Microglia, complement, and synaptic pruning: Relevance to schizophrenia

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Abstract

During development, the brain undergoes synaptic pruning, in which some synapses are eliminated whereas other synapses are maintained and strengthened. Microglia are phagocytic cells within the central nervous system that express receptors for molecular signals that target synapses for microglial engulfment and destruction. Complement proteins, which function peripherally as activators of the immune system, serve as one type of molecular signal, and multiple complement proteins have been shown to be necessary for the normal trajectory of synaptic pruning. The gene encoding complement component 4 (*C4*), in particular, exhibits a strong association with risk of schizophrenia, where variants that increase expression of the C4A protein are associated with increased synaptic engulfment by microglia in both human and mouse studies. Mouse models that overexpress the *C4A* variant associated with schizophrenia also display impairment in behavioral domains, similar to the negative symptoms of schizophrenia. However, a recent genomic and transcriptomic analysis has called into question whether increased *C4A* expression is directly responsible for schizophrenia pathology, versus other downstream genetic pathways that are downregulated with increased copies of *C4A*. This review will describe recent advances in microglia-component protein signaling and its relationship to schizophrenia, and will provide recommendations for future research.

Background

Synaptic pruning is a developmental phenomenon in which a large number of synapses that form during early development are eliminated while a subset of synapses are maintained.[1] Over the last decade, the role of the immune system in synaptic pruning has become increasingly apparent. In the peripheral immune system, phagocytic cells interact with several molecular signaling pathways, mediating clearance of cellular debris. These molecular signals include the complement cascade, which consists of proteins that can bind receptors expressed by phagocytic cells.[2] Specifically, upstream proteins such as C1q and C4 promote C3 activation, allowing C3 to bind to targets and promote their phagocytosis (Figure 1).[1] During development, C3 tagging is required for synapse engulfment by microglia, which are phagocytic cells within the central nervous system and the only cells in the brain that express complement receptors.[2,3]

Synaptic pruning has been implicated in pathological states in humans, including schizophrenia and other disorders.[4] Schizophrenia is a common neuropsychiatric condition that encompasses both positive symptoms (e.g., delusions and hallucinations) and negative symptoms (e.g., amotivation, social withdrawal, and cognitive deficits).[5] The onset of schizophrenia symptoms tends to occur during late adolescence, which coincides with later stages of synaptic pruning in the prefrontal cortex (PFC).[6] Schizophrenia has been associated with reduced gray matter volume in the PFC,[7] as well as decreased dendritic spine density on layer 3 pyramidal neurons in the PFC.[8,9] These findings could be consistent with excessive synapse elimination and resulting impaired PFC circuitry in schizophrenia.[4]

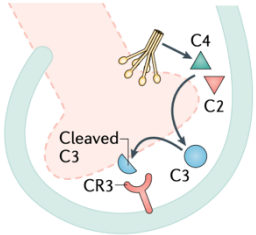

Figure 1. Microglial synaptic pruning through the complement signaling cascade. Faust et al., 2021.

This review will describe key studies over the last decade that have informed our understanding of the role of complement protein in synaptic pruning and its relationship to schizophrenia. Specifically, complement proteins are necessary for synapse engulfment by microglia.[3] Complement proteins have also been implicated in genetic studies of schizophrenia, in which more copies of the *C4A* are associated with increased risk of disease.[10,11] Behavioral studies using mouse lines overexpressing *C4A* show deficits in social motivation similar to the negative symptoms of schizophrenia.[12,13] However, a recent genomic analysis has questioned whether the pathogenesis of schizophrenia is related to complement and inflammatory signaling, versus other mechanisms related to synapse machinery in neurons.[14] Finally, this review will highlight remaining questions regarding the role of complement and synaptic pruning in the cellular pathogenesis of schizophrenia.

Microglia shape neural circuits in an activity- and complement-dependent manner

For decades, it has been understood that neuronal activity plays a role in synaptic pruning during development. However, the precise function of microglia within synapse remodeling and the mechanism of microglia-synapse interactions remained elusive until 2012, when Schafer and colleagues demonstrated microglial engulfment of presynaptic inputs.[3] They hypothesized that complement proteins act as signals to induce microglial phagocytosis of select dendritic spines, and that this phagocytosis was regulated by neuronal activity and developmental timing.

To test this hypothesis, Schafer and colleagues used the mouse retinogeniculate system, in which retinal ganglion cells (RGCs) compete for territory throughout the dorsolateral geniculate nucleus (dLGN) during development.[15] Schafer and colleagues used CXC3CR1+/EGFP mice, in which all microglia express enhanced green fluorescent protein (EGFP) under the control of fractalkine receptor (CX3CR1) expression. They labeled presynaptic inputs from retinal ganglion cells via intraocular injection of anterograde cholera toxin b subunit tracers. Schafer and colleagues observed numerous labeled RGC inputs within the processes and soma of microglia in the dLGN, colocalizing with CD68 (a marker of lysosomes specific to microglia), suggesting that microglia engulf RGC inputs undergoing synaptic remodeling. Engulfment was highest at postnatal day (P)5, but reduced at P9, when pruning is largely complete,[2] suggesting that microglia-mediated engulfment of RGC inputs is temporally correlated with a known period of synaptic pruning. To test whether phagocytosis was activity-dependent, Schafer and colleagues injected either tetrodotoxin (TTX), which blocks activity, or forskolin, which increases activity, into the left eye, and vehicle into the right eye. They observed that microglia phagocytosed more inputs from the TTX- treated eye compared to the vehicle-treated eye, and more inputs from the vehicle-treated eye compared to the forskolin-treated eye, indicating that microglia preferentially engulf inputs with decreased activity.

Next, the researchers aimed to confirm whether the microglia were engulfing presynaptic elements from RGC projections. Using immunohistochemical electron microscopy (immunoEM) for the microglia marker iba-1, they observed membrane-bound structures consistent with presynaptic vesicles completely surrounded by microglial cytoplasm. By staining for presynaptic machinery specific to RGCs such as vesicular glutamate transporter 2 (VGluT2) in the P5 dLGN of CX3CR1+/EGFP mice, then imaging using 3D structural illumination microscopy,[16] Schafer and colleagues observed VGluT2 immunoreactivity within the EGFP-positive cytoplasm of microglial cells, confirming that the engulfed tissue originated from RGCs.

Finally, given their previous observation that complement component C3 is necessary for synaptic pruning in the retinogeniculate system,[2] Schafer and colleagues hypothesized that

interaction between C3 and its receptor, CR3, was one mechanism by which microglia engulf RGC inputs. To test whether C3-CR3 interaction was necessary for microglial engulfment, Schafer and colleagues used an in vivo phagocytosis assay to measure engulfment in P5 mice that were lacking functional CR3 (CR3 KO) or deficient in the C3 ligand (C3 KO). Microglia from CR3 KO and C3 KO mice had a decreased capacity to engulf RGC inputs compared to WT littermate controls, suggesting that the C3-CR3 interaction was necessary for synaptic pruning. At P10 and P30, there was increased overlap between ipsilateral and contralateral inputs in the dLGN of CR3 KO mice compared to WT littermate controls, with CR3 KO mice displaying aberrant RGC inputs in inappropriate regions, suggesting a deficit in synaptic pruning. Quantification of retinogeniculate synaptic density using array tomography[17] found that adult CR3 KO mice exhibited significantly increased RGC synaptic density compared to WT controls. Schafer and colleagues concluded that C3-CR3 interaction is necessary for microglial synapse engulfment in the dLGN.

Overall, this work from Schafer and colleagues[3] demonstrated the role of microglia in activity-dependent synaptic pruning within the mouse retinogeniculate system. They found that microglia are responsible for the phagocytic engulfment of retinal inputs undergoing synaptic pruning, and that this engulfment is dependent on developmental timing and neuronal activity. Finally, they identified interaction between complement component C3 and its receptor, CR3, as a necessary mechanism for microglial synapse engulfment. This work paved the way for future studies that have further described the microglial-complement system in synaptic pruning.[18]

Schizophrenia risk is associated with variants of complement component 4

A convergent line of research has investigated the role of the immune system in the pathology of schizophrenia. Data from multiple genetic studies have implicated the Major Histocompatibility Complex (MHC), a highly polymorphic region related to immune function, as one of the most significant genetic loci associated with risk of schizophrenia.[19–22] However, the relationship between schizophrenia and the MHC was unclear, as it did not correspond to linkage disequilibrium around any known variant within the MHC.[19,22] This challenge led Sekar and colleagues[10] to investigate genetic influences that might lead to unconventional genetic signals. Consistent with a known association between schizophrenia and *CSMD1*, a gene within the same region as the MHC that encodes a regulator of complement component 4 (*C4*),[19,22] Sekar and colleagues observed strong genetic associations near a complex, multi-allelic locus that affects the *C4* gene. They hypothesized that variation at this locus might provide mechanistic insight into the pathogenesis of schizophrenia.

The human *C4* gene exists as two functionally distinct isoforms, *C4A* and *C4B*, which can each vary in structure (long [L] or short [S] versions) and copy number. Humans typically have one to three total copies of *C4* within the MHC region. Sekar and colleagues[10] developed a method of imputing *C4* "structural haplotypes," including information about the copy number of *C4A* and *C4B* as well as the L / S status of each *C4* copy. They found that of the 222 chromosomes sampled, 90% of individuals had one of the 4 most common C4 structural haplotypes: AL-BL, AL-BS, AL-AL, and BS. In a sample of human brain tissue, they found that RNA expression of C4A and C4B proteins were positively associated with copy number of *C4A* and *C4B* genes, respectively. Based on the data from human tissue, they created a model that could use genetic data to predict levels of *C4A* and *C4B* expression in the brain. Using data from the Psychiatric Genomics Consortium,[19] Sekar and colleagues found that this predictor of *C4A* expression was strongly associated with risk of schizophrenia, even after adjusting for multiple

confounds. In addition, the same structural haplotypes of *C4* that were associated with higher *C4A* expression were also associated with increasing risk of schizophrenia.

Sekar and colleagues next tested the distribution of *C4A* expression in the brain. In a sample of brain tissue from 35 human schizophrenia patients and 70 individuals without schizophrenia, tissue from schizophrenia patients exhibited greater *C4A* expression in each of the five brain regions tested, even after adjusting for age, postmortem interval, and average *C4A* copy number among the brain donors with schizophrenia. Immunohistochemistry revealed that C4 was present in subsets of NeuN+ neurons as well as a subset of astrocytes, and tended to colocalize with synaptic markers such as VGluT1/2 and PSD95, suggesting that C4 is presented on neurons near synapses.

Finally, Sekar and colleagues found that mice deficient in C4 exhibited reduced C3 immunostaining in the dLGN, as well as greater overlap between RGC inputs from the two eyes compared to WT littermate controls. These results suggest that in the C4 deficient mice, impaired synthesis and deposition of C3 resulted in deficits in synaptic pruning of the retinogeniculate system. These findings were consistent with prior work regarding C3,[2,3] as well as other studies of mice deficient in C1q (another upstream component of the complement cascade),[23] suggesting a convergent influence of these complement proteins on synaptic refinement.

This work from Sekar and colleagues[10] was the first investigation to show that the longstanding genetic association of schizophrenia with the MHC locus arises in part from *C4*. Increased copy numbers of *C4* (particularly *C4AL*) were associated with increased C4A protein levels as well as risk of schizophrenia. In addition, Sekar and colleagues found that human C4A protein localized to neuronal synapses, and mediated synapse elimination in the mouse retinogeniculate system during development. Taken together, these results provided an insight into the role of C4 protein in synaptic pruning in humans, and a relationship to schizophrenia pathology in the human brain.

Increased microglial synapse elimination in schizophrenia patient-derived models of synaptic pruning

The work by Sekar and colleagues,[10] while instrumental in establishing an association between the C4A protein and schizophrenia, did not specifically demonstrate increased microglial engulfment of synaptic material. Accordingly, Sellgren and colleagues[11] sought to examine whether microglial uptake of synaptic material was increased in induced microglial (iMG) cells derived from schizophrenia patients compared to matched healthy controls, and whether this relationship was modulated by C4 genetic risk variants.

Sellgren and colleagues used monocytes that were isolated from either male schizophrenia patients or age-matched male healthy controls. They then induced differentiation into iMG cells that were morphologically and transcriptomically similar to microglia. To model interactions between microglia and synapses, they next reprogrammed induced pluripotent stem cells (iPSCs) from dermal fibroblasts of both male schizophrenia patients and matched controls. From these iPSCs, Sellgren and colleagues were able to isolate synaptic nerve terminals (SYNs), which they validated through both structural and functional analyses.

Sellgren and colleagues first aimed to compare phagocytosis in schizophrenia-derived iMG cells and SYNs. Using a dye that fluoresces in post-phagocytic phagolysosome compartments, they observed increased fluorescence in schizophrenia derived cultures compared to control-derived cultures. In coculture experiments, the iPSC-derived neurons from patients with schizophrenia exhibited decreased spine density compared to those derived from controls.

In addition, schizophrenia-derived iMG cells displayed increased uptake of control-derived SYNs, indicating that microglial factors contributed to the increased uptake of synaptic structures. Finally, Sellgren and colleagues assessed the relationship between *C4* schizophrenia risk variants and C3 protein deposition on neurons and synapse engulfment, and found a significant positive correlation between *C4AL* copy number and C3 complement deposition, as well as a significant correlation between *C4AL* copy number and iMG synapse uptake in the schizophrenia-derived cultures. Overall, these results demonstrated that schizophrenia risk variants in the *C4* locus are associated with excessive complement deposition, which contributes to increased synaptic engulfment in iMG cells derived from patients with schizophrenia.

Increased C4 expression leads to PFC hypoconnectivity and reduced social interaction

More recently, the relationship between *C4* expression and development of specific circuits related to schizophrenia pathology has been an ongoing question. Based on prior work showing decreased gray matter volume and dendritic spine density in the medial prefrontal cortex (mPFC) in schizophrenia,[8,9] Comer and colleagues[12] aimed to investigate whether increased *C4* expression induced changes to L2/3 excitatory neurons in the mPFC, as well as changes to microglial synapse engulfment and social behavior.

Human C4 protein (hC4) is encoded by two genetic isotypes, *C4A* and *C4B*, whereas mouse C4 protein (mC4) is encoded by a single gene (*mC4*) that is similar to both *hC4* isotypes.[*24,25] To induce *C4* overexpression in L2/3 mPFC pyramidal neurons, Comer and colleagues used in utero electroporation in mice at embryonic day 16, using plasmids with the CAG promotor to co-express GFP and *mC4*. In addition to the controls and *mC4* condition, Comer and colleagues also electroporated *hC4A* for comparison. IUEs led to increased mC4 transcript and C4 protein at P21, but there was not a significant increase at P60 (Figure 2). Similarly, apical tuft dendritic spine density in L2/3 mPFC neurons was lower at P21 in *hC4A*- and *mC4*-overexpressing neurons compared to controls, but there was no difference in density at earlier or later timepoints (P7, P14, and P60, respectively). Increased expression of *mC4* led to an increased number of microglia colocalizing with GFP+ L2/3 neurons, as well as increased percentage of microglia positive for engulfment using an assay labeling PSD-95 (a postsynaptic protein) colocalized with iba-1 and CD68. Therefore, IUE causing overexpression of *mC4* led to increased synaptic loss in the mouse PFC.

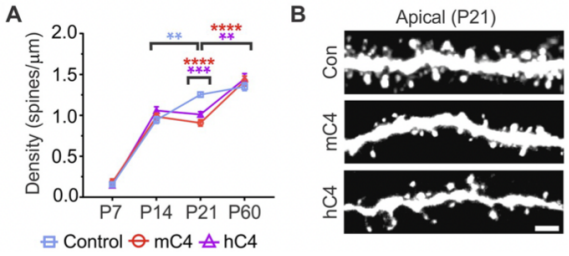

Figure 2. C4 overexpression causes dendritic spine alterations in L2/3 mPFC neurons. Comer et al., 2020.

Next, Comer and colleagues aimed to test whether overexpression of *mC4* in the frontal cortex led to impairment in early social behaviors. When exposing control and *mC4* mice at P18 to an arena containing nest bedding, an empty wire mesh cup, and a wire mesh cup containing the mother, *mC4*-overexpressing mice spent less time exploring the cup containing the mother relative to controls, suggesting lower interest in interacting with their mother. Similarly, when adult mice (P60-70) were placed in an arena with an empty wire mesh cup in one corner and a wire mesh cup containing a novel mouse in the opposite corner, *mC4*-overexpressing mice spent a lower

[*] **Note.** Because most of the studies discussed in this review refer only to the human *C4* gene, the notation "*C4*" can be assumed to refer to the human variant. For ease of interpretation, when describing studies that distinguish between human and mouse *C4*, the notation "*hC4*" and "*mC4*" will be used.

proportion of their time with the novel mouse compared to control mice. In separate tasks, mC4-overexpressing mice and control mice spent similar amounts of time interacting with nest bedding and novel objects, and spent similar amounts of time in the open arms of the elevated-zero maze, indicating that the observed differences in social behavior were not accompanied by differences in general sensorimotor abilities or anxiety-like behavior.

Overall, this study demonstrated that overexpression of either *mC4* or *hC4A* caused increased microglial phagocytosis of synaptic material leading to spine loss in the mouse mPFC. Additionally, increased expression of *mC4* in frontal L2/3 cortical neurons led to a reduction in social interactions in both juvenile and adult mice. This work was the first to show that C4-driven pathways are sufficient to cause both cellular and behavioral phenotypes consistent with prior observations in schizophrenia.[8] However, one limitation was the focus on *mC4* rather than *hC4* for the behavioral tasks.

Overexpression of human C4A promotes excessive synaptic loss and behavioral change

To specifically probe whether overexpression of *hC4A* could affect brain development and behavior, Yilmaz and colleagues[13] introduced *hC4A* or *hC4B* genes into the mouse genome using bacterial artificial chromosome DNA transgenesis, which allows human genes to be delivered along with their introns and cis-regulatory elements. Resulting mouse strains were backcrossed onto a *mC4*-deficient background to create *hC4A/-* and *hC4B/-* mice. In P5 dLGN sections stained for hC4 and VGluT2, *hC4A/-* mice displayed greater numbers of C4+ puncta colocalized with VGluT2+ synapses compared to *hC4B/-* mice (with *C4-/-* mice showing only background levels of staining). Similarly, when fluorescently tagged cholera toxin subunit b anterograde tracers were injected intravitreally to trace RGC projections, *hC4A/-* mice showed a lower percentage of overlapping territories compared to *C4-/-* littermates, and similar levels to WT controls, whereas *hC4B/-* mice had a similar percentage of overlap to *C4-/-* littermates, which was higher than controls. Based on these results, Yilmaz and colleagues concluded that *hC4A* was more specifically responsible for developmental synaptic refinement, and the rest of their investigation focused specifically on *hC4A*.

To create an overexpression model for *hC4A*, Yilmaz and colleagues backcrossed *hC4A/-* mice together to create an *hC4A/A* line with eight *hC4A* copies per strain (compared to 1-3 copies in typical humans). These *hC4A/A* mice displayed reduced overlapping territories in the dLGN compared with their *hC4A/-* littermates, and increased microglial engulfment of cholera toxin b-labeled RGC inputs at P5, indicating that the overexpression model resulted in increased synaptic pruning in the retinogeniculate system. Focusing on the PFC due to its relationship to schizophrenia, Yilmaz and colleagues measured C4 protein levels in *hC4A/A* mice and *hC4A/-* mice, at different timepoints from development to adulthood, and found that *hC4A/A* mice had significantly higher levels of C4 compared with *hC4A/-* mice at P10 (development) and P40 (adolescence), but not P60. Bulk-sequencing analysis found that the RNA profiles of microglia from *C4-/-*, *hC4B/-*, *hC4A/-*, and *hC4A/A* mice were unchanged compared to WT mice, indicating that the increased synapse engulfment was not related to a change in microglial transcription but rather to increased deposition of complement on synapses.

To test whether *hC4A*-overexpressing mice have decreased synaptic density in the mPFC, Yilmaz and colleagues stained for pre- and post-synaptic markers at P10, P40, and P60. *hC4A/A* mice did not exhibit a decrease in synapse density at P10 and P40 compared to the other mouse lines, but had decreased synaptic density at P60 (Figure 3a-c). Interestingly, though the authors hypothesized that the *hC4-/-* mice would exhibit increased synaptic density relative to WT mice,

synapse density did not differ between these two strains, suggesting that other mechanisms may be involved in the synaptic pruning process. Finally, to test whether the reduced synapse density in P60 *hC4A/A* mice persisted through later adulthood, Yilmaz and colleagues performed a dendritic spine density analysis in the mPFC at P180, and found that *hC4A/A* mice still exhibited lower dendritic spine density in L2/3 pyramidal neurons compared to *hC4A/-* mice (Figure 3d).

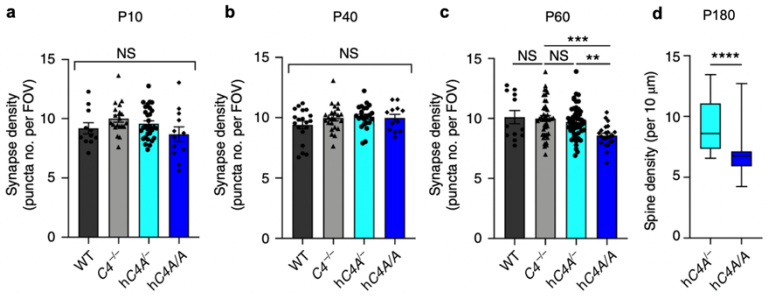

Figure 3. Adult hC4A-overexpressing mice have decreased mPFC synaptic density. Yilmaz et al., 2021.

Finally, Yilmaz and colleagues sought to test whether the *hC4A*-overexpressing mice showed behavioral alterations consistent with schizophrenia. In 10-12 week old mice, *C4-/-* and *hC4A/-* mice showed social tendencies similar to WT mice, whereas *hC4A/A* mice showed decreased preference toward interacting with a mouse compared to an object in the three-chamber social interactions test. *hC4A/A* mice also displayed more anxiety-like behavior compared with WT, *C4-/-* and *hC4A/-* mice in the open-field test. In addition, when exposed to the novel arm in the novel Y-maze, *hC4A/A* mice showed no preference toward the novel arm of the maze whereas WT, *C4-/-* and *hC4A/-* mice showed similar preference toward exploring the novel arm. These behavioral tasks displayed reduced social motivation, increased anxiety, and decreased interest in novel stimuli in the *hC4A*-overexpressing mice compared to all other mice, consistent with some of the behavioral and mood symptoms of schizophrenia.[5]

In this work, Yilmaz and colleagues successfully generated a mouse model that overexpressed the human isoforms of *hC4A* and *hC4B*. In addition, they showed that *hC4A* overexpression led to a greater degree of synaptic engulfment in the dLGN and mPFC, as well as abnormal mouse behaviors in social, emotional, and cognitive contexts. Notably, the normal synaptic density in mice without C4 (C4-/-) implied that complement might not be required for normal synaptic pruning, though increased deposition does lead to pathologically high amounts of pruning. Taken together, these findings support a model for schizophrenia in which increased complement deposition leads to altered synapse refinement in development and adolescence, causing downstream behavioral impairment.

Genetic co-expression networks link complement with synaptic pathology in schizophrenia

Within months of the work by Yilmaz and colleagues,[13] Kim and colleagues[14] uncovered a separate pathway by which *C4* risk variants might confer risk for schizophrenia. Their investigation aimed to assess the functional role of the complement system and *C4A* in the human brain, as well as the relationship between *C4A* and other parts of the complement system and other schizophrenia risk factors. Kim and colleagues used genetic and transcriptomic data from PsychENCODE[26,27] and the Genotype-Tissue Expression (GTEx) project[28] to create co-expression networks that represent covarying biological processes,[29] and investigated genes whose expression was either positively or negatively correlated with *C4A* expression.

First, Kim and colleagues aimed to test whether there was genetic evidence for an association between any of the 57 genes annotated within the complement system and schizophrenia. Of these 57 genes, six were within 1 Mb of genome-wide significant loci[19,30] and

expressed in the brain.[26] After accounting for genes within the same genomic region, and data from Hi-C interactions and Mendelian randomization analyses, Kim and colleagues concluded that there was a moderate amount of evidence for an association between up to four of the candidate genes within the complement system and schizophrenia. However, after using stratified linkage disequilibrium score regression[31] as well as a Multi-marker Analysis of GenoMic Annotation (MAGMA),[32] two methods of testing whether these associations are greater than expected by chance, they found no significant enrichment of single nucleotide polymorphism (SNP)-based heritability in schizophrenia, even after expanding annotations to also include all high-confidence protein-protein interactions for the complement system. This lack of enrichment was surprising given the known role of *C4A* in the complement system and the strong genetic association between *C4A* and schizophrenia, and implied that dysregulation of the complement system might not be the main pathway for schizophrenia pathogenesis.

Next, Kim and colleagues aimed to look at patterns of gene expression that covaried with expression of *C4A*. To examine these patterns, they used gene co-expression network analysis, which provides annotations based on correlated gene expression patterns across samples.[29] They started by "seeding" the network with *C4A* expression from frontal cortex samples of neurotypical controls in the PsychENCODE dataset.[26,27] Kim and colleagues generated a network consisting of 1,869 genes whose expression was positively correlated with *C4A* expression (*C4A*-positive), and 1,152 genes whose expression was negatively correlated with *C4A* expression (*C4A*-negative). Investigation of Gene Ontology terms showed that *C4A*-positive genes were most strongly enriched for "immune effector process" and "response to cytokine" whereas *C4A*-negative genes were most strongly enriched for "anterograde trans-synaptic signaling" and "chemical synaptic transmission" terms. *C4A*-negative genes, but not *C4A*-positive genes, were strongly enriched for SNP-based heritability in schizophrenia as well as other neuropsychiatric disorders. Taken together, these findings indicated a stronger statistical relationship between *C4A*-negative genes and schizophrenia rather than *C4A*-positive genes, implying a possible pathway in which increased *C4A* expression is related to lower expression of genes related to synaptic function, which then influence schizophrenia pathogenesis more directly.

Next, Kim and colleagues aimed to better understand the biological pathways represented by these *C4A*-seeded networks. Overlapping these gene sets with previously characterized brain co-expression modules[26] showed that *C4A*-positive genes were enriched for modules previously shown to represent astrocyte, microglial, and NF-κB signal pathways, whereas *C4A*-negative genes were enriched for neuronal and synaptic processes, including several glutamate receptors, calcium regulators, and potassium channels, among others. Using single cell and single nucleus RNA-seq data,[33] Kim and colleagues found that *C4A*-positive genes were strongly associated with astrocytes at low copy numbers, though with higher copy numbers they became more broadly associated with microglia and endothelial cells. *C4A*-negative genes were most highly expressed in cortical interneurons, hippocampal CA1 pyramidal neurons, somatosensory cortex pyramidal neurons, medium spiny neurons, and interneurons; all of these cell types are known to be enriched for schizophrenia GWAS signals.[34] In addition, *C4A* co-expression networks were stronger in males, stronger in frontal cortical brain regions, and accentuated by smoking; these relationships are consistent with existing knowledge related to schizophrenia epidemiology.[5]

Overall, this study was notable for its use of large genomic and transcriptomic datasets to examine associations with *C4A* expression in the human brain. Despite a large body of work relating the complement system to synaptic pruning and impaired social behavior,[12,13] Kim and

colleagues did not find a genetic association for schizophrenia within the complement system, instead finding enrichment among *C4A*-negative genes that are associated with neuronal, synaptic pathways. The authors suggested that the complement system may not be directly responsible for the pathophysiology of schizophrenia, and that instead research should focus on these downstream synaptic pathways that are downregulated with increasing *C4A* copy numbers.

Conclusions and Future Directions

Synaptic pruning is a complex phenomenon in which a subset of synapses are eliminated while another subset are maintained and strengthened.[1] Microglia play a key role in this phenomenon, responding to molecular "eat me" signals such as complement proteins that target synapses for phagocytosis.[35] Genetic associations between *C4A* genetic variants and schizophrenia[10] have uncovered a putative mechanism by which increased C4A protein expression can cause increased synaptic engulfment during key developmental timepoints,[11] leading to behavioral patterns similar to those seen in schizophrenia.[12,13] However, genomic co-expression network analysis has raised questions about associated pathways that may be downregulated in individuals with high *C4A* expression,[14] which could provide an alternate mechanism for the decreased dendritic spine density seen in schizophrenia.[8,9]

Investigating *C4A*-negative genetic pathways. The recent paper from Kim and colleagues[14] suggests that the role of *C4A* in schizophrenia pathogenesis is not specifically due to the complement pathway itself, but rather other synaptic proteins that are negatively correlated with increased *C4A* expression. Indeed, Schafer and colleagues[3] noted that the engulfed synaptic material visualized in microglia tended to lack mitochondria, and postulated that a lack of mitochondria could be one factor leading to complement deposition on synapses. In this way, it is possible that under-expression of synaptic genes implicated by Kim and colleagues[14] could lead to impaired synapse maturation, which then would be associated with increased complement deposition as these immature synapses are tagged for removal.

Addressing these relationships would require a specific experimental manipulation in which the key *C4A*-negative genes identified by Kim and colleagues are knocked out or otherwise manipulated and synapse maturation and engulfment are assessed. In addition, measuring expression of these synaptic genes in the brains of *C4A*-overexpressing mice, such as the *hC4A/A* strain used by Yilmaz and colleagues,[13] could provide insights into related pathways that are affected in these models. Overall, the work by Kim and colleagues[14] points to a need for more granular understanding of the synaptic pathways that lead to microglial engulfment.

Translation from mouse models to human phenotype. Mouse models of *C4A* overexpression are associated with behavioral symptoms mainly related to social motivation.[12,13] These phenotypes are similar to the negative symptoms associated with schizophrenia,[5] but other aspects of the translational validity of this model have yet to be tested. Experiments that show relationship to more of the positive symptoms of schizophrenia (e.g., hyperactivity), as well as "rescue" with treatments such as antipsychotic medications, would provide additional evidence for the relevance of the *C4A* overexpression model to schizophrenia in humans.[36]

Timing- and region-specific features of synaptic pruning. Research characterizing synaptic pruning has clearly illustrated the heterogeneity of this phenomenon in time and space, i.e., different brain networks undergo synaptic pruning at different times during development. For example, during adolescence in non-human primates, layer 3 cortical synapses undergo more extensive pruning than those in layers 5 and 6,[37] mirroring the observed decreased dendritic spine density in layer 3 but not layer 5 or 6 of the PFC in humans with schizophrenia.[8,9] The

studies that induced *C4A* overexpression in this review found somewhat conflicting results regarding timing of changes in synaptic density. Comer and colleagues,[12] who used in utero electroporation at embryonic day 16, found decreased synaptic density in L2/3 pyramidal cells from the mPFC of *mC4A*- and *hC4A*-overexpressing mice at P21 only. In contrast, Yilmaz and colleagues,[13] who used bacterial artificial chromosome DNA transgenesis on fertilized eggs, observed no difference in L2/3 mPFC synaptic density in *hC4A/A* mice compared to controls at P10 and P40, but decreased density at P60 and P180. Therefore, it remains to be seen how manipulations of C4A expression at varying timepoints throughout development might affect this process.

Despite these lingering questions, the complement system clearly plays a role in microglia-dependent engulfment of synaptic proteins. In addition, there is a clear association between C4 variants, synaptic loss, and schizophrenia, though the exact mechanism by which the C4 risk variant is associated with microglial synapse engulfment warrants further investigation. The relationship between synaptic pruning and neuropsychiatric diseases such as schizophrenia will surely continue to be a key area of research within both basic and clinical areas of neuroscience.

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