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COMPLEMENT MEDIATED SYNAPSE ELIMINATION IN SCHIZOPHRENIA

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Karolinska Institutet

Stockholm 2023

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8016-911-0
Cover illustration by **İlknur Şafak Demirel**: "In this illustration, you see the green cell in the head, microglia, pruning the neuron endings in a patient's brain. This is a normal process but too much of it can be one of the ways people develop schizophrenia. Around the head, colorful splashes symbolize the colorful chaos and symptoms like hallucinations that Schizophrenia patients experience in their daily life."

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Biomedicum (room Granit), Karolinska Institutet, Solna on the 17th of March 2023 at 10 am.

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To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

Schizophrenia is perceived as a disease of the mind: “schizo” means ‘splitting’ and “phren” means ‘mind’. It is a devastating disorder that affects approximately 20 million people worldwide. Schizophrenia is largely heritable, and a growing number of genetic risk factors has been identified. The available pharmacological treatment options are still unsatisfying in terms of effect and side-effects, which makes it difficult for people to adhere to the treatment plan. A major reason for this is the lack of basic understanding of the mechanisms that are causing schizophrenia and hence a lack of targeted drugs. In this thesis, we tried to answer a basic question: what is different in the development of the brain in an individual with schizophrenia as compared to in a healthy brain? In teenage years, excessive nerve connections are normally removed, and schizophrenia usually presents in late adolescence. Hence one of the main working hypotheses of what is causing schizophrenia is that something goes wrong during this removal of nerve connections as people with schizophrenia have fewer connections between the nerve cells.

Given the limited access to brain tissue from patients, we collected skin tissue and blood from individuals with schizophrenia and used cellular reprogramming to generate so-called ‘induced pluripotent stem cells’ that can differentiate into more or less any cell in the human body. We then developed a cellular model in a dish that made it possible to study how microglia, the main immune cells of the brain, ‘eat’ connections between nerve cells. In our models, we observed that when we used cells from individuals with schizophrenia more nerve connections were taken up by microglia. To understand in more details what was causing this, we generated models in which we combined nerve connections from individuals with schizophrenia and microglia from healthy control subjects, and then nerve connections from healthy controls with microglia from individuals with schizophrenia. This made us understand that the fault in removal of nerve connections is caused both by factors related to microglia and factors related to the nerve cells. One of the most strongly associated genetic risk factors for schizophrenia is repeats of a gene coding for complement factor 4A (*C4A*). In our models, we could now show that the protein coded for by this gene contributes to a more extensive uptake of nerve connections in microglia. These results are presented in **Study I**.

Zooming out, we decided to take a different approach in **Study II**. We developed a method to measure protein levels of C4A in spinal fluid of individuals who get hospitalised for the first

time with an episode of psychosis. Psychosis is when a person loses touch with reality and is usually characterised by hallucinations and delusions. Approximately half of the individuals that develop psychosis will later on be diagnosed with schizophrenia. The individuals that participated in this study had been follow-up over time and we therefore knew which ones that were to develop schizophrenia in later years. By measuring C4A levels in spinal fluid when the patients first got psychotic symptoms, we could show that C4A was specifically elevated in individuals that would go on to develop schizophrenia. Interestingly, the elevation of C4A protein in this group could not fully be explained by repeats of the *C4A* gene. Therefore, we performed additional experiments to show that interleukin (IL)-1beta and IL-6 (two signaling proteins in the immune system) can increase the expression of the *C4A* gene. In spinal fluid from the individuals with schizophrenia, higher C4A levels also correlated with higher levels of IL-1beta as well as lower levels of markers for nerve connections.

In **Study III** we focused on developing more advanced models to study how microglia remove nerve connections. Instead of using cell cultures that are grown in two dimensions, we generated three-dimensional models that more accurately capture processes in the developing human brain. In these so-called ‘mini-brains’ (or cerebral) organoids we could, by using advanced molecular techniques capture microglial ‘eating’ of nerve terminals in a model that also capture important other features of the early developing human brain. These models will facilitate future studies that aim to address this mechanism in more detail and hopefully generate new target treatment approaches for schizophrenia.

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LIST OF ABBREVIATIONS

AAVS1	Adeno-associated virus integration site 1
AIF1	Allograft inflammatory factor 1
C1q	Component 1q
C3	Complement component 3
C4	Complement component 4
C4A	Complement component 4A
C4B	Complement component 4B
CD68	Cluster of Differentiation 68
CD47	Cluster of Differentiation 47
CI	Confidence interval
CN	Copy numbers
CNS	Central nervous system
CR3	Complement component 3 receptor
CSF	Cerebrospinal fluid
CSF1R	Colony stimulating factor 1 receptor
CX3CR1	CX3C chemokine receptor 1
ddPCR	Droplet digital PCR
DIV	Days in vitro
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EB	Embryoid body
FBS	Fetal bovine serum
FEP	First episode psychosis
FEP-nSCZ	Patients with first episode psychosis who later do not develop schizophrenia
FEP-SCZ	Patients with first episode psychosis who later develop schizophrenia
FGA	First generation antipsychotic
GABA	Gamma-aminobutyric acid
GRIP	Gothenburg research and investigation on psychosis
GWAS	Genome wide association study
HC	Healthy control
HERV	Human endogenous retrovirus
IL-1beta	Interleukin 1 beta
IL-6	Interleukin 6
iPSCs	Induced pluripotent stem cells
iMG	Induced microglia-like cells
KaSP	Karolinska schizophrenia project
LAI	Long-acting injectable antipsychotics
LAPTM5	lysosomal-associated multispanning membrane protein 5
MACS	Magnetic-activated cell sorting

MHC	Major Histocompatibility complex
mRNA	Messenger RNA
NCAM	Neural Cell Adhesion Molecule
NGN2	Neurogenin 2
NIS	Neural induction supplement
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cells
P2RY6	G-protein coupled pyrimidinergic receptor 6
PAX-6	Paired box protein 6
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PET	Positron emission tomography
PMP	Pre-macrophage precursors
PSA-NCAM	Polysialic Acid Neural Cell Adhesion Molecule
PSD-95	Post synaptic density 95
PU.1	Transcription factor PU.1
SCZ	Schizophrenia
SGA	Second generation antipsychotics
Sirpα	Signal regulatory protein alpha
SMAD	Suppressor of Mothers against Decapentaplegic
SNAP-25	Synaptosomal-associated protein 25
SOX1	SRY-box transcription factor 1
SOX2	SRY-box transcription factor 2
SPP1	Secreted Phosphoprotein 1
SV2A	Synaptic vesicle glycoprotein 2A
SYN	Synaptosomes
TDP-43	Transactive response DNA binding protein of 43 kDa
TMEM 119	Transmembrane protein 119
TREM2	Triggering receptor expressed on myeloid cells 2
TYROBP	Tyrosine kinase binding protein
UDP	Uridine diphosphate
VEH	Vehicle treatment

SCIENTIFIC ABSTRACT

Schizophrenia (SCZ) is a devastating psychiatric disorder with a typically age of onset in late adolescence. The heritability is estimated to be in between 60-80% and large-scale genome-wide studies have revealed a prominent polygenic component to SCZ risk and identified more than three-hundred common risk variants. Despite a better understanding of which genetic risk variants that increases SCZ risk, it has been challenging to map out the pathophysiology of the disorder. This has stalled the development of target drugs and current treatment options display moderate efficacy and are prone to produce side-effects. SCZ is generally considered a neurodevelopmental disorder and it was proposed more than forty years ago that physiological removal of less active synapses in adolescence, i.e., synaptic pruning, is increased in SCZ and hereby causes the core symptoms of the disorder. This theory has then been supported by post-mortem brain tissue and imaging studies displaying decreased synapse density in SCZ. More recently, it was then shown that the most strongly associated risk loci can largely be explained by copy numbers of a gene coding for the complement factor 4A (*C4A*). As microglia prune synapses with the help of complement signalling, we therefore decided to use a recently developed human 2D *in vitro* assay to assess microglial uptake of synaptic structures in models based on cells from individuals with SCZ and healthy controls (**study I**). We observed excessive uptake of synaptic structures in SCZ models and by mixing synapses from healthy controls with microglia from SCZ patients, and vice versa, we showed the contribution of microglial and neuronal factors contributing to this excessive uptake of synaptic structures. We then developed an *in vitro* assay to study neuronal complement deposition dependent on copy numbers of *C4A* in the neuronal lines. Complement 3 (C3) deposition increased by *C4A* copy numbers but was independent of *C4B* copy numbers (also unrelated to SCZ risk). Similar *C4A* copy numbers correlated with the extent of microglial uptake of synapses. Microglial uptake of synaptic structures could also be inhibited by the tetracycline minocycline that also decreased risk of developing SCZ in an electronic health record cohort. In **study II**, we cerebrospinal fluid (CSF) from first-episode psychosis patients to measure protein levels of C4A. In two independent cohorts, we observed elevated C4A levels (although not C4B levels) in first-episode patients that later were to develop SCZ and could show correlations with markers of synapse density. However, elevated C4A levels could not fully be explained by more copy numbers of C4A in individuals with SCZ and *in vitro* experiments revealed that SCZ-associated cytokines can induce *C4A* mRNA expression while also correlating with C4A in

patient-derived CSF. In **study III**, we set-up a 3D brain organoid models to more fully comprehensively capture processes in the developing human brain and then also included innately developing microglia. We display synaptic pruning within these models and use single cell RNA sequencing to validate them.

In conclusion, this thesis uses patient-derived cellular modelling to uncover a disease mechanism in SCZ that link genetic risk variants with *bona fide* protein changes in living patients.

LIST OF SCIENTIFIC PAPERS

I. Carl M. Sellgren, **Jessica Gracias**, Bradley Watmuff, Jonathan D. Biag, Jessica M. Thanos, Paul B. Whittredge, Ting Fu, Kathleen Worringer, Hannah E. Brown, Jennifer Wang, Ajamete Kaykas, Rakesh Karmacharya, Carleton P. Goold, Steven D. Sheridan, Roy H. Perlis. Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. Nat Neuroscience 22, 374–385 (2019). https://doi.org/10.1038/s41593-018-0334-7

II. **Jessica Gracias**, Funda Orhan, Elin Hörbeck, Jessica Holmén-Larsson, Neda Khanlarkani, Susmita Malwade, Sravan K. Goparaju, Lilly Schwieler, İlknur Ş. Demirel, Ting Fu, Helena Fatourus-Bergman, Aurimantas Pelanis, Carleton P. Goold, Anneli Goulding, Kristina Annerbrink, Anniella Isgren, Timea Sparding, Martin Schalling, Viviana A. Carcamo Yañez, Jens C. Göpfert, Johanna Nilsson, Ann Brinkmalm, Kaj Blennow, Henrik Zetterberg, Göran Engberg, Fredrik Piehl, Steven D. Sheridan, Roy H. Perlis, Simon Cervenka, Sophie Erhardt, Mikael Landen, Carl M. Sellgren Cerebrospinal fluid concentration of complement component 4A is increased in first episode schizophrenia. Nat Communications 13, 6427 (2022). https://doi.org/10.1038/s41467-022-33797-6

III. **Jessica Gracias***, Susmita Malwade*, Asimenia Gkokga, Ana O. Oliveira, Marja Koskuvi, Roy H. Perlis, Jari Tiihonen, Jari Koistinaho, Samudyata, Carl M. Sellgren. High-resolution characterizations of innately developing microglia in human brain organoids. *Manuscript in preparation.* *These authors contributed equally.

INTRODUCTION

1.1 Schizophrenia

SCZ (in Greek, “schizo” means ‘splitting’ and “phren” means ‘mind’) is a largely heritable and chronic debilitating psychiatric disorder that affects close to 20 million individuals worldwide (James et al., 2018). Typically, the first symptoms occur in adolescence and results in a chronic course of illness with a lifelong disability. Compared to the general population, the lifespan of individuals suffering from SCZ is decreased by approximately 14.5 years (average), and the decreased life expectancy is largely due to suicide or somatic co-morbidities such as diabetes or cardiovascular complications (Hjorthøj et al., 2017). Additionally, the socioeconomic impact of SCZ is staggering considering the costs of healthcare as well as loss of productivity (Jin & Mosweu, 2017). In Sweden, the estimated annual cost of this disorder per individual in is estimated to be EUR 55,100 (Ekman et al., 2013).

1.2 Symptoms and clinical presentation

SCZ is characterised by three main clusters of symptoms: positive (disorganised thinking, hallucinations, and delusions), negative (social withdrawal, apathy, and deceased motivation for everyday tasks), as well as cognitive symptoms (e.g., memory and attention deficits, as well as impaired executive functioning). The negative and cognitive symptoms typically occur already in the prodromal phase, while the occurrence of stable positive psychotic symptoms confirms the diagnosis (Häfner et al., 2013; Häfner & An Der Heiden, 1999).
In a large-scale epidemiological study conducted over 25 years, a steep peak in SCZ incidence in men was noted at the age of 15-24 years, while in women there was a less pronounced peak in between 15-29 years followed by an additional peak at 45-49 years (menopausal age). Various studies in animal models (Gattaz et al., 1992; Y. C. Wu et al., 2013) have also suggested a protective role of estrogen that has been proposed as a therapeutical target in some severe cases (Crider & Pillai, 2017; Kulkarni et al., 2008). Nonetheless, from the perspective of common risk variants, sex-specific associations have been hard to identify (Bergen et al., 2014). Twin and sibling studies (Albus et al., 1994; Canuso & Pandina, 2007; Delisi, 1992; Ochoa et al., 2012) indicates that the strong genetic component overrides the potential protective effect of estrogen.

1.3. Treatments

Since the introduction of chlorpromazine in the 1950s, antipsychotics (also known as neuroleptics) have remained the mainstay in the pharmacological treatment of SCZ (Carlsson & Lindqvist, 1963; Tandon, 2011). To date, a large number of antipsychotics are available, and these drugs are typically classified into two major groups: first-generation antipsychotics (FGA, e.g., chlorpromazine, haloperidol, and perphenazine) and second-generation antipsychotics (SGA, e.g., clozapine, olanzapine, and risperidone). All antipsychotics to a variable degree have an antagonistic (or a partial agonistic) effect on the dopamine D2 receptor, and this pharmacological property is believed to be needed to generate the antipsychotic effect (which is then also the basis for the dopamine hypothesis of SCZ). In terms of efficacy, most of the available antipsychotics convincingly reduce the overall symptom burden more than placebo in randomized clinical trials (with mean effect sizes around 0.40 in more recent analyses) (Huhn et al., 2019; Lieberman et al., 2005), while observational studies suggest the use of an antipsychotic agent also decreases the risk of rehospitalization and decreased the all-cause mortality (Correll, Solmi, et al., 2022; Taipale, Mehtälä, et al., 2018; Taipale, Mittendorfer-Rutz, et al., 2018; Tiihonen et al., 2017). Given the still moderate efficacy of antipsychotics, it is noteworthy that patients in clinical trials then more commonly drop out due to inefficacy rather than adverse events (Huhn et al., 2019). Nonetheless, side-effects are still very common for all available antipsychotics and introduce further limitations. In comparison to efficacy, antipsychotics display more differences regarding the side-effect profiles making it an important factor for the clinician in selecting the most appropriate treatment for the individual patient. In general, FGAs are more associated with extrapyramidal side-effects and prolactin elevation (likely due to a higher D2 occupancy), while metabolic side-effects and sedation are more evenly distributed between FGAs and SGAs (Huhn et al., 2019).

Clozapine was introduced in the 1970s and is generally considered the prototypic SGA and gold-standard treatment in SCZ (Siskind et al., 2016; Stroup, 2019). In clinical trials, clozapine also typically ranks as one of the antipsychotics with best efficacy (Huhn et al., 2019; Lieberman et al., 2005; Siskind et al., 2016), a finding that is also mimicked by observational studies based on real-world data (Leucht & Tiihonen, 2021). Unfortunately, clozapine-induced agranulocytosis limits the use of this drug (Mijovic & MacCabe, 2020).

While this is a rare side-effect, the potential serious complications to this condition has led to recommendations that clozapine should only be used for treatment-resistant cases (usually defined as insufficient effect on at least two other antipsychotics) as well as in conjunction with stringent blood monitoring (Stroup, 2019).

Another factor to consider when administering an antipsychotic is which pharmaceutical form to use. Non-adherence to treatment is a major problem which has introduced long-acting injectable antipsychotics (LAIs) as an alternative to oral administration (available for both FGAs and SGAs). As discussed above, inefficacy as well as adverse events are important factors to consider in non-adherent patients, but it is also likely that disease related symptoms such as lack of motivation, cognitive impairments, and lack of insight contribute (Correll et al., 2016). Therefore, many guidelines recommend the use of LAIs at least in cases where it is suspected that non-adherence is caused by the introduction of such factors (Correll, Martin, et al., 2022). Observational studies then also support that the effectiveness of LAIs is comparable to clozapine (only available for oral administration) (Leucht & Tiihonen, 2021).

Along with the discovery that even in healthy individuals, SCZ-like symptoms can be induced by NMDA antagonists like ketamine and phencyclidine (Krystal et al., 1994), it has within the framework of 'glutamate hypothesis' of SCZ been suggested that glutamatergic acting drugs could further reduce the symptoms in SCZ when added to an antipsychotics. However, clinical trials so far have not been able to show this hypothetical additive effect in the positive symptoms and the improvement of negative symptoms is very modest (Tiihonen & Wahlbeck, 2006).

In addition to pharmacological treatments, psychosocial approaches have more recently also been introduced as part of the treatment for SCZ. Cognitive-behavioural therapy-based interventions (Jauhar et al., 2019), as well as psychoeducation (Xia et al., 2011), still hold limited evidence for efficacy but are typically included in updated treatment programs.

In conclusion, although strong evidence for efficacy of antipsychotics in SCZ, many patients at the best achieve partial remission of symptoms while the complicated act of balancing risks versus benefits further complicated the clinical use. Given the serendipitous discovery

of the antipsychotic effect of these drugs, this is hardly surprising as targeted treatment for obvious reasons heavily relies on identifying the underlying mechanisms of the disorder.

1.4 Etiology

1.4.1 Genetic risk factors for schizophrenia

SCZ is considered to be multifactorial in origin, with a large number of contributing genetic and environmental risk factors. Twin and family studies have indicated the heritability to be up to 80% (Hilker et al., 2018; Riecher-Rössler et al., 2018), and like many other common disorders, the genetic contribution can largely be explained by an outstanding polygenic component (Ripke et al., 2014a; Trubetskoy et al., 2022). Approximately one-third of the genetic liability to SCZ has been linked to more than 200 common risk variants (through GWAS) (Trubetskoy et al., 2022). In addition to common genetic variants, a growing number of rare genetic variants, then displaying large individual effect sizes, have also been shown to contribute to SCZ risk and include inherited as well as *de novo* copy number variants (Genovese et al., 2016; Marshall et al., 2017; Purcell et al., 2014; Singh et al., 2022). Recently, fine-mapping combined with transcriptomic analyses and functional genomic annotations have been utilized to prioritize common as well as rare genetic variants and connect genetic risk variants to plausible pathophysiology in SCZ (Singh et al., 2022; Trubetskoy et al., 2022). These studies have indicated convergences between rare variants and fine-mapped GWAS loci (Trubetskoy et al., 2022), and genes involved in synaptic transmission as well as immune-related pathways (Ripke et al., 2014a; Singh et al., 2022; Trubetskoy et al., 2022). Interestingly, many of the identified loci are also pleiotropic, i.e., overlap between the major psychiatric disorders, and display enrichment for genes regulating how synaptic connections are established in the developing brain (Lee et al., 2019; Smoller et al., 2013).

1.4.2 Environmental risk factors for schizophrenia

Several environmental risk factors have been linked to SCZ. Although many of these factors can be broadly defined as adverse life events (e.g., migration, child and adult abuse, social adversity), or pertain to substance abuse (Dean & Murray, 2005), they also include maternal infection during pregnancy and obstetric complications (Blomström et al., 2012; Brown et al., 2001, 2005; Limosin et al., 2003; Mortensen et al., 2007; Suvisaari et al., 2013; Yolken et al., 2011). Regarding infectious risk factors, and as discussed below, it is then often difficult to disentangle if transfer to the fetus, or maternal inflammatory responses, is the main culprit.

Further, several gene-by-environment interaction have been described in SCZ, including infectious agents (genetic risk variants within the complement factor (*C4*) locus and toxoplasma gondii or cytomegalovirus infection (Severance et al., 2021)) as well as polygenic risk scores for SCZ and cannabis use (Malone et al., 2010; Wainberg et al., 2021).

1.5 Schizophrenia as a neurodevelopmental disorder

1.5.1 The neurodevelopmental theory

As discussed above, many of the recently identified genetic and environmental risk factors pertain to the fetal or postnatal period. The idea of a neurodevelopmental origin of SCZ was however introduced more than forty years ago. In 1982, Irwin Feinberg conceptualized SCZ as a product of excessive elimination of synapses during brain development (Feinberg, 1982). Feinberg argued that a programmed decrease in synapse density occurs in late adolescence and that "an error or defect in this process gives rise to schizophrenia", although noting that "the idea of searching for quantitative abnormalities of synaptic arrangements in a brain with ten billion neurons, each capable of several hundred thousand synaptic connections, is unlikely to inspire even the most obsessive neuroanatomist". To a large extent, Feinberg's hypothesis was inspired by the recently discovered principal of systematic reduction of neural redundancy observed in early neurodevelopment (Hahn et al., 1983), and the fact that Peter Huttenlocher had reported that synapse density sharply decrease in late adolescence as a function of age (Huttenlocher et al., 1982; Peter R., 1979). A few years later, more systematic imaging, postmortem, and epidemiological studies had been performed in SCZ and it was suggested on a more general level by Daniel R. Weinberger that SCZ is a consequence of "fixed structural defects that occurs long before the diagnosis is made" (Weinberger, 1987).

In the following three decades, the evidence supporting a neurodevelopmental origin of SCZ grew stronger. Large-scale epidemiological studies revealed associations between maternal immune activation (either by stress, infection with herpes simplex virus 1, influenza, etc. as discussed above) and developing SCZ (Brown et al., 2004, 2005; Mortensen et al., 2007; Yolken et al., 2011), especially then if exposure occured in the first trimester (Brown et al., 2004). In parallel, pre-clinical studies in animal models added further support reporting that offspring of pregnant mice exposed to human influenza developed neuropathological deficits in corticogenesis, decrease in GABA markers and decrease in hippocampal volume (Fatemi et al., 1999), as well as behavioral deficits, especially a decrease in pre-pulse inhibition of

startle response (Shi et al., 2003). Interestingly, some of the behavioral abnormalities later in life could be rescued by treatment with clozapine or chlorpromazine (Shi et al., 2003). Post-mortem brain tissue obtained from individuals with SCZ has also shown a marked decrease in dendritic spine density (especially in the prefrontal cortex) (Garey et al., 1998; Glantz & Lewis, 2000; Kolluri et al., 2005), while advances in imaging techniques allowed for detection of morphological changes in alive SCZ patients with reproducible functional (fMRI) and structural (measured by MRI, DTI, PET etc.) data (Schmitt et al., 2011)). Further, longitudinal imaging studies indicated that the decrease in cortical thickness early in the disorder, more specifically during the transition to psychosis (Borgwardt et al., 2008; Cannon et al., 2015; Meyer-Lindenberg et al., 2005; Pantelis et al., 2003; Sun et al., 2009; T. Takahashi et al., 2009; Ziermans et al., 2012). With the recent introduction of PET-ligands targeting a synapse protein (SV2A), data from alive SCZ patients has also been generated and reveal a decrease *in vivo* synapse density (Onwordi et al., 2020). In summary, this accumulated data through the past decades has then consolidated the notion a neurodevelopmental origin of SCZ while supporting the original mechanistic hypothesis proposed by Irwin Feinberg.

1.5.2 Microglia in neurodevelopment

Microglia are tissue macrophages residing in the brain parenchyma. Early in neurodevelopment and before vascularization, the first wave of primitive macrophages from the yolk sac (at about embryonic day 7.5 in mice) enter the developing central nervous system (CNS) and give rise to the microglia population in a *Myb*-independent manner (Ginhoux et al., 2010; Ginhoux & Prinz, 2015; Gomez Perdiguero et al., 2015; Palis et al., 1999; Schulz et al., 2012). In the human adult brain, microglia are then unevenly distributed and local density can vary from less than a percentage to more than 15% (Mittelbronn et al., 2001). Microglia are highly receptive to their immediate surroundings and possess motile pseudopodia that can actively scan the extracellular space (Haynes et al., 2006; Raivich, 2005). Microglia adopt a wide range of transient or more long-lasting functional states depending on the presented local extracellular cues as well as the given developmental phase and region. Recent single-cell characterizations have revealed that this context-dependent heterogeneity of microglia goes far beyond the simplified M1/M2 classification system of macrophages (M1 being the pro-inflammatory or activated state and M2 being the anti-inflammatory or resting state) (Hammond et al., 2019; Ransohoff, 2016). In line with the plethora of functional microglial states, a constantly increasing number of housekeeping

functions have been added to the more classical ‘first defense’ functions of microglia (infections, injuries, or degeneration). To a large extent, such housekeeping functions pertain to the developmental phase and include key events such as synaptogenesis and developmentally primed activity-dependent synapse remodeling (Li & Barres, 2018; Stephan et al., 2012; M. È. Tremblay et al., 2011). As a consequence, elimination of microglia, or functional disruption through genetic or pharmacological methods, significantly increases synapse numbers and leads to aberrations in behavior among adult animals (Filipello et al., 2018; Ji et al., 2013; Nelson & Lenz, 2017; Paolicelli et al., 2011; Parkhurst et al., 2013; Schafer et al., 2012; Zhan et al., 2014). *In vivo* imaging studies in rodents have also revealed a direct communication between neurons and microglia in which microglia can sense and respond to the activity of neurons (Akiyoshi et al., 2018; Li & Barres, 2018).

While the exact molecular and cellular events that define the interaction between neurons and microglia are still largely unknown, several receptors and signaling pathways have been indicated, both on the neuronal side as well as on the microglial side. These include microglial receptors involved in mediating phagocytosis of synaptic structures, e.g., the triggering receptor expressed on myeloid cells 2 (TREM2), CX3C chemokine receptor 1 (CX3CR1), the G-protein coupled pyrimidinergic receptor 6 (P2RY6), and the complement component 3 receptor (CR3). *TREM2* knockout mice display a significant decrease in microglial activation and number, as well as a decreased synaptic uptake in microglia with increased number of dendritic spines (Filipello et al., 2018). “Spared” synapses are then also more often unstable or inefficient in these mice while defects in repetitive and social behaviors are more common (Filipello et al., 2018), hereby indicating that physiological synaptic pruning is impaired. CX3CR1 responds to signals from the neuron-expressed chemokine fractalkine, and although the exact mechanism of this interaction remains largely elusive, it has been shown in mice that genetic removal of fractalkine increases the number of dendritic spines (Hoshiko et al., 2012; Paolicelli et al., 2011). The P2RY6 receptor on the other hand is activated by UDP released by neurons and acts as an ‘eat me’ signal. In animal models, P2RY6 has been shown to increase phagocytosis by microglia (Koizumi et al., 2007). CR3 recognizes complement C3b (or cleaved iC3b), a product of the classical complement pathway (Schartz & Tenner, 2020). This pathway is initiated by the sensory molecule C1q. Binding of C1q to one of its targets (e.g., a pathogen) then activates the C1 complex that promotes the cleavage of C4 (either the C4A or C4B isotype) into C4a (anaphylatoxin) and C4b (opsonin), as well as C2 into C2a and C2b. C4bC2a then forms a C3

convertase that cleaves C3 into C3a (anaphylatoxin) and C3b (opsonin). C3b binds to C4bC2a to form a C5 convertase that cleaves C5 to then produce the anaphylatoxin C5a as well as C5b that initiates the assembly of the membrane attack complex). While initially identified as part of the innate immune system, this part of the complement system is now also recognized as a signalling cascade that facilitate microglial removal of synapses (Stephan et al., 2012).

It is hypothesized that to balance neuronal 'eat me' signals, the so-called 'don't eat me' signals protect synapses from microglial removal by strengthening functional synaptic connections (Elward & Gasque, 2003). In 2018, a study by Lehrman and colleagues described CD47 (on neurons), as well as SIRPα, as protective signals in the visual thalamus at the peak of pruning, and removing both these proteins increased microglia mediated pruning and as a consequence loss of synapses in the dorsal lateral geniculate nucleus (Lehrman et al., 2018). Whether this is specific to the visual system or applicable more broadly, remains to be investigated. In addition, Paolicelli et al. also found that TDP-43, a transcription factor expressed in microglia, regulates phagocytosis in microglia as well as neuronal synapse numbers (Paolicelli et al., 2017).

Why are some developing synapses protected from microglial phagocytosis while other synapses are removed? To some extent, the answer may lie in activity. Several decades of pharmacological, genetic and electrophysiological approaches have proven that uneven activity in neurons is required for circuit refinement and changing the activity levels of certain neurons cause a shift in loss of "weaker" neurons in the peripheral nervous system (Balice-Gordon & Lichtman, 1994; Barber & Lichtman, 1999; Buffelli et al., 2003; Busetto et al., 2000; O'Brien et al., 1978; Thompson et al., 1979) as well as in the CNS (Butts et al., 2007; Penn et al., 1998; Shatz & Stryker, 1988; Sretavan & Kruger, 1998; Stellwagen & Shatz, 2002). Elegant experiments using the rodent visual system have revealed that if one eye is blocked from receiving light inputs, there is increased loss of synapses in the dorsal lateral geniculate nucleus, and further, if mice are bred in the dark, there is increased microglial activity and phagocytosis of weak neurons (M. Ě. Tremblay et al., 2010).

1.5.3 Complement-dependent synaptic pruning in schizophrenia

In 2016, Sekar et al. (Sekar et al., 2016) performed a fine-mapping study of the most strongly associated GWAS locus, located in the extended Major Histocompatibility Complex (MHC) region on chromosome 6. This locus contains multiple copies of two closely related genes that codes for variants of C4: *C4A* and *C4B*. While the C4A and C4B proteins are more than 99% similar on amino-acid level, they however display distinct molecular affinity (Isenman & Young, 1984; Law et al., 1984). Segregated by the insertion of a human endogenous retroviral (*HERV*) in intron 9, both genes can also exist in a long or short version then creating a complex genetic variation (Blanchong et al., 2001). Using ddPCR to define copy numbers of *C4A* and *C4B* (including the *HERV* status), Sekar et al. could (with acceptable accuracy) impute *C4A* and *C4B* copy numbers in a large GWAS cohort of individuals with SCZ and healthy controls. Their analyses revealed that *C4A* copy numbers, as well as other structural variance leading to increased *C4A* mRNA expression, to a large degree explained SCZ risk originating from this locus. However, and intriguingly, SCZ risk was not influenced by copy numbers of the closely related *C4B* gene. *C4A* mRNA expression was then also increased in post-mortem brain tissue obtained from 35 schizophrenia patients as compared to in brain tissue from 70 controls. While rodents lack specified *C4* genes, the authors evaluated a possible effect of C4 on synaptic pruning using a *C4* knockout mice and observed decreased microglial engulfment of presynaptic inputs in the developing mouse retinogeniculate system. This finding very much mimicked what have been observed for other complements in the classical pathway (C1q and C3) as well as C3R (Wilton et al., 2019), then providing a possible link between SCZ risk variants and excessive complement-dependent microglial synapse pruning in SCZ.

1.6 Schizophrenia risk in a human model system

Current knowledge of the underlying molecular mechanisms of SCZ is mostly based on experimental animal models or observational human studies utilizing post-mortem brain tissue or neuroimaging. Animal studies have a great advantage in that they easily can be genetically and pharmacologically modified to allow mechanistic studies under very tightly controlled conditions. However, and even with the development of precise gene editing, animal models becomes severely limited in SCZ where common variance typically contributes to risk in a non-deterministic fashion and needs to be studied in the context of other human risk alleles (Nestler & Hyman, 2010). In addition, some risk genes may not be

expressed or may not be as complex in animals as compared to humans. For example, and as described above, rodents lack the schizophrenia risk gene *C4A*. The ability of mouse models to capture the complex phenotypes observed in patients are therefore questioned (Nestler & Hyman, 2010). Similar, post-mortem samples are limited as they cannot be manipulated, there is a high variability in the quality of the sample (depending on fixation methods, storage conditions, etc.), and it is difficult to dissect whether the observations are an effect of comorbidities and attempts to treat the disease, all of which interfere with interpretation of the results (Mistry & Pavlidis, 2010). Post-mortem samples also have the inherent flaw of being the result of lifelong illness, and hence, these studies miss the neurodevelopment aspect of SCZ.

In 2006, a new window to study human development *in vitro* opened up when Yamanaka and Takahashi reprogrammed somatic mouse cells (fibroblasts) into induced pluripotent stem cells (iPSCs) (K. Takahashi & Yamanaka, 2006). Soon, many groups successfully applied this technology to convert e.g., human fibroblasts (collected through skin biopsies) into human induced pluripotent stem cells (hiPSCs) (Lowry et al., 2008; K. Takahashi et al., 2007; Yu et al., 2007) that have the potential to differentiate into different cell types given the right cues and growth conditions. Protocols have then rapidly developed from 2D differentiation to 3D culture and cover a large number of somatic cell types.

With limited access to diseased tissue in SCZ, and the limited value of genetically engineered mouse lines as disease models, the ability to reprogram, or transdifferentiate, easily accessible human somatic cells into neurons and glia cells has in recent years gained increased attention in the context of SCZ disease modeling. Therefore, iPSC-based models has rapidly become an important complement to animal models in studying human neurodevelopment and neurodevelopmental disorders (Amin & Pașca, 2018). However, it is important to also acknowledge the limitations of these models, including the influence of *in vitro* conditions *per se* on cellular states as well as a general high line-to-line variability (Carcamo-Orive et al., 2017; DeBoever et al., 2017; Kilpinen et al., 2017; Strano et al., 2020; Youhanna et al., 2022a). Despite these limitations, iPSCs-based model system has only grown in popularity as a platform to model disease and is becoming widely used in all areas of research, especially when the target cell type is inaccessible or hard to obtain.

While the protocol for generating different types of neurons from iPSCs quickly became prioritized in neuroscience, the first 2D protocols for generating microglia-like cells from iPSCs, or through trans-differentiation, became available first in 2017 (Sellgren et al., 2017) (Abud et al., 2017; Haenseler et al., 2017; Pandya et al., 2017). These protocols differed largely in regard to use of reprogramming or trans-differentiation as the initiating step and with reference to total days in culture and external patterning factors. Nonetheless, transcriptomically, they produce rather similar cells. As noted elsewhere, all protocols also propose serum-free conditions with the addition of interleukin (IL)-34 (Hammond & Stevens, 2016).

Organotypic 3D brain culture models aim to more precisely capture the complex procedures that define the functions of the developing brain tissue (Pas, 2018; Youhanna et al., 2022a). By limiting interactions between the cells and the plastic surfaces, such models are thought to strengthen interactions between cells as well as between cells and the extracellular matrix, while also providing opportunities to study cellular interactions long-term. Such models (commonly referred to as 'cerebral' or 'brain organoids') can either be optimized regarding culture conditions to support intrinsic cues for neuroectoderm formation (undirected approaches) or more strongly focus on external inductive signals (directed approaches) that instruct the cells towards an neuroectodermal faith (Youhanna et al., 2022a).

In 2013, the Knoblich laboratory generated the first cerebral organoid model by maintaining neuroectoderm of embryoid bodies in 3D culture and embed them in an extracellular matrix (Matrigel) followed by culture in bioreactors (Lancaster et al., 2013; Lancaster & Knoblich, 2014). These 3D cultures, usually referred to as undirected whole-brain organoids, already after 20-30 days displayed a cellular diversity and architecture that in many aspects recapitulates the structure of the developing human brain. Later single cell transcriptomic analyses have then confirmed a mixture of region specific ectodermal derived cells as well as a significant number of mesodermal cell types that are not present in the *in vivo* brain (Samudyata et al., 2022; Uzquiano et al., 2022). More recently, Mansour et al grafted undirected human cerebral organoids into living mouse brains with functional synaptic connectivity and vascularization (Mansour et al., 2018). Because of a large variability between organoids, including the extent of the mesodermal component, several modifications have more recently been proposed to reduce the degree of stochasticity and strengthen the neuronal induction in these models (Camp et al., 2015; Quadrato et al., 2017). Instead, newer

studies were able to increase the percentage of mesodermal progenitors (which leads to generation of microglia-like cells within these organoids) by lowering the concentration of heparin (a neuroectoderm stimulator) and also delaying the matrigel embedding step (Ormel et al., 2018; Samudyata et al., 2022).

Directed approaches instead focus more on the addition of external patterning factors and can also include more region-specific neural cell types (Eiraku et al., 2008; Kadoshima et al., 2013). Region specific organoids can be obtained by a vast number of new protocols to either obtain patterned organoids of the cortex, midbrain, cerebellum pituitary gland or hypothalamus (Youhanna et al., 2022b). Fusion of region-specific organoids, often referred to as ‘assembloids’, have also been introduced to enable modeling of functional connectivity (Birey et al., 2017).

RESEARCH AIMS

The overall aims of this thesis were to 1) investigate if complement-dependent microglial synaptic pruning is increased in a human model system based on cells from individuals with SCZ, 2) if a potential increase in synaptic pruning, then can be linked to the SCZ risk gene *C4A*, and 3) if individuals with SCZ display elevated levels of C4A in CSF.

Specific aims:

1. Use 2D human cellular modelling to quantify microglial uptake of synaptic structures in models based on cells from individuals with SCZ and healthy controls, B) investigating how copy numbers of *C4A* (versus *C4B*) influence complement deposition on synapses and microglial uptake of synaptic structures.
2. Use targeted mass spectrometry to measure C4A and C4B levels in CSF obtained from first episode psychosis patients and healthy controls.
3. Generate and characterize brain organoids with innately developing microglia.

MATERIALS AND METHODS

3.1 Study I

3.1.1 Induced microglia-like cells

In brief, whole blood was centrifuged in tubes containing Ficoll (1500xg for 20 minutes with the brake off). The peripheral blood mononuclear cell (PBMCs) layer was then pipetted off, and after addition of phosphate buffered saline (PBS) the samples were centrifuged at 300xg for 10 minutes. Cells were frozen down in cryovials with fetal bovine serum (FBS) containing 10% Dimethyl sulfoxide (DMSO). Thawed PBMCs were diluted with RPMI-1640 medium containing 10% FBS and centrifuged at 300xg (5 minutes with break off). 24 well plates precoated with Geltrex were used to plate the cells at a density of $1x10^6$ cells per well. After twenty-four hours in culture, medium was replaced with RPMI-1640 supplemented with Glutamax, IL-34, and GM-CSF. Assays were performed after 14 days in culture

3.1.2 Generation of iPSCs

Skin biopsies were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS for about a week. We passaged the cells three times and then cryopreserved with 10% DMSO in DMEM. All fibroblast lines were tested for Mycoplasma and then reprogrammed under xeno-free conditions using mRNA reprogramming as previously described (Warren et al., 2010).

3.1.3 Generating neuronal cultures

For generating neuronal cultures in which we isolated synaptosomes (SYN), a ‘stochastic’ approach was used. In brief, differentiation was initiated with neurobasal medium (NBM) supplemented with 1× NIS. Confluent cells were then passaged with Accutase (100 ml; and purified using Magnetic-Activated Cell Sorting (MACS) and Anti-PSA-NCAM MicroBeads. We then used a neural expansion medium combined with advanced DMEM/F-12 with 1× NIS) for further expansion. Putative neural progenitor cells (NPCs) by MACS ($CD271^-$ and $CD133^+$). We confirmed that the cells stained positive for nestin, the transcription factors SOX1 and SOX2, as well as paired box protein (Pax-6). Cells were then plated at a seeding density of 40,000 cells per cm^2 on poly-l-ornithine/laminin-coated 6-well tissue culture plates in neuronal differentiation medium supplemented with N-2 Supplement and B-27 supplement. Twenty-four hours before we collected the neural cultures, the medium was then changed to astrocyte-conditioned medium.

For the neural cultures we used in the co-culture experiments, we instead generated inducible *neurogenin 2* (*NGN2*) expressing NPC lines using TALEN-based plasmids as described in the publication (Sellgren et al., 2019). Briefly, a doxycycline-inducible *NGN2* construct was inserted into the adeno-associated virus integration site 1 (AAVS1) safe-harbor locus. For each transfection, cells were pelleted at 300*g* and then resuspended in 100 µl prewarmed nucleofector solution with *NGN2* plasmid and 1R and 1L plasmids were added directly to the resuspended cells, or negative control without plasmid, followed by nucleofection. After nucleofection, we plated the cells onto Geltrex-coated 6-well plates in neural expansion medium. Stable lines were then expanded by selection with the addition of G418.

For the cortical excitatory neurons used in the complement deposition assay, we passaged iPSCs in TeSR-E8 medium on vitronectin recombinant human protein and then induced differentiation by using a standard dual SMAD inhibition protocol with some slight modifications as described in the publication (Sellgren et al., 2019).

3.1.4 Generating labeled synaptosomes

SYNs were isolated using a synaptic protein extraction reagent (Syn-PER) according to the manufacturer's instructions (Sellgren et al., 2017). To confirm the purity of the freeze thawed SYNs, we then performed several validation steps. First, we used immunocytochemistry and Western blot to check for pre- and postsynaptic markers. SYNs were then also assessed by transmission electron microscopy, as well analyzed for synaptic vesicle endocytosis as previously described (Daniel et al., 2012). After quality control, the resulting SYNs were labelled with a pH-sensitive dye (pHrodo Red), indicating uptake of SYNs in the acidic phagolysosome of microglia-like cells, for real-time live imaging experiments. In the fixed experiments, we instead labelled SYNs with the fixable pH-sensitive dye CypHer5E NHS Ester.

3.1.5 Quantifying the uptake of synaptosomes in microglia-like cells

These experiments were performed using an IncuCyte ZOOM live imaging system as previously described (Sellgren et al., 2017). Briefly, sonicated and pHrodo-labelled SYNs were added to induced microglia-like cells and imaged. For the minocycline experiments, the

cells were pre-treated with either 1 μM, 10 μM, 60 μM, or vehicle (VEH) for 30 min before imaging. Phagocytic indexes were generated based on the total amount of phagocytized particles divided by number of cells.

3.1.6 Quantifying dendritic spines in co-cultures

Induced microglia-like cells were collected and pelleted. 1,000 cells were then added to each neuronal culture. After 48 h, co-cultures were washed and fixed followed by staining with Alexa Fluor 488 phalloidin. The IN-Cell Analyzer 6000 was used for imaging. 10 μm sections of phalloidin-stained dendritic spines were identified on randomly selected images and counted manually (blinded).

3.1.7 Quantifying *C4* copy numbers

To quantify copy numbers of *C4A, C4B, C4L*, and *C4S* we used ddPCR on a QX200 Droplet Digital PCR System with the specific and reference primers and probes as previously described (Y. L. Wu et al., 2007). We then imputed *C4AL*, *C4AS*, *C4BL*, and *C4BS* copy numbers based on the assumption that *C4A* copy numbers + *C4B* copy numbers = *C4L* copy numbers + *C4S* copy numbers, and that all *C4L* were *C4A* copy numbers as long as *C4L* copy numbers were less or equal to *C4A* copy numbers (given the rarity of *C4AS* copy numbers).

3.1.8 Assessing neuronal complement deposition

To determine the effect of structural variation within the *C4* locus on neuronal complement deposition we treated neuronal cultures (from haplotyped individuals with schizophrenia) with an IgM anti-NCAM antibody to sensitize complement activation. We then supplemented the cultures with C1, C2, and C3 to specifically test how the neuronal synthesis of C4A and C4B influenced complement deposition. C3 (representing complement deposition) and NCAM were then visualized using immunocytochemistry on an IN-Cell Analyzer 6000.

3.1.9 The effect on minocycline exposure on risk for developing schizophrenia

Here we used de-identified data from electronic health records to identify individuals that during adolescence had been exposed for minocycline treatment (at least 90 days) due to Acne Vulgaris. To increase power, we also included individuals that had been exposed for the chemically (and functionally) similar tetracycline doxycycline. Non-exposed individuals had been prescribed erythromycin, clarithromycin, or trimethoprim/sulfa-methoxazole on the same indication. In the primary analysis, we used survival methodology (via a Kaplan–Meier

log-rank test) with results stratified by age at index antibiotic, sex, self-reported ethnicity, and calendar year of study entry. Hazard ratios were also obtained using Cox regression adjusted for these features.

3.2 Study II

3.2.1 Cohorts

In this study we recruited individuals with first-episode psychosis (FEP) from two similar longitudinal FEP studies: Karolinska Schizophrenia Project (KaSP) and the Gothenburg research and investigation on psychosis (GRIP) project.

In brief, KaSP recruits patients from all the major psychiatric clinics in Stockholm (Sweden). Included patients are antipsychotic naïve or have been treated with an antipsychotic agent for less than 30 days. Patients with a severe somatic disease deemed to influence brain function, current substance abuse, or autism spectrum disorder, are excluded. Collected clinical data (here we used baseline data and data collected from a 1.5-year follow-up) is based on a semi-structured interview performed by a research physician and complemented by targeted rating scales. Healthy matched controls (age and sex) are recruited by advertisement and and evaluated using The Mini International Neuropsychiatric Interview (MINI) to exclude previous or current psychiatric illness. Individuals with current use of illegal drugs, first-degree relatives with psychotic illness or bipolar disorder, or with a neurologic disease or other severe somatic disease are excluded.

GRIP recruits patients at the psychosis clinic at Sahlgrenska University Hospital (Mölndal, Sweden). The initial diagnostic assessments are performed by treating clinicians and to secure the diagnosis for research case conferences are held on which a minimum of two board-certified psychiatrists need to participate. As no matched healthy controls are recruited in this project, we here used healthy controls collected within the larger “St. Göran projektet”. These controls are randomly selected by Statistics Sweden and undergo the same clinical investigations as the patients.

3.2.3 Lumbar punctures

Lumbar punctures were performed in the morning after a night of rest (and fasting). A non-cutting (atraumatic) spinal needle was used. Not more than 1 h after collection, CSF was centrifuged at 3500 rpm for 10 min, then divided into aliquots and stored at –80 °C until analysis. All CSF samples were individually analyzed.

3.2.4 Targeted mass spectrometry (C4A and C4B)

The protein concentration in the CSF samples was measured in triplicates using the Micro BCA Protein Assay Kit. The measurements were performed in a 96-well plate and fluorescence read by plate reader FLUOstar Omega. Sample preparation was performed on the Agilent AssayMAP Bravo Platform and details can be found in the publication (Gracias et al., 2021). The LC-MS analysis was performed on a Tribrid mass spectrometer Fusion equipped with a Nanospray Flex ion source, coupled to an EASY-nLC 1000 ultra-high pressure liquid chromatography (UHPLC) system. Tryptic peptides spiked with AQUA peptides of each trypsin-digested CSF sample was injected. We ran pooled CSF samples throughout all the samples as quality control. The linear range of each heavy peptide was measured in pooled CSF digest. A mixture of heavy AQUA peptides was spiked at different concentrations into pooled CSF samples. Each sample was analyzed three times and the peak areas of heavy peptides were plotted against the spiked heavy peptides concentrations.

3.2.5 Targeted mass spectrometry (neuronal pentraxins)

Preparation of samples and LC-MS/MS analyses were performed as previously described using micro-high-performance liquid chromatography-mass-spectrometry (6495 Triple Quadrupole LC/MS system, Agilent Technologies) (Nilsson et al., 2021).

3.2.6 CSF cytokine analyses

IL-1beta and IL-6 had previously measured as part of the Novex Human Ultrasensitive Cytokine 10-Plex Panel on the Luminex immunoassay platform. The measurement was performed on a FM3D instrument. Standard curves were generated using weighted ($1/y^2$) 5-parameter logistics and the cytokine concentrations in the samples were calculated based on the standard curves. In this study, we initially only analyzed IL-1beta and IL-6 concentrations but based upon request from one of the reviewers, data on all cytokines were then extracted from the database for post-hoc analyses.

3.2.7 Molecular analyses of *C4* structural elements

Within the GRIP cohort, C4 structural elements were quantified by ddPCR as described in 3.1.7. Within KaSP, copy numbers of *C4* structural elements were imputed from MHC genotypes computed from whole genome sequencing data as suggested by Sekar et al. (Sekar et al., 2016).

3.2.8 iPSC generation and neuronal differentiation

iPSCs were generated as described in 3.1.2, and neuronal cultures were derived through *NGN2* expressing NPCs as described in 3.1.3.

3.2.9 Cytokine stimulation experiments

For these experiments we used cultures with mature *NGN2* neurons. After exposure IL-1β, IL-6, or the combination, we extracted total RNA. The quantity and purity of total RNA was measured using a NanoDrop® 1000 spectrophotometer and complementary DNA was synthesized. qPCR was performed with TaqMan Gene Expression Assays using the StepOnePlus Real-time PCR system (Applied Biosystems). Primers are specified in the publication (Gracias et al., 2021). ΔCt values were obtained by comparison to Ct values of the housekeeping gene GAPDH (for each technical replicate), and fold changes ($2 - \Delta\Delta Ct$) were generated by normalization to the average ΔCt value for vehicle treated wells per plate.

3.3 Study III

3.3.1 Generating iPSCs

iPSCs were either generated using mRNA reprogramming (as described in 3.1.2) or the CyooTune-iPS 2.0 Sendai reprogramming kit according to manufacturer's instructions. All iPSCs were MACS sorted for tra 1-60 (Miltenyi) according to manufacturer's instructions, were negative for mycoplasma, and stained positive for OCT4 by immunocytochemistry.

3.3.2 Generating undirected brain organoids with microglia

The unguided approach was performed as described by Ormel et al. (Ormel et al., 2018) but with adjustments to the use of custom-made miniaturized bioreactors. Briefly, we seeded iPSCs at 9000 cells per well in low attachment 96 wells U bottom plates in mTeSR medium containing rock inhibitor. EBs emerged 24 hours later and were allowed to grow for five days. Medium was then exchanged with neural induction media containing 0.1μg/ml of Heaprin and fed every other day till day 12. On day 13, growth factor reduced matrigel was used to embed each EB which were then transferred to a 24 well plate (one EB per well) in cerebral organoid differentiation medium containing 1% B27 minus vitamin A. Media was changed every day for four days and on day 17 we moved the organoids to a 12 well mini bioreactor.

In the guided approach we combined pre-macrophage precursors (PMPs) and neural progenitor cells (NPCs) , using a protocol that is very similar to the now published one by Xu et al. (Xu et al., 2020). Briefly, 10,000 iPSCs per well were seeded on ultra-low attachment V bottom 96 well plates in mTeSR medium supplemented by rock inhibitor. Twenty-four hours later, EBs were formed. Media was carefully changed to 'EB media' containing mTeSR supplemented with 1mM rock-inhibitor, BMP-4, SCF and VEGF. EBs were carefully observed over the next four days and then plated onto a tissue culture treated 6 well plate in media containing X-VIVO15, M-CSF, and IL-3. After about three weeks, 'pre' pMAC cells started to appear in the suspension and were harvested and combined with NPCs.

For generation of iPSC-derived NPC we seeded Tra-1-60 purified iPSCs on ultra-low attachment V bottom 96 well plates. EBs were formed within 24 hours and the media was changed to DMEM F12 supplemented with 1XN2, dorsomorphin and SB431542. EBs were then transferred to growth factor reduced matrigel coated 6 well plate containing media

composed of DMEM F12 supplemented with 1x N2, and laminin 521. After 7 days, neural rosetted were observed and harvested. These were plated on growth factor reduced matrigel coated 6 well plates in media composed of Neurobasal media, DMEM/F12, 1x N2, 1x B27-RA, FGF2, CHIR99021, hLIF, SB431542, and ROCK inhibitor Y-27632. PSA-NCAM positive cells were then selected by MACS, followed by MACS for $CD271^{-}CD133^{+}$ cells.

PMPs and NPCs were then combined to generate the organoids. Seven thousand pNPCs and 3000 PMPs cells were plated per well of an ultra-low attachment 96 well plate and centrifuged at 150xg for 5 minutes. Each organoid was then transferred to an ultra-low attachment 24 well plate and proliferation media was changed daily until day 27 when the organoids were moved to an ultra-low attachment 6 well plate and placed on a shaker at 90 rpm in the incubator. At day 35, the media was changed to Neurobasal medium, combined with DMEMF12, Brainphys medium supplemented with BDNF, GDNF, dibutyryl cyclic AMP, IL-34 and GM-CSF.

3.3.3 Single cell sequencing

Unguided brain organoids were dissociated into single cell suspensions using the Neural dissociation kit from Miltyeni Biotec. We then enriched for microglia using CD11b microbeads and MACS. High quality suspension (viability >80%) were loaded onto the Chromium controller chip v3.1 from 10X Genomics (target output of 6000-7000 cells per lane). Gel Beads in emulsion (BEDs) were generated and processed with the Single Cell 3' Gel Bead and Library v3.1 kit from10X Genomics followed by sequencing on the Illumina Nova-seq (S2-100) flow cell (2x50 bp and 100 cycles) at SciLifeLab (Sweden) as previously described (Samudyata et al., 2022).

Guided brain organoids were dissociated into single cell suspensions using cold mechanical homogenization with trituration. The cell suspensions were further subjected to cellular lysis according to protocol as provided by 10x genomics (CG000366, RevD) to isolate intact nuclei. Isolated nuclei suspensions were processed according to Chromium Next GEM Single Cell Multiome ATAC + Gene Expression (GEX) protocol (CGOOO338) and with a target recovery of 10000 cells per individual. Prepared libraries were pooled and processed on Illumina Nova-seq (S2-100) flow cell.

Publicly available single-cell transcriptomes were obtained from the respective publications and preprocessed similarly. Gene sets for microglia core signatures were obtained from Patir et al.(Patir et al., 2019) and cells were scored for each gene set using ‘AddModuleScore’ function in Seurat. We integrated data using identified anchors in microglia clusters across datasets and canonical correlation analysis implemented in Seurat. Trajectory analyses and gene module identification was performed using monocle package.

RESULTS

4.1 Study I: Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning

In this study, we assessed microglial engulfment of synaptic structures in 2D models obtained from individuals with SCZ and healthy controls. The study was based on a protocol that we had developed a few years earlier (Sellgren et al., 2017). Given our initial findings, we also studied genetic contribution to excess microglial pruning in our SCZ models.

4.1.1 Increased uptake of synaptic structures in schizophrenia-derived models

The model we used in this study (Sellgren et al., 2017) is based on isolation of synaptic nerve terminals (SYNs) from iPSC-derived neuronal cultures and generation induced microglia-like cells. SYNs are then conjugated to a pH sensitive dye that fluorescence when entering the lysosome compartment in microglia-like cells. After adding SYNs to microglia-like cells, the signal of the pH-sensitive dye is quantified by live-imaging to represent the phagocytic index. After live-imaging, the cells are then also fixed and stained for a synaptic marker (here the post-synaptic marker PSD-95) to confirm that the engulfed particles contained synaptic material. Compared to traditional co-culture approach, this model system has several advantages. First, throughput increases as it is possible to generate a large amount of SYNs, freeze them down, and then thaw as needed for assay (induced microglia-like cells can be generated in approximately two weeks). Second, quantification of the uptake of synaptic structures in the phagocytic cell can be done with a much higher reliability (we observe r^2 values above 0.90 between experimental replicates).

In addition to our previous validations (Sellgren et al., 2017), we in this study performed additional validation steps to assure the purity of the SYN preparations (electron microscopy, western blot analysis for the synaptic marker SNAP-25, functional labelling showing that they are active synapses, and immunocytochemistry showing the presence of the post synaptic protein PSD-95). Given that SYNs were also derived from iPSC-derived neuronal cultures, as compared to brain tissue, these validations confirmed a high purity of the generated SYN preparations.

We then used validated SYNs from 3 individuals with SCZ and 3 matched healthy controls combined with microglia-like cells from 13 individuals with SCZ and 9 matched healthy

controls. In real time live imaging (Figure 1), as well as in quantifications using fixed cultures (PSD-95^{+} particles), we then observed a significantly increased uptake of SYNs in models derived from individuals with SCZ.

To validate our findings using SYNs, we also derived more traditional co-cultures (3 individuals with SCZ and 3 healthy controls, Figure1) by adding microglia-like cells to iPSC-derived neuronal cultures. Before adding microglia-like cells, we observed a similar spine density in both groups, but forty-eight hours after adding microglia-like cells the spine density decreased in both groups and was significantly lower in cultures from individuals with SCZ as compared to healthy controls.

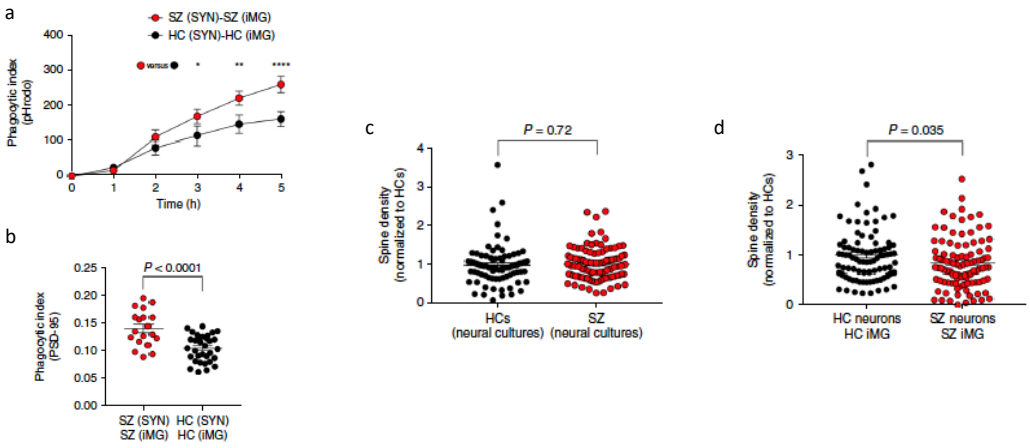

Figure 1: *Increased engulfment of synaptic structures in schizophrenia-derived models.* **a,** Quantification of pHrodo (red)-labeled SYN uptake in iMG cells during live imaging. The phagocytic index represents the mean pHrodo+ area per iMG cell. **b**, Quantification of phagocytic inclusions (PSD-95+ inclusions, 0.5–1.5 μ m) using confocal microscopy. The phagocytic index represents the number of PSD-95+ particles (0.5–1.5 μ m) per iMG cytoplasm area. **c**, Quantification of spine density (spines per 10-μ m dendrite) in neural lines derived from SCZ patients versus HCs. **d**, Quantification of spine density in the same neural lines but cocultured with iMG cells. Data in both graphs are normalized to the HC group. All reported P values are two-sided; the mean ± s.e.m. is indicated for each group in all graphs. *P < 0.05, **P < 0.01, ****P < 0.0001. ***Reproduced with permission from Springer Nature*** (Sellgren et al., 2019)**.**

4.1.2 Both neuronal and microglial factors contribute to excessive pruning

To understand to what extent microglia-like cells, or the synaptic structures, contributed to the excessive elimination of synaptic structures in SCZ-derived cultures we designed ‘crossover’ experiments, i.e., to answer the question what happens if microglia-like cells are ‘healthy’, but synaptic structures originate from individuals with SCZ, and the opposite, if microglia-like cells are SCZ-derived and synaptic structures originate from healthy controls (Figure 2). In these experiments, both the crossover models generated mean phagocytic indexes in between the ‘pure’ disease model and the model completely derived from healthy

controls (Figure 2). Therefore, these results suggested that both microglia and synaptic factors might be contributing to the increase in synaptic elimination observed in SCZ.

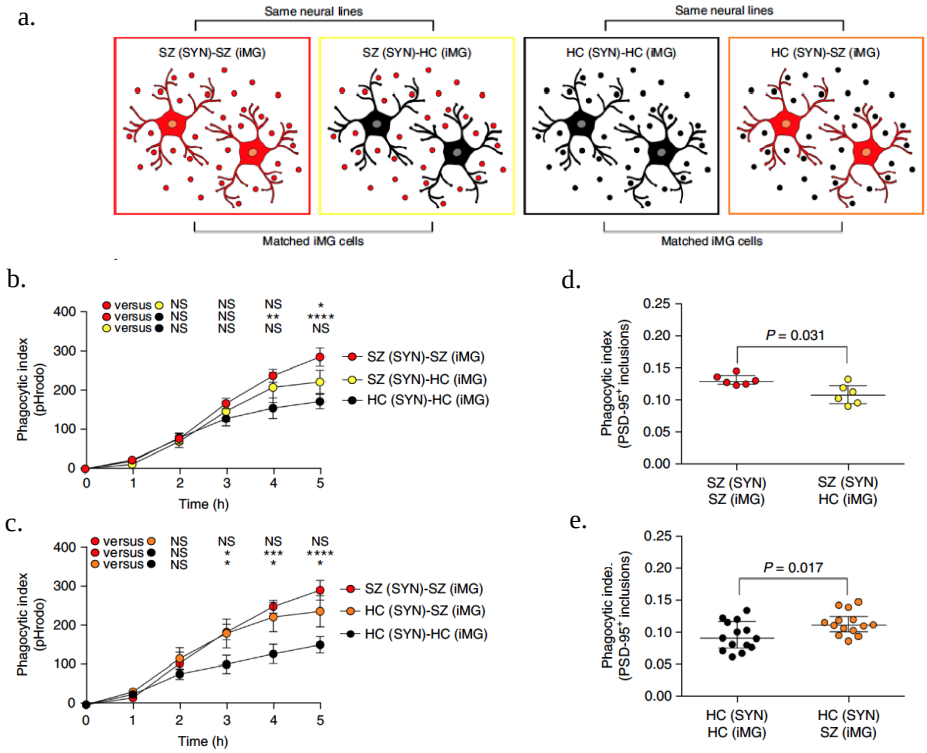

Figure2: *Microglial factors influence synapse engulfment.* **a**, Schematic of 'pure' disease models, derived from patients with SCZ, that were compared to 'mixed' models in which the same synaptic structures from SCZ patients were added to iMG cells derived from matched HCs, as well as 'pure' HC models compared to 'mixed' models in which the same synaptic structures from HC patients were added to iMG cells derived from matched SCZ patients. For both experimental designs, as a reference, we included matched 'pure' HC and SCZ models, respectively. **b**, Quantification of pHrodo (red)-labeled SYN uptake in iMG cells during live imaging sessions of 5 h. **c**, Identical live imaging design as in b but in this experiment comparing HC-HC models with HC-SCZ models based on the same SYN from HCs but iMG cells derived from matched SCZ patients. **d**, Quantification of phagocytic inclusions (PSD-95+ inclusions, 0.5–1.5 μ m) using confocal microscopy and SYNs derived from SCZ lines matched with iMG cells from SCZ patients or matched HCs. **e**, Quantification of PSD-95+ inclusions using confocal microscopy and SYNs derived from HC lines matched with iMG cells from HCs or SCZ patients. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ***Reproduced with permission from Springer Nature*** (Sellgren et al., 2019).

4.1.3 Risk variants within the *C4* locus contribute to excessive synaptic pruning

What could then be the mechanism behind the excessive elimination of synapses in SCZ? Given that copy numbers of *C4A* increases SCZ risk (Sekar et al., 2016), and that complement signaling plays an important role in microglia-mediated pruning (Stephan et al., 2012; Tyler & Boulanger, 2012), we decided to study how copy numbers of *C4A*, as compared to copy numbers of *C4B* (not increasing SCZ risk) (Sekar et al., 2016), influenced complement deposition on synaptic structures. For this, we used iPSC lines from 13 individuals with SCZ and known copy numbers of *C4A* and *C4B*. We then developed a

complement deposition assay to indirectly measure complement activation dependent on predicted neuronal production of C4A or C4B. Briefly, iPSC derived neurons were first treated with an anti NCAM antibody to sensitize them to complement action and used media deficient in complement components. We then saturated the media with the complement factors C1, C2 and C3 to make activation of cascade dependent on the amount of C4A or C4B produced by the neurons and measured C3 deposition as a marker for complement deposition on neurons. We then observed increased C3 deposition dependent on *C4A* copy numbers while no correlation could be observed between C4B copy numbers and *C4B* copy numbers. This observation was then paralleled in our functional pruning assay, with more uptake of SYNs in microglia-like cells dependent on C4A copy numbers and no correlation between C4B copy numbers and the extent of SYN uptake in microglia-like cells (Figure 3).

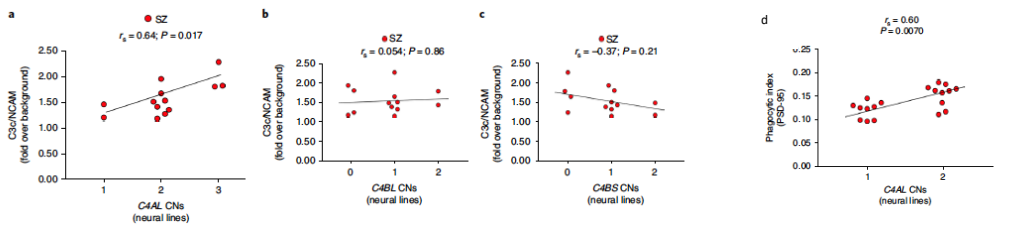

Figure 3: *C4 SCZ risk variants increase complement deposition on neurons and increase synapse engulfment in in vitro models derived from SCZ patients.* **a,** Significant correlation between neural C4AL CNs and neuronal C3 complement deposition; no effect of C4BL **(b)** or C4BS **(c)** CN on C3 deposition was observed. **d,** Similar correlation as in 'a' but between C4AL CNs and SYN uptake as measured by PSD-95+ phagocytic inclusions. All reported P values are two-sided; correlation coefficients are Spearman's. ***Reproduced with permission from Springer Nature*** (Sellgren et al., 2019).

4.1.4 Minocycline decreases engulfment of synapses and reduces schizophrenia risk

In the set of experiments, we focused on ways to inhibit microglia-mediated synaptic pruning. Minocycline, a brain penetrant tetracycline, has been hypothesized to target synaptic remodeling (Inta et al., 2017), although the exact mechanism of action remains unknown. We therefore pre-treated microglia-like cells with different concentrations of minocycline (1, 10, and 60 μM) and then added SYNs. Live imaging as well as in fixed cultures quantifying uptake of PSD-95$^+$ particles revealed a significant (and dose dependent) decrease in microglial SYN uptake (Figure 4).

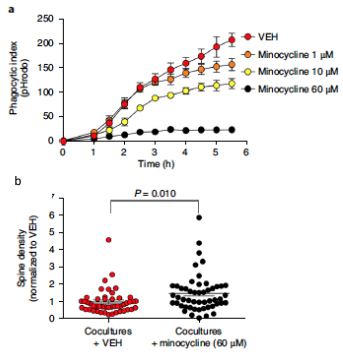

Figure 4: *Minocycline inhibits synapse engulfment in vitro* **a**, Quantification of pHrodo (red)-labeled SYN uptake in iMG cells during live imaging sessions. iMG cultures were pre-treated for 30 min with VEH or minocycline at different concentrations. **b**, Quantification of spine density in co-cultures (neurons and iMG cells) derived from an HC line and treated with 60 µM minocycline or VEH as indicated. Data are normalized to the VEH group in the graph. ***Reproduced with permission from Springer Nature*** (Sellgren et al., 2019).

As these results suggested that minocycline also could have an effect on microglial engulfment of synaptic structures in humans under *in vivo* conditions, we decided to study risk of developing SCZ in electronic health records depending on exposure for minocycline during adolescence. Minocycline can be prescribed against Acne Vulgaris and to optimize power we here then also included individuals that had been prescribed doxycycline, another tetracycline that chemically and mechanistically is similar to minocycline. At least 90 days of exposure was required and as comparison we used individuals that had been prescribed another antibiotic (erythromycin, clarithromycin, or trimethoprim/sulfa-methoxazole) with the same indication. Adjusting for age, sex, self-reported ethnicity, and year of entry, among the 22,027 individuals in the cohort, we detected a significantly decreased risk of incident psychosis in the minocycline-exposed group.

Study II: Elevated CSF C4A levels in first-episode schizophrenia

Despite several key papers showing evidence in experimental models linking *C4A* copy numbers to increased synapse elimination, several factors prevented the measurements of C4A protein levels in the human CNS: first, C4A and C4B protein are 99% identical in amino acid sequences, differing by only 9 amino acids. Second, *in vivo* protein measurements in CNS are usually performed in CSF that typically requires a low limit of detection. Third, to detect subtle changes that occur in a patient versus control design, further sensitive to the method is required. Therefore, in study II, we develop and validated a new targeted mass spectrometry method to detect C4A and C4B proteins in CSF in the femtomolar range. We then applied this assay to CSF obtained from individuals with FEP and healthy controls.

4.2.1 Elevated CSF C4A levels in first-episode schizophrenia

First, we used a discovery cohort consisting of 44 individuals with FEP and 20 HCs (age and sex matched). The FEP group was further divided dependent on if they obtained a SCZ diagnosis at follow-up (FEP-SCZ) or another psychiatric diagnosis (FEP-nSCZ). FEP-SCZ group displayed a significantly higher CSF C4A levels compared to the HCs as well as the individuals with FEP-nSCZ (Figure 5). However, when measuring C4B levels the levels were similar between groups (Figure 5). We could then replicate our findings from the discovery cohort using an independent cohort of 31 individuals with FEP and 21HCs (Figure 5).

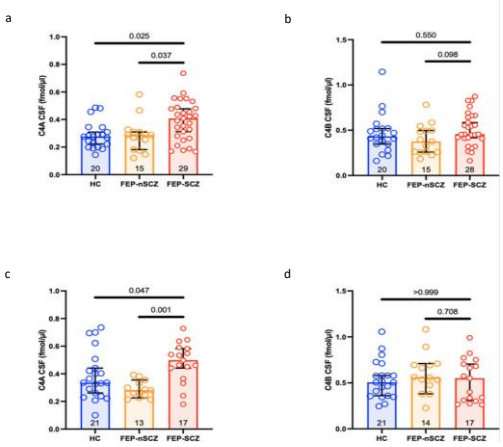

Figure 5: *Cerebrospinal fluid levels of C4A are increased in patients with first episode psychosis who develop schizophrenia.* **a** In the discovery cohort (KaSP), FEP-SCZ displayed significantly higher CSF C4A concentrations as compared to HCs or FEP-nSCZ **b** CSF C4B concentrations were similar across groups. **c** In the replication cohort (GRIP), FEP-SCZ displayed significantly higher CSF C4A concentrations as compared to HCs or FEPnSCZ, while (**d**) CSF C4B concentration were similar across groups. Bar graphs represent medians and error bars represent 95% CIs. Data were analyzed using Kruskal–Wallis H tests followed by post-hoc tests. Significance was set to $P < 0.05$. All reported p-values are two-sided. ***Reproduced from*** (Gracias et al., 2021)

4.2.2 Disease-associated cytokines contribute to the elevation of C4A levels

The original analysis of CSF C4A concentration across the three groups were then adjusted for *C4A* copy numbers. We observed that CSF C4A levels per *C4A* copy numbers remained significantly increased in the FEP-SCZ group. Additionally, a previous study by Kim *et al* suggested that *C4A* expression in SCZ increases much more than expected levels (that are based on genetic risk variance in the *C4* locus) (Kim et al., 2021a).Taken together, this indicated that there might be other factors that can increase CSF C4A levels that are not related to genetic risk variability within the C4 locus. SCZ-brain co-expression network analyses observed an enrichment of cytokine signaling genes (in the positively co-expressed gene module) when *C4A* was used as the 'seed gene' (Kim et al., 2021b). CSF of individuals with SCZ also showed an increase in the cytokines- IL1-beta and IL-6 (Schwieler et al., 2015; Söderlund et al., 2009). To test if these cytokines could contribute to increasing C4A levels in SCZ, we generated iPSC derived cortical glutamatergic neurons and stimulated them with either IL1-beta, IL-6 or a combination of IL1-beta and IL-6, for 24 hours. Neuronal cultures stimulated with a combination of IL1-beta and IL-6 displayed a significant increase in *C4A* mRNA expression. To study *in vivo* relevance, we then turned to CSF and looked at correlations between these cytokines and C4A levels. Controlling for genetically predicted *C4A* mRNA expression, we then observed a significant correlation between CSF IL-1beta and CSF C4A levels although not between CSF levels of IL-6 and C4A. This suggests that foremost IL-1beta can influence C4A levels also under *in vivo* conditions and independent of C4A copy numbers.

4.2.4 C4A correlates with measurements of synapse density in CSF

If our *in vitro* assays reflected *in vivo* mechanisms, then *in vivo* CNS C4A levels should reflect *in vivo* measurements of synapse density. To test this, we measured CSF markers of synapse density-neuronal pentraxins 1,2 and the neuronal pentraxin receptor. These markers have previously been shown to inversely correlate to cortical thickness (by MRI), and correlate to loss of synapses in neurodegenerative diseases like Alzheimer's disease and dementia (Nilsson et al., 2021; van der Ende et al., 2020). In CSF from individuals with FEP and healthy controls we also observed significant negative correlations between C4A levels and all these markers of synapse density.

Study III: High-resolution characterizations of innately developing microglia in human brain organoids

In this study we wanted to develop and validate more advanced 3D models that more comprehensively captured the interplay between microglia and developing synapses. First we derived undirected organoids with innately developing microglia as suggested by Ormel at al. (Ormel et al., 2018), then we focused on a directed approach, combining NPCs and 'pre-microglia', similar to the protocol recently proposed by Xu et al. (Xu et al., 2020). Single cell transcriptomic sequencing was used to characterize the organoids from the two protocols and for further comparison we also used a publicly available single-cell transcriptomes dataset from organoids derived using another recently published protocol (Cakir et al., 2022).

4.3.1 Cellular composition of brain organoids with innately developing microglia.

Both directed and undirected protocols had the presence (by immunohistochemistry) of neurons, astrocytes, microglia, radial glia, as well as neural progenitor cells, although the guided organoids displayed a more accelerated differentiation of neurons expressing mature markers (Figure 6). Nonetheless, both protocols exhibited a general reduction in the expression of progenitors and immature cellular markers and an increase in markers of mature neurons and astrocytes proportional to time in culture.

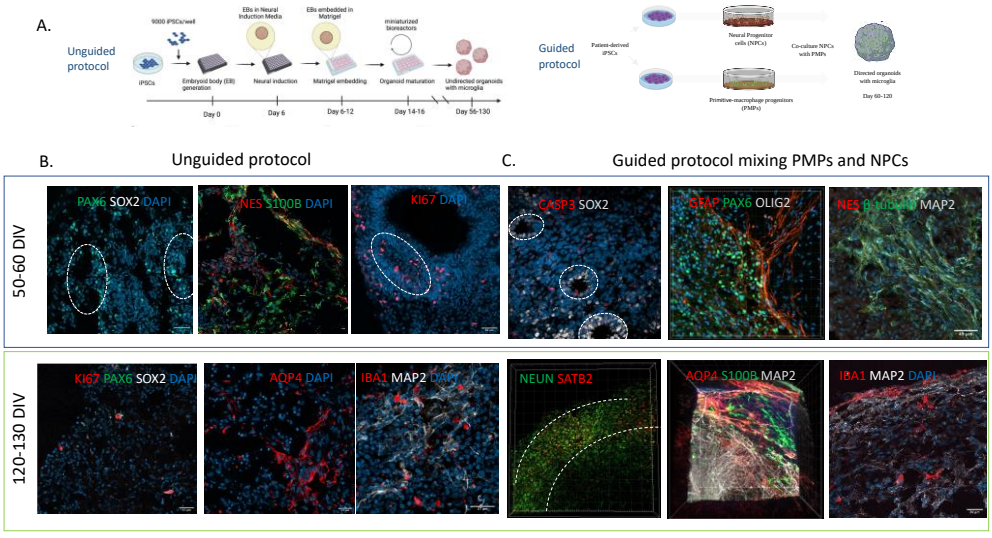

Figure 6. (A) Schematic for generating cerebral organoids with the unguided protocol (left) and the guided protocol by mixing PMPs and NPCs (right) harvested at 50-60 DIV and 120-130 DIV. Representative immunostained confocal images for key cell type markers in **(B)** unguided COs sections and **(C)** guided COs sections showing the presence of immature and mature neurons (BIII-tubulin, MAP2, NEUN, SATB2), astrocytes (GFAP, AQP4), neural precursor cells/progenitors (PAX6, SOX2), Radial glia (GFAP, NES, KI67), and microglia (IBA1), at 50-60 DIV (top) and 120-130 DIV (bottom).

Next, we used high-resolution single cell RNA-seq to characterize the repertoire and fidelity of cell types within the organoids generated with the two different protocols. A single cell transcriptomic dataset was generated from the directed brain organoids at the later timepoint and for comparisons with the unguided approach we took advantage of our previously generated scRNA-seq dataset from undirected organoids (Samudyata et al., 2022). After filtering out low-quality cells, cells from the unguided organoids (n=39808) and the guided organoids (n=21512) were processed and projected onto UMAP space individually. Organoids from both the guided and unguided approach had a relatively similar composition of neuroectodermal populations, including radial glia, intermediate progenitors, glutamatergic and GABAergic neurons, astrocytes, proliferative neural precursors and glioblasts (Figure 7). However, unguided organoids displayed an enrichment of non-ectodermal cell types exhibiting characteristics of cells with a mesodermal origin whereas guided organoids were mainly composed of neuroectodermal cell types (Figure 7). Among the non-ectodermal clusters in the unguided organoids, we also observed signatures of muscle differentiation, smooth muscle cells, endothelium, mural cells, ectomesenchyme, epithelial cells, and peripheral Schwann cells, in addition to microglia.

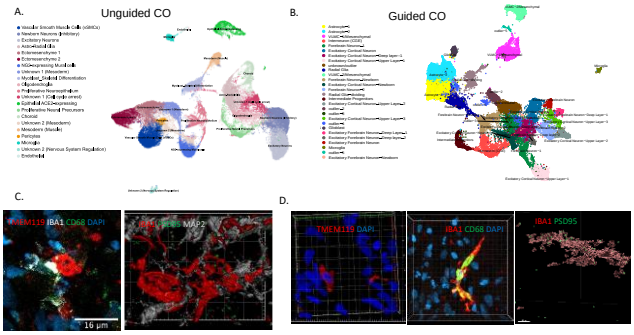

Figure 7. (A) UMAP plots of unguided COs (n=18 organoids, 39808 cells) demonstrating cell type clusters found in organoids generated with the unguided protocol. Clusters were annotated according to cluster-specific marker genes (Supplementary table 1). **(B)** UMAP plots of guided COs (n=36 organoids, 21512 cells) demonstrating cell type clusters found in organoids generated with the unguided protocol. Clusters were annotated according to cluster-specific marker genes (Supplementary table 1). Super-resolution immunostained images of microglia markers (IBA1, TMEM119, CD68) in **(C)** unguided COs, and **(D)** guided COs, with 3D reconstruction (right) of IBA1+ cells (red) with engulfed PSD95+ synaptic puncta (green) respectively.

4.3.2 Microglia signature in undirected and directed brain organoids

Organoids from both protocols displayed cells that stained positive for TMEM119 as well as CD68. Similar to our previous observations from unguided organoids (Samudyata et al., 2022), we observed spontaneous uptake of synaptic material in unguided as well as in guided organoids. Next, we benchmarked the microglial signature in the two datasets against human fetal microglia obtained during gestational weeks 8-39 (Nowakowski et al., 2017), using a

core microglial score based on a previously curated core microglia transcriptomic signature in humans (Patir et al., 2019). We also included transcriptomic data from a recently published guided protocol containing PU.1-induced microglia (Cakir et al., 2022). Microglia obtained from both the unguided protocol and the guided protocol mixing PMPs and NPCs displayed a high core microglial score, similar to primary fetal microglia, whereas microglia from guided PU.1-induced organoids showed limited expression of core microglial genes. However, microglia exhibit limited expression of homeostatic signature genes in all of the protocols. Reduced expression of homeostatic genes was also observed in transcriptomes of primary fetal microglia incorporated exogenously into organoids upon comparison. We observed the expression of known microglial genes such as *SPP1, LAPTM5, CD68, TYROBP, CSF1R* in microglia from both unguided and guided protocol mixing PMPs and NPCs whereas microglia from the guided PU.1-induced organoids exhibited *AIF1* expression but low to no expression of other markers.

We used an unbiased approach to compare microglial signatures using data integration. Again, microglia developed within unguided organoids and guided organoids mixing PMPs and NPCs demonstrated a strong transcriptional overlap with primary fetal microglia, while we observed a modest transcriptional separation between primary fetal microglia and microglia from PU.1-induced organoids in the integrated space. We then also investigated the epigenome of microglia using single cell ATAC-seq data generated from directed organoids (mixing PMPs and NPCs) at 130 DIV and confirmed open chromatin or accessible peaks at microglial marker genes.

GENERAL DISSCUSSION

Unbiased large-scale genetic studies have in many ways revolutionist SCZ research in the last 20 years. With polygenic risk scores that successively become more accurate in capturing SCZ risk and a growing number of confirmed SCZ risk loci, it may seem inevitable that biological insights and new the discovery of novel therapeutic targets is to follow. However, SCZ remains a brain disorder, with all the obvious obstacles, and the polygenicity is even larger than expected. To meet these challenges, experimental approaches and disease model systems need to adjust (Hyman, 2018). As argued elsewhere (Hyman, 2012; Khodosevich & Sellgren, 2023), patient-derived cellular models hold potential to address some of the limitations seen in classical disease models.

In **Study I**, we use a reductionist approach to address the more than forty years old hypothesis of excessive synaptic pruning in SCZ. Utilizing a protocol that we developed with the aim to provide a model that have acceptable cost-effectiveness and within-individual variability (Sellgren et al., 2017), i.e., feasibility for disease modeling, we were able to compare microglial uptake of synaptic structures in models based on cells from a relatively large number of patients and controls. Using cells derived from individuals also made studying the human *C4* locus and its effect on SCZ risk not only possible but we were further able to address cell type specific contribution in a disease context. The main limitation with this study is however *in vivo* relevance, i.e., is the *in vitro* assay really reflecting mechanisms in the brain of patients with SCZ? While there is no clear answer to this question, some more recent studies provide additional information. First, Onwordi *et al*. in 2020 used [^{11}C]UCB-J positron emission tomography to show reduced synaptic terminal protein levels in living SCZ patients (Onwordi et al., 2020). While the cause of this observation is left unanswered, this finding at least excludes that loss of synapse in SCZ is related to processes taking place in late life. In 2021, Yilmaz *et al*. also developed a mouse model with the primate-lineage-specific *C4A* isoform (Yilmaz et al., 2021). This study was able to successfully mimic the findings in **Study I** *in vivo*: that C4A is capable of binding to synapses more efficiently than C4B, and further that C4A is more active in microglial mediated pruning than C4B.

In **Study II**, we returned to the living patients and measured C4A and C4B protein levels in CSF. In accordance with our *in vitro* findings, we observed elevated C4A (although not C4B) levels in individuals with FEP that later developed SCZ. However, the elevation in CSF C4A

levels in SCZ exceeded our predictions based *C4* haplotypes. This led us to investigate SCZ-related factors that could increase *C4A* mRNA expression per given haplotype. Utilizing an iPSC-based models, *C4A* mRNA expression can be induced by a combination of the cytokines- IL-1beta and IL-6. This data is echoed in vivo where IL-1beta and C4A levels display a positive correlation in CSF. C4A also inversely correlated with markers of synapse density, further strengthening the notion that C4A is involved in synaptic pruning in the living human brain. The strength of this study is that we were able to replicate the elevation of C4A in two independent cohorts and the fact that our mechanistic *in vitro* findings were largely mimicked by observational *in vivo* data. The need for both, IL-1beta and IL-6 to stimulate neuronal *C4A* mRNA expression *in vitro* however complicated the interpretation of the *in vivo* correlation data, in which only IL-1beta correlated with C4A levels. To some extent, this may be a reflection of using a neuronal mono-culture system that foremost relied on the addition of cytokines without a stable innate production.

In **Study III**, we evaluated 3D brain organoid models in the context of modeling microglial synaptic pruning in SCZ. While still preliminary data, some initial conclusions can be drawn. First, in both the direct and undirected approach we observe spontaneous phagocytosis of synaptic structures by microglia-like cells. Second, the models display differences both in regard to microglial signature and the composition of other cell types. Third, while the undirected approach and one of the directed approaches displayed microglia that acceptably clustered with primary fetal microglia, homeostatic genes were low as compared to *in vivo* microglia. This emphasizes that the 3D approaches now available cannot completely by-pass the alteration of the human microglia transcriptome that occurs when switching to *in vitro* conditions (Gosselin et al., 2017). Chimeric mouse models to support human organoid development and maturation may partly address these limitations but further decreases throughput in the context of disease modeling.

The work presented in this thesis raises several questions that warrant further studies. Perhaps most important, while the included studies, as well as work by other laboratories, provide plausible evidence for a role of C4A in synaptic pruning, the variants within the *C4* loci only slightly increase SCZ risk. Do other genetic risk variants, or environmental risk factors, then converge on excessive synaptic pruning as a more broader disease mechanism in SCZ? Animal models suggest that synaptic pruning is activity-dependent (Wilton et al., 2019), and many of the implicated genes in GWAS have a role at the synapse (Ripke et al., 2013, 2014b;

Trubetskoy et al., 2022). In regard to environmental risk factors, murine models also suggest that infection with RNA viruses can induce complement-dependent phagocytosis of synaptic inputs by microglia that leads to cognitive impairments (Chhatbar & Prinz, 2021; Garber et al., 2019; Vasek et al., 2016). Recently, our laboratory also used undirected brain organoids to display excessive microglial uptake of synaptic structures after infection with SARS-CoV-2 (Samudyata et al., 2022), then highlighting the utility of these models also for studying environmental risk factors in SCZ.

In conclusion, the present thesis suggests that microglia excessively engulf synapses in SCZ and that this can partly be explained by elevated C4A levels. Further, to some extent, it provides evidence that SCZ risk variants can be studied by using patient-derived cellular modeling as well as provides mechanistic data that then can be confirmed in observational patient studies.

ACKNOWLEDGEMENTS

The PhD journey has been the most challenging thing I have ever done, a roller-coaster with so any highs and definitely a lot of lows. I have a lot of people to thank over the last (almost) five years and I hope I can manage to mention everyone. In case I miss to mention anyone by name, I am very sorry in advance- it is one hour to my printer deadline, and I am trying to recall 5 years of memories XD- please know that I am very thankful to you all.

I would first like to thank Dr. Carl Sellgren Majkowitz- for the opportunity to do the PhD in your lab and dragging me all the way to Sweden. I have learned so much from the last 7 years of working together. Thank you to my co-supervisors, Dr. Sophie Erhardt and Dr. Roy Perlis for your support scientifically. Steven Sheridan- thanks for your mentorship and guidance over the years!

To all the present and former members of the lab- Neda, Ruth, Sraven, Sanna, Eleonora, Funda, Şafak, Gkraseila, Ana, Asemenia, Marja, Samudayata, Susmita, Martin- thanks for the support over the years. A very special thank you to İlknur Şafak Demirel for making my cover art! Thanks to present and former members of the Erhardt lab and Maria Lee- for your close collaboration and friendship over the years. Florian- from the biomedicum imaging core facility- thank you for always answering my questions promptly and for teaching me a lot about microscopy.

Sophia Pettersson- Thanks for being the best administrator for the PhD students at Fyfa. Your organisation and knowledge on navigating KI's complex documents and procedures have been invaluable. In addition, I would also like to thank past and current members of fyfa HR (especially Diana Mattsson) and fyfa finance admin Zahra Adburahman.

To Kent Jardemark (director of doctoral studies at FyFa)- thanks for the guidance during my doctoral studies and answering every question I have promptly. It was a pleasure working with you in my role as student representative in FyFa for two years.

Members of the past and present Doctoral Student Association at KI- especially Leonie, Jan, Sebastian, Valeria, Mirco, Alicia, Julia, Manouk, Ines, Dina, Ioanna, Lea Van Husen, Weiyingqi Cui, Dominyka, Sara- It was a pleasure working with you to help improve the

experiences of doctoral students at KI. We achieved great things together and more importantly your friendship and peer support has been amazing throughout my PhD journey. Special thanks to Ingeborg Van Der Ploeg- it was a very fulfilling experience being the student representative in your 'Kurs-och programmkommitte' at KI. Thank you for all that you do for doctoral education at KI.

Ayla De Paepe (former career service manager) – thank you for the career guidance over the years, from individual coaching sessions to the career skills course. You made me more confident in my skills and opened my eyes to a whole world of opportunities outside of academia. It was because of your coaching and encouragement that I landed the job at Procella Therapeutics even before I completed my PhD studies.

The 'C5' crew: Paula, Ada, Theresa, Merve- thanks for all the random tea breaks, venting sessions and support over the years. We have shared a lot of tears, laughter, and triumphs, and I hope this continues even after the PhD.

A special thanks to Paula- you have seen me at my worst and my best, and have been the best support throughout my PhD, my personal cheerleader when things got tough. I don't think I have enough words to thank you for everything.

Dina Dabaghie- We met in the most random of ways through the DSA, but now I cannot imagine life without your friendship. We have been through some life changing things together during our PhD. I can confidently say I wouldn't have made it through without you. I am also so happy we are defending on the same day!

Jill, Lourdes and Stelia- we met in my last year of PhD on the career course. Thank you for the peer support and listening to my rants. Stelia- I am so happy we are now colleagues at Procella therapeutics!

Filipa Bouçanova- We met very early in my PhD studies when we organized the neuroscience winter conference. Since then, you have become one of my closest friends. I will always remember our random coffee sessions in Biomedicum and your dinner and board game nights!

Walter, Megan, Martin- thanks for all the support especially in 2020. I am so grateful to have you all in my life.
Kerri O'Regan- My closest friend from Boston- you have been one of my best friends from the start of this project way back in Boston even before I started my PhD. Thank you for always being there for me, even though we live on different continents.

Malkom Thorén, my psychologist at Bergshamra Vårdcentral, I would definitely not be able to cross the finish line without you. Your counselling not only helped me navigate the twists and turns of my PhD but also helped me understand myself better and become a better version of myself- for this I am eternally grateful.

I am thankful to my Swedish family- Lena & Niklas, Rebecka & Henrik, Robin & Elsa, Nils-Bertil & Ammi, Ulla & Björn, Lucas, Emma, Annika, Inga & Lars, Ellenor & Björn, -for being part of my support system here in Sweden and welcoming me into your family- for all the family fikas, Christmas trips, and dinners that have sustained me through my PhD journey- thank you!

A special thanks to my aunt (Iona) and uncle ('tipa' Rony)- 12 years ago you supported a crazy dream of mine of going to the US to pursue biomedicine. That road lead me here. This PhD would not be possible without you.

I am eternally grateful to my parents, Andre and Mariola Gracias- for always being supportive and putting up with me over the last 34 years. You have always sacrificed a lot for me, and I would have never gotten here without your love and support. Special thanks to Michael, Jennifer, Anisha, Rochelle- even though we all live on different continents the bond of family always keeps us close and supporting each other in times of need.

Last but most important, my husband Rickard- I do not know how you found the strength and patience to deal with me throughout the journey of getting my PhD. Thank you for always being my rock. I love you.

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