Complex Complement 4: Where the Immune System Ends and Schizophrenia Begins

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Schizophrenia (SCZD) is an incurable chronic mental disorder that affects approximately 1% of the US population. Previous studies found an association between C4A expression and SCZD risk (1). Currently, there are no specific pathological or molecular mechanisms linking the protein to the disorder. In order to determine the direct relationship between C4A expression and SCZD pathology, we will examine the three previously identified C4 alleles (1) which correlate with the highest risk of SCZD. We hypothesize transgenic mice and neuronal cell lines expressing the AL-AL variant of C4 should show the most severe SCZD symptoms. First, we will create mice models of the three C4 genes and overexpress the most potent gene in dopaminergic, glutamatergic and GABA-ergic neurons. We will also study neurons with the three C4 alleles in vitro. By examining the C4A interaction with other proteins related to the immune system or to the SCZD phenotype, we will illuminate potential SCZD molecular pathways. Lastly, we will create an iPSC-derived neurons and evaluate their relationship with the pathology of the disorder. We predict that expressing the three alleles in associated pathways will lead to a development of SCZD. Also we hope to demonstrate rescued function in transgenic mice treated with healthy iPS-derived cells.

Introduction

Schizophrenia (SCZD) is a chronic mental disorder that affects approximately 1% of the US population (2). SCZD is two-times more prevalent than Alzheimer's disease and five-times more prevalent than multiple sclerosis (3). The positive symptoms include hallucinations, delusions, and movement disorders (2). In addition, many patients exhibit cognitive symptoms such as deficits in decision making, attention, learning, and memory (2). Lastly, negative symptoms are characterized by a disruption of positive emotions and an expression of depressive behaviors (2). Previous researchers have evaluated that various environmental factors such as drug abuse, physical abuse and the area of residence might place some people at higher risk of getting SCZD than others (6, 7, 8). Currently, scientific research has been focused on the role of genes in the SCZD development.

SCZD is associated with the dysfunction of synapses and several neurotransmitters. According to the dopamine hypothesis, hyperactivity of the dopaminergic system results in SCZD. Alternatively, the glutamate hypothesis suggests that SCZD is due to hypoactivity of the glutamatergic system (2). Lastly the interneuron dysfunction hypothesis suggests the deficiency in GAD67, the primary GABA-synthesizing enzyme, leads to SCZD pathology (9). Each hypothesis explains a different facet of SCZD. While hyperactivity of the dopaminergic system may explain positive symptoms such as hallucinations (4), the hypoactivity of the glutamatergic systems correlates with negative symptoms (5), and the dysfunction of GABA-ergic systems are connected to both types of symptoms.

Single nucleotide polymorphisms (SNPs) are genetic variations that results in a single base change in the DNA sequence. Transitions are an exchange between purine-purine or pyrimidine-pyrimidine. Transversions are an exchange between purine-pyrimidine or pyrimidine-purine bases (10). Several SNPs are distinctly tied to SCZD, and have been targeted by several SCZD laboratories. For example, Papaleo et. al used a Tet-OFF system to systematically over-express neuregulin 1 (NRG1-IV) in a neuronal specific manner. Using the model they created, they used a broad range of methods to determine that NRG1-IV affects learning, memory, prepulse inhibition, and neuroanatomy in a way mirroring SCZD. Alternatively, Belforte et. al (2009) used a Cre-Lox system to systematically knockout the NR1 subunit of NMDA receptors (11). They also showed deficits in memory, prepulse inhibition, as well as a decrease in neural synchrony. Using a combination of methods and measurements from these papers, we will study complex complement 4 (C4) and it's relationship to SCZD pathology.

Several SNP haplotypes on the major histocompatibility complex (MHC) locus have been highly correlated with risk of SCZD development (12). The MHC locus encodes for several immune-related molecules, some of which are connected to assorted autoimmune disorders (13, 14). The polygenic MHC locus has two classes of genes: MHC I and MHC II. The locus is also highly polymorphic, leading to several variants of each gene. These two qualities work together to create a more diverse and effective immune system in the human population. Different versions of the Human Leukocyte Antigen (HLA) genes are in both MHC classes. The C4 gene separates the MHC classes on the locus. The resulting HLA and C4 proteins are responsible for localizing and tagging various targets of the immune system (15). C4 is known to be a part of the recruitment pathway involving C3 that triggers phagocytosis of infected cells by white blood cells. Inside the CNS, C3 triggers synaptic elimination by microglia (16, 17). This is an essential and healthy part of neurodevelopment. Unlike the rest of the animal kingdom, humans go through an additional stage of synaptic pruning during late adolescence, where the frontal lobe becomes fully developed.

Sekar et. al (2016) hypothesized that SCZD arises in late adolescence because C4 is over-activated in this final stage of human neurodevelopment. The mechanics behind this theory are yet to be revealed (1). The C4 gene is made up of one-to-three tandem alleles (1, 17). There are four possible alleles: A-Long (AL), A-Short (AS), B-Long (BL), and B-Short (BS). The A and B alleles encode for functionally different proteins, C4A and C4B (18, 19). The long alleles of both C4A and C4B are derived from the insertion of a human endogenous retroviral (HERV) segment. The HERV segment doesn't alter the function of the C4 protein in either case (20), but it does alter expression of the C4 protein (1). Sekar et. al found AL-AL, AL-BL, AL-BS, and BS are the four most prevalent variants of C4 in the human population. The AL-AL, AL-BL, AL-BS genes produce more C4A than the BS gene, which encodes for just C4B, and also have a higher association with SCZD than BS. This SCZD association is proportional to the amount of C4A produced by each gene. AL-AL produces the most C4A protein and demonstrates the highest risk. Since a direct relationship between C4A expression and SCZD pathology has yet to be identified, we propose to generate transgenic mice, neuronal cell lines, and iPS-derived neurons expressing the four most prevalent alleles. We hypothesize that the AL-AL gene should show the most severe SCZD symptoms, AL-BL should show moderate SCZD symptoms, and AL-BS should show mild if any SCZD symptoms. The BS gene will serve as our control and shouldn't demonstrate any SCZD symptoms. Our findings will hopefully demonstrate a new therapeutic target, and show iPS-derived neurons effectively rescue the transgenic mice.

Relevance

Broader relevance

Schizophrenia is one of the most prevalent psychiatric disorders that prevents 78% those diagnosed from living productive, independent lives. 12% of those diagnosed are in jail or homeless. Current treatments and therapy have varied results because there hasn't been a specific cellular or molecular pathway found to directly cause SCZD. None of the current treatments cure SCZD, but instead treat symptoms and potentially halt the progress of the disease. By discovering a specific molecular pathway tied to SCZD, we will provide a direct target for pharmacological studies hopefully leading to a more successful treatment for those with malfunctioning C4A. We will also demonstrate that iPS cells could be used as an effective treatment for SCZD.

Intellectual relevance

Not only is there no causal pathway discovered, but there are also two dominant theories for SCZD development: the glutamate hypothesis and the dopamine hypothesis. By examining glutamatergic, dopaminergic, and GABA-ergic pathways, we will be able to determine the one most affected by the C4A protein, leading to more targeted studies. In addition, the C4 protein has a clear function outside of the central nervous system, and we will elucidate its role in the brain through in vitro studies. We will also demonstrate that iPS cells are sufficient for creating SCZD models, broadening the methods available for neurological and psychiatric research.

Aims

1. *Evaluate C4 Risk Alleles in Transgenic Mice*

a. Create mice models of the four C4 genes

To evaluate the relationship of C4A and SCZD, we will breed four strains of transgenic mice; three with the C4 alleles correlated with the highest SCZD risk and one with the control gene BS. To compare the mouse lines, we will evaluate behavioral, anatomical and cellular differences.

b. Create mice with tissue-specific overexpression in the most potent gene

To elucidate which neuronal pathway is most affected by C4A, we will overexpress the gene that demonstrated the most severe SCZD symptoms (from aim 1a) in three separate zones: dopaminergic, glutamatergic, and GABA-ergic neurons. Using the same behavioral, anatomical and cellular tests as the previous aim, we can determine which neuronal pathway is most affected by C4A and therefore demonstrates the best model of SCZD.

2. *Evaluate biochemical interactions of C4A in vitro*

To examine the molecular pathway and cellular alterations due to C4A, we will introduce AL-AL, AL-BL, and AL-BS alleles into the SH-SY5Y cell line, using the BS gene for a control. We will measure C4A protein expression and the neuron morphology. Additionally by using immunofluorescence, we will examine the localization of proteins C4A, C3, PSD 95, SYNGAP1, Shank 3, Neuregulin 1,DR6, and CASP6. We will evaluate which proteins are more likely to interact with C4A by analyzing the protein localizations and by employing binding assays.

3. *Create and evaluate iPS-derived neurons for SZCD models*

a. Generate iPS-derived neurons from fibroblasts of SCZD patients and inject into healthy mice

To evaluate the relationship between C4A and the SCZD pathology, we will generate dopaminergic, glutamatergic, and GABA-ergic iPS-derived neurons from fibroblasts of SCZD patients expressing C4 risk genes, and transplant the neurons into healthy mice. To evaluate the iPS-derived neurons, we will use the same measurements as aim 2. Additionally, to measure the effect of the transplantation of the SCZD neurons in healthy mice, we will use the same measurements as described in aim 1b.

b. Generate generic iPS-derived neurons from fibroblasts of healthy individual and inject into aim 1a transgenic mice:

In addition, healthy human-derived neurons will be transplanted into the transgenic mouse models created in aim 1a. Using the same behavioral, anatomical and cellular tests as before, we will evaluate whether pathological symptoms can be rescued in the transgenic mice.

Research Methods and Design

1. *Evaluate C4 Risk Alleles in Transgenic Mice*

Rationale: Sekar et. al (2016) discovered that the four most prominent genes had a SCZD risk factor proportional to the amount of C4A expressed (1). We aim to express the four genes to determine if heightened expression of C4A is sufficient for SCZD development. In addition, we aim to overexpress the gene that lead to the most severe pathology in each of the three pathways implicated in SCZD to determine whether C4A is most harmful in glutamatergic, dopaminergic, or GABA-ergic neurons.

a. Create transgenic mice models

A total of 40 transgenic mice will be developed; 10 expressing each gene. We will use the method described (21) to microinject the purified transgene DNA construct into the harvested zygotes, and then implant the zygotes into a pseudo-pregnant recipient. After genotyping, we will evaluate behavioral, anatomical and cellular differences using the following measures. The behavioral assessment will include three tests. First, we will use a startle and prepulse inhibition (PPI) test to measure sensorimotor gating in mice. We will then record the startle amplitude to quantify PPI (11). To measure recognition memory and social anxiety, we will calculate a discrimination ratio based on behavior during the novel-mouse discrimination test (22, 23). To evaluate spatial memory and learning in mice, we will record the time latency in the Morris water maze test (24). The anatomical assessment will also be derived from three measures (25). We will measure lateral ventricle size to evaluate loss of brain matter in the transgenic mice. Additionally, we will stain for PV cells in both the prefrontal cortex and hippocampus according to the method described (22, 25, 26). Since C4 was shown to be involved in synaptic pruning in mice (1), we will use the immunohistochemistry-based assay described (26) to quantify the synaptic density of the brain. The three assessments of cellular differences will be C4A and C3 localization (30), RNA expression analysis (27), and neuronal connectivity (27). Using immunofluorescence techniques, we will co-stain for C4A, C3, PSD95, and SYNGAP1 which will determine where C4A and C3 are most prevalent (28). Using the method described (27) we will assess the mRNA levels of Shank 3 and Neuregulin 1 which are already associated with SCZD. Finally, we will use the rabies virus to measure neuronal connectivity because transmission of the virus occurs through synapses, and this would indicate synaptic strength (29).

b. Create mice with tissue-specific overexpression in the most potent gene

Additionally, a total of 30 transgenic mice will be developed; 10 for each pathway. We will use tissue specific promoters to overexpress the gene that demonstrated the best SCZD model in dopaminergic, glutamatergic, and GABA-ergic pathways using the tetracycline controlled transcriptional activation (30). Similar to the method described, we will create a transgenic mouse line expressing TET on of the neuronal pathways and cross with a tetO-C4 transgenic mouse line (11, 31). For overexpression in dopaminergic neurons, we will place the TET gene after the tyrosine hydroxylase promoter (32, 33). For glutamatergic neurons, we will place the TET gene after the vGlut1 promoter (32, 34). For GABA-ergic neurons, we will place the TET gene after the GABA transporter 1 promoter (32, 35). We will evaluate the tissue specific expression with co-immunofluorescence with tyrosine hydroxylase, vGlut1, and GABA transporter 1, and comparison of neural connectivity of the pathways in each line (30). Then using the same behavioral, and anatomical tests we will compare these models to the transgenic and control mouse from aim 1a.

Predictions

The AL-AL gene is associated with the highest risk and produces the most C4A (1). Therefore, we predict that the AL-AL transgenic mice will demonstrate severe SCZD pathology, AL-BL mice will demonstrate a moderate SCZD pathology, and AL-BS mice will demonstrate mild is any SCZD pathology. Since PPI is attenuated in SCZD patients, we predict that the startle amplitude will be higher and therefore the PPI will be less in the SCZD mice compared to WT, especially in the AL-AL mice. The SCZD mice should not demonstrate any difference in interactions with the familiar mouse versus the stranger mouse, which would be evident in a decrease in the discrimination ratio compared to BS mice. The transgenic mice should take longer to solve the Morris water maze due to a deficit in spatial memory typical of SCZD. Since both human patients and SCZD mice models show enlarged lateral ventricles resulting from brain tissue loss, the AL-AL transgenic mouse should show the largest lateral ventricles. Since PV cell loss is typical of SCZD models (21, 22, 25), we expect to see a significant reduction in PV cells in the AL-AL transgenic mice as well as a moderate reduction in PV cells in AL-BL mice. Furthermore, synaptic density should be most reduced in AL-AL mice. Additionally, we predict C3 and C4A to be localized around dendrites and axonal projections in all mouse lines (1). With the knowledge that glutamatergic hypofunction leads to SCZD and C4A aids in synaptic pruning and possibly cell loss, we predict that the glutamatergic neuronal pathway will be most affected by C4A and will therefore produce that model with the most severe symptoms. Overexpression in dopaminergic neurons should lead to a deficiency in prepulse inhibition, an increase in time taken in the Morris water maze, and a decrease in parvalbumin cells. Overexpression in GABA-ergic neurons should mirror the effects of the other two mouse lines because they are interneurons, and a deficiency in GABA-ergic neurons will affect both dopaminergic and glutamatergic pathways.

2. *Evaluate biochemical interactions of C4A in vitro:*
Rationale:

C4A protein expression is elevated in SCZD patients who carry AL-AL, AL-BL, and AL-BS genes (1). Therefore, it is important to learn

more about the cellular alterations and the protein-protein interactions to further understand the SCZD pathology. We will investigate the interactions of the C4A protein with the the C3A protein since C3A and C4A work together outside the brain to catalyze the elimination of pathogens by phagocytes (15). Also, we will investigate how C4A interacts with proteins Shank 3 and Neuregulin 1 which are linked to SCZD (37, 24). Additionally, our goal is to use protein-protein interactions to explain the cellular morphology. We will investigate C4A interactions with synapse-associated proteins SYNGAP1 and PSD-95 (37, 38) and pruning proteins DR6 and CASP6 (34) because SCZD neurons have a reduced number of synapses (40) and C4 mediates axon pruning (1).

a. Express three alleles in a neuronal cell culture

Using Lipofectamine 2000 system, we will create three cell lines by transfecting the SH-SY5Y cells with a pcDNA3 vector containing one of the three C4 genes (30). As our control group, we will use cells transfected with empty pcDNA3 vector and cells transfected with BS gene. A Western Blot will evaluate C4A protein expression. We will use a confocal microscope to assess dendritic spine density, axon length, and the number of branchings. Additionally, we will use the immunofluorescence to examine C4A colocalization with proteins C3, Neuregulin 1, PSD95, SYNGAP1, Shank 3, DR6 and CASP 6. Based on which of these proteins are co-localized with C4A, we will then test for protein-protein interactions.

b. Assess C4A protein-protein interactions

The recombinant proteins will be produced by using pET vectors and then purified according to protocol (41). The first step of this procedure is to place AL-AL, AL-BL, and AL-BS genes fused with a polyhistidine-tag into a plasmid (42). Similarly we will put BS gene into a pET vector for our negative control. To create the four cell cultures, we will place the four plasmids into separate E. coli BL21(DE3) strains. We will use immobilized metal affinity chromatography (IMAC) to purify the produced proteins. If additional purification is required, we will use size-exclusion chromatography (SEC) or ion exchange chromatography. In order to examine the protein bindings, we will use the coimmunoprecipitation assay. We will place purified C4A protein into the affinity column and add other purified proteins, such as DRD6, PSD95, etc (42). To eliminate proteins which didn't interact with C4A, we will wash with a buffer and collect the what leaves the column. We will run the immunoprecipitated product on the Western blot to determine with which proteins C4A interacted. Additionally, we will use a yeast two-hybrid assay (43) to assess the protein interaction. Lastly, immunofluorescence will be used to localize the bindings in the cells.

Predictions

We expect the introduction of the three alleles into the SH-SY5Y cells will lead to the elevated levels of C4A protein. The AL-AL will exhibit the highest level of C4A and the most severe SCZD morphology. We predict decreased dendritic spine density, axon length, and number of branchings compared to the controls. Since AL-AL gene is associated with the highest SCZD risk, we anticipate that the AL-AL cell line will have C4A most or least localized to C3, SCZD-associated proteins, and pruning proteins. We will examine protein-protein interactions based on the proximity to C4A. Similarly, we would expect that the AL-AL cell line will exhibit the strongest or the weakest interaction with C3, SCZD-associated proteins, and synaptic pruning compared to the other genes.

3. *Create and evaluate an iPS-derived neurons for SZCD models: Rationale:*

Researchers are trying to develop new therapies that will help treat patients with genetic disorders. Due to possible immune rejection of human embryonic stem cells, scientists examined the therapeutic potential of induced pluripotent stem cells (iPS cells). The risk of tissue rejection is reduced because the cell contains the patient's own genetic material (44). Similar to embryonic stem cells, iPS cells have the ability to differentiate into various types of neurons (45). Previous studies have shown that iPS have been used as therapies against diseases such as sickle cell anemia, Rett syndrome, and long-QT syndrome (44, 46, 47). In addition, SCZD has been modeled in humans using iPS cells with an unknown risk factor gene (31). The use of iPS cells will allow us to further asses the SCZD pathology. To generate our iPS cells, we will use a mixture of transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, because it was shown to work for somatic cells and for mammals, including mice, rhesus monkeys, and humans

a. Generate iPS-derived neurons from fibroblasts of SCZD patients and inject into healthy mice

We will perform a skin biopsy to isolate the fibroblasts from three SCZD patients expressing AL-AL, AL-BL, AL-BS genes. Fibroblasts will also be biopsied from a healthy individual expressing BS gene. Then, we will reprogram these cells into iPS cells by adding the transcription factors Oct3/4, Sox2, Klf4, and c-Myc (48). iPS cells will be then placed in HUES medium for 28 days to generate colonies. The neural progenitor cell (NPC) medium will be used to differentiate the colonies into neural progenitor cells (NPCs). During the immunofluorescence test, the presence of the visible rosettes marked with NPC indicators Nestin and SOX2 will confirm the formation of NPCs (31). To generate dopaminergic neurons from NPCs, we will use exogenous fibroblast growth factor 8 (FGF8), proto-oncogene protein WNT1, low dose retinoic acid, and a high activity form of sonic hedgehog protein (SHH) (45, 49). GABA-ergic neurons will be created by using restricted dorso ventral marker Nkx2.1 in conjunction with the transcription factor Foxg1 (50). Lastly, glutamatergic neurons will be generated with the inhibition of SHH pathway and the stimulation with FGF-2 growth factor (51). We will demonstrate the creation of the correct neurons by fluorescent expression of dopaminergic marker such as vesicular monoamine transporter 2 (VMAT2) and DA transporter (DAT) (52), GABA-ergic marker GAD67 (31), and glutamatergic markers VGLUT1 and TBR1 (31). To demonstrate that we specifically created SCZD dopamine, glutamate, and GABA-ergic neurons, we will perform the measurements described in aim 2a. Transplantation of the brain neurons into the healthy will be done by using 3D microtopographic scaffolds. The neurons will be loaded onto the scaffolds that will be injected into specific mouse brain regions using Hamilton syringe. We will inject dopamine neurons into substantia nigra, glutamatergic into prefrontal cortex, and GABA into middle layers of prefrontal cortex. Previous findings have shown that this method of transplantation demonstrated the improvement in neurite outgrowth, increased firing of action potential and cell survival compared to other techniques (53). To measure the effect of the transplantation of the SCZD neurons in healthy mice, we will use the same measurements as described in aim 1b and compare the mice to those from aim 1b.

b. Generate generic iPS-derived neurons from fibroblasts of healthy individual and inject into transgenic mice

Using the same method as described in 3a, we will generate NPCs. Next we will dissociate NPCs with Accutase and place cells in neuronal differentiation medium. To demonstrate the creation of the correct neurons, we will use βIII tubulin marker along with the dendritic marker MAP2AB (31). We will transplant the neurons into either the hippocampus or prefrontal cortex of transgenic mice created in aim 1a. Both of these regions are implied in SCZD and are previously assessed in aim 1. To observe corrected pathology of transgenic mice, we will use the same behavioral, pathological, and cellular tests as described in aim 1a.

Predictions

The iPS-derived neurons from SCZD patients should show similar deficiencies in axon length, dendritic spine density, and dendritic branching to the neuroblasts in aim 2a. This would confirm that we had successfully made SCZD neurons in aim 2, and show which type of iPS-derived neuron is pathologically similar to those created with just the pathogenic C4 gene. We will also use the same behavioral, anatomical and cellular tests as in aim 1b, and we expect to see similar but not as severe of results for each neuronal pathway transplanted with SCZD iPS-derived neurons. Mice injected with AL-AL glutamatergic neurons should theoretically show the worst symptoms in behavioral, anatomical and cellular levels compared to the other neuronal pathways. AL-AL dopaminergic neurons should demonstrate the deficiency in prepulse inhibition test, slower performance in Morris water maze and a decrease in parvalbumin cells. AL-AL GABA-ergic neurons should induce the same symptoms as both glutamatergic and dopaminergic neurons because they are the interneurons. We also expect to see the ability of the transgenic mice expressing AL-BL and AL-BS introduced with healthy neurons to have a significant increase in prepulse inhibition test, faster performance in Morris water maze and the increase in discrimination ratio for novel

mouse recognition task. We hope to also observe their improvement on anatomical and cellular levels. Because the AL-AL mice are expected to have the most severe symptoms, we expect that the transplantation will not rescue as much function as in the other transgenic mice.

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