the underlying mechanisms is of great importance. Despite the extensive investigation over the past several decades, the precise cause of schizophrenia remains elusive. Our research and study is primed on investigating these mechanisms and elucidating the
fundamental aspects by tracing back towards the basis of the brain’s extracellular matrix (ECM). The ECM is a significant component of the brain which initiates chemical and mechanical signals, orchestrates tissue and cellular organization and functions (Naba, et al., 2016). In addition to providing biochemical cues, ECMs also empower signaling cascades that dictate cell survival, cell propagation, stem cell states, and differentiation (Campbell & Humphries, 2011; Rozario & DeSimone 2010; Wickström, et al., 2011).

Increasing evidence has suggested that the brain ECM is implicated in the pathophysiology of several brain disorders and critically involved in the regulation of synaptic plasticity (Dzyubenko, et al., 2016; Pantazopoulos & Berretta, 2016). Of specific interest, schizophrenia has been notably linked through studies in genetics, animal models, and human postmortem studies in the pathophysiology of schizophrenia (Pantazopoulos, et al., 2013; Berretta, 2012; Pantazopoulos, et al., 2010; Mauney, et al., 2013; Buxbaum, et al., 2008; Muhleisen, et al., 2012; So, et al., 2010).

Interestingly, developing data and research from our group and others have shown that these very CSPGs may play a central role in the pathophysiology of psychiatric disorders, specifically schizophrenia (Pantazopoulos & Berretta, 2016). Moreover, our group has revealed that systematized perisynaptic ECM structures enriched in CSPGs, mainly perineuronal nets (PNNs), have diminished counts in various regions throughout the brains of people with schizophrenia and bipolar disorder (Pantazopoulos, et al., 2010; Pantazopoulos, et al., 2015). Within this context, recent findings have pointed to CS-6 clusters, a novel form of ECM/CSPG aggregate, to be markedly reduced within the amygdala of individuals with schizophrenia (Pantazopoulos, 2015). Accounting for the fact that these CS-6 clusters play a critical role in
synaptic plastic mechanisms, our aim is to test our hypothesis using a fear conditioning paradigm.

Our main goal of the proposed studies was to investigate and provide evidence that these CS-6 cluster microenvironments have a potential linkage to experience-induced active sites of structural synaptic plasticity which contribute to dendritic spine pathology in schizophrenia.

To test this hypothesis, we delineated the mice models into four groups of the home-caged littermates and four groups of the conditioned mice, the latter of which were accordingly exposed to a single session of auditory fear conditioning. Auditory fear conditioning, used as a model of behavioral training, was the comparative procedure that was used for analysis amongst the two groups. The overall sequence that was followed involved habituation and conditioning of the mice, harvesting the brain, and staining the tissues using the CS-56 antibody; this would ensure that there was proper marking of the CS-6 clusters of interest—rosette and diffuse.

Once we categorized the tissues accordingly to their morphological characteristics, setting forth a precise criterion, and delineated brain regions of interest, we began our counting and analysis of CS-6 clusters within the hippocampus, amygdala, and auditory cortex. Through our observations and much of our analysis, we noticed that there were slight differences and distinctions in the overall size and density of rosettes and diffuse clusters. For instance, in specific cortical layers, particularly in L1 of the auditory cortex and L6 auditory cortex, we consistently observed lower numbers of overall clusters and the overall size of these clusters tended to be smaller than those of other layers (L2/3, L4, L5).

After analyzing and computing the total number of clusters, rosette and diffused, in the specified regions of interest, we used t-test and g-test for assessing whether there was a significant difference between one set of data from one group versus another set of data from
another group. The t-test enabled us to decide as to whether there was a significant difference in the number of CS-6 clusters in the regions/layers of one of the groups which was being examined and then parallel it with the other group and its regions/layers. Similarly, g-test was also utilized to measure for overall effect size.

Our data provided insight into the differences in the number of clusters in the two groups, conditioned and home-caged littermates, and the various brain regions/layers of interest. On the basis of existing studies and research on the role of CSPGs in synaptic plasticity and morphological observations, our hypothesis was that CS-6 clusters may represent synaptic microdomains involved in structural remodeling.

Based off the data from our results and the significance through the t-test and g-test in the brain regions, our counting revealed hippocampal, DG and CA2 of rosette clusters, CA2 of diffused clusters, BL of the amygdala regions in rosette and diffused clusters, and L2/3 and L/4 of both diffused and rosette clusters from the auditory cortex; all had data which had either a significant difference due to the t-test or an effect size from the g-test (see above Graphs 2-4).

It can be ascertained that the numbers of CS-6 clusters appear to be dynamically modulated by experience. Furthermore, these distinct morphologies of CS-6 clusters may signify and characterize specific time-points in the development of CS-6 clusters in response to experience. We believe that these findings symbolize the preliminary and groundwork evidence for the role of CS-6 clusters in modulating synaptic plasticity. The data and conclusions offer further support for a disruption of the interactions between ECM, neurons, and glial cells as possible underlying mechanism in contributing to synaptic pathology in schizophrenia.
Ultimately, our findings shed light on the precise nature of CS-6 clusters, a unique ECM structure abundant throughout the cortical and subcortical brain regions. Additionally, we expect that these findings will continue to elucidate an unknown mechanism which may potentially play a major role in regulating synaptic plasticity within segregated microenvironments. These results will further allow development of tools in analyzing and investigating how experience affects groups of adjacent synapses and in process reveal compelling insight on the prospective contribution of CS-6 cluster density/volume to the pathophysiology of several brain disorders, including schizophrenia and bipolar disorder.


