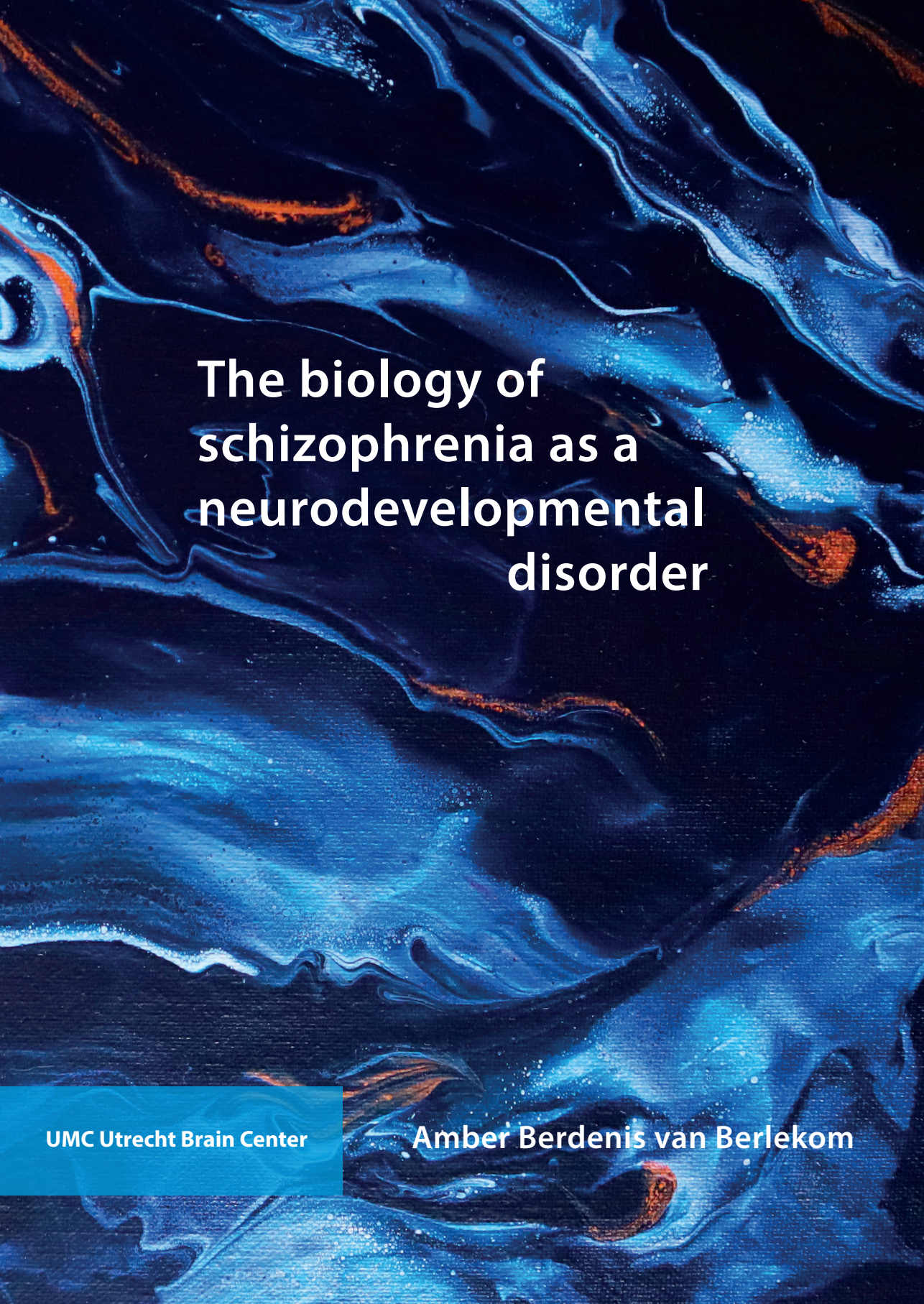


The biology of schizophrenia as a neurodevelopmental disorder

UMC Utrecht Brain Center

Amber Berdenis van Berlekom

Voor mijn ouders

About the cover

Acryl painting by Amber Berdenis van Berlekom inspired by the research performed in this PhD thesis: searching for molecular and cellular neurodevelopmental phenotypes in schizophrenia. If you look closely you can find small cells everywhere and strings of gold and light-blue, resembling the protrusions of astrocytes and microglia.

Colofon

PhD thesis, Utrecht University, The Netherlands
Copyright © 2022 Amber Berdenis van Berlekom

ISBN: 978-90-393-7490-0
DOI: 10.33540/1269

Author: Amber Berdenis van Berlekom
Cover: Amber Berdenis van Berlekom
Layout: Proefschrift-All In One | www.proefschrift-aio.nl

The biology of schizophrenia as a neurodevelopmental disorder

De biologie van schizofrenie als een ontwikkelingsstoornis
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 1 juli 2022 des middags te 12.15 uur

door

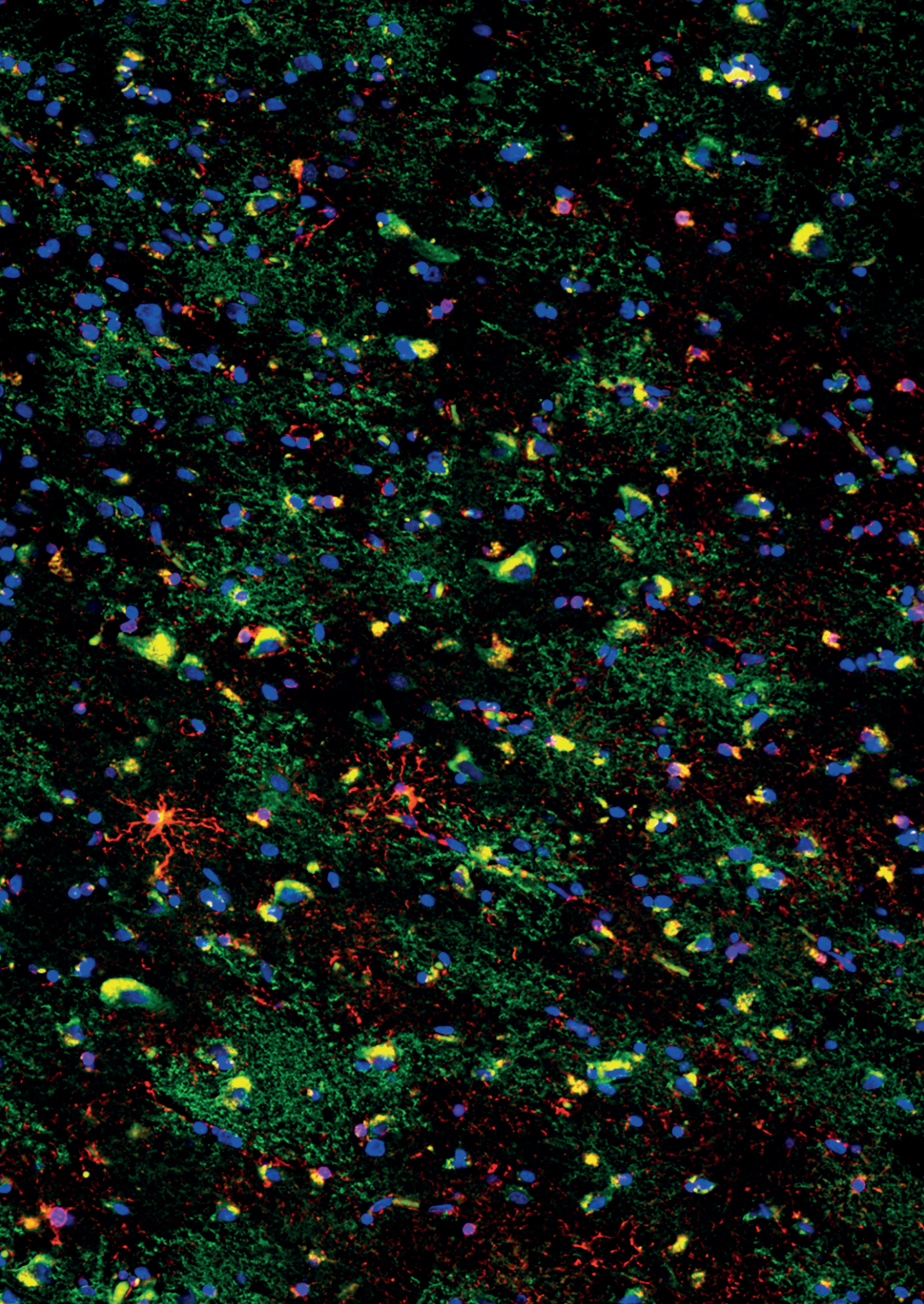
Amber Berdenis van Berlekom

geboren op 8 februari 1992
te Utrecht

Promotoren:
Prof. dr. E.M. Hol
Prof. dr. R.S. Kahn

Co-promotoren:
Dr. L.D. de Witte
Dr. M.P. Boks

Table of contents



Chapter 1

General introduction

Prefix

Schizophrenia is a neuropsychiatric disorder causing severe morbidity and high levels of mortality. Neurodevelopment has been implicated in the pathophysiology of schizophrenia for many years. Genetic and environmental influences affect both prenatal and postnatal neurodevelopmental processes, leading to an increased risk of developing schizophrenia.

The genetic architecture of schizophrenia is highly polygenic, with many common and rare genetic variants explaining only a small proportion of the disease risk. Non-genetic environmental factors are considered important contributors to schizophrenia development. The environment during life, but also prenatal environmental disturbances, contribute to increased risk of developing schizophrenia in epidemiological studies.

There is still a dire need to further elucidate the specific cellular and molecular mechanisms underlying the disturbances in neurodevelopmental processes suggested to be involved in schizophrenia pathology. To this end, the study of postmortem brain material and the use of 3D neurodevelopmental *in vitro* models provides opportunity to study brain alterations of schizophrenia patients on a microscopic level.

This thesis reports studies on neurodevelopmental phenotypes of schizophrenia. In particular, on postnatal synaptic loss and prenatal environmental risk factors in schizophrenia. As an introduction to this, the basic concepts and current literature related to these subjects are described. First, we discuss the clinical characteristics of schizophrenia, and genetic and environmental risk factors involved (chapter 1.1). Followed by a brief overview of the two main topics studied in this thesis related to cortical neurodevelopment in schizophrenia: the dysconnectivity hypothesis, and prenatal environmental risk factors (maternal inflammation and prenatal nutritional deficits) (chapter 1.2). The next paragraphs introduce glial cells (microglia and astrocytes) and provide an overview of their role in cortical neurodevelopment and functioning, and their possible involvement in schizophrenia etiology (chapter 1.3). Then we describe translational models for mechanistic study of the brain in schizophrenia (chapter 1.4). Finally, the outline of the thesis is presented.

1.1 Schizophrenia

Clinical characteristics of schizophrenia

The term schizophrenia was first described by Kraeplin in 1896. Schizophrenia is a chronic psychiatric disorder affecting 20 million people worldwide[1]. The lifetime prevalence of schizophrenia is estimated at 0.5-1% in the general population[2–4]

Patients suffer from a broad spectrum of symptoms encompassing negative symptoms such as flattened affect, positive symptoms such as psychosis, accompanied by cognitive symptoms such as impairments in memory function[5]. Previously, the diagnosis of schizophrenia knew several subtypes (such as catatonic, disorganized, and paranoid). However, currently schizophrenia is viewed as a spectrum disorder, with patients presenting with varying combinations and intensities of symptoms, which can also change over the disease course[6]. Schizophrenia is associated with higher medical morbidity and a decrease in life expectancy[7,8]. Also, psychiatric comorbidities are frequent in schizophrenia, especially depression, anxiety, and substance abuse are common[9,10]. Besides symptoms of the disease impacting patient life on a physical, social, and occupational level, stigma, discrimination, and violation of human rights of people with schizophrenia is common, contributing to disease burden[11].

Classification of schizophrenia is based on the diagnostic and statistical manual of mental disorders (DSM). To be diagnosed with schizophrenia, patients must experience at least two core symptoms for a significant proportion of time for one month, from which one must be delusions, hallucinations, or disorganized speech[6].

The disease is generally recognized and diagnosed in adolescence or early adulthood during the active phase, when the first psychosis manifests**.** Prodromal symptoms, such as a decline from social activities and in cognitive functioning, occur in a substantial proportion of patients, but often go unnoticed[12]. The current treatment consists of a holistic approach including psychological treatment, antipsychotic medication, and rehabilitation[13,14]. However, antipsychotic medication is not effective for all patients, often causes major side effects, and generally target positive, but not negative symptoms of the disease[15].

Genes and environment in schizophrenia

Schizophrenia is a highly heritable disease, with family and twin studies showing an incidence risk between 60 and 80 percent[16,17]. To identify and understand the underlying genetics, the disease has been studied using family cohorts, linkage studies, and more recently, genome wide association studies (GWAS)[18–23]. These studies have led to the identification of many schizophrenia risk loci. However, they also revealed that the

genetic architecture of schizophrenia is highly polygenic, with many common and rare genetic variants explaining only a small proportion of the risk[24–26].

Besides the genetic risk, non-genetic environmental factors are important contributors to disease development in schizophrenia. Indeed, environmental factors such as (childhood) trauma, substance abuse, and pregnancy complications (like maternal infection and malnutrition) have been associated with an increased risk of developing schizophrenia[27,28]. Epigenetic regulation, modifying DNA accessibility trough both genetic and environmental influences, is thought to act as a mediator of environmental risk factors involved in schizophrenia pathology[29–34]. Both candidate- and epigenome-wide association study approaches have led to the identification of genetic loci that are differentially regulated epigenetically in schizophrenia[35–38].

Altogether this has led to the gene-environment hypothesis in schizophrenia: stating that genetic background, environmental risk factors, and their interaction contribute to rendering an individual more vulnerable for manifestation of the disease.

From symptoms and risk genes to disease mechanism

Altogether, the heterogeneity of the disease presentation in patients, the multitude of genetic and environmental risk factors for developing schizophrenia, and the lack of biological markers for the disease make it challenging to elucidate schizophrenia etiology. It is evident that there is a need for translation to underlying cellular and molecular phenotypes in the brain for better understanding of the disease and the development of novel treatment strategies.

1.2 Schizophrenia as a neurodevelopmental disorder

Because schizophrenia is not characterized by gross brain pathology, it was infamously called the 'graveyard of neuropathologists'[39]. However, improved methods and the development of novel techniques have resulted in ample evidence of macroscopic and microscopic brain changes in schizophrenia. Neuro-imaging and postmortem brain studies have identified a decrease in grey and white matter volume and density, disruption of white matter connectivity, and changes in gyrification and cortical thickness[9,40–50]. Over the past decades many theories on the biological underpinnings of schizophrenia disease etiology have been proposed, relating to neurodegeneration, neurotransmitter disturbances (including glutamatergic, serotonergic, dopaminergic, and GABAergic transmitter systems), and increased immune activation.

For the past 25 years, schizophrenia has increasingly been considered a neurodevelopmental disorder. During the prodromal phase of the disease subclinical features encompassing cognitive symptoms (such as IQ deficits) and neuropsychological symptoms are detectable, in some cases already before the age of 8 years[51–55]. Also, structural brain changes have been found premorbidly in frontal regions, with additional changes occurring over the transition to illness during adolescence and early adulthood[56]. Furthermore, schizophrenia risk loci identified by GWAS are shown to be enriched for transcript features that are involved in developmental regulation of the prefrontal cortex[57,58]. Altogether, development and maturation of the prefrontal cortex especially seems to be implicated in schizophrenia.

Importantly, cortical neurodevelopment is not only something that happens prenatally, but is a process that continues postnatally (Figure 1 and BOX 1)[59]. In the context of schizophrenia, the neurodevelopmental theory proposes that the consequences of prenatal genetic predispositions and/or early adverse events during mid-gestation are latent throughout the first decades of life and become "unmasked" during normative maturational changes as psychosis becomes evident in early adulthood[60,61]. However, neurodevelopment is a continuing process, with neural network formation, optimization, and maturation in the frontal cortex extending beyond the perinatal period well into adolescence [62–64]. Schizophrenia typically has its onset during late adolescence or early adulthood, coincident in time with cognitive maturation of the prefrontal cortices.

Therefore, disruptions (genetic or environmental) affecting both pre- and postnatal stages of neurodevelopment are central in the neurodevelopmental theory of schizophrenia pathology.

Dysconnectivity hypothesis

One of the most popular theories of postnatal neurodevelopmental origins of schizophrenia is related to the dysconnectivity hypothesis. The concept of schizophrenia being a disease of altered integration has been around for a long time, with psychiatrist Eugen Bleuler redefining schizophrenia as a splitting (*schizen)* of the mind's (*phren*) normally integrated processes in 1911[65]. The dysconnectivity hypothesis proposes that symptoms of schizophrenia arise from disturbed signaling within and between different brain regions, due to changes in neuronal connectivity and network organization[66–70]. A wealth of research has identified alterations in both structural (using magnetic resonance imaging and diffusion tensor imaging) and functional (using functional magnetic resonance imaging) measures of brain connectivity in schizophrenia, with many involving prefrontal brain regions[71]. Furthermore, brain network organization is associated with cognitive function in healthy subjects[72–76] and symptom severity in schizophrenia[77–81].

Accumulating evidence from genetic and neuropathological studies implies that changes in synapse density underlie these alterations in macroscale connectome organization in schizophrenia[82–86]. Repeatedly, studies have reported alterations in the density of pre- and post-synaptic elements in schizophrenia, and this phenotype is considered one of the most robust cellular aberrations found in schizophrenia postmortem tissue to date[84].

Synapse formation and elimination are dynamic neurodevelopmental processes. Synapses are generated in large amounts during childhood, upon which many less functional/essential synapses are eliminated during early adolescence[87]. Already in 1983, the hypothesis was stated that schizophrenia could be caused by increased synaptic elimination during postnatal development[88].

However, although literature on the role of synapse pathology in the dysconnectivity phenotype of schizophrenia is extensive, questions remain. Studies quantifying synapse number in schizophrenia show conflicting results. Heterogeneity exists between studies on several levels, such as variety in pre-and postmortem confounders, differences in methodological approaches, and brain regions studied. Therefore, it remains challenging to infer conclusions about (sub-)brain region specificity, and the robustness of the phenotype in general. Furthermore, not much is known about either the cell autonomous or non-cell autonomous processes causing synaptic deficits in schizophrenia.

Prenatal environmental risk factors

Environmental influences during early life, when the brain is rapidly developing, have found to be important for healthy brain development. Both epidemiological and preclinical studies have shown that various prenatal disturbances during pregnancy are associated to the increase of psychiatric disease in offspring, such as maternal infection, and prenatal nutritional deficits.

Maternal immune activation

Prenatal infections have long been associated to the development of psychiatric disorders. Birth cohort studies have shown that offspring from mothers who experienced an infection during pregnancy (shown by the presence of antibodies or elevated c-reactive protein level) are at increased risk to develop schizophrenia later in life[89–91]. These findings are corroborated by rodent and non-human primate studies of maternal immune activation. Inducing an inflammatory response in a pregnant animal, increasing peripheral cytokine expression using viral or bacterial inflammatory stimuli, leads to a plethora of symptoms in the offspring. Behavioural alterations include deficits in sensorimotor gating, repetitive behaviour, and atypical social interactions[92–99]. Furthermore, prenatal inflammation leads to long lasting alterations in neurodevelopment, involving general reductions in brain volume, cortical and hippocampal abnormalities, neuronal loss, deficient neuronal

migration, changed dopaminergic and serotonergic neurotransmitter system functioning, astrogliosis, and microglia activation[98,99]. However, it remains to be determined how these changes, observed in animal studies, translate to the human situation.

Prenatal nutrition

It is known that brain development is vulnerable to nutritional changes during early gestation[100–102]. Epidemiological studies have shown that maternal nutritional alterations during pregnancy increase the risk on psychiatric disease, such as schizophrenia, in the offspring[102–107]. For example, maternal famine increases the chance of schizophrenia by twofold[108]. Animal studies show that prenatal nutrition influences the developing brain in a structural way. Prenatal under- and over-nutrition, the availability of specific micronutrients (such as folate and vitamin A), and the composition of the diet, can affect neurogenesis, dendritic arborization, and glial phenotype[109–112]. However, "prenatal nutrition" is a broad concept, including many different components, leading to much heterogeneity among studies. Furthermore, studies disseminating the effect of maternal nutrition on specific signaling cascades and neurodevelopmental processes are scarce. Amino acids are a key component of nutrition and the essential amino acids need to be provided through the diet. Amino acids are best known as the building blocks of proteins, but also have an important regulatory function in the cell[113]. Specific amino acids have shown to influence mTOR activity. mTOR is involved in processes like cell growth, metabolism, and autophagy, and in neurodevelopment, regulating cortical structure formation through outer radial glia, timing of the gliogenic switch, axon formation and dendritic arborization[114–118]. Deregulation of mTOR function, due to genetic mutations or altered protein expression is involved in brain diseases, particularly developmental neuropsychiatric disorders such as autism spectrum disorder, schizophrenia, and tuberous sclerosis[114,119–122]. Therefore unravelling the effect of dietary amino acid changes on human neurodevelopment is of interest.

Basics of human cortical development.

Corticogenesis starts at the ventricular zone of the brain. Ventricular radial glia ($PAX6^+$) are formed at the ventricular zone around 15-17 gestational weeks (GW), making full basal-apical contacts, extending a long protrusion towards the cortical plate. Asymmetric division of these apical progenitors generates intermediate progenitors ($TBR2^+$), which migrate alongside a radial protrusion through the subventricular zone and intermediate zone towards the cortical plate. This process continues until mid-gestation (20 GW). In the cortical plate, the intermediate progenitors further develop into mature pyramidal neurons ($NEUN^+$). Cortical inhibitory neurons arise from the ganglionic eminences and migrate tangentially to integrate into the cortex. Importantly, outer radial glia ($PAX6^+$ and $HOPX^+$), present in the outer subventricular zone, increase the progenitor pool and the number of radial fibers that neurons can use to migrate towards the cortical plate in humans. The process of cortical layer formation is regulated in an "inside-out" manner, a process regulated by reelin signaling. Early-born intermediate progenitors end up forming the deeper layers of the cortical plate ($CTIP2^+$), while the late-born intermediate progenitors form the upper layers of the cortical plate ($SATB2^+$), later giving rise to the six distinct layers of the cortex. After cortical neurogenesis, in the second half of human fetal development, radial glia switch to producing glial progenitors, which develop into astrocytes and oligodendrocytes. A process known as the gliogenic switch, which continues postnatally. Mesodermal derived microglia progenitors invade the brain parenchyma from the blood stream as early as 4 GW and are fully colonizing the entire cerebral cortex from the middle of the second trimester. Neural network formation, including synaptogenesis, synaptic pruning, dendritic arborization, myelination, and development/finetuning of both excitatory and inhibitory neurotransmitter systems, are processes that continue well into adolescence, which is accompanied by an increase in cognitive executive function. The development of the brain postnatally is also emphasized by the great increase in brain weight in the first decade of life. (See Figure 1)[59,123–131]

Box 1. Basics of human cortical development.

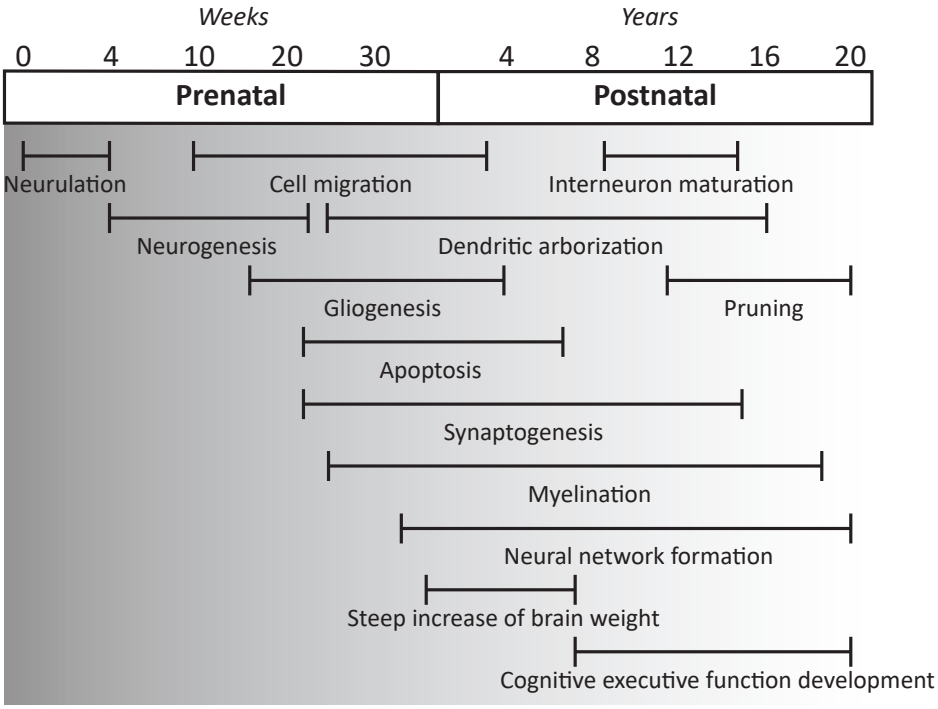


Figure 1. Schematic depiction of prenatal and postnatal neurodevelopmental processes.
During prenatal cortical development, a radial scaffold is formed in which neurons, astrocytes and oligodendrocytes are generated and migrate towards their accurate position in the six layered cortex to mature. During postnatal development the brain increases significantly in size, while processes concerning the interconnection of cells and different brain regions occur, coinciding with an increase in cognitive executive function development. This exemplifies that cortical neurodevelopment is a protracted process encompassing both prenatal and postnatal stages.

1.3 Role for glia

Classically, hypothesis-driven research about schizophrenia etiology has focused on neuronal disfunction. However, recently an increasing amount of research has been dedicated towards the contribution of glial cells, like astrocytes and microglia, in this disease.

Astrocytes

Astrocytes are often referred to as "the stars of the brain". Not only because of their highly ramified, starshaped appearance, but also because of the numerous functions they exert in the brain. Astrocytes originate from the neuroectoderm. After a process called the gliogenic switch, sub-ventricular zone radial glia cells switch from producing neuronal precursors, to generating glial progenitors[126]. Astrocytes are involved in maintaining

homeostasis, provide structural and metabolic support for other brain cells, are a component of the blood brain barrier, immunologically protect the brain parenchyma, and influence neuronal connectivity by chemically and physically interacting with synapses[132,133] (Figure 2).

Microglia

Microglia are known as the innate immune cells of the brain. These resident macrophages originate from mesodermal lineage in the yolk sac, from where primitive macrophages generated during primitive hematopoiesis infiltrate the brain parenchyma, where they further develop into microglia[134–136]. They are well-known for their function in inflammation, which includes secretion of pro- and anti-inflammatory cytokines, and phagocytosis of pathogens[137,138]. However, microglia are also found to be key players of neurodevelopment, as they eliminate excess neuronal progenitors, but also for their interaction with synapses and ability to eliminate them[139–142] (Figure 2).

Astrocyte and microglia involvement in schizophrenia

A role for glia in schizophrenia was first proposed by Bogerts in 1983: *'If the functional unit of the brain is not the neuron but rather the neuron-glial complex, then both neuronal and glial cells could be involved in mental diseases'*[143].

Glia are especially interesting as the evolutionary appearance of uniquely human disorders, such as schizophrenia, parallels that of glia evolution. Glia evolution accelerated with the appearance of hominids, as astroglial complexity and pleomorphism increased significantly, also microglia acquired human specific transcriptomic programs[144–146].

Genes

Large genetic and transcriptome studies have identified both astrocytic and microglial schizophrenia risk genes. The GWAS study on schizophrenia in 2014 showed that the major histocompatibility complex class II (MHC-II) locus, involved in the immune response, was the strongest genetic association with schizophrenia[147]. Later, a study assessing multiple psychiatric disorders, found a shared association among pathways involved in (among histone methylation and synaptic function) immune related genes[23]. Because of their important role in the immune system of the CNS, these findings point to a genetic fundament for microglial involvement in schizophrenia. Furthermore, functional gene set analysis, testing for combined effects of genetic variants in glial genes in schizophrenia GWAS data, showed a high significance for the astrocyte gene set[148]. The study of Gandal (2018b), including both schizophrenia GWAS and transcriptomic data, also identified an astrocyte gene cluster to be upregulated in schizophrenia[86]. These genetic studies underline the involvement of astrocytes in schizophrenia.

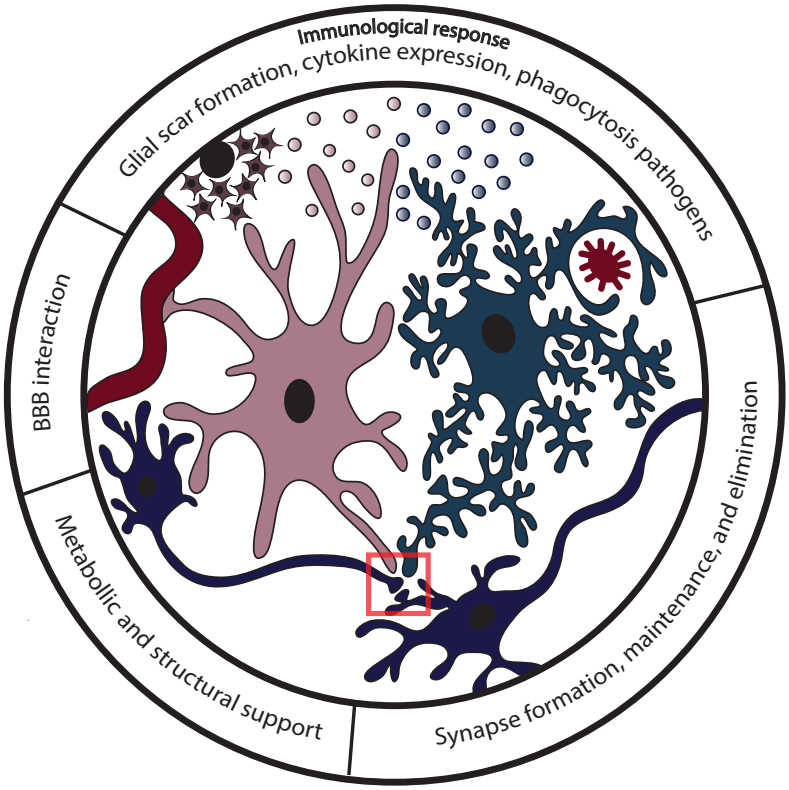


Figure 2. Functions of astrocytes and microglia.
Astrocytes (pink), and microglia (blue) are involved in many brain functions, such as structural and metabolic support, interaction with the blood-brain-barrier (BBB), the immunological response (containing glial scar formation, cytokine expression, and phagocytosis of foreign pathogens), and interact with neuronal (purple) synapses, forming the quad-partite synapse (red square) facilitating synapse formation, maturation, and elimination.

Immune activation in schizophrenia?

Astrocytes and microglia have been studied extensively in relation to the immune hypothesis in schizophrenia pathogenesis, posing that microglia and/or astrocytes are immunologically activated, leading to disease. The theory is based on above-described genetic findings, the co-occurrence of auto-immune and infectious disorders, and increased immunological markers (such as cytokines) in blood and CSF in patients with schizophrenia[149–155]. However, this field has been losing playing field because of many reports showing no widespread microglia activation and/or astrogliosis in postmortem human brain tissue of patients with schizophrenia[156,157]. Now, the focus on the role of astrocytes and microglia in schizophrenia has shifted to different perspectives.

Synapse elimination

Astrocytes and microglia both interact closely with synapses, forming the quad-partite synapse. In their own way, astrocytes and microglia are involved in the formation, maintenance, and pruning of synapses during neurodevelopment[158,159]. Therefore, these glia are considered key suspects regarding the decreased density of synapses identified in postmortem brain of schizophrenia patients. Especially their role in synaptic pruning is hypothesized to be involved in schizophrenia pathology.

Microglia are well-known as the "gardeners" of the brain, as for many years their role in synapse elimination has been observed[160]. Microglia prune away synapses through complement signaling. Complement factors C1q and C3 are expressed as "eat me" signals on synapses of specific neurons, which are recognized by the C3 receptor on microglia, which induces phagocytosis of the synapse[142,159]. Interestingly, astrocytes are also involved in this process. Astrocyte produced TGF-beta, inducing C1q expression in neurons, thereby promoting the elimination of synapses through microglial pruning[161,162]. However, astrocytes are also capable of actively pruning synapses themselves through the MEGF10 and MERTK phagocytic pathway[158].

The complement cascade has been strongly implied in schizophrenia pathogenesis. The major histocompatibility complex (MHC) stands out as the strongest region of association in multiple schizophrenia GWAS[147,163,164]. Complement factor 4a (C4a) was identified as an affected gene in this locus, as schizophrenia risk is associated to different C4a copy number variants, leading to increased expression of the gene[165–167].

Interestingly, a robust increase of glial mediated synaptic pruning during adolescence coincides with the general onset of psychotic symptoms in schizophrenia, further implicating glial functioning in the pathogenesis of schizophrenia.

Susceptibility to environmental change during neurodevelopment

Because of their reactive nature, astrocytes and microglia are found to be important relay stations between the outside environment and the CNS. Also, this makes them highly susceptible to environmental stimuli, such as stress, but also, inflammation and altered nutrition, especially during prenatal development[112,168–174].

Deviations in the glial developmental trajectory are thought to influence neurodevelopment actively, by phenotypically changing to their activated state, releasing cytokines, and impairing neurogenesis. But also, passively, by not exerting their regular functions, thereby reducing neuronal support[102,175–178]. Furthermore, these environmental effects on glial development, can cause a shift in cell type ratio's by changing the timing of the gliogenic switch, or affecting microglial regulation of the neural progenitor pool.

1.4 Translational approaches in schizophrenia

Studying the brain in psychiatry comes with challenges. Although informative on a macroscale level, *in vivo* neuroimaging studies do not provide the spatial resolution necessary for the study of microscale (sub-)cellular processes. And due to obvious ethical and functional implications, brain biopsies from living donors are not performed. Therefore, the field often relies on preclinical animal models to study disease mechanisms. However, the use of animal models to study symptoms related to higher cognitive functioning in schizophrenia is controversial. Furthermore, rodent and human brain differentiate from each other significantly, not just in size and complexity, but also in cellular and molecular processes involved during development, and in form and function of various types of brain-cells[144–146,179,180]. Altogether, translational approaches for the study of schizophrenia are necessary.

Postmortem human brain tissue

Postmortem human brain studies currently form the golden standard in research on the cellular and molecular basis of psychiatric disease. These tissues provide a valuable resource to study the diseased brain directly and in detail, and this approach has therefore been performed in schizophrenia quite extensively. The Netherlands Brain Bank (NBB) collects, processes, and stores postmortem human brain materials from premortem registered donors for research worldwide. Since 2012, the NBB started a program recruiting donors with psychiatric disorders specifically: NBB-Psychiatry (NBB-PSY). This has led to a novel cohort of schizophrenia postmortem brain tissue from the NBB-PSY initiative, which has been studied in this thesis. However, postmortem research comes with its own challenges, considering many confounders[181]. Importantly, this approach can only be applied for static "end-point" measurements and does not provide opportunity to study early neurodevelopmental disease processes.

Cerebral organoids

In 2013, Lancaster and colleagues introduced a novel 3D *in vitro* model, enabling the study of the human developing brain in a dish: the cerebral organoid model[182]. Induced pluripotent stem cells (iPSC), generated from human blood or skin cells, are grown in spheres. The spheres are grown in an environment in which they develop neuroectoderm, after which they further self-assemble into 3D brain structures. The cerebral organoids are shown to resemble the human development of the neocortex on transcriptomic, epigenomic and structural level[182–185]. These studies have shown that cerebral organoids cultured up to 100 days simulate fetal brain development at post-conceptional week 17-24. Furthermore, cerebral organoids originated from human iPSC show neurostructural phenotypes which are specific for the human developing cortex and not present in rodents, like the presence of an outer sub ventricular zone, with outer radial

glia[182,183]. As cerebral organoids mature, the system contains a multitude of different cell types: neurons, astrocytes, but as recently discovered, also microglia[182,186,187]. These different cell types form an integrated network in 3D, opposed to single cell type 2D *in vitro* iPSC derived approaches. Upon its discovery, multiple different variants of "brain organoids" have been developed, each with their own (dis)advantages[188–192]. The cerebral organoid model, and its derivatives, have previously been utilized to study neuropsychiatric disorders, among which (specific genetic variants of) schizophrenia, resulting in the identification of developmental abnormalities leading to cellular disfunction[193–196]. Furthermore, approaches exploring the effect of environmental stimuli on neurodevelopment have also been applied in these type of models, such as exposure to alcohol[197], nicotine[198], and drugs such as methamphetamine[199]. Altogether, the cerebral organoid model provides a unique translational opportunity to study human neurodevelopment *in vitro* in a dynamic system of alive cells.

Aim of this thesis

Aim

The aim of this thesis is to elucidate underlying neurodevelopmental molecular and cellular mechanisms of schizophrenia pathology in the (developing) cortex, with a specific focus on tissue specificity and environmental risk factors.

Hypothesis

We hypothesize that specific molecular and/or cellular neurodevelopmental processes are affected in schizophrenia pathogenesis.

Approach

We research tissue specific cortical abnormalities in schizophrenia using postmortem human brain tissue related to the dysconnectivity phenotype and DNA methylation (part I) and study the effect of potential prenatal environmental risk factors for developing schizophrenia on cortical development in an *in vitro* cerebral organoid model (part II).

Main research questions

Can we identify tissue specific cellular/molecular phenotypes related to postnatal neurodevelopment in the postmortem brain of schizophrenia patients?

- Are synapse density changes more prevalent in schizophrenia postmortem brain tissue compared to controls?
- Are synapse, microglia, or astrocyte number or phenotype altered in schizophrenia, and are they related to each other?
- Are tissue type specific DNA methylation patterns affected in schizophrenia?

Can we identify cellular/molecular phenotypes which are affected during early human neurodevelopment upon exposure to environmental risk factors for schizophrenia in cerebral organoids?

- Are early neurodevelopmental processes, and especially glial function, affected upon immune stimulation?
- Is there an mTOR mediated effect on neurodevelopment upon changed dietary exposure?

Thesis outline

Part 1 Tissue specific cortical abnormalities in schizophrenia using postmortem human brain tissue

Chapter 2. Changed synapse density has been suggested to be involved in altered brain connectivity underlying schizophrenia pathology. However, postmortem studies addressing this topic are heterogeneous and it is not known whether changes are restricted to specific brain regions. We systematically described and meta-analysed published literature on quantitative measures of postsynaptic elements in both subcortical and cortical postmortem human brain tissue comparing patients with schizophrenia and non-psychiatric controls.

Chapter 3. Glial cells, like microglia and astrocytes, have repeatedly been implicated to contribute to the synapse decrease found in schizophrenia. However, few studies have investigated synapses and glia simultaneously, and the importance of spatial heterogeneity of the cortical layers has been underappreciated. In this study we performed an extensive phenotypical analysis of synapses, microglia, astrocytes and their association in cortical grey matter, and layer III specifically, in postmortem human cortical brain tissue comparing patients with schizophrenia and non-psychiatric controls.

Chapter 4. Epigenetic regulation, such as DNA methylation, which functions as a mediator of both genetic differences and environmental risk factors involved in schizophrenia pathology, is hypothesized to contribute to changes in cortical functioning in schizophrenia. Therefore, we performed an explorative epigenome-wide DNA methylation study, separating grey and white matter, in cortical postmortem human brain tissue, comparing patients with schizophrenia and non-psychiatric controls.

Part II The effect of potential prenatal environmental risk factors for developing schizophrenia on neurodevelopment in cerebral organoids

Chapter 5. Maternal infection during pregnancy is a risk factor for developing schizophrenia and other psychiatric illness in the offspring. It remains difficult to investigate how early human development is affected in human subjects or with the use of animal models. We investigated the effect of prenatal immune activation on neurodevelopment by exposing developing cerebral organoids to lipopolysaccharide.

Chapter 6. Prenatal nutrition is a key modifier of neurodevelopment and affects the risk on a range of neuropsychiatric conditions such as schizophrenia. Specific alterations in amino acid ratios show to affect mTOR activity in cells and change mouse behaviour. In

our study, we researched the effect of specific amino acid alterations on mTOR activation, general growth, and transcriptomics in human neurodevelopment by exposing cerebral organoids to increased concentrations of the three amino acids Histidine, Lysine, and Threonine.

Chapter 7. Here, a summary of the findings from this thesis is given and the conclusions, limitations, and recommendations for further research are discussed.

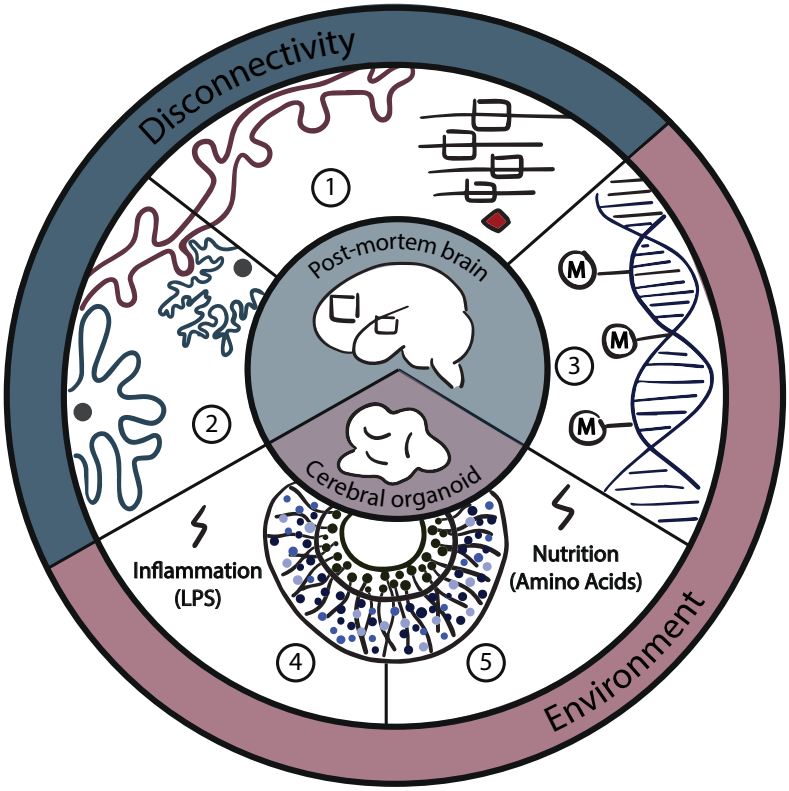


Figure 3. Schematic overview of the different chapters of this thesis and their connection.
The different thesis chapters are studies on post-and pre-natal neurodevelopmental phenotypes of schizophrenia which were performed using postmortem human brain tissue or cerebral organoids, studying the dysconnectivity phenotype or environmental risk factors.

References

1. James, S. L. *et al.* Global, regional, and national incidence, prevalence, and years lived with disability for 354 Diseases and Injuries for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet* **392**, 1789–1858 (2018).
2. Saha, S., Chant, D., Welham, J. & McGrath, J. A systematic review of the prevalence of schizophrenia. *PLoS medicine* **2**, e141 (2005).
3. McGrath, J., Saha, S., Chant, D. & Welham, J. Schizophrenia: A Concise Overview of Incidence, Prevalence, and Mortality. *Epidemiologic Reviews* **30**, 67–76 (2008).
4. Simeone, J. C., Ward, A. J., Rotella, P., Collins, J. & Windisch, R. An evaluation of variation in published estimates of schizophrenia prevalence from 1990–2013: a systematic literature review. *BMC Psychiatry* **15**, 193 (2015).
5. Kahn, R. S. *et al.* Schizophrenia. *Nature Reviews Disease Primers* **1**, (2015).
6. *Substance Abuse and Mental Health Services Administration. Impact of the DSM-IV to DSM-5 Changes on the National Survey on Drug Use and Health [Internet]. Rockville (MD): Substance Abuse and Mental Health Services Administration (US); 2016 Jun. PMID: 3019*.
7. Lambert, T., Velakoulis, D. & Pantelis, C. Medical comorbidity in schizophrenia. *The Medical journal of Australia* **178**, (2003).
8. Laursen, T., Nordentoft, M. & Mortensen, P. Excess early mortality in schizophrenia. *Annual review of clinical psychology* **10**, 425–448 (2014).
9. Green, A. I., Canuso, C. M., Brenner, M. J. & Wojcik, J. D. Detection and management of comorbidity in patients with schizophrenia. *Psychiatric Clinics* **26**, 115–139 (2003).
10. Buckley, P., Miller, B., Lehrer, D. & Castle, D. Psychiatric comorbidities and schizophrenia. *Schizophrenia bulletin* **35**, 383–402 (2009).
11. WHO Schizophrenia - key facts (2019). Available at https://www.who.int/news-room/fact-sheets/detail/schizophrenia. https://www.who.int/news-room/fact-sheets/detail/schizophrenia.
12. Kahn, R. S. & Keefe, R. S. E. Schizophrenia Is a Cognitive Illness: Time for a Change in Focus. *JAMA Psychiatry* **70**, 1107–1112 (2013).
13. Kahn, R. S. *et al.* Schizophrenia. *Nature Reviews Disease Primers 2015 1:1* **1**, 1–23 (2015).
14. McDonagh, M. S. *et al.* Psychosocial Interventions for Adults With Schizophrenia: An Overview and Update of Systematic Reviews. *Psychiatric services (Washington, D.C.)* appips202000649 (2021) doi:10.1176/appi.ps.202000649.
15. Stępnicki, P., Kondej, M. & Kaczor, A. Current Concepts and Treatments of Schizophrenia. *Molecules (Basel, Switzerland)* **23**, (2018).
16. Sullivan, P. F., Kendler, K. S. & Neale, M. C. Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch Gen Psychiatry* **60**, 1187–1192 (2003).
17. Lichtenstein, P. *et al.* Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *Lancet (London, England)* **373**, 234–239 (2009).
18. DeLisi, L. E. An Overview and Current Perspective on Family Studies of Schizophrenia. *Handbook of Neurochemistry and Molecular Neurobiology* 493–504 (2009) doi:10.1007/978-0-387-30410-6_15.
19. Allen, N. *et al.* Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. *Nature genetics* **40**, 827–834 (2008).
20. The Schizophrenia Working Group of the Psychiatric Genomics Consortium, Ripke, S., Walters, J. T. & O'Donovan, M. C. Mapping genomic loci prioritises genes and implicates synaptic biology in schizophrenia. *medRxiv* 2020.09.12.20192922 (2020) doi:10.1101/2020.09.12.20192922.

21. Marshall, C. R. *et al.* Contribution of copy number variants to schizophrenia from a genome-wide study of 41,321 subjects. *Nature genetics* **49**, 27 (2017).

22. Purcell, S. *et al.* A polygenic burden of rare disruptive mutations in schizophrenia. *Nature* **506**, 185–190 (2014).

23. Gandal, M. J. *et al.* Shared molecular neuropathology across major psychiatric disorders parallels polygenic overlap. *Science (New York, N.Y.)* **359**, 693–697 (2018).

24. Sullivan, P. F. The Psychiatric GWAS Consortium: Big science comes to psychiatry. *Neuron* vol. 68 182–186 (2010).

25. Lee, S. H. *et al.* Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nature Genetics* **45**, 984–994 (2013).

26. Purcell, S. M. *et al.* Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* **460**, 748–752 (2009).

27. Stilo, S. & Murray, R. Non-Genetic Factors in Schizophrenia. *Current psychiatry reports* **21**, (2019).

28. Robinson, N. & Bergen, S. E. Environmental Risk Factors for Schizophrenia and Bipolar Disorder and Their Relationship to Genetic Risk: Current Knowledge and Future Directions. *Frontiers in Genetics* **12**, (2021).

29. Boks, M. P. *et al.* The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* **4**, 6767 (2009).

30. Kofink, D., Boks, M. P. M., Timmers, H. T. M. & Kas, M. J. *Epigenetic dynamics in psychiatric disorders: Environmental programming of neurodevelopmental processes*. (Neuroscience and Biobehavioral Reviews, 2013).

31. Labrie, V., Pai, S. & Petronis, A. Epigenetics of major psychosis: Progress, problems and perspectives. *Trends in Genetics* vol. 28 427–435 (2012).

32. Pidsley, R. *et al.* Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology* **15**, (2014).

33. Rutten, B. P. F. & Mill, J. Epigenetic mediation of environmental influences in major psychotic disorders. *Schizophrenia Bulletin* vol. 35 1045–1056 (2009).

34. Chen, Q. *et al.* Research Progress on the Correlation Between Epigenetics and Schizophrenia. *Frontiers in neuroscience* **15**, (2021).

35. Perzel Mandell, K. A. *et al.* Genome-wide sequencing-based identification of methylation quantitative trait loci and their role in schizophrenia risk. *Nature Communications 2021 12:1* **12**, 1–12 (2021).

36. Montano, C. *et al.* Association of DNA Methylation Differences With Schizophrenia in an Epigenome-Wide Association Study. *JAMA Psychiatry* **73**, 506–514 (2016).

37. Grayson, D. *et al.* Reelin promoter hypermethylation in schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 9341–9346 (2005).

38. Abdolmaleky, H. M. *et al.* Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *Human molecular genetics* **15**, 3132 (2006).

39. Plum, F. Prospects for research on schizophrenia. 3. Neurophysiology. Neuropathological findings. *Neurosci Res Program Bull.* **10**, 384–8. (19972).

40. Olabi, B. *et al.* Are there progressive brain changes in schizophrenia? *A meta-analysis of structural magnetic resonance imaging studies* **70**, 88–96 (2011).

41. Wright, I. *et al.* Meta-analysis of regional brain volumes in schizophrenia. *Am J Psychiatry* **157**, 16–25 (2000).

42. Cahn, W. *et al.* Brain volume changes in the first year of illness and 5-year outcome of schizophrenia. *British Journal of Psychiatry* **189**, 381–382 (2006).

43. Haijma, S. V. *et al.* Brain Volumes in Schizophrenia: A Meta-Analysis in Over 18 000 Subjects. *Schizophrenia Bulletin* **39**, 1129–1138 (2013).

44. Kyriakopoulos, M., Vyas, N. S., Barker, G. J., Chitnis, X. A. & Frangou, S. A Diffusion Tensor Imaging Study of White Matter in Early-Onset Schizophrenia. *Biological Psychiatry* **63**, 519–523 (2008).

45. Mandl, R. C. W. *et al.* Altered white matter connectivity in never-medicated patients with schizophrenia. *Human Brain Mapping* **34**, 2353–2365 (2013).

46. Harrison, P. J. Postmortem studies in schizophrenia. *Dialogues in clinical neuroscience* **2**, 349–57 (2000).

47. Brown, R. *et al.* Postmortem Evidence of Structural Brain Changes in Schizophrenia: Differences in Brain Weight, Temporal Horn Area, and Parahippocampal Gyrus Compared With Affective Disorder. *Archives of General Psychiatry* **43**, 36–42 (1986).

48. Flynn, S. W. *et al.* Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. *Molecular Psychiatry* **8**, 811–820 (2003).

49. Lin, D. *et al.* Network modules linking expression and methylation in prefrontal cortex of schizophrenia. *Epigenetics* 1–18 (2020) doi:10.1080/15592294.2020.1827718.

50. Abé, C. *et al.* Genetic risk for bipolar disorder and schizophrenia predicts structure and function of the ventromedial prefrontal cortex. *Journal of psychiatry & neuroscience : JPN* **46**, E441–E450 (2021).

51. Jones, P., Murray, R., Jones, P., Rodgers, B. & Marmot, M. Child developmental risk factors for adult schizophrenia in the British 1946 birth cohort. *The Lancet* **344**, 1398–1402 (1994).

52. Cannon, M. *et al.* Evidence for Early-Childhood, Pan-Developmental Impairment Specific to Schizophreniform Disorder: Results From a Longitudinal Birth Cohort. *Archives of General Psychiatry* **59**, 449–456 (2002).

53. Seidman, L. J. *et al.* Neuropsychological performance and family history in children at age 7 who develop adult schizophrenia or bipolar psychosis in the New England Family Studies. *Psychological Medicine* **43**, 119–131 (2013).

54. Khandaker, G. M., Barnett, J. H., White, I. R. & Jones, P. B. A quantitative meta-analysis of population-based studies of premorbid intelligence and schizophrenia. *Schizophrenia Research* **132**, 220–227 (2011).

55. Schulz, J., Sundin, J., Leask, S. & Done, D. J. Risk of Adult Schizophrenia and Its Relationship to Childhood IQ in the 1958 British Birth Cohort. *Schizophrenia Bulletin* **40**, 143–151 (2014).

56. Pantelis, C., Yücel, M., Wood, S. J., McGorry, P. D. & Velakoulis, D. Early and late neurodevelopmental disturbances in schizophrenia and their functional consequences. *Australian and New Zealand Journal of Psychiatry* vol. 37 399–406 (2003).

57. Birnbaum, R. & Weinberger, D. R. Genetic insights into the neurodevelopmental origins of schizophrenia. *Nature Reviews Neuroscience* vol. 18 727–740 (2017).

58. Jaffe, A. E. *et al.* Developmental and genetic regulation of the human cortex transcriptome illuminate schizophrenia pathogenesis. *Nature Neuroscience* **21**, 1117–1125 (2018).

59. Catts, V. S. *et al.* Rethinking schizophrenia in the context of normal neurodevelopment. *Frontiers in Cellular Neuroscience* vol. 7 60 (2013).

60. Weinberger, D. R. Implications of Normal Brain Development for the Pathogenesis of Schizophrenia. *Archives of General Psychiatry* **44**, 660–669 (1987).

61. Insel, T. R. Rethinking schizophrenia. *Nature 2010 468:7321* **468**, 187–193 (2010).

62. Collin, G. & van den Heuvel, M. The ontogeny of the human connectome: development and dynamic changes of brain connectivity across the life span. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **19**, 616–628 (2013).

63. Dennis, E. *et al.* Development of brain structural connectivity between ages 12 and 30: a 4-Tesla diffusion imaging study in 439 adolescents and adults. *NeuroImage* **64**, 671–684 (2013).

64. Chini, M. & Hanganu-Opatz, I. Prefrontal Cortex Development in Health and Disease: Lessons from Rodents and Humans. *Trends in neurosciences* **44**, 227–240 (2021).

65. Bleuler, E. Dementia praecox or the group of schizophrenias. *International Universities Press.* (1950).

66. van den Heuvel, M. P. *et al.* Abnormal Rich Club Organization and Functional Brain Dynamics in Schizophrenia. *JAMA Psychiatry* **70**, 783 (2013).

67. Pettersson-Yeo, W. *et al.* Using genetic, cognitive and multi-modal neuroimaging data to identify ultra-high-risk and first-episode psychosis at the individual level. *Psychological Medicine* **43**, 2547–2562 (2013).

68. Anticevic, A. *et al.* Connectivity, pharmacology, and computation: toward a mechanistic understanding of neural system dysfunction in schizophrenia. *Frontiers in psychiatry* **4**, 169 (2013).

69. Friston, K. J. & Frith, C. D. Schizophrenia: a disconnection syndrome? *Clinical neuroscience (New York, N.Y.)* **3**, 89–97 (1995).

70. Stephan, K. E., Friston, K. J. & Frith, C. D. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. *Schizophrenia bulletin* **35**, 509–27 (2009).

71. Van Den Heuvel, M. P. & Fornito, A. Brain networks in schizophrenia. *Neuropsychology Review* vol. 24 32–48 (2014).

72. Bassett, D. *et al.* Cognitive fitness of cost-efficient brain functional networks. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 11747–11752 (2009).

73. Li, Y. *et al.* Brain anatomical network and intelligence. *PLoS computational biology* **5**, (2009).

74. van den Heuvel, M., Stam, C., Kahn, R. & Hulshoff Pol, H. Efficiency of functional brain networks and intellectual performance. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 7619–7624 (2009).

75. Cole, M., Yarkoni, T., Repovs, G., Anticevic, A. & Braver, T. Global connectivity of prefrontal cortex predicts cognitive control and intelligence. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 8988–8999 (2012).

76. Reijmer, Y. *et al.* Disruption of cerebral networks and cognitive impairment in Alzheimer disease. *Neurology* **80**, 1370–1377 (2013).

77. Skudlarski, P. *et al.* Brain connectivity is not only lower but different in schizophrenia: A combined anatomical and functional approach. *Biological psychiatry* **68**, 61 (2010).

78. Wang, Q. *et al.* Anatomical insights into disrupted small-world networks in schizophrenia. *NeuroImage* **59**, 1085–1093 (2012).

79. Yu, Q. *et al.* Altered Topological Properties of Functional Network Connectivity in Schizophrenia during Resting State: A Small-World Brain Network Study. *PLoS ONE* **6**, (2011).

80. Cole, M., Anticevic, A., Repovs, G. & Barch, D. Variable global dysconnectivity and individual differences in schizophrenia. *Biological psychiatry* **70**, 43–50 (2011).

81. Repovs, G., Csernansky, J. & Barch, D. Brain network connectivity in individuals with schizophrenia and their siblings. *Biological psychiatry* **69**, 967–973 (2011).

82. Schijven, D. *et al.* Comprehensive pathway analyses of schizophrenia risk loci point to dysfunctional postsynaptic signaling. *Schizophrenia Research* **199**, 195–202 (2018).

83. Soler, J. *et al.* Genetic variability in scaffolding proteins and risk for schizophrenia and autism-spectrum disorders: a systematic review. *Journal of psychiatry & neuroscience : JPN* **43**, 223–244 (2018).

84. Moyer, C. E., Shelton, M. A. & Sweet, R. A. Dendritic spine alterations in schizophrenia. *Neuroscience letters* **601**, 46–53 (2015).

85. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular Psychiatry* (2018) doi:10.1038/s41380-018-0041-5.

86. Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science (New York, N.Y.)* **362**, eaat8127 (2018).

87. Petanjek, Z. *et al. Extraordinary neoteny of synaptic spines in the human prefrontal cortex*. *Proceedings of the National Academy of Sciences of the United States of America* vol. 108 (Proc Natl Acad Sci U S A, 2011).

88. Feinberg, I. Schizophrenia: Caused by a fault in programmed synaptic elimination during adolescence? *Journal of Psychiatric Research* **17**, 319–334 (1982).

89. Canetta, S. *et al.* Elevated maternal C-reactive protein and increased risk of schizophrenia in a national birth cohort. *American Journal of Psychiatry* **171**, 960–968 (2014).

90. Brown, A. S. & Derkits, E. J. Prenatal infection and schizophrenia: A review of epidemiologic and translational studies. *American Journal of Psychiatry* vol. 167 261–280 (2010).

91. Debost, J. C. P. G. *et al.* Joint effects of exposure to prenatal infection and peripubertal psychological trauma in schizophrenia. *Schizophrenia Bulletin* **43**, 171–179 (2017).

92. Machado, C. J., Whitaker, A. M., Smith, S. E. P., Patterson, P. H. & Bauman, M. D. Maternal immune activation in nonhuman primates alters social attention in juvenile offspring. *Biological Psychiatry* **77**, 823–832 (2015).

93. Meyer, U. Prenatal Poly(I:C) exposure and other developmental immune activation models in rodent systems. *Biological Psychiatry* vol. 75 307–315 (2014).

94. Meyer, U., Feldon, J. & Fatemi, S. H. In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders. *Neuroscience and Biobehavioral Reviews* vol. 33 1061–1079 (2009).

95. Schwartzer, J. J. *et al.* Behavioral impact of maternal allergic-asthma in two genetically distinct mouse strains. *Brain, Behavior, and Immunity* **63**, 99–107 (2017).

96. Malkova, N. v., Yu, C. Z., Hsiao, E. Y., Moore, M. J. & Patterson, P. H. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, Behavior, and Immunity* **26**, 607–616 (2012).

97. Bauman, M. D. *et al.* Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological Psychiatry* vol. 75 332–341 (2014).

98. Brown, A. S. & Conway, F. Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Frontiers in Psychiatry* vol. 10 430 (2019).

99. Bergdolt, L. & Dunaevsky, A. Brain changes in a maternal immune activation model of neurodevelopmental brain disorders. *Progress in Neurobiology* vol. 175 1–19 (2019).

100. Cheatham, C. L. Nutritional Factors in Fetal and Infant Brain Development. *Annals of Nutrition and Metabolism* **75**, 20–32 (2019).

101. Georgieff, M. K., Ramel, S. E. & Cusick, S. E. Nutritional Influences on Brain Development. *Acta paediatrica (Oslo, Norway : 1992)* **107**, 1310 (2018).

102. Bordeleau, M., Fernández de Cossío, L., Chakravarty, M. M. & Tremblay, M.-È. From Maternal Diet to Neurodevelopmental Disorders: A Story of Neuroinflammation. *Frontiers in Cellular Neuroscience* **0**, 461 (2021).

103. de Rooij, S. R., Wouters, H., Yonker, J. E., Painter, R. C. & Roseboom, T. J. Prenatal undernutrition and cognitive function in late adulthood. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 16881–16886 (2010).

104. Hoek, H. W., Brown, A. S. & Susser, E. The Dutch Famine and schizophrenia spectrum disorders. in *Social Psychiatry and Psychiatric Epidemiology* vol. 33 373–379 (Soc Psychiatry Psychiatr Epidemiol, 1998).

105. Kang, Y. *et al.* Nutritional Deficiency in Early Life Facilitates Aging-Associated Cognitive Decline. *Current Alzheimer Research* **14**, (2017).

106. Li, M., Francis, E., Hinkle, S. N., Ajjarapu, A. S. & Zhang, C. Preconception and prenatal nutrition and neurodevelopmental disorders: A systematic review and meta-analysis. *Nutrients* vol. 11 (2019).

107. Peretti, S. *et al.* Diet: the keystone of autism spectrum disorder? *Nutritional Neuroscience* vol. 22 825–839 (2019).

108. St Clair, D. *et al.* Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. *Journal of the American Medical Association* **294**, 557–562 (2005).

109. Salas, M., Torrero, C., Rubio, L. & Regalado, M. Effects of perinatal undernutrition on the development of neurons in the rat insular cortex. *Nutritional Neuroscience* **15**, 20–25 (2012).

110. Watanabe, K., Furumizo, Y., Usui, T., Hattori, Y. & Uemura, T. Nutrient-dependent increased dendritic arborization of somatosensory neurons. *Genes to Cells* **22**, 105–114 (2017).

111. Marques, A. H., Bjørke-Monsen, A. L., Teixeira, A. L. & Silverman, M. N. Maternal stress, nutrition and physical activity: Impact on immune function, CNS development and psychopathology. *Brain Research* **1617**, 28–46 (2015).

112. Abbink, M. R., van Deijk, A. L. F., Heine, V. M., Verheijen, M. H. & Korosi, A. The involvement of astrocytes in early-life adversity induced programming of the brain. *GLIA* vol. 67 1637–1653 (2019).

113. Wu, G. Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1–17 (2009).

114. Bockaert, J. & Marin, P. mTOR in Brain Physiology and Pathologies. *Physiological Reviews* **95**, 1157–1187 (2015).

115. Swiech, L., Perycz, M., Malik, A. & Jaworski, J. Role of mTOR in physiology and pathology of the nervous system. *Biochimica et Biophysica Acta - Proteins and Proteomics* vol. 1784 116–132 (2008).

116. Andrews, M. G., Subramanian, L. & Kriegstein, A. R. Mtor signaling regulates the morphology and migration of outer radial glia in developing human cortex. *eLife* **9**, 1–21 (2020).

117. Cloëtta, D. *et al.* Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis. *Journal of Neuroscience* **33**, 7799–7810 (2013).

118. Blair, J. D., Hockemeyer, D. & Bateup, H. S. Genetically engineered human cortical spheroid models of tuberous sclerosis. *Nature Medicine* **24**, 1568–1578 (2018).

119. Winden, K. D., Ebrahimi-Fakhari, D. & Sahin, M. Abnormal mTOR Activation in Autism. *Annual Review of Neuroscience* vol. 41 1–23 (2018).

120. Howell, K. R. & Law, A. J. Neurodevelopmental concepts of schizophrenia in the genome-wide association era: AKT/mTOR signaling as a pathological mediator of genetic and environmental programming during development. *Schizophrenia Research* **217**, 95–104 (2020).

121. Curatolo, P., Moavero, R. & de Vries, P. J. Neurological and neuropsychiatric aspects of tuberous sclerosis complex. *The Lancet Neurology* vol. 14 733–745 (2015).

122. Zimmer, T. S. *et al.* Tuberous Sclerosis Complex as Disease Model for Investigating mTOR-Related Gliopathy During Epileptogenesis. *Frontiers in Neurology* **0**, 1028 (2020).

123. Howard, B. M. *et al.* Radial Glia Cells in the Developing Human Brain. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **14**, 459 (2008).

124. Lui, J. H., Hansen, D. v. & Kriegstein, A. R. Development and evolution of the human neocortex. *Cell* vol. 146 18–36 (2011).

125. Pollen, A. A. *et al.* Molecular Identity of Human Outer Radial Glia during Cortical Development. *Cell* **163**, 55–67 (2015).

126. Holst, C. B., Brøchner, C. B., Vitting-Seerup, K. & Møllgård, K. Astrogliogenesis in human fetal brain: complex spatiotemporal immunoreactivity patterns of <scp>GFAP</scp> , S100, <scp>AQP</scp> 4 and <scp>YKL</scp> -40. *Journal of Anatomy* **235**, 590–615 (2019).

127. Monier, A., Evrard, P., Gressens, P. & Verney, C. Distribution and differentiation of microglia in the human encephalon during the first two trimesters of gestation. *The Journal of Comparative Neurology* **499**, 565–582 (2006).

128. Menassa, D. A. & Gomez-Nicola, D. Microglial dynamics during human brain development. *Frontiers in Immunology* vol. 9 1014 (2018).

129. Anderson, S. A., Eisenstat, D. D., Shi, L. & Rubenstein, J. L. R. Interneuron migration from basal forebrain to neocortex: Dependence on Dlx genes. *Science* **278**, 474–476 (1997).

130. Tamamaki, N., Fujimori, K. E. & Takauji, R. Origin and Route of Tangentially Migrating Neurons in the Developing Neocortical Intermediate Zone. *Journal of Neuroscience* **17**, 8313–8323 (1997).

131. Ayala, R., Shu, T. & Tsai, L.-H. Trekking across the Brain: The Journey of Neuronal Migration. *Cell* **128**, 29–43 (2007).

132. Verkhratsky, A. & Butt, A. Glial Physiology and Pathophysiology. *Glial Physiology and Pathophysiology* (2013) doi:10.1002/9781118402061.

133. Escartin, C. *et al.* Reactive astrocyte nomenclature, definitions, and future directions. *Nature neuroscience* **24**, 312–325 (2021).

134. Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)* **330**, 841–5 (2010).

135. Kierdorf, K. *et al.* Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nature neuroscience* **16**, 273–80 (2013).

136. Goldmann, T. *et al.* Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature immunology* **17**, 797–805 (2016).

137. Helmut, K., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiological Reviews* **91**, 461–553 (2011).

138. Verkhratsky, A., Sun, D. & Tanaka, J. Snapshot of microglial physiological functions. *Neurochemistry International* **144**, 104960 (2021).

139. Miyamoto, A. *et al.* Microglia contact induces synapse formation in developing somatosensory cortex. *Nature communications* **7**, 12540 (2016).

140. Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* **333**, 1456–1458 (2011).

141. Stevens, B. *et al.* The classical complement cascade mediates CNS synapse elimination. *Cell* **131**, 1164–78 (2007).

142. Stevens, B. *et al.* The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131**, 1164–1178 (2007).

143. Bogerts, B., Häntsch, J. & Herzer, M. A morphometric study of the dopamine-containing cell groups in the mesencephalon of normals, Parkinson patients, and schizophrenics. *Biol Psychiatry.* **18**, 951–69 (1983).

144. Verkhratsky, A. & Nedergaard, M. The homeostatic astroglia emerges from evolutionary specialization of neural cells. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **371**, (2016).

145. Oberheim, N. A. *et al.* Uniquely hominid features of adult human astrocytes. *Journal of Neuroscience* **29**, 3276–3287 (2009).

146. Geirsdottir, L. *et al.* Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell* **179**, 1609-1622.e16 (2019).

147. Ripke, S. *et al.* Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421–427 (2014).

148. Goudriaan, A. *et al.* Specific glial functions contribute to schizophrenia susceptibility. *Schizophrenia bulletin* **40**, 925–35 (2014).

149. Benros, M. E., Eaton, W. W. & Mortensen, P. B. The epidemiologic evidence linking autoimmune diseases and psychosis. *Biological psychiatry* **75**, 300–6 (2014).

150. Benros, M. E., Eaton, W. W. & Mortensen, P. B. The epidemiologic evidence linking autoimmune diseases and psychosis. *Biological psychiatry* **75**, 300–6 (2014).

151. Pedersen, M. S., Benros, M. E., Agerbo, E., Børglum, A. D. & Mortensen, P. B. Schizophrenia in patients with atopic disorders with particular emphasis on asthma: a Danish population-based study. *Schizophrenia research* **138**, 58–62 (2012).

152. Miller, B. J. & Goldsmith, D. R. *Towards an immunophenotype of schizophrenia: progress, potential mechanisms, and future directions*. (Neuropsychopharmacology, 2016).

153. Trépanier, M. O., Hopperton, K. E., Mizrahi, R., Mechawar, N. & Bazinet, R. P. Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. *Molecular psychiatry* **21**, 1009–26 (2016).

154. Trépanier, M. O., Hopperton, K. E., Mizrahi, R., Mechawar, N. & Bazinet, R. P. Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. *Molecular psychiatry* **21**, 1009–26 (2016).

155. Mansur, R. B. *et al.* Cytokines in schizophrenia: possible role of anti-inflammatory medications in clinical and preclinical stages. *Psychiatry and clinical neurosciences* **66**, 247–60 (2012).

156. Trépanier, M. O., Hopperton, K. E., Mizrahi, R., Mechawar, N. & Bazinet, R. P. Postmortem evidence of cerebral inflammation in schizophrenia: A systematic review. *Molecular Psychiatry* vol. 21 1009–1026 (2016).

157. Snijders, G. J. L. J. *et al.* A loss of mature microglial markers without immune activation in schizophrenia. *Glia* **69**, 1251–1267 (2021).

158. Chung, W. S. *et al.* Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* **504**, 394–400 (2013).

159. Schafer, D. P. *et al.* Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74**, 691–705 (2012).

160. Hughes, V. Microglia: The constant gardeners. *Nature* **485**, 570–572 (2012).

161. Bialas, A. R. & Stevens, B. TGF-β Signaling Regulates Neuronal C1q Expression and Developmental Synaptic Refinement. *Nature neuroscience* **16**, 1773 (2013).

162. Schafer, D. P., Lehrman, E. K. & Stevens, B. The "quad-partite" synapse: Microglia-synapse interactions in the developing and mature CNS. *GLIA* **61**, 24–36 (2013).

163. International Schizophrenia Consortium *et al.* Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* **460**, 748–52 (2009).

164. Shi, J. *et al.* Common variants on chromosome 6p22.1 are associated with schizophrenia. *Nature* **460**, 753–7 (2009).

165. Sekar, A. *et al.* Schizophrenia risk from complex variation of complement component 4. *Nature* **530**, 177–83 (2016).

166. Woo, J. J., Pouget, J. G., Zai, C. C. & Kennedy, J. L. The complement system in schizophrenia: where are we now and what's next? *Molecular Psychiatry 2019 25:1* **25**, 114–130 (2019).

167. Yilmaz, M. *et al.* Overexpression of schizophrenia susceptibility factor human complement C4A promotes excessive synaptic loss and behavioral changes in mice. *Nature Neuroscience 2020 24:2* **24**, 214–224 (2020).

168. Hanamsagar, R. & Bilbo, S. D. Environment Matters: Microglia Function and Dysfunction in a Changing World. *Current opinion in neurobiology* **47**, 146 (2017).

169. Frank, M. G., Fonken, L. K., Watkins, L. R. & Maier, S. F. Microglia: Neuroimmune-sensors of stress. *Seminars in Cell & Developmental Biology* **94**, 176–185 (2019).

170. Murphy-Royal, C., Gordon, G. R. & Bains, J. S. Stress-induced structural and functional modifications of astrocytes-Further implicating glia in the central response to stress. *Glia* **67**, 1806–1820 (2019).

171. Ozaki, K. *et al.* Maternal immune activation induces sustained changes in fetal microglia motility. *Scientific Reports 2020 10:1* **10**, 1–19 (2020).

172. Lago-Baldaia, I., Fernandes, V. M. & Ackerman, S. D. More Than Mortar: Glia as Architects of Nervous System Development and Disease. *Frontiers in Cell and Developmental Biology* **0**, 1527 (2020).

173. Canales, C. P. *et al.* A temporal map of maternal immune activation-induced changes reveals a shift in neurodevelopmental timing and perturbed cortical development in mice. *bioRxiv* 2020.06.13.150359 (2020) doi:10.1101/2020.06.13.150359.

174. Chagas, L. da S. *et al.* Environmental Signals on Microglial Function during Brain Development, Neuroplasticity, and Disease. *International Journal of Molecular Sciences* **21**, (2020).

175. Cunningham, C. L., Martínez-Cerdeño, V. & Noctor, S. C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *Journal of Neuroscience* **33**, 4216–4233 (2013).

176. Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481–487 (2017).

177. Wallace, J., Lord, J., Dissing-Olesen, L., Stevens, B. & Murthy, V. N. Microglial depletion disrupts normal functional development of adult-born neurons in the olfactory bulb. *eLife* **9**, (2020).

178. Windrem, M. S. *et al.* Human Glial Progenitor Cells Effectively Remyelinate the Demyelinated Adult Brain. *Cell reports* **31**, 107658 (2020).

179. Hodge, R. D. *et al.* Conserved cell types with divergent features in human versus mouse cortex. *Nature 2019 573:7772* **573**, 61–68 (2019).

180. Lui, J. H., Hansen, D. V. & Kriegstein, A. R. Development and evolution of the human neocortex. *Cell* vol. 146 18–36 (2011).

181. Lewis, D. A. The Human Brain Revisited: Opportunities and Challenges in Postmortem Studies of Psychiatric Disorders. *Neuropsychopharmacology 2002 26:2* **26**, 143–154 (2002).

182. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).

183. di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. *Nature Reviews Neuroscience* **18**, 573–584 (2017).

184. Kelava, I. & Lancaster, M. A. Dishing out mini-brains: Current progress and future prospects in brain organoid research. *Developmental Biology* vol. 420 199–209 (2016).

185. Luo, C. *et al.* Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. *Cell Reports* **17**, 3369–3384 (2016).

186. Quadrato, G. *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* **545**, 48–53 (2017).

187. Ormel, P. R. *et al.* Microglia innately develop within cerebral organoids. *Nature Communications* **9**, (2018).

188. Sloan, S. A. *et al.* Human Astrocyte Maturation Captured in 3D Cerebral Cortical Spheroids Derived from Pluripotent Stem Cells. *Neuron* **95**, 779-790.e6 (2017).

189. M, B. *et al.* Human iPSC-Derived Cerebral Organoids Model Cellular Features of Lissencephaly and Reveal Prolonged Mitosis of Outer Radial Glia. *Cell stem cell* **20**, 435-449.e4 (2017).

190. Kadoshima, T. *et al.* Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 20284–9 (2013).

191. Velasco, S. *et al.* Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature* **570**, 523–527 (2019).

192. Sidhaye, J. & Knoblich, J. A. Brain organoids: an ensemble of bioassays to investigate human neurodevelopment and disease. *Cell Death & Differentiation 2020 28:1* **28**, 52–67 (2020).

193. Benson, C. A. *et al.* Immune Factor, TNFα, Disrupts Human Brain Organoid Development Similar to Schizophrenia—Schizophrenia Increases Developmental Vulnerability to TNFα. *Frontiers in Cellular Neuroscience* **14**, 233 (2020).

194. Zuiki, M. *et al.* Luteolin attenuates interleukin-6-mediated astrogliosis in human iPSC-derived neural aggregates: A candidate preventive substance for maternal immune activation-induced abnormalities. *Neuroscience Letters* **653**, 296–301 (2017).

195. Kathuria, A. *et al.* Transcriptome analysis and functional characterization of cerebral organoids in bipolar disorder. *Genome Medicine 2020 12:1* **12**, 1–16 (2020).

196. Khan, T. A. *et al.* Neuronal defects in a human cellular model of 22q11.2 deletion syndrome. *Nature Medicine* **26**, 1888–1898 (2020).

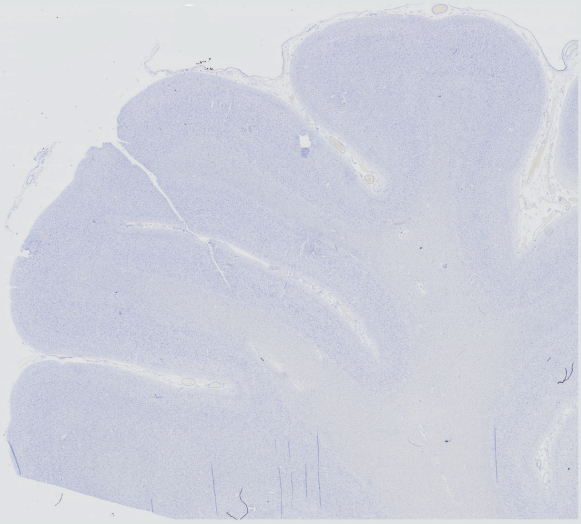
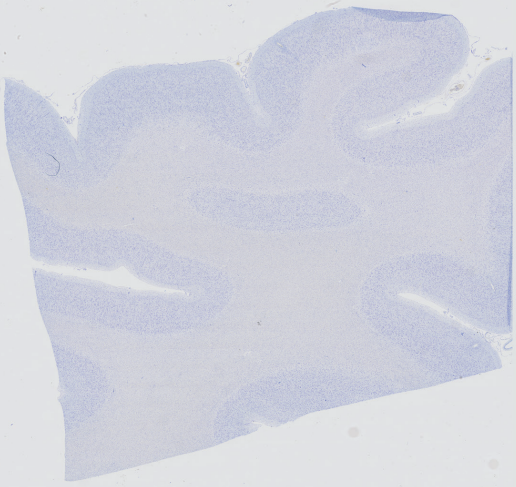
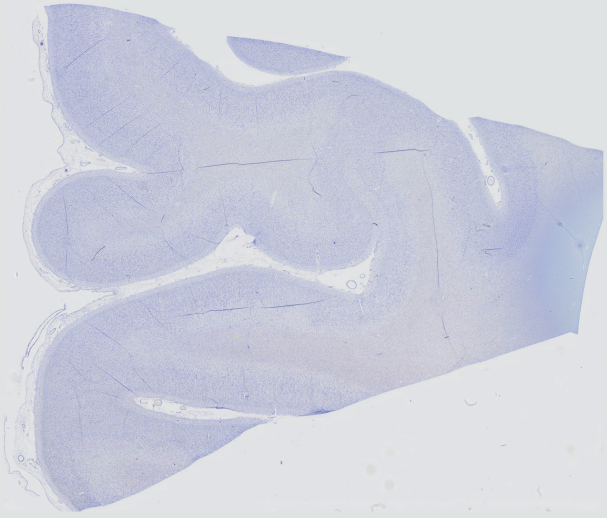
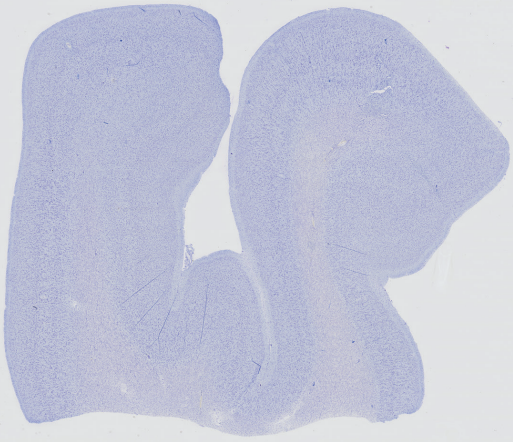
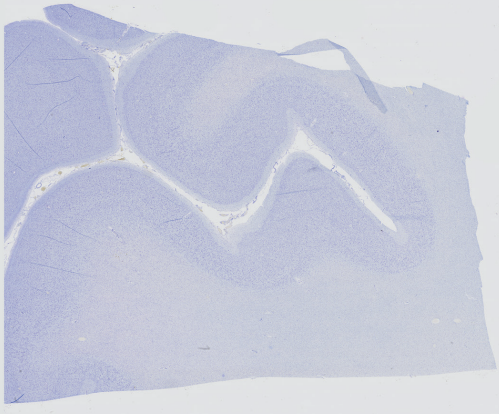
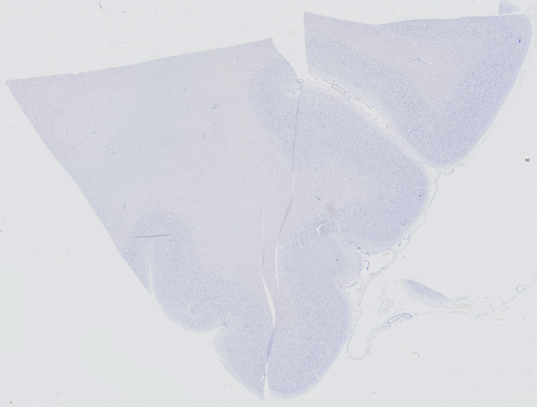
197. Arzua, T. *et al.* Modeling alcohol-induced neurotoxicity using human induced pluripotent stem cell-derived three-dimensional cerebral organoids. *Translational Psychiatry* **10**, (2020).

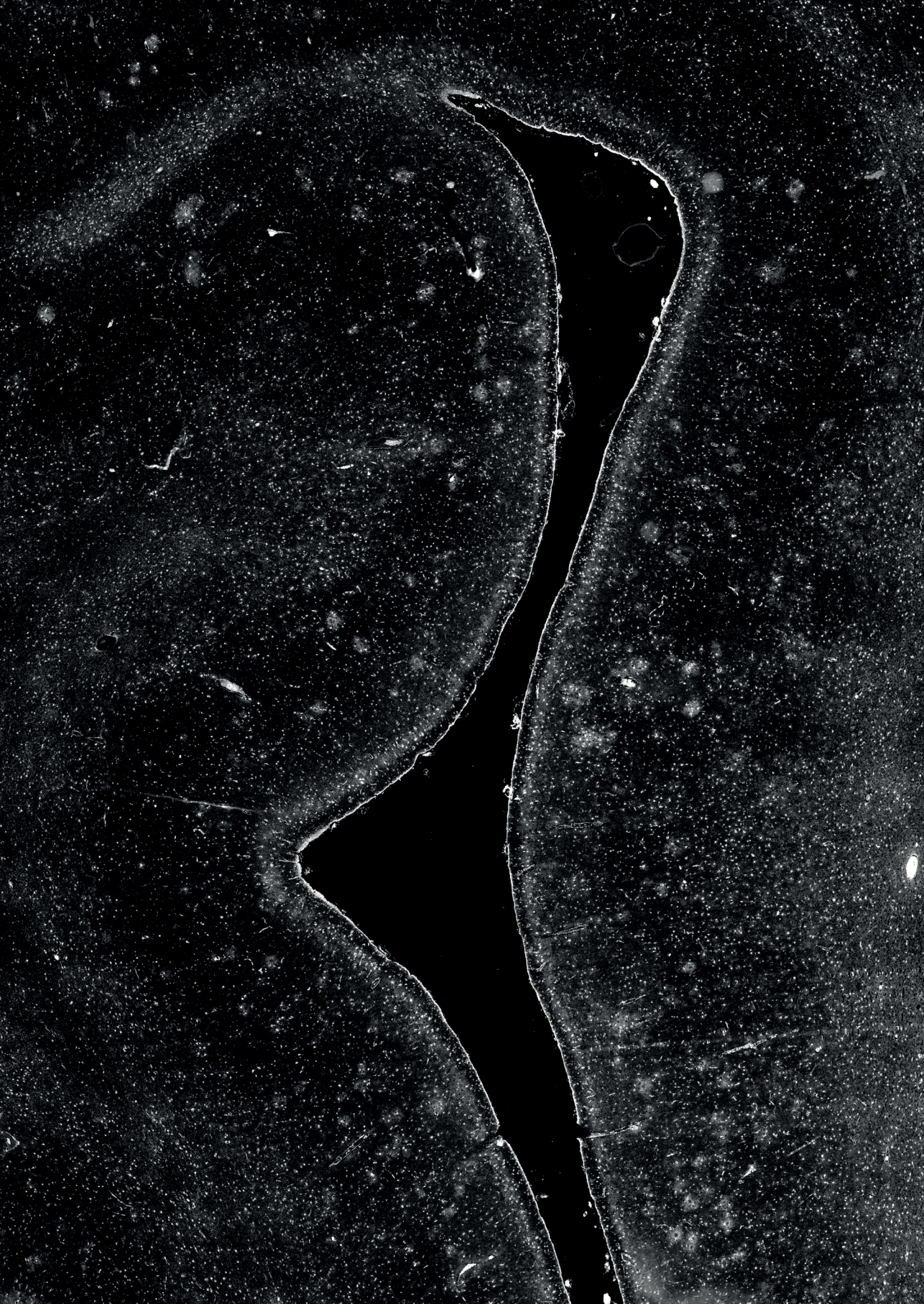
198. Wang, Y., Wang, L., Zhu, Y. & Qin, J. Human brain organoid-on-a-chip to model prenatal nicotine exposure. *Lab on a Chip* **18**, 851–860 (2018).

199. Dang, J. *et al.* Glial cell diversity and methamphetamine-induced neuroinflammation in human cerebral organoids. *Molecular Psychiatry* (2020) doi:10.1038/s41380-020-0676-x.

Part I

Tissue specific cortical abnormalities in schizophrenia using postmortem human brain tissue



Chapter 2

Synapse pathology in schizophrenia: A meta-analysis of postsynaptic elements in postmortem brain studies

Amber Berdenis van Berlekom[a,b], Cita H. Muflihah[b,c], Gijsje J.L.J. Snijders[a,b], Harold D. MacGillavry[d], Jinte Middeldorp[b], Elly M. Hol[b], René S. Kahn[a,e,f], and Lot D. de Witte[a,e,f]

[a] Department of Psychiatry, University Medical Center Utrecht Brain Center, Utrecht University, Utrecht, The Netherland

[b] Department of translational neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, Utrecht, The Netherlands

[c] Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Sukoharjo, Indonesia

[d] Cell Biology, Department of Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands

[e] Department of psychiatry, Icahn School of Medicine at Mount Sinai, New York City, New York, USA

[f] Mental Illness Research, Education and Clinical Center (MIRECC), James J Peters VA Medical Center, Bronx, NY, USA

Schizophrenia Bulletin 2020

Abstract

Changed synapse density has been suggested to be involved in the altered brain connectivity underlying schizophrenia (SCZ) pathology. However, postmortem studies addressing this topic are heterogeneous and it is not known whether changes are restricted to specific brain regions. Using meta-analysis, we systematically and quantitatively reviewed literature on the density of postsynaptic elements in postmortem brain tissue of patients with schizophrenia compared to healthy controls. We included three outcome measurements for postsynaptic elements: dendritic spine density, postsynaptic density number, and postsynaptic density protein expression levels. Random-effects meta-analysis (31 studies) revealed an overall decrease in density of postsynaptic elements in schizophrenia (Hedges's g: -0.33; 95%CI: -0.60 - -0.05; p = 0.020). Subgroup analyses showed reduction of postsynaptic elements in cortical, but not subcortical tissues (Hedges's g: -0.44; 95%CI: -0.76 - -0.12; p = 0.008, Hedges's g: -0.11; 95%CI: -0.54 – 0.35 p = 0.671), and specifically a decrease for the outcome measure dendritic spine density (Hedges's g: -0.81; 95%CI: -1.37 - -0.26; p = 0.004). Further exploratory analyses showed a significant decrease of postsynaptic elements in the prefrontal cortex and cortical layer 3. In all analyses, substantial heterogeneity was present. Meta-regression analyses showed no influence of age, sex, postmortem interval or brain bank on the effect size. This meta-analysis shows a region-specific decrease in the density of postsynaptic elements in schizophrenia. This phenotype provides an important cellular hallmark for future preclinical and neuropathological research in order to increase our understanding of brain dysconnectivity in schizophrenia.

1. Introduction

Schizophrenia (SCZ) is a severe psychiatric disorder affecting approximately 0.5-1% of the general population, causing high morbidity and mortality rates[1–4]. Core symptoms of SCZ are characterized by hallucinations, lack of motivation, and cognitive impairments and are thought to result from altered brain connectivity and network organization[5–9]. Accumulating evidence from genetic and neuropathological studies implies that changes in synapse density underlie these alterations in macroscale connectome organization in SCZ[10–19]. This is supported by studies reporting grey matter volume reductions in SCZ patient brains, caused by a decrease in neuropil rather than a loss of cell number[20–23]. Furthermore, it was recently shown that levels of the presynaptic protein synaptophysin are decreased in SCZ hippocampus, frontal cortex and cingulate cortex[19]. However, a combined systematic analysis of changes in the expression of postsynaptic proteins, and the density of postsynaptic elements such as dendritic spines is lacking.

Dendritic spines are small bulges on dendrites, forming the primary site of input for most excitatory synapses in the brain[24–26]. The number of dendritic spines is dynamic, particularly during development, showing a rapid increase during childhood followed by a prominent decrease during adolescence[27]. Interestingly, changes in spine pruning rate during adolescence have been implicated in the development of schizophrenia[28–31]. Dendritic spines contain many different proteins involved in neuro-chemical signalling. In particular, neurotransmitter receptor proteins such as NMDARs and AMPARs are anchored in the postsynaptic density (PSD) by numerous scaffolding proteins such as PSD95[32]. Thus, the PSD has an important role in arranging and coordinating receptor function and is essential for efficient synaptic transmission.

The density of postsynaptic structures in postmortem brain tissue can be determined using several approaches (Figure 1A-D). Dendritic spine density can be quantified with Golgi-staining and immunohistochemistry (IHC) (Figure 1B)[33]. At the ultrastructural level, electron microscopy studies can identify the number of PSD's that are separated by a synaptic cleft from a presynaptic membrane, forming functional synapses (Figure 1C)[34]. Also, PSD protein expression levels, measured with Western blot or IHC, although varying with the size of the PSD, are thought to reflect the number of synapses (Figure 1D)[35,36]. Therefore, all these measures (dendritic spine density, PSD number and PSD protein expression), which we collectively refer to as "postsynaptic elements", can be used as proxies for the number of excitatory synapses in postmortem brain tissue.

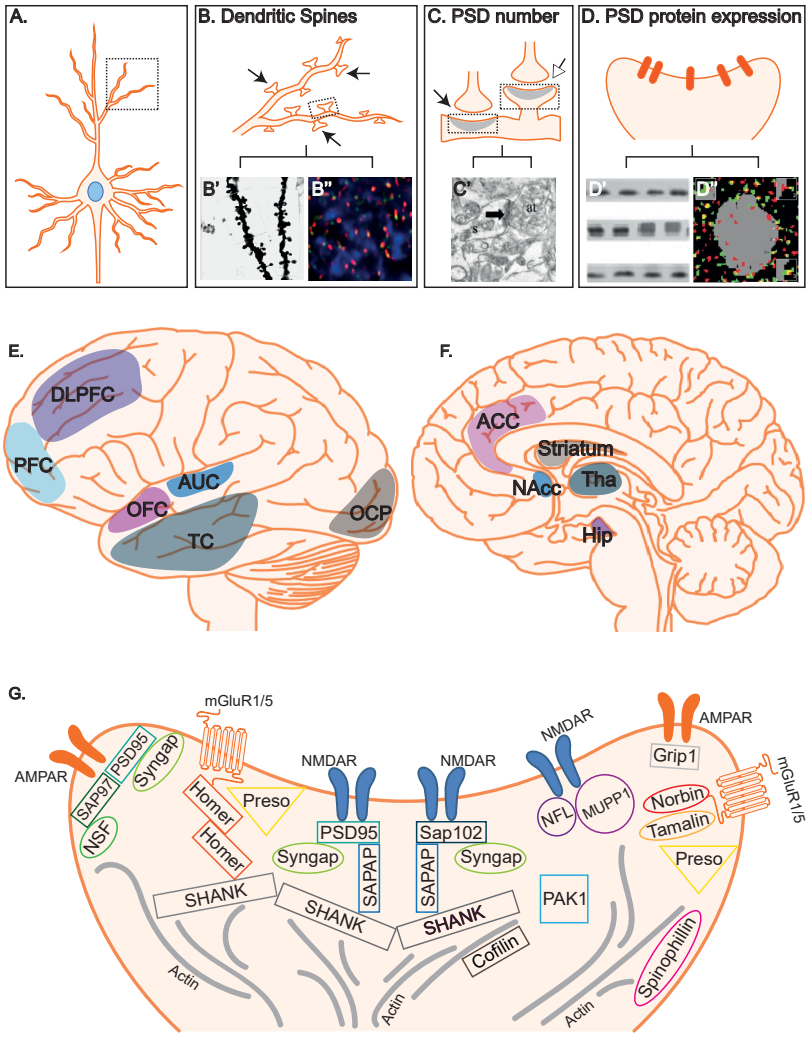


Figure 1. Schematic representation of postsynaptic element measurements and brain regions included in the meta-analysis.

Panel A-D show measurements that are used to quantify postsynaptic elements in postmortem brain tissue. (A) shows a neuron with its dendritic tree. The enlargement in (B) shows that each dendrite contains numerous dendritic spines (arrows), which can be quantified using Golgi staining (B' from Glantz and Lewis, 2000[37]) or immunohistochemistry (B" from Shelton et al., 201516). In (C) pre-synaptic terminals innervate postsynaptic-densities on a dendritic spine (white arrow), forming an axospinous synapse, or directly on the dendrite (black arrow), forming an axodendritic synapse. The number of these PSD can be measured with electron microscopy (C' from Roberts et al., 2015). The postsynaptic-density in (D) is an accumulation of many postsynaptic proteins at the postsynaptic membrane, which can be quantified by western blot (D' from Clinton et al., 2006) or immunohistochemistry (D" from Chung et al., 2016). (E-G) provide a simplified representation of brain regions and proteins in the postsynaptic-density that are assessed in studies included in our meta-analysis: PFC-Prefrontal Cortex; DLPFC-Dorsolateral Prefrontal Cortex; OFC-Orbitofrontal Cortex; OC-Olfactory Cortex; AC-Auditory Cortex; TC-Temporal Cortex; OCP-Occipital Cortex; ACC-Anterior Cingulate Cortex; NAcc-Nucleus Accumbens; Tha-Thalamus; Hip-Hippocampus.

Although literature on the density of postsynaptic elements in SCZ is quite extensive, findings are often conflicting. Most postmortem brain studies included a limited number of subjects, due to restricted availability of material and the labor intensiveness of performing histological studies. Furthermore, a large variety of pre- and postmortem confounders contributes to the high heterogeneity observed between postmortem studies[38]. In addition, it is difficult to draw conclusions on regional effects, as studies are performed using different methodological approaches, and assess different brain regions. Altogether, these factors limit the understanding of the contribution of changes in postsynaptic element in the pathophysiology of SCZ.

While literature on dendritic spine density, PSD number, and PSD protein expression in SCZ postmortem brain tissue has been reviewed individually[13–18,39], an integrated assessment, combining these different types of synapse density measurements in multiple brain areas in SCZ using meta-analysis has not been performed before. Although not often performed in the context of preclinical studies, meta-analysis provides a powerful tool to synthesize data on a specific topic.

The primary aim of this study was to review the evidence for alterations in the density of postsynaptic elements in SCZ postmortem brain tissue. The second aim was to analyze whether changes in the density of postsynaptic elements are specific to certain brain regions. To this end, we performed a systematic search to qualitatively and quantitatively review available literature on dendritic spine density, PSD density, and PSD protein expression in SCZ.

2. Methods

2.1 Search strategy

This quantitative review is performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[40], following Meta-analysis of Observational Studies in Epidemiology (MOOSE)[41] guidelines throughout. Two systematic searches were performed in PubMed: 1- (spine OR dendritic spine OR spine*) AND (density) AND ((Schizophrenia OR Psychosis OR Psychotic)), 2- (schizophreni* OR psychosis OR schizophrenia) AND (post synapse OR PSD OR post-synapse OR post-synapt* OR post synapt* OR postsynap*). The search was updated until April 30th, 2018. Pre-specified inclusion criteria were set as: human postmortem studies; comparing patients with healthy controls; measuring a structural outcome of postsynaptic elements (dendritic spine density, PSD number, or PSD protein expression); original research, published in a peer-reviewed journal; written in English. Exclusion criteria were: presence of other neurological disorders; animal studies; review articles; re-analysis of previously published

data; proteomic/transcriptomic approaches; studies that reported data incompletely and did not provide the information upon request. Furthermore, as messenger ribonucleic acid (mRNA) measurements provide no direct structural readout of the number of postsynaptic elements, and post translational modifications can result in a poor relation between transcript and protein expression, we excluded studies focussing on RNA only.

2.2 Data extraction

ABvB and LDW independently performed title and abstract screening for both systematic searches and reviewed full text for eligibility. Data extraction was performed by ABvB and checked independently by CHM. In addition to main outcome variables (dendritic spine density, PSD number and PSD protein expression), following variables were extracted for effect size (ES) calculation and potential moderator analyses: sample size, methods, brain-bank, brain-area, sub-region, age, sex, postmortem interval (PMI), and pH. When data records in the original article were not sufficient to generate ES, corresponding authors were asked to provide the raw data. Reference lists were checked for cross-references. In case of follow-up data or re-analysis of previously reported data[42–45], we only included outcomes of the original research. Studies using partly overlapping samples, studying different brain areas or different proteins, were included separately. Where data were not reported numerically, data were extracted using https://automeris.io/WebPlotDigitizer/.

2.3 Quality-control

Methodology, study design, and reporting were assessed to evaluate quality of included studies. Methodology was checked for complete description of technical methods and analyses. Study design was rated by researches blinded to diagnosis, whether they checked for neuropathology, the degree of matching of control and patient population, and the assessment/correction of general (age/PMI) and other confounding factors (such as: medication use, suicide or smoking). For reporting, we assessed whether studies fully described the method of psychopathological examination, population demographics and main outcome variables.

2.4 Statistical analysis

Meta-analyses were performed using the Comprehensive Meta-Analysis software (Biostat, Englewood, NJ USA). Change in dendritic spine density, PSD number, or PSD protein expression per brain (sub-)region was used to quantify ES between SCZ and the control group. Sample size, mean and standard deviation (SD) were used to generate ES. When mean and/or SD were unavailable, sample size and exact p-value were used to generate ES. Hedges'g and the upper/lower limit of the 95% confidence interval (95%CI) were used to express ES. A random effects model was used, as heterogeneity between studies was to be expected. Heterogeneity between studies was measured with Cochran's Q-test and I^2

statistic to provide an estimation of the variation attributed to differences in true effects. Q (weighted sum of squares) is, equal to *df* if studies share a common effect. I^2 reflects the proportion of observed variance reflecting real differences in ES, by dividing the excess dispersion (Q-*df*) by the total dispersion (Q). I^2 was considered low at 25%, moderate at 50% and high at 75%. Publication bias was assessed by visual inspection of the Funnel Plot and calculated with Egger's test (significance level: $p < 0.1$). Random effects meta-regression analyses were performed to analyse the role of potential confounding factors (brain bank, age, sex and PMI).

As we expected that different measurements within the same study are not independent of each-other, we nested data from these studies in a conservative approach, computing combined scores from all measurement within one study.

The primary meta-analysis was performed pooling all included studies to assess a brain wide effect on the density of postsynaptic elements in SCZ. We further stratified the analysis with subgroup analysis of a-priori selected variables, analysing biological- (subcortical/cortical) and technical variation (outcome measures), to assess sources of heterogeneity. Data of the same study were included in multiple sub-categories when data were reported separately for these categories (indicated with *). As we assume a common among-study variance across different subgroups, we pooled within group estimates of tau-squared. Between-group differences were tested using the Q-test based analysis of variance to determine whether the variance within subgroups was significantly smaller than the variance of all the combined data ($Q_{between} = Q_{total} - (Q_{SubgroupA} + Q_{SubgroupB})$. Exploratory subgroup meta-analysis, separating data based on sub-brain area, were performed when at least five independent studies (recommended for random-effects meta-analysis) could be included[46]. Throughout the study, forest plot figures show random effects meta-analysis, representing ES in Hedges's g with 95%CI for each study. Square size is proportional to study weight and the grey diamond indicates pooled effect size. Schematic images were produced using Motifolio.

3. Results

3.1 Database Search

Database searches in PubMed and cross-referencing yielded a total of 1527 records (Figure 2). After title and abstract screening, 116 studies remained for full text assessment. Of these, we excluded 81 studies (Supplementary Table 1). Authors of four studies[47–50] were contacted for additional information, a reply was received from one[50].

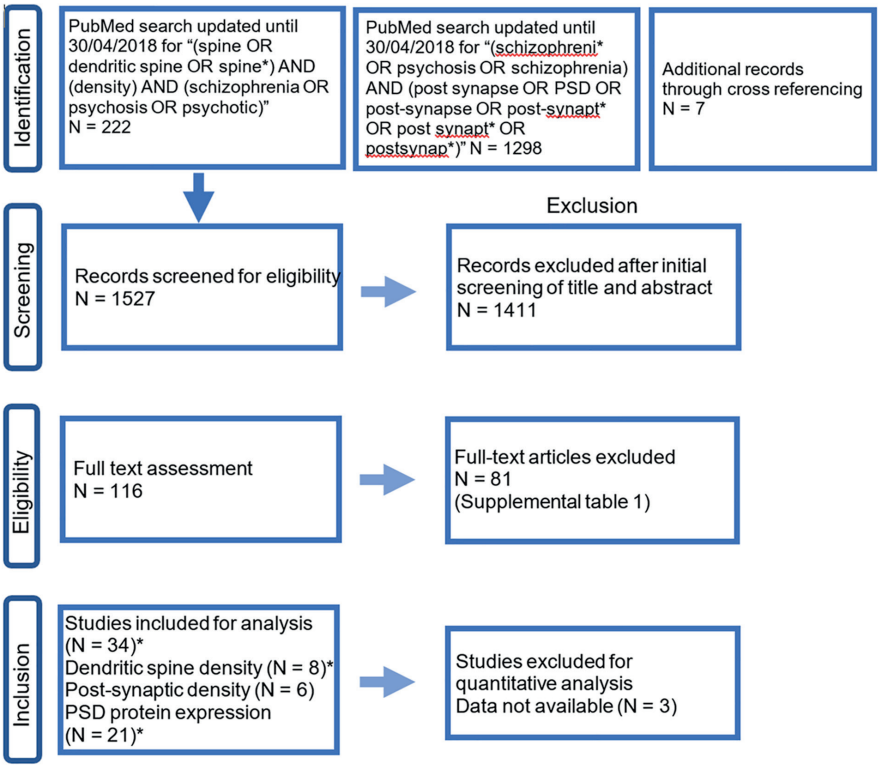


Figure 2. PRISMA flowchart.
Diagram of the systematic search strategy. *One paper reported data on both dendritic spine density and PSD protein expression.

We identified 34 individual studies assessing structural measurements of postsynaptic elements: dendritic spine density (8), PSD number (6), and PSD protein expression (21) for qualitative analysis. One study measured both dendritic spine density and PSD protein expression[51]. These studies considered 12 different brain regions (Figure 1E) and a variety of PSD proteins (Figure 1F). Replication studies, analysing the exact same measurement in the same region in at least three separate cohorts, were scarce. Only PSD95 measurements were replicated in the hippocampus[51–54], ACC[55–57] and DLPFC[52,55,56,58,59]. This limits the opportunity to perform separate analyses of specific brain regions. Therefore, we assessed all data together, to then further explore where possible sources of heterogeneity in sub-analyses. A*n overview of included studies and extracted data can be found in Supplementary Table 2*[12,50,51,53–55,57–80].

3.2 Qualitative analysis

We performed a quality assessment for all 34 studies assessing methodology, study design and reporting (Supplementary Table 3). Although postmortem studies are labour intensive and involve many pre- and postmortem confounders, our assessment showed that in general the included studies were of good quality. Most studies reported the demographics in full, described their applied methods extensively, performed matching, and controlled for important confounders (age/PMI/sex). However, 16 studies did not report on neuropathological examinations. As changes in synapse number have been described in a number of neurodegenerative diseases[81], neurologic comorbidity could be an important confounder. Moreover, we found that 16 studies did not report on blinding the experiment, and six studies did not report on the method of SCZ diagnosis.

3.3 Primary analysis: association of postsynaptic element density in SCZ postmortem brain

We performed a random-effects meta-analysis on 31 separate studies including all brain regions and all three study categories (comprising 98 individual datapoints). To prevent overrepresentation of studies including multiple measurements, estimated ES within each study were nested. Meta-analysis of the nested data showed that the density of postsynaptic elements is lower in SCZ patients then in control subjects (Figure 3; ES: -0.33; 95%CI: -0.60 – -0.05; p = 0.020). A similar result was obtained performing the analysis with un-nested data (Supplementary Figure 1; ES: -0.22; 95%CI: -0.37 – -0.07; p = 0.004).

We detected high between study heterogeneity (I^2: 78.39%; Q: 138.90; $p < 0.001$). Sensitivity analysis, excluding studies with a residual z-core +/-1.96[16], showed no significant, but trend level decrease in postsynaptic elements (Supplementary Figure 2; ES: -0.24; 95%CI: -0.48 – -0.003; p = 0.053). Although decreased, heterogeneity remained moderate (I^2: 70.59%; Q: 98.61; $p < 0.001$).

Publication bias was assessed based on visual inspection of the funnel plot and Egger's regression test. No asymmetry was observed by visual inspection, which was confirmed by Egger's regression test (p = 0.42) (Supplementary Figure 3).

We performed meta-regression analyses to check potential continuous (age, sex distribution and PMI) and categorical (brain bank) confounder variables. Age, sex, PMI and brain bank showed no moderating effects on outcome measurements (Supplementary Figure 4; p > 0.05).

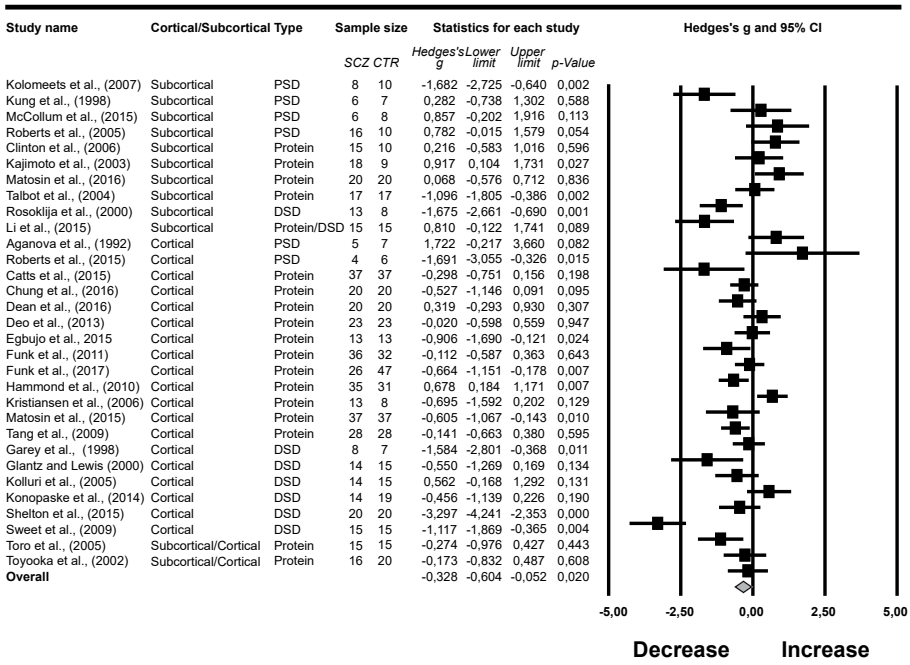


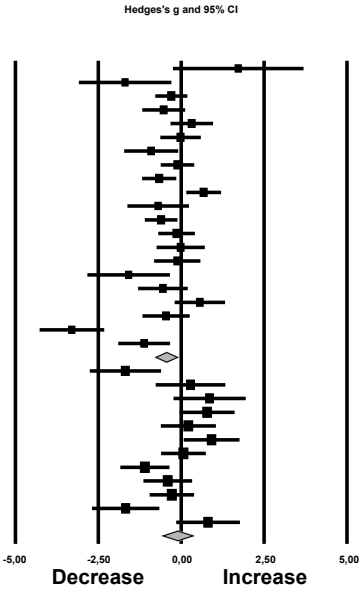
Figure 3. Forest plot of primary meta-analysis on density of postsynaptic elements in SCZ.
The pooled effect size of all studies on postsynaptic elements indicates that the density of postsynaptic elements is decreased in SCZ (p < 0.05). PSD-PSD number; Protein-PSD protein expression level; DSD-dendritic-spine density.

3.4 Subgroup analysis: stratified by brain region and study category

To assess possible sources of variation, we performed subgroup analyses. Data from the same study were included in both analyses when data were reported separately for each group[51,53,60,82.] First, we separated cortical and subcortical studies. Subgroup analyses revealed a significant decrease in density of postsynaptic elements in cortical tissues (Figure 4a; ES: -0.44; 95%CI: -0.76 – -0.12; p = 0.008), but no change in subcortical tissues (Figure 4a; ES: -0.11; 95%CI: -0.54 – 0.35 p = 0.671). However, the Q-test based analysis

A. Stratified by brain region

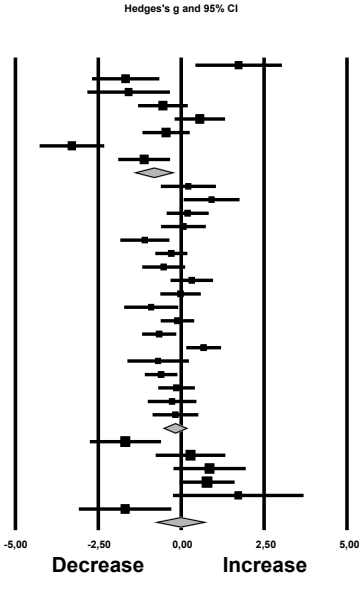
Study name	Cortical/Subcortical	Type	Sample size SCZ	Sample size CTR	Hedges's g	Lower limit	Upper limit	p-Value
Aganova et al., (1992)	Cortical	PSD	5	7	1,722	-0,217	3,660	0,082
Roberts et al., (2015)	Cortical	PSD	4	6	-1,691	-3,055	-0,326	0,015
Catts et al., (2015)	Cortical	Protein	37	37	-0,298	-0,751	0,156	0,198
Chung et al., (2016)	Cortical	Protein	20	20	-0,527	-1,146	0,091	0,095
Dean et al., (2016)	Cortical	Protein	20	20	0,319	-0,293	0,930	0,307
Deo et al., (2013)	Cortical	Protein	23	23	-0,020	-0,598	0,559	0,947
Egbujo et al., 2015	Cortical	Protein	13	13	-0,906	-1,690	-0,121	0,024
Funk et al., (2011)	Cortical	Protein	36	32	-0,112	-0,587	0,363	0,643
Funk et al., (2017)	Cortical	Protein	26	47	-0,664	-1,151	-0,178	0,007
Hammond et al., (2010)	Cortical	Protein	35	31	0,678	0,184	1,171	0,007
Kristiansen et al., (2006)	Cortical	Protein	13	8	-0,695	-1,592	0,202	0,129
Matosin et al., (2015)	Cortical	Protein	37	37	-0,605	-1,067	-0,143	0,010
Tang et al., (2009)	Cortical	Protein	28	28	-0,141	-0,663	0,380	0,595
Toro et al., (2005)	Cortical	Protein	15	15	-0,015	-0,711	0,682	0,967
Toyooka et al., (2002)	Cortical	Protein	14	23	-0,118	-0,786	0,550	0,730
Garey et al., (1998)	Cortical	DSD	8	7	-1,584	-2,801	-0,368	0,011
Glantz and Lewis (2000)	Cortical	DSD	14	15	-0,550	-1,269	0,169	0,134
Kolluri et al., (2005)	Cortical	DSD	14	15	0,562	-0,168	1,292	0,131
Konopaske et al., (2014)	Cortical	DSD	14	19	-0,456	-1,139	0,226	0,190
Shelton et al., (2015)	Cortical	DSD	20	20	-3,297	-4,241	-2,353	0,000
Sweet et al., (2009)	Cortical	DSD	15	15	-1,117	-1,869	-0,365	0,004
Overall Cortical					-0,437	-0,759	-0,114	0,008
Kolomeets et al., (2007)	Subcortical	PSD	8	10	-1,682	-2,725	-0,640	0,002
Kung et al., (1998)	Subcortical	PSD	6	7	0,282	-0,738	1,302	0,588
McCollum et al., (2015)	Subcortical	PSD	6	8	0,857	-0,202	1,916	0,113
Roberts et al., (2005)	Subcortical	PSD	16	10	0,782	-0,015	1,579	0,054
Clinton et al., (2006)	Subcortical	Protein	15	10	0,216	-0,583	1,016	0,596
Kajimoto et al., (2003)	Subcortical	Protein	18	9	0,917	0,104	1,731	0,027
Matosin et al., (2016)	Subcortical	Protein	20	20	0,068	-0,576	0,712	0,836
Talbot et al., (2004)	Subcortical	Protein	17	17	-1,096	-1,805	-0,386	0,002
Toro et al., (2005)*	Subcortical	Protein	15	15	-0,404	-1,108	0,300	0,260
Toyooka et al., (2002)*	Subcortical	Protein	21	16	-0,283	-0,924	0,359	0,388
Rosoklija et al., (2000)	Subcortical	DSD	13	8	-1,675	-2,661	-0,690	0,001
Li et al., (2015)	Subcortical	Protein/DSD	15	15	0,810	-0,122	1,741	0,089
Overall Subcortical					-0,096	-0,537	0,345	0,671



Test for subgroup differences: Q-between = 1.498 (p = 0.221)

B. Stratified by study category

Study name	Cortical/Subcortical	Type	Sample size SCZ	Sample size CTR	Hedges's g	Lower limit	Upper limit	p-Value
Li et al., (2015)*	Subcortical	DSD	7	5	1,732	0,458	3,005	0,008
Rosoklija et al., (2000)	Subcortical	DSD	13	8	-1,675	-2,661	-0,690	0,001
Garey et al., (1998)	Cortical	DSD	8	7	-1,584	-2,801	-0,368	0,011
Glantz and Lewis (2000)	Cortical	DSD	14	15	-0,550	-1,269	0,169	0,134
Kolluri et al., (2005)	Cortical	DSD	14	15	0,562	-0,168	1,292	0,131
Konopaske et al., (2014)	Cortical	DSD	14	19	-0,456	-1,139	0,226	0,190
Shelton et al., (2015)	Cortical	DSD	20	20	-3,297	-4,241	-2,353	0,000
Sweet et al., (2009)	Cortical	DSD	15	15	-1,117	-1,869	-0,365	0,004
Overall DSD					-0,811	-1,366	-0,255	0,004
Clinton et al., (2006)	Subcortical	Protein	15	10	0,216	-0,583	1,016	0,596
Kajimoto et al., (2003)	Subcortical	Protein	18	9	0,917	0,104	1,731	0,027
Li et al., (2015)	Subcortical	Protein	21	21	0,195	-0,410	0,799	0,528
Matosin et al., (2016)	Subcortical	Protein	20	20	0,068	-0,576	0,712	0,836
Talbot et al., (2004)	Subcortical	Protein	17	17	-1,096	-1,805	-0,386	0,002
Catts et al., (2015)	Cortical	Protein	37	37	-0,298	-0,751	0,156	0,198
Chung et al., (2016)	Cortical	Protein	20	20	-0,527	-1,146	0,091	0,095
Dean et al., (2016)	Cortical	Protein	20	20	0,319	-0,293	0,930	0,307
Deo et al., (2013)	Cortical	Protein	23	23	-0,020	-0,598	0,559	0,947
Egbujo et al., 2015	Cortical	Protein	13	13	-0,906	-1,690	-0,121	0,024
Funk et al., (2011)	Cortical	Protein	36	32	-0,112	-0,587	0,363	0,643
Funk et al., (2017)	Cortical	Protein	26	47	-0,664	-1,151	-0,178	0,007
Hammond et al., (2010)	Cortical	Protein	35	31	0,678	0,184	1,171	0,007
Kristiansen et al., (2006)	Cortical	Protein	13	8	-0,695	-1,592	0,202	0,129
Matosin et al., (2015)	Cortical	Protein	37	37	-0,605	-1,067	-0,143	0,010
Tang et al., (2009)	Cortical	Protein	28	28	-0,141	-0,663	0,380	0,595
Toro et al., (2005)	Cortical/Subcortical	Protein	15	15	-0,274	-0,976	0,427	0,443
Toyooka et al., (2002)	Cortical/Subcortical	Protein	16	20	-0,173	-0,832	0,487	0,608
Overall Protein					-0,171	-0,509	0,166	0,320
Kolomeets et al., (2007)	Subcortical	PSD	8	10	-1,682	-2,725	-0,640	0,002
Kung et al., (1998)	Subcortical	PSD	6	7	0,282	-0,738	1,302	0,588
McCollum et al., (2015)	Subcortical	PSD	6	8	0,857	-0,202	1,916	0,113
Roberts et al., (2005)	Subcortical	PSD	16	10	0,782	-0,015	1,579	0,054
Aganova et al., (1992)	Cortical	PSD	5	7	1,722	-0,217	3,660	0,082
Roberts et al., (2015)	Cortical	PSD	4	6	-1,691	-3,055	-0,326	0,015
Overall PSD					-0,011	-0,718	0,696	0,976



Test for subgroup differences: Q-between = 4.445 (p = 0.108)

Figure 4. Forest plots of subgroup meta-analyses on density of postsynaptic elements in SCZ. Subgroup meta-analyses for postsynaptic density in SCZ stratified per (A) brain region (cortical/subcortical) and (B) study category. The pooled effect size of studies on the density of postsynaptic elements in cortical tissues is decreased in SCZ ($p<0.05$), but not significantly changed in studies on subcortical tissues ($p > 0.05$). PSD-PSD number; Protein-PSD protein expression level; DSD-dendritic-spine density.

of variance for subgroup differences indicated no significant difference between the two groups ($Q_{between}$ = 1.50; p = 0.221). No publication bias (Supplementary Figure 5) or confounding effects of age, sex, PMI and brain bank (Supplementary Figure 6) were found. High between-study heterogeneity remained in both cortical (I^2: 77.98%; Q: 90.82; p < 0.001) and subcortical (I^2: 76.18%; Q: 46.17; p < 0.001) studies.

A subgroup analysis was also performed separating the three study categories (dendritic spine density, PSD number, and PSD protein expression). We found a significant decrease in dendritic spine density (Figure 4b; ES: -0.81; 95%CI: -1.37 – -0.26; p = 0.004), and no difference for PSD protein expression (Figure 4b; ES: -0.17; 95%CI: -0.51 – 0.16; p = 0.320) or PSD number (Figure 4b; ES: -0.01; 95%CI: -0.72 – 0.70; p = 0.98). However, no difference between groups was detected as shown by the Q-test based analysis of variance ($Q_{between}$ = 4.45; p = 0.108). No publication bias (Supplementary Figure 7) or confounding effects of age, sex, PMI, aor brain bank (Supplementary Figure 8) were found. Moderate to high heterogeneity was observed in all study categories; dendritic spine density (I^2: 88.72%; Q: 62.07; p < 0.001), synapse density (I^2: 80.31%; Q: 25.39; p < 0.001) and PSD protein expression (I^2: 61.44%; Q: 44.09; p < 0.001).

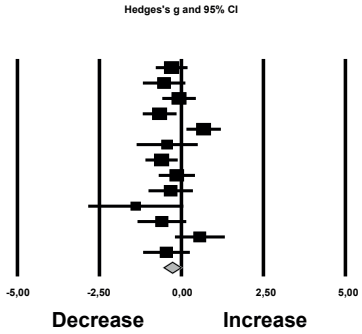
Surprisingly, we identified a study reporting significant opposite effect directions in the expression of PSD proteins: showing an upregulation for Homer1a and Preso, and downregulation for PSD95 and Homer1b/c in the hippocampus[54]. This was also the case at a non-significant level in other studies[50,51,55,60,63–65]. To visualize the variation in expression of different PSD proteins in SCZ postmortem tissue, we generated a forest plot with un-nested data of all PSD protein expression studies (Supplementary Figure 9).

3.5 Exploratory sub-analyses: specific brain areas

Lastly, we performed an exploratory subgroup-analyses when five or more studies were performed on the same brain area. These analyses showed a significant decrease of postsynaptic elements in the prefrontal cortex (Figure 5a; ES: -0.27; 95%CI: -0.53 – -0.01; p = 0.043) and cortical layer 3 (Figure 5b; ES: -1.39; 95%CI: -2.24 – -0.54; p = 0.001). No change was found in the ACC (Figure 5c; ES: -0.25; 95%CI: -0.97 – 0.47; p = 0,50) and the hippocampus (Figure 5d; ES: -0.57; 95%CI: -1.17 – 0.02; p = 0.059). A graphical representation of these results is depicted in Figure 6. Heterogeneity in these analyses was moderate in the PFC (I^2: 58.42%; Q: 28.86; p = 0.004) and high in cortical layer 3 (I^2: 81.79%; Q: 27.46; p < 0.001), the ACC (I^2: 71.14%; Q: 13.86; p = 0.008) and hippocampus (I^2: 75.21%; Q: 24.20; p < 0.001).

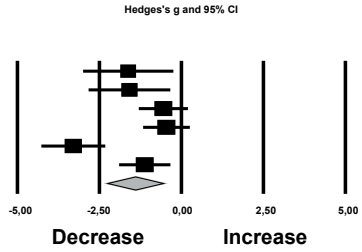
A. Prefrontal Cortex

Study name	Cortical/Subcortical	Type	Sample size SCZ	CTR	Hedges's g	Lower limit	Upper limit	p-Value
Catts et al., (2015)	Cortical	Protein	37	37	-0,298	-0,751	0,156	0,198
Chung et al., (2016)	Cortical	Protein	20	20	-0,527	-1,146	0,091	0,095
Funk et al., (2011)	Cortical	Protein	35	31	-0,072	-0,552	0,408	0,768
Funk et al., (2017)	Cortical	Protein	26	47	-0,664	-1,151	-0,178	0,007
Hammond et al., (2010)	Cortical	Protein	35	31	0,678	0,184	1,171	0,007
Kristiansen et al., (2006)	Cortical	Protein	13	8	-0,433	-1,334	0,467	0,345
Matosin et al., (2015)	Cortical	Protein	37	37	-0,605	-1,067	-0,143	0,010
Tang et al., (2009)	Cortical	Protein	28	28	-0,141	-0,663	0,380	0,595
Toyooka et al., (2002)	Cortical	Protein	14	25	-0,327	-0,975	0,321	0,322
Garey et al., (1998)	Cortical	DSD	3	5	-1,393	-2,813	0,026	0,054
Glantz and Lewis (2000)	Cortical	DSD	15	15	-0,596	-1,309	0,117	0,101
Kolluri et al., (2005)	Cortical	DSD	14	15	0,562	-0,168	1,292	0,131
Konopaske et al., (2014)	Cortical	DSD	14	19	-0,456	-1,139	0,226	0,190
Overall					-0,267	-0,526	-0,008	0,043



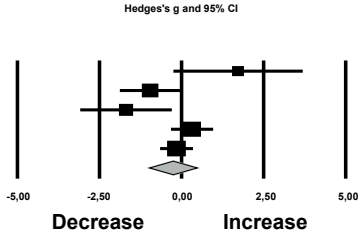
B. Cortical Layer 3

Study name	Cortical/Subcortical	Type	Sample size SCZ	CTR	Hedges's g	Lower limit	Upper limit	p-Value
Roberts et al., (2015)	Cortical	PSD	4	6	-1,622	-2,968	-0,276	0,018
Garey et al., (1998)	Cortical	DSD	8	7	-1,584	-2,801	-0,368	0,011
Glantz and Lewis (2000)	Cortical	DSD	14	15	-0,550	-1,269	0,169	0,134
Konopaske et al., (2014)	Cortical	DSD	14	19	-0,456	-1,139	0,226	0,190
Shelton et al., (2015)	Cortical	DSD	20	20	-3,297	-4,241	-2,353	0,000
Sweet et al., (2009)	Cortical	DSD	15	15	-1,117	-1,869	-0,365	0,004
Overall					-1,393	-2,242	-0,544	0,001



C. Anterior Cingulate Cortex

Study name	Cortical/Subcortical	Type	Sample size SCZ	CTR	Hedges's g	Lower limit	Upper limit	p-Value
Aganova et al., (1992)	Cortical	PSD	5	7	1,722	-0,217	3,660	0,082
Kristiansen et al., (2006)	Cortical	Protein	13	8	-0,957	-1,851	-0,063	0,036
Roberts et al., (2015)	Cortical	PSD	4	6	-1,691	-3,055	-0,326	0,015
Dean et al., (2016)	Cortical	Protein	20	20	0,319	-0,293	0,930	0,307
Funk et al., (2011)	Cortical	Protein	36	33	-0,153	-0,622	0,317	0,525
Overall					-0,250	-0,971	0,470	0,496



D. Hippocampus

Study name	Cortical/Subcortical	Type	Sample size SCZ	CTR	Hedges's g	Lower limit	Upper limit	p-Value
Kolomeets et al., (2007)	Subcortical	PSD	8	10	-1,682	-2,725	-0,640	0,002
Matosin et al., (2016)	Subcortical	Protein	20	20	0,068	-0,576	0,712	0,836
Talbot et al., (2004)	Subcortical	Protein	17	17	-1,096	-1,805	-0,386	0,002
Toro et al., (2005)	Subcortical	Protein	15	15	-0,404	-1,108	0,300	0,260
Toyooka et al., (2002)	Subcortical	Protein	21	16	-0,283	-0,924	0,359	0,388
Rosoklija et al., (2000)	Subcortical	DSD	13	8	-1,675	-2,661	-0,690	0,001
Li et al., (2015)	Subcortical	Protein/DSD	15	15	0,810	-0,122	1,741	0,089
Overall					-0,572	-1,166	0,022	0,059

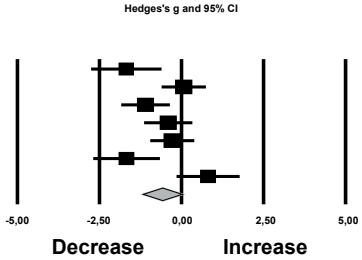


Figure 5. Forest plots of brain area specific exploratory subanalyses.
Exploratory subgroup meta-analyses for postsynaptic elements in SCZ in the (A) Prefrontal cortex (PFC), (B) Cortical layer 3, (C) Anterior Cingulate Cortex (ACC) and (D) Hippocampus. The pooled effect sizes of studies on the density of postsynaptic elements in the PFC and layer 3 are significantly decreased ($p<0.05$), but not changed in studies on the ACC and hippocampus ($p > 0.05$). PSD-PSD number; Protein-PSD protein expression level; DSD-dendritic-spine density.

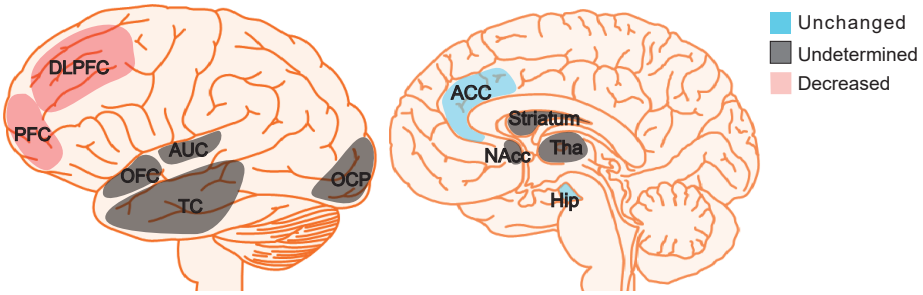


Figure 6. Schematic representation of changes in postsynaptic elements.
The schematic represents changes in postsynaptic elements in SCZ for brain regions tested with meta-analyses (unchanged or decreased) and shows which brain regions could not be tested (undetermined).

4. Discussion

In this study, we quantitatively investigated three outcome measures reflecting the number of postsynaptic elements in SCZ postmortem brain tissue: dendritic spine density, PSD number and PSD protein expression. Our meta-analysis showed a significant decrease in density of postsynaptic elements in SCZ patients compared to healthy controls. However, sensitivity analyses showed high heterogeneity, suggesting the presence of sub-groups. No evidence was found for publication bias, or confounding factors (age, PMI, sex and brain bank). With our meta-analysis we quantitatively assessed, to our knowledge, the largest sample size to date on structural abnormalities of postsynaptic elements in postmortem brain tissue of SCZ patients, providing an extensive overview of the current literature on this topic.

At the same time, we recognise that several of the included studies were performed on sample populations from the same brain bank or cohort[12,37,75,76,78,83,84]. It was not feasible to determine which parts of the samples were overlapping, to compute separate ES. This could result in an overrepresentation of specific populations in our meta-analysis. Furthermore, our research design provided evidence that alterations in postsynaptic elements were not due to age, sex or PMI of the studied subjects. However, given the limited availability of data, several potential confounding factors such as suicide rate, severity of symptoms, and antipsychotic use could not be considered. Confounding by these factors is unavoidable in SCZ postmortem research and should therefore be addressed in future analyses. In particular, the use of antipsychotics has been suggested to influence synapse density[85,86]. Although some studies (10) did not/could not correct for medication use[50,55,59,60,73,75,76,78,80,83], most studies included in our meta-analyses (18) found no association between medication use and the outcome measurement[12,49,51,53,58,63–66,68–70,72,74,77,79,87]. Thus, while our study shows a decrease in density of postsynaptic elements in SCZ, future research will need to address the contribution of these confounding factors.

High heterogeneity was observed among included studies in the primary analysis. Although this is common in meta-analyses on preclinical data[41], it should be considered and explored. A-priori, we defined brain region and study category as potential sources of heterogeneity. Our subgroup and exploratory sub-analyses showed a significant decrease of postsynaptic elements in cortical regions, specifically in the PFC and cortical layer 3. We did not observe this effect in subgroup analysis for subcortical regions or in analyses of the ACC and hippocampus. Although this suggests that the effect is most pronounced in cortical tissues, subgroup differences between cortical and subcortical studies were not statistically significant. Regional heterogeneity of postsynaptic element deficits in SCZ has been hypothesized before[13,16]. An earlier study showed that spine density was decreased in cortical layer 3, but not in layer 5/6 of the same cohort[83]. Studies of the basal

ganglia show an opposite effect, with an increase of PSD number[76,79]. These changes also seem to be specific to sub-regions, as increases are exclusively found in the core compartment of the NAcc[76], and in the caudate but not the putamen of the striatum[79]. It should be considered that we were unable to perform meta-analysis for each brain region separately, as most are underrepresented in our dataset. Strikingly, electron microscopy studies are almost exclusively performed on subcortical tissues, while most dendritic spine studies are performed selectively in cortical layer 3. Other cortical layers were researched in two separate studies[75,83]. Systematic analysis of different brain regions and replication studies with large cohorts, recently shown feasible for transcriptomic studies[88,89], are necessary to compare specific brain areas to fully identify sources of heterogeneity.

Understanding local heterogeneity could also help determine the neuronal populations most vulnerable to pathology in SCZ. Our study has focussed primarily on excitatory synapses. Dendritic spines form the primary source of excitatory input[24–26], and the structural proteins of the PSD in our analysis are almost exclusively found in excitatory synapses[32,90]**.** Furthermore, with exception of one study[91], most electron microscopy studies show that effects are specific for excitatory (asymmetric and axospinous) synapses[44,75,76,79]. Although impaired inhibition also has been hypothesized to affect cognition in SCZ, few structural postmortem studies have been performed to asses this[92].

Our subgroup analysis identified no significant difference between the three study categories, dendritic spine density, synapse density, or PSD protein expression. However, we identified a significant decrease in dendritic spine density, suggesting that the effect is most pronounced in dendritic spines. Some electron microscopy studies indeed show a specific decrease of axospinous synapses in SCZ[75,77]. An alternative explanation for these findings could be the brain regions represented in each category. Subcortical studies are overrepresented in the category of PSD number and are less prevalent in dendritic spine density studies.

Our subgroup meta-analysis showed no significant difference in PSD protein expression in SCZ. Unexpectedly, some studies show opposite regulation of different individual PSD proteins, a phenomenon masked in our analysis because we nested the data. It suggests that the expression level of some PSD proteins is actively regulated in SCZ, and not only a consequence of the number of synapses.

Possible mechanisms explaining the decrease in density of postsynaptic elements found in our meta-analysis include deficits in synapse formation, maintenance, or elimination. Defects in synapse formation are suggested by studies identifying SCZ risk genes encoding for PSD scaffolding proteins like DISC1, SHANK, and HOMER[11]. Altered synapse stabilization is implicated by a study showing that especially smaller, transient dendritic

spines are decreased in SCZ[45]. SCZ risk genes like CACNB2 and CACNB4[93] could affect local calcium transients at dendritic spines, necessary for their stabilization[45,94,95]. Alternatively, non-cell autonomous involvement of glia (microglia and astrocytes) might play a role. Studies have shown altered secretion of astrocytic gliotransmitters, necessary for synapse stability[96,97]. Furthermore, increased glial pruning of synapses is suggested by a recent in vitro study[98], and because of the high association between complement 4 genes in the MHC locus and risk of developing SCZ[93,99].

A recent elaborate transcriptomic study from the PsychENCODE consortium, using cortical brain tissue, could provide insight in postsynaptic element disfunction in SCZ[89]. Most genes coding for proteins assessed in our meta-analyses were not differentially expressed (Supplementary Table 4). However, many novel SCZ risk genes, related to synaptic or glial function, were suggested. For example, the kinase *DCLK3*, which enhances dendritic remodelling and synapse maturation[89,100]. Also, the astrocytic glutamate transporters *SLC1A3* and *SLC1A2*, which dysfunction could affect astrocyte-synapse interaction at excitatory synapses[89,101]. More SCZ risk genes were identified within the modules of PSD/ trans-synaptic signaling, astrocytes and microglia that need to be further explored in future studies assessing the relation to synapse dysfunction.

In general, the observed decrease of postsynaptic elements in the cortex, and layer 3 specifically, could be related to the clinical phenotype of SCZ. Cortical layer 3 contains pyramidal neurons, important for corticocortical projections[102]. These projections are required for higher cognitive functions, like working memory, which are affected in SCZ[92,103]. A decrease in excitatory synapses is predicted to result in a reduced excitatory drive, possibly resulting in hypoactivity of layer 3 neurons. Previously, decreases in spine density were shown to be associated with alterations in connectome architecture as measured with diffusion tensor imaging[104]. Therefore, microscale deficits in synapse structure and function could influence brain connectivity at macroscale, potentially underlying the symptoms observed in SCZ. Altogether, the overall decrease in postsynaptic elements in the cortex also provides a specific cellular hallmark for translational research in SCZ that could be studied in human cell culture systems, brain organoid models, and animal studies. However, study approaches extending histological analyses to integrate cellular phenotypes with proteomic, transcriptomic, genomic and clinical data in large cohorts are imperative for translational research.

Furthermore, this phenotype also provides a possible target for diagnostics and novel therapeutics. Interestingly, several positron-emission tomography (PET) tracers visualizing presynaptic elements *in vivo* have been developed[105,] providing means to analyse synapse density in the living human brain. Currently, PET tracers for postsynaptic elements are targeted towards receptor proteins, like the NMDA and dopamine receptor, which are

suspected to be actively regulated in SCZ. The development of intracellular PET tracers for postsynaptic scaffolding proteins would contribute to the analysis of postsynaptic element dynamics during disease states and could provide a biological outcome measurement for diagnostic purposes. Eventually, strategies that target postsynaptic elements, for instance stabilizing PSD integrity, could present a novel therapeutic approach in the treatment of SCZ.

Acknowledgements

The authors declared no conflicts of interest. CHF was supported by an LPDP (Indonesian Endowment Fund for Education) scholarship. Funding was also provided by the Mental Illness, Education, and Clinical Center (MIRECC) of the JJPVA Medical Center in the Bronx, NY. We would like to thank Linda McPhee for editing the manuscript.

Author contributions

A.B.B. and L.D.W. designed the study. G.J.L.J.S, H.D.M, J.M. and E.M.H. critically reviewed the study design. A.B.B. performed literature search, which was checked by C.H.M. A.B.B. did the meta-analysis and figure design. A.B.B. wrote the manuscript with supervision of L.D.W. J.M. and E.M.H. provided critical feedback in reviewing and editing of the manuscript. Funding of the project was obtained by L.D.W. and R.S.K.

Supplementary material

Supplementary material is available at the online version of the paper:
https://doi.org/10.1093/schbul/sbz060

References

1. Saha, S., Chant, D., Welham, J. & McGrath, J. A systematic review of the prevalence of schizophrenia. *PLoS medicine* **2**, e141 (2005).
2. McGrath, J., Saha, S., Chant, D. & Welham, J. Schizophrenia: A Concise Overview of Incidence, Prevalence, and Mortality. *Epidemiologic Reviews* **30**, 67–76 (2008).
3. Chang, W. C. *et al.* Lifetime Prevalence and Correlates of Schizophrenia-Spectrum, Affective, and Other Non-affective Psychotic Disorders in the Chinese Adult Population. *Schizophrenia bulletin* **43**, 1280–1290 (2017).
4. Simeone, J. C., Ward, A. J., Rotella, P., Collins, J. & Windisch, R. An evaluation of variation in published estimates of schizophrenia prevalence from 1990–2013: a systematic literature review. *BMC Psychiatry* **15**, 193 (2015).
5. van den Heuvel, M. P. *et al.* Abnormal Rich Club Organization and Functional Brain Dynamics in Schizophrenia. *JAMA Psychiatry* **70**, 783 (2013).
6. Pettersson-Yeo, W. *et al.* Using genetic, cognitive and multi-modal neuroimaging data to identify ultra-high-risk and first-episode psychosis at the individual level. *Psychological Medicine* **43**, 2547–2562 (2013).
7. Anticevic, A. *et al.* Connectivity, pharmacology, and computation: toward a mechanistic understanding of neural system dysfunction in schizophrenia. *Frontiers in psychiatry* **4**, 169 (2013).
8. Friston, K. J. & Frith, C. D. Schizophrenia: a disconnection syndrome? *Clinical neuroscience (New York, N.Y.)* **3**, 89–97 (1995).
9. Stephan, K. E., Friston, K. J. & Frith, C. D. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. *Schizophrenia bulletin* **35**, 509–27 (2009).
10. Schijven, D. *et al.* Comprehensive pathway analyses of schizophrenia risk loci point to dysfunctional postsynaptic signaling. *Schizophrenia Research* **199**, 195–202 (2018).
11. Soler, J. *et al.* Genetic variability in scaffolding proteins and risk for schizophrenia and autism-spectrum disorders: a systematic review. *Journal of psychiatry & neuroscience : JPN* **43**, 223–244 (2018).
12. Shelton, M. A. *et al.* Loss of Microtubule-Associated Protein 2 Immunoreactivity Linked to Dendritic Spine Loss in Schizophrenia. *Biological psychiatry* **78**, 374–85 (2015).
13. Glausier, J. R. & Lewis, D. A. Dendritic spine pathology in schizophrenia. *Neuroscience* **251**, 90–107 (2013).
14. Coley, A. A. & Gao, W.-J. PSD95: A synaptic protein implicated in schizophrenia or autism? *Progress in neuro-psychopharmacology & biological psychiatry* **82**, 187–194 (2018).
15. Harrison, PaulJ. The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. *Psychopharmacology* **174**, 151–162 (2004).
16. Moyer, C. E., Shelton, M. A. & Sweet, R. A. Dendritic spine alterations in schizophrenia. *Neuroscience letters* **601**, 46–53 (2015).
17. Fornito, A., Yücel, M., Dean, B., Wood, S. J. & Pantelis, C. Anatomical abnormalities of the anterior cingulate cortex in schizophrenia: bridging the gap between neuroimaging and neuropathology. *Schizophrenia bulletin* **35**, 973–93 (2009).
18. Parker, E. M. & Sweet, R. A. Stereological Assessments of Neuronal Pathology in Auditory Cortex in Schizophrenia. *Frontiers in neuroanatomy* **11**, 131 (2017).

19. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular Psychiatry* (2018) doi:10.1038/s41380-018-0041-5.

20. Honea, R., Crow, T. J., Passingham, D. & Mackay, C. E. Regional Deficits in Brain Volume in Schizophrenia: A Meta-Analysis of Voxel-Based Morphometry Studies. *American Journal of Psychiatry* **162**, 2233–2245 (2005).

21. Haijma, S. V. *et al.* Brain Volumes in Schizophrenia: A Meta-Analysis in Over 18 000 Subjects. *Schizophrenia Bulletin* **39**, 1129–1138 (2013).

22. Heckers, S., Heinsen, H., Geiger, B. & Beckmann, H. Hippocampal neuron number in schizophrenia. A stereological study. *Archives of general psychiatry* **48**, 1002–8 (1991).

23. Thune, J. J., Uylings, H. B. & Pakkenberg, B. No deficit in total number of neurons in the prefrontal cortex in schizophrenics. *Journal of psychiatric research* **35**, 15–21.

24. Rochefort, N. L. & Konnerth, A. Dendritic spines: from structure to in vivo function. *EMBO reports* **13**, 699–708 (2012).

25. GRAY, E. G. Electron Microscopy of Synaptic Contacts on Dendrite Spines of the Cerebral Cortex. *Nature* **183**, 1592–1593 (1959).

26. Megías, M., Emri, Z., Freund, T. F. & Gulyás, A. I. Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. *Neuroscience* **102**, 527–40 (2001).

27. Chen, C.-C., Lu, J. & Zuo, Y. Spatiotemporal dynamics of dendritic spines in the living brain. *Frontiers in Neuroanatomy* **8**, 28 (2014).

28. Chung, W.-S., Welsh, C. A., Barres, B. A. & Stevens, B. Do glia drive synaptic and cognitive impairment in disease? *Nature neuroscience* **18**, 1539–1545 (2015).

29. Chen, X. *et al.* Apoptotic engulfment pathway and schizophrenia. *PloS one* **4**, e6875 (2009).

30. Sellgren, C. M. *et al.* Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nature Neuroscience* **22**, 374–385 (2019).

31. Forrest, M. P., Parnell, E. & Penzes, P. Dendritic structural plasticity and neuropsychiatric disease. *Nature Reviews Neuroscience* **19**, 215–234 (2018).

32. Sheng, M. & Hoogenraad, C. C. The Postsynaptic Architecture of Excitatory Synapses: A More Quantitative View. *Annual Review of Biochemistry* **76**, 823–847 (2007).

33. Holtmaat, A. & Svoboda, K. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nature reviews. Neuroscience* **10**, 647–58 (2009).

34. Burette, A., Collman, F., Micheva, K. D., Smith, S. J. & Weinberg, R. J. Knowing a synapse when you see one. *Frontiers in neuroanatomy* **9**, 100 (2015).

35. Walaas, S. I., Jahn, R. & Greengard, P. Quantitation of nerve terminal populations: synaptic vesicle-associated proteins as markers for synaptic density in the rat neostriatum. *Synapse (New York, N.Y.)* **2**, 516–20 (1988).

36. Masliah, E., Terry, R. D., Alford, M. & DeTeresa, R. Quantitative immunohistochemistry of synaptophysin in human neocortex: an alternative method to estimate density of presynaptic terminals in paraffin sections. *Journal of Histochemistry & Cytochemistry* **38**, 837–844 (1990).

37. Glantz, L. A. & Lewis, D. A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of general psychiatry* **57**, 65–73 (2000).

38. Harrison, P. J. Postmortem studies in schizophrenia. *Dialogues in clinical neuroscience* **2**, 349–57 (2000).

39. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular psychiatry* (2018) doi:10.1038/s41380-018-0041-5.

40. Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS medicine* **6**, e1000097 (2009).

41. Stroup, D. F. Meta-analysis of Observational Studies in Epidemiology<SUBTITLE>A Proposal for Reporting</SUBTITLE>. *JAMA* **283**, 2008 (2000).

42. McKinney, B., Ding, Y., Lewis, D. A. & Sweet, R. A. DNA methylation as a putative mechanism for reduced dendritic spine density in the superior temporal gyrus of subjects with schizophrenia. *Translational psychiatry* **7**, e1032 (2017).

43. Roberts, R. C., Roche, J. K. & Conley, R. R. Differential synaptic changes in the striatum of subjects with undifferentiated versus paranoid schizophrenia. *Synapse* **62**, 616–627 (2008).

44. Kolomeets, N. S., Orlovskaya, D. D., Rachmanova, V. I. & Uranova, N. A. Ultrastructural alterations in hippocampal mossy fiber synapses in schizophrenia: a postmortem morphometric study. *Synapse (New York, N.Y.)* **57**, 47–55 (2005).

45. MacDonald, M. L. *et al.* Selective Loss of Smaller Spines in Schizophrenia. *The American journal of psychiatry* **174**, 586–594 (2017).

46. Jackson, D. & Turner, R. Power analysis for random-effects meta-analysis. *Research Synthesis Methods* **8**, 290–302 (2017).

47. Leber, S. L. *et al.* Homer1a protein expression in schizophrenia, bipolar disorder, and major depression. *Journal of Neural Transmission* **124**, 1261–1273 (2017).

48. Dean, B., Thomas, N., Lai, C. Y., Chen, W. J. & Scarr, E. Changes in cholinergic and glutamatergic markers in the striatum from a sub-set of subjects with schizophrenia. *Schizophrenia Research* **169**, 83–88 (2015).

49. Talbot, K. *et al.* Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. *The Journal of clinical investigation* **113**, 1353–63 (2004).

50. Clinton, S. M., Haroutunian, V. & Meador-Woodruff, J. H. Up-regulation of NMDA receptor subunit and post-synaptic density protein expression in the thalamus of elderly patients with schizophrenia. *Journal of neurochemistry* **98**, 1114–25 (2006).

51. Li, W. *et al.* Synaptic proteins in the hippocampus indicative of increased neuronal activity in CA3 in schizophrenia. *The American journal of psychiatry* **172**, 373–82 (2015).

52. Toyooka, K. *et al.* Selective reduction of a PDZ protein, SAP-97, in the prefrontal cortex of patients with chronic schizophrenia. *Journal of neurochemistry* **83**, 797–806 (2002).

53. Toro, C. & Deakin, J. F. W. NMDA receptor subunit NRI and postsynaptic protein PSD-95 in hippocampus and orbitofrontal cortex in schizophrenia and mood disorder. *Schizophrenia research* **80**, 323–30 (2005).

54. Matosin, N. *et al.* Molecular evidence of synaptic pathology in the CA1 region in schizophrenia. *npj Schizophrenia* **2**, 16022 (2016).

55. Kristiansen, L. V., Beneyto, M., Haroutunian, V. & Meador-Woodruff, J. H. Changes in NMDA receptor subunits and interacting PSD proteins in dorsolateral prefrontal and anterior cingulate cortex indicate abnormal regional expression in schizophrenia. *Molecular psychiatry* **11**, 737–47, 705 (2006).

56. Funk, A. J., McCullumsmith, R. E., Haroutunian, V. & Meador-Woodruff, J. H. Abnormal activity of the MAPK- and cAMP-associated signaling pathways in frontal cortical areas in postmortem brain in schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **37**, 896–905 (2012).

57. Dean, B. *et al.* Changes in cortical N-methyl-d-aspartate receptors and post-synaptic density protein 95 in schizophrenia, mood disorders and suicide. *Australian and New Zealand Journal of Psychiatry* **50**, 275–283 (2016).

58. Funk, A. J. *et al.* Postsynaptic Density-95 Isoform Abnormalities in Schizophrenia. *Schizophrenia bulletin* **43**, 891–899 (2017).

59. Chung, D. W., Fish, K. N. & Lewis, D. A. Pathological Basis for Deficient Excitatory Drive to Cortical Parvalbumin Interneurons in Schizophrenia. *The American journal of psychiatry* **173**, 1131–1139 (2016).

60. Toyooka, K. *et al.* Selective reduction of a PDZ protein, SAP-97, in the prefrontal cortex of patients with chronic schizophrenia. *Journal of neurochemistry* **83**, 797–806 (2002).

61. Kajimoto, Y. *et al.* Synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP) is expressed differentially in phencyclidine-treated rats and is increased in the nucleus accumbens of patients with schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **28**, 1831–9 (2003).

62. Funk, A. J., Rumbaugh, G., Harotunian, V., McCullumsmith, R. E. & Meador-Woodruff, J. H. Decreased expression of NMDA receptor-associated proteins in frontal cortex of elderly patients with schizophrenia. *Neuroreport* **20**, 1019–22 (2009).

63. Tang, J. *et al.* Dysbindin-1 in dorsolateral prefrontal cortex of schizophrenia cases is reduced in an isoform-specific manner unrelated to dysbindin-1 mRNA expression. *Human Molecular Genetics* **18**, 3851–3863 (2009).

64. Hammond, J. C., McCullumsmith, R. E., Funk, A. J., Haroutunian, V. & Meador-Woodruff, J. H. Evidence for abnormal forward trafficking of AMPA receptors in frontal cortex of elderly patients with schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **35**, 2110–9 (2010).

65. Deo, A. J. *et al.* PAK1 Protein Expression in the Auditory Cortex of Schizophrenia Subjects. *PLoS ONE* **8**, (2013).

66. Egbujo, C. N. *et al.* Molecular evidence for decreased synaptic efficacy in the postmortem olfactory bulb of individuals with schizophrenia. *Schizophrenia research* **168**, 554–62 (2015).

67. Catts, V. S., Derminio, D. S., Hahn, C.-G. & Weickert, C. S. Postsynaptic density levels of the NMDA receptor NR1 subunit and PSD-95 protein in prefrontal cortex from people with schizophrenia. *NPJ schizophrenia* **1**, 15037 (2015).

68. Matosin, N. *et al.* Alterations of mGluR5 and its endogenous regulators Norbin, Tamalin and Preso1 in schizophrenia: towards a model of mGluR5 dysregulation. *Acta Neuropathologica* **130**, 119–129 (2015).

69. Konopaske, G. T., Lange, N., Coyle, J. T. & Benes, F. M. Prefrontal cortical dendritic spine pathology in schizophrenia and bipolar disorder. *JAMA psychiatry* **71**, 1323–31 (2014).

70. Sweet, R. A., Henteleff, R. A., Zhang, W., Sampson, A. R. & Lewis, D. A. Reduced dendritic spine density in auditory cortex of subjects with schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **34**, 374–89 (2009).

71. Kolluri, N., Sun, Z., Sampson, A. R. & Lewis, D. A. Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. *American Journal of Psychiatry* **162**, 1200–1202 (2005).

72. Glantz, L. A. & Lewis, D. A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry* **57**, 65–73 (2000).

73. Garey, L. J. *et al.* Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *Journal of Neurology, Neurosurgery & Psychiatry* **65**, 446–453 (1998).

74. Rosoklija, G. *et al.* Structural abnormalities of subicular dendrites in subjects with schizophrenia and mood disorders: preliminary findings. *Archives of general psychiatry* **57**, 349–56 (2000).

75. Roberts, R. C., Barksdale, K. A., Roche, J. K. & Lahti, A. C. Decreased synaptic and mitochondrial density in the postmortem anterior cingulate cortex in schizophrenia. *Schizophrenia research* **168**, 543–53 (2015).

76. McCollum, L. A., Walker, C. K., Roche, J. K. & Roberts, R. C. Elevated Excitatory Input to the Nucleus Accumbens in Schizophrenia: A Postmortem Ultrastructural Study. *Schizophrenia bulletin* **41**, 1123–32 (2015).

77. Kolomeets, N. S., Orlovskaya, D. D. & Uranova, N. A. Decreased numerical density of CA3 hippocampal mossy fiber synapses in schizophrenia. *Synapse (New York, N.Y.)* **61**, 615–21 (2007).

78. Kung, L., Conley, R., Chute, D. J., Smialek, J. & Roberts, R. C. Synaptic changes in the striatum of schizophrenic cases: A controlled postmortem ultrastructural study. *Synapse* **28**, 125–139 (1998).

79. Roberts, R. C., Roche, J. K. & Conley, R. R. Synaptic differences in the postmortem striatum of subjects with schizophrenia: a stereological ultrastructural analysis. *Synapse (New York, N.Y.)* **56**, 185–97 (2005).

80. Aganova, E. A. & Uranova, N. A. Morphometric analysis of synaptic contacts in the anterior limbic cortex in the endogenous psychoses. *Neuroscience and behavioral physiology* **22**, 59–65.

81. Sheng, M., Sabatini, B. L. & Südhof, T. C. Synapses and Alzheimer's disease. *Cold Spring Harbor perspectives in biology* **4**, (2012).

82. Wu, J. *et al.* Dietary interventions that reduce mTOR activity rescue autistic-like behavioral deficits in mice. *Brain, Behavior, and Immunity* (2017) doi:10.1016/j.bbi.2016.09.016.

83. Kolluri, N., Sun, Z., Sampson, A. R. & Lewis, D. A. Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. *The American journal of psychiatry* **162**, 1200–2 (2005).

84. Roberts, R. C., Roche, J. K. & Conley, R. R. Synaptic differences in the patch matrix compartments of subjects with schizophrenia: A postmortem ultrastructural study of the striatum. *Neurobiology of Disease* **20**, 324–335 (2005).

85. Konradi, C. & Heckers, S. Antipsychotic drugs and neuroplasticity: insights into the treatment and neurobiology of schizophrenia. *Biological psychiatry* **50**, 729–42 (2001).

86. Huang, X.-F. & Song, X. Effects of antipsychotic drugs on neurites relevant to schizophrenia treatment. *Medicinal Research Reviews* **39**, 386–403 (2019).

87. Catts, V. S., Derminio, D. S., Hahn, C.-G. & Weickert, C. S. Postsynaptic density levels of the NMDA receptor NR1 subunit and PSD-95 protein in prefrontal cortex from people with schizophrenia. *npj Schizophrenia* **1**, 15037 (2015).

88. Gandal, M. J. *et al.* Shared molecular neuropathology across major psychiatric disorders parallels polygenic overlap. *Science (New York, N.Y.)* **359**, 693–697 (2018).

89. Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science (New York, N.Y.)* **362**, eaat8127 (2018).

90. Sheng, M. & Kim, E. The postsynaptic organization of synapses. *Cold Spring Harbor perspectives in biology* **3**, a005678 (2011).

91. Aganova, E. A. & Uranova, N. A. Morphometric analysis of synaptic contacts in the anterior limbic cortex in the endogenous psychoses. *Neuroscience and behavioral physiology* **22**, 59–65 (1990).

92. Hoftman, G. D., Datta, D. & Lewis, D. A. Layer 3 Excitatory and Inhibitory Circuitry in the Prefrontal Cortex: Developmental Trajectories and Alterations in Schizophrenia. *Biological psychiatry* **81**, 862–873 (2017).

93. Schizophrenia Working Group of the Psychiatric Genomics Consortium. Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421–7 (2014).

94. Lohmann, C. & Bonhoeffer, T. A Role for Local Calcium Signaling in Rapid Synaptic Partner Selection by Dendritic Filopodia. *Neuron* **59**, 253–260 (2008).

95. Sheng, L., Leshchyns'ka, I. & Sytnyk, V. Neural Cell Adhesion Molecule 2 Promotes the Formation of Filopodia and Neurite Branching by Inducing Submembrane Increases in Ca2+ Levels. *Journal of Neuroscience* **35**, 1739–1752 (2015).

96. Halassa, M. M., Fellin, T. & Haydon, P. G. The tripartite synapse: roles for gliotransmission in health and disease. *Trends in Molecular Medicine* **13**, 54–63 (2007).

97. Lin, H., Jacobi, A. A., Anderson, S. A. & Lynch, D. R. D-Serine and Serine Racemase Are Associated with PSD-95 and Glutamatergic Synapse Stability. *Frontiers in Cellular Neuroscience* **10**, 34 (2016).

98. Mallya, A. P. & Deutch, A. Y. (Micro)Glia as Effectors of Cortical Volume Loss in Schizophrenia. *Schizophrenia Bulletin* **44**, 948–957 (2018).

99. Sekar, A. *et al.* Schizophrenia risk from complex variation of complement component 4. *Nature* **530**, 177–83 (2016).

100. Shin, E. *et al.* Doublecortin-like kinase enhances dendritic remodelling and negatively regulates synapse maturation. *Nature communications* **4**, 1440 (2013).

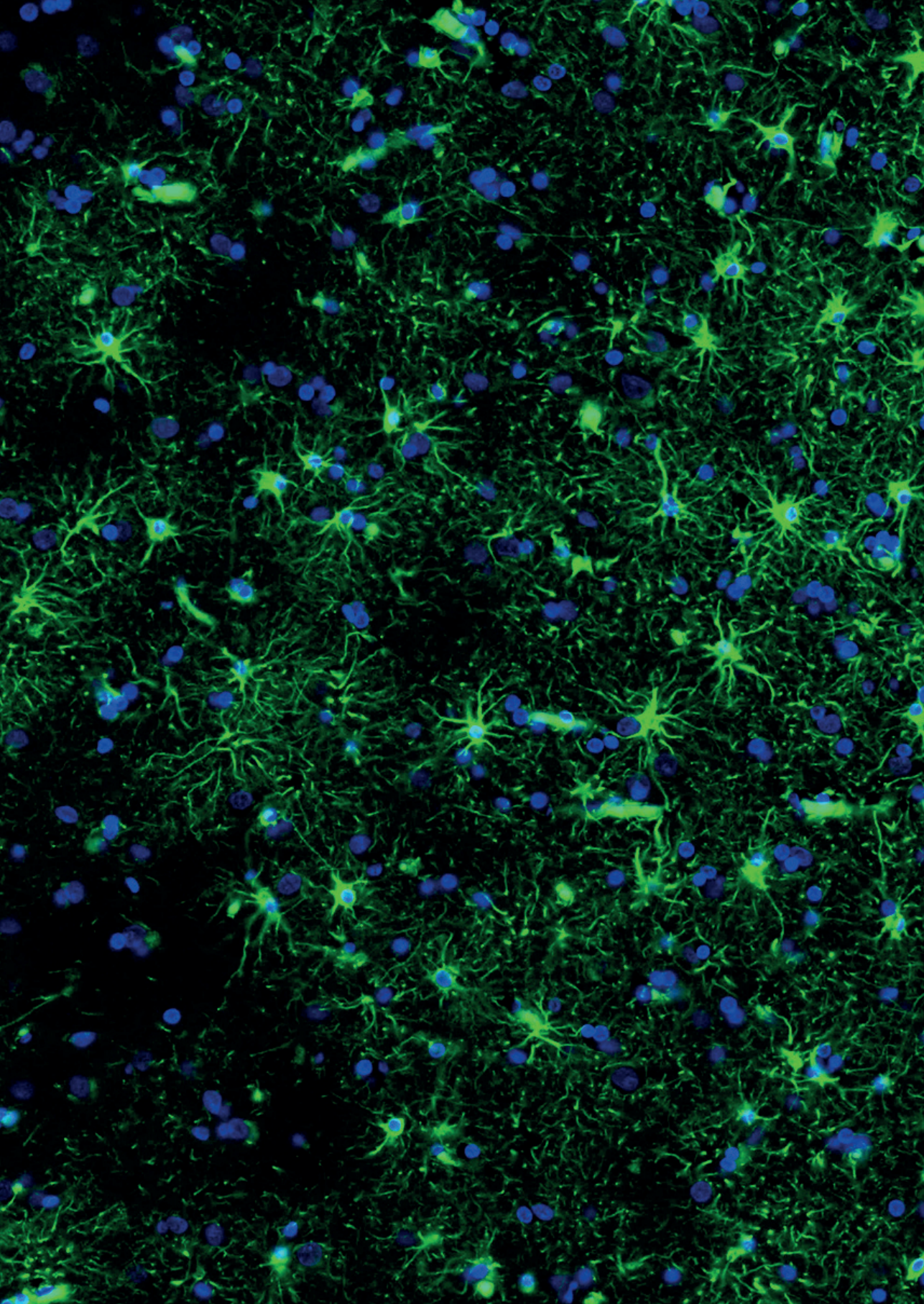
101. O'Donovan, S. M., Sullivan, C. R. & McCullumsmith, R. E. The role of glutamate transporters in the pathophysiology of neuropsychiatric disorders. *npj Schizophrenia* **3**, 32 (2017).

102. Melchitzky, D. S., Sesack, S. R., Pucak, M. L. & Lewis, D. A. Synaptic targets of pyramidal neurons providing intrinsic horizontal connections in monkey prefrontal cortex. *The Journal of comparative neurology* **390**, 211–24 (1998).

103. Reichenberg, A. *et al.* Static and Dynamic Cognitive Deficits in Childhood Preceding Adult Schizophrenia: A 30-Year Study. *American Journal of Psychiatry* **167**, 160–169 (2010).

104. van den Heuvel, M. P., Scholtens, L. H., de Reus, M. A. & Kahn, R. S. Associated Microscale Spine Density and Macroscale Connectivity Disruptions in Schizophrenia. *Biological psychiatry* **80**, 293–301 (2016).

105. Finnema, S. J. *et al.* Imaging synaptic density in the living human brain. *Science translational medicine* **8**, 348ra96 (2016).



Chapter 3

A postmortem study on cortical synapses, microglia, and astrocytes in schizophrenia

Amber Berdenis van Berlekom[a,b], Jesca E. de Jager[b], Marjolein A.M. Sneeboer[a,b], Gijsje J.L.J. Snijders[a,c], Jinte Middeldorp[b], Elly M. Hol[b], René S. Kahn[a,c,d], Marco P. Boks[a], and Lot D. de Witte[a,c,d]

[a] Department of Psychiatry, University Medical Center Utrecht, Brain Center, Utrecht University, Utrecht, The Netherlands

[b] Department of translational neuroscience, University Medical Center Utrecht, Brain Center, Utrecht University, Utrecht, The Netherland

[c] Department of psychiatry, Icahn School of Medicine at Mount Sinai, New York City, New York, USA

[d] Mental Illness Research, Education and Clinical Center (MIRECC), James J Peters VA Medical Center, Bronx, NY, USA

Under Review

Abstract

Elucidating the cellular and molecular changes in schizophrenia patients is essential for progress in diagnosis and treatment of this disabling disorder. Mounting evidence suggests that synaptic changes are at the root of the disorder. Decreases in cortical synaptic density have been repeatedly identified, particularly in cortical layer III. Glial cells, like microglia and astrocytes, are suggested to impact synapse number in schizophrenia, as they play a role in synapse formation, maintenance, and elimination. Despite the importance of the interaction between synapses and glia, few studies have investigated them simultaneously and the importance of layer specificity has been underappreciated. In this study the phenotype and number of synapses, microglia, astrocytes, and their association in cortical grey matter and layer III in schizophrenia are analysed. For that purpose, we performed analyses for gene and protein expression, and morphometric analyses based on Nissl staining and immunohistochemistry in human postmortem brain tissue of the *gyrus frontalis medialis* in schizophrenia patients and unaffected controls. Altogether, despite rigorous efforts, we did not find evidence that synapses, glia, or their association are different in schizophrenia patients, except for a decrease in mRNA expression of microglial receptor genes *CX3CR1* and *ITGAM* in total grey matter. These results show the need for additional postmortem studies addressing glia synapse interactions, as well as studies that can bridge the gap between preclinical mechanistic findings and static human postmortem specimens.

1. Introduction

Although the pathogenesis of schizophrenia (SCZ) is largely unknown, there is an increasing support for the hypothesis that changes in connectivity and network organization form the basis of the disease[1–5]. Changes in synapse number are suspected to contribute to this altered connectivity, based on genetic (GWAS), gene-expression (RNAseq) and histological postmortem brain studies[6–9]. A decrease in cortical synaptic density has been repeatedly reported and is considered a robust cellular aberration in postmortem tissue of SCZ patients[9–11]. Interestingly, synapse decrease seems to be specifically localized in cortical layer III, as studies examining cortical layer V and VI and subcortical areas did not show this phenomenon[9,12–15].

Although previous studies predominantly focused on neuronal dysfunction in SCZ synapse pathology, mounting research suggests a contribution of glial cells, such as astrocytes and microglia[16–20]. Astrocytes and microglia are abundantly present in the brain and play an essential role in many central nervous system functions in health and disease[21]. Astrocytes are involved in blood flow regulation, metabolic and structural support, and modulating inflammatory response[22–25]. Microglia function as the resident immune cells of the brain, involved in responding to neuronal injury and pathogen invasion. Moreover, they also have a role in neurodevelopment by eliminating excess neuronal progenitors [26–28]. Both glial cell-types are in close contact with synapses, interacting chemically and physically, forming the quad-partite synapse[29,30]. They have been shown to be involved in the formation, maintenance, and pruning of synapses during development[31,32].

Compelling evidence suggests that the interaction between glia and neurons, and more specifically the role of glia in synaptic pruning, is linked to SCZ. Genetic studies identified an association between SCZ and different variants of the complement component 4 (C4) gene, which is involved in microglial mediated synaptic pruning[7,32–34]. In the same line, microglia derived from induced pluripotent stem cells of SCZ patients showed an increase in synaptosome uptake[35]. Further, studies suggest alterations in synapse supporting functions by astrocytes in SCZ. Dysregulation of astrocytic glutamate related genes and proteins, important for synaptic functioning, have been observed in SCZ postmortem tissue[36,37]. Furthermore, chimeric mice containing human glial cells from SCZ patients showed impaired structural maturation of astrocytes[38]. Lastly, the pruning phase during early adolescence, when glia remove less functional synapses generated during childhood, coincides with the general onset of SCZ[39]. Altogether, this has led to the hypothesis that alterations in astrocyte and microglia form and function influence synapse density in SCZ.

Astrocytes and microglia have been studied extensively in postmortem human brain tissue of SCZ patients, especially in relation to reactive gliosis and immune activation[40,41]. However, the relation between glia and synaptic density has not yet been assessed and cortical layer specificity has been underappreciated. The synapse deficit observed in postmortem brain tissue of SCZ patients seems to possess specific spatial heterogeneity. Glia also express strong spatial heterogeneity, with evident differences between cells in the grey and white matter or even between cortical layers[42–45].

To assess the association between glial number and synaptic density in SCZ, it is essential to assess both readouts within the same tissue and take layer specificity into account. Therefore, in our study we apply a multi-modal approach using gene and protein expression analyses, and morphometric analyses to assess microglia, astrocytes, synapses, and glia-synapse interaction in cortical grey matter of the *gyrus frontalis medialis*, and more specifically layer III, using human postmortem brain tissue.

2. Methods and Materials

2.1 Donors

Postmortem brain tissue of SCZ patients and control (CTR) donors was obtained from the Netherlands Brain Bank (NBB, www.brainbank.nl). Ante-mortem written informed consent was obtained for brain autopsy, and the use of tissue and clinical information for research purposes. We obtained fresh frozen (SCZ N = 12, CTR N = 11) and paraffin embedded (SCZ N = 13, CTR N= 12) tissue from the *gyrus frontalis medialis* (area 1 and 2) (GFM). A detailed description of the anatomical dissection boundaries of these regions used during autopsy can be found on the website of the NBB (www.brainbank.nl/brain-tissue/autopsy/). At the NBB, the diagnoses of participants were confirmed by trained research assistants using standardized instruments (like the Structured Clinical Interview for DSM-IV (SCID-I), Mini International Neuropsychiatric Interview (M.I.N.I.) plus, Comprehensive Assessment of Symptoms and History (CASH), or Composite International Diagnostic Interview (CIDI))[46]. Furthermore, the clinicopathological information from all donors of the NBB was reviewed by two individual psychiatrists in our group (LdW and GS) to confirm a SCZ diagnosis. CTR donors did not have a history of major neurological or psychiatric disorders. SCZ and CTR donor groups were matched based on age and sex to ensure diagnostic groups did not differ in mean age and sex (table 1). All donors were checked for neuropathology by a pathologist and excluded in case of neurodegenerative pathology in the frontal cortex. For each donor we subtracted information on PMD, pH, age, sex, onset of disease, cause of death, Braak and amyloid score, substance dependency (smoking, alcohol and drugs), neuropathology score, somatic and DSM comorbidities, when available (Table 1 and Supplementary Table 1). Four main possible confounding factors (sex, age, pH and PMD)

were assessed for difference on group level with Mann-Whitney and Chi-square tests where appropriate (significance level $p < 0.05$).

2.2 Ethics statement

The Netherlands Brain Bank received permission to perform autopsies, and to use donor tissue and medical records, from the VU medical center Ethical Committee (VUMC, Amsterdam, The Netherlands).

2.3 Panel design

We designed a gene panel based on literature[11,23,47–49], including general markers for synapses (*SYN1, SYP, PPP1R9B* and *PSD95*), microglia (*TREM2, TGFB1, P2RY12, AIF1, TMEM119, HLA-DRA, IL-6, CSF1R, CSF1,* and *C1Q*) and astrocytes (*GFAP, GJA1, VIM, S100B, AQP4, ALDH1L1, GRM3, SOX9, GJB6, GLUL,* and *C4*), and for interacting factors of microglia and synapses (*CX3CR1, ITGAM,* and *ITGAX)* and astrocytes and synapses (*SLC1A1, MERTK, SLC1A3,* and *MEGF10*). We selected three markers for markers for synapses, astrocytes, and microglia for which we were able to detect specific bands with western blot (Synaptophysin, Spinophilin, PSD95, ALDH1L1, GFAP, VIM, CX3CR1, IBA1 and HLA-DRA), for protein analysis.

2.4 Processing fresh tissue for RNA and protein extraction

Fresh frozen tissue was sectioned (100µm) using the cryostat (Leica Biosystems, Germany) and grey matter dissected manually. Tissue was dissociated in TRIzol reagent (Thermo Life Technologies, USA) for RNA isolation, or suspension buffer and 2x SDS loading buffer for protein extraction. Tissue was dissociated using the Ultra Turrax Homogenizer (IKA) and stored at -80°C. Protein samples were heated at 95 °C for 5 minutes and DNA fractions broken up with a 25-gauge needle.

2.5 RNA expression

RNA extraction and cDNA preparation were performed as described before [49] using the chloroform/isopropanol method and the Quantitect Reverse Transcription Kit (QIAGEN, Germany). For quantitative real-time polymerase chain reaction (qPCR) 3.5ng input cDNA per reaction was used with SYBR green PCR mix (FastStart Universal, Roche, Germany). Primers (intron spanning) were designed using the NCBI primer-BLAST tool (Supplementary table 4). QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies Corporation, USA) was used with the following cycle conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds. All qPCR experiments were performed in triplo and averaged. Absolute fluorescence levels were calculated ($2^{\Delta CT}$) and normalized using the geomean of reference genes Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and 18S ribosomal RNA (*18S*), stable for brain tissue[50]. Expression levels of *GAPDH* and *18S* was not different between CTR and SCZ

subjects. Samples with an undetermined Cycle Threshold were included in the rank-based non-parametric analyses as 0. SCZ and CTR samples were processed together by placing them alternately on each qPCR plate and master mixes with the cDNA were added to all plates simultaneously.

2.6 Protein expression

Proteins were separated by SDS-PAGE gel electrophoresis on 7.5%, 10%, or 15% gels, and blotted on a 0.45 or 0.22 μm pore size nitrocellulose membrane (GE Healthcare) using wet blotting. Blots were incubated in blocking buffer and stained with primary antibody (Supplementary table 5) in blocking buffer at 4°C over-night. Blots were washed in TBS-T and incubated in secondary antibody in blocking buffer at RT for one hour. Blots were washed in TBS-T and rinsed with demineralized-H_2O before scanning. Both scanning and quantification were performed using the Odyssey CLx Western blot Detection System (LI-COR biosciences, USA). Each protein of interest was normalized against a reference protein on the same blot (GAPDH or beta-actin). SCZ and CTR samples were placed alternately on each western blot.

2.7 Tissue preparation paraffin tissue

Paraffin embedded tissue was sectioned (7μm) on the microtome (Leica Microsystems) and mounted on glass slides. Sections were cut in series of four adjacent sections for layer determination (Nissl) and analysis of different markers in cortical layer III (GFAP, IBA1 and Spinophilin).

2.8 Nissl staining

Tissue sections were deparaffinized through xylene and alcohol series and stained using 0.1% cresyl fast violet solution (0.14%, Clin-Tech, UK) for 7 minutes, rinsed with tap water and dehydrated through an alcohol series and mounted using Entellan (Merk Millipore, Germany).

2.9 Immunohistochemistry

Tissue sections were deparaffinized through xylene and alcohol series, washed in 1x PBS and quenched for endogenous peroxidases (1% H_2O_2 (Sigma-Aldrich, the Netherlands) in 1x PBS) for 10 minutes and washed in 1x PBS/0.05% Tween (PBST). For antigen retrieval, slides were heated for 15 minutes in citrate buffer, in demineralized H_2O, pH 6, followed by incubation in blocking buffer 2 for 30 minutes. Sections were incubated at 4°C overnight with primary antibody (GFAP, 1:1000; IBA1, 1:1000; Spinophilin; 1:1000, see Supplementary table 5) in blocking buffer 2. The next day, slides were washed in PBST and processed for DAB (glia) or immunofluorescence (synapse). For DAB, sections were incubated with secondary goat-anti-rabbit biotinylated antibody (1:400 (Jackson ImmunoReserach, UK)) in blocking buffer 2 for one hour, washed in PBST and treated for 30 minutes in avidin-bi-

otin-peroxidase complex solution (1:50, (Avidin-Biotin Complex Peroxidase Staining kit, Vector Laboratories) in blocking buffer 2). After PBST wash, slides were incubated for 10 minutes in DAB-nickel solution. Sections were rehydrated in ethanol/xylene series and mounted in Entellan. For immunofluorescence, sections were incubated in secondary goat-anti-rabbit Alexa 488 antibody (1:1000, Jackson ImmunoResearch, UK) and Hoechst (1:1000, Fisher Scientific, USA) for nuclei staining at RT for two hours, washed in PBST and incubated in Sudan-black. After a final PBS wash, slides were mounted in prolong-gold (Invitrogen, UK). All samples (SCZ and CTR) were processed simultaneously in the same run. Supplementary table 3 describes the composition of the buffers and solutions.

2.10 Microscopy

Both the Nissl and DAB-stainings were imaged using the NanoZoomer-XR (Hamamatsu Photonics, Pathology department UMC Utrecht, The Netherlands) to obtain whole-slide brightfield images (40x). Illumination time and settings were kept constant for all sections.

For the synapse immunohistochemistry, a confocal microscope (LSM 880 Zeiss) was used (63x). For each section, five image regions within cortical layer III were randomly selected within the Hoechst channel. Exposure times for the 488-nm excitation wavelength were pre-set and kept consistent. Maximum intensity projections were generated from image stacks of five (0.2µm step size).

2.11 Quantification

Open-source software ImageJ/FIJI and Nanozoomer Digital Pathology View (NDP view, (Hamamatsu Photonics, Japan) were used to pre-process and quantify images. In NDP view the Nissl staining was used to determine cortical layer III based on layer features and cell density for each donor. To determine the relative thickness of layer III, three random sites within each section were measured for the width of layer III, dived by the total distance between the pia and the white matter, and averaged per donor. For each section, 10 image regions containing cortical layer III were randomly selected at 2.5x in NDP View and exported as TIFF images for further processing. For quantification of the IBA1 and GFAP positive cell bodies in cortical layer III, an overlay was created with the Nissl staining using the TrakEM tool in FIJI. For each glial image, a region of interest (ROI) was generated for layer III. To minimize quantification bias, we developed a macro to determine the number of GFAP positive cell bodies within the ROI using automated thresholding, which was validated by counting 10 random samples by hand by J.E.J and A.BvB. The number of IBA1 positive cell bodies within each ROI was quantified by systematic manual counting in FIJI, as heterogeneity between donors did not allow for automated threshold determination. Images were excluded when: the image was not in focus, we did not observe any positive staining in the image, or when the tissue was damaged. One image region was used for the determination of the number of glial nuclei

in cortical layer III based on the Nissl staining, by designing a macro in FIJI quantifying the amount of round and smaller glial nuclei, and the total amount of nuclei within cortical layer III per mm^2.

The puncta-analyzer plugin was used to quantify Spinophilin immunofluorescence. Maximum intensity projections were converted to RGB using FIJI and processed through the puncta-analyzer plugin pipeline in ImageJ (V1.29X). Output masks were saved and used for area coverage determination in ImageJ.

We calculated and removed image outliers within each donor using the interquartile range for glia and synapse immunohistochemistry (images excluded: IBA1 CTR: 3 SCZ: 3; GFAP CTR: 5 SCZ: 7; Spinophilin CTR: 5 SCZ: 8). This resulted in the analysis of 112 SCZ and 96 CTR GFAP images (average: 8; minimum: 4), 84 SCZ and 85 CTR IBA1 images (average: 7; minimum 3), and 55 SCZ and 55 CTR Spinophilin images (average: 4; minimum: 3). Results of the analyzed pictures were averaged.

2.12 Statistics

Statistical analysis and figure design were performed with Graphpad software (version 8.4) and R studio (version 4.0.0, R core team (2020)). We assessed the influence of six main confounding factors, i.e. age, sex, brain pH, PMD, Braak- and amyloid score for each datapoint with Spearman Rho correlation or the Kruskal Wallis test as appropriate (supplemental table 6). When a significant association was found ($p < 0.05$), the variable was entered as a covariate in post-hoc analyses using logistic regression. Unpaired Mann-Whitney U tests were used to compare gene expression, protein expression, and the number of astrocytes, microglia, and between SCZ and CTR donors because data were not normally distributed. Spearman Rho correlation analyses were applied to determine the main correlation effect between numerical values (glia- and synapse number, genes, or effect sizes) and Fisher's r to z transformation was applied to assess the interaction effect. For the gene expression association analysis, we grouped the genes based on cell-type enrichment expression [47] into three clusters: microglia, astrocyte and synapse. Expression data were z-transformed, and the mean z-value was calculated for each cluster. Subsequently gene-module correlations were performed using the *corrplot* package (Taiyun Wei & Viliam Simko (2018)). Given limited number and the inherent relation between the different transcripts, and small sample size no correction for multiple testing was applied this increasing type I error rate of the study.

2.13 Data availability statement

Relevant data supporting the discussed findings are included in the paper and its supplementary information files. Extended information and data are available from the corresponding author upon request.

3. Results

In order to study the phenotype and number of synapses, astrocytes, microglia, and their association in cortical grey matter and layer III specifically, we performed gene (qPCR) and protein (western blot) expression analyses, and morphometric analyses using Nissl staining and immunohistochemistry (Figure 1).

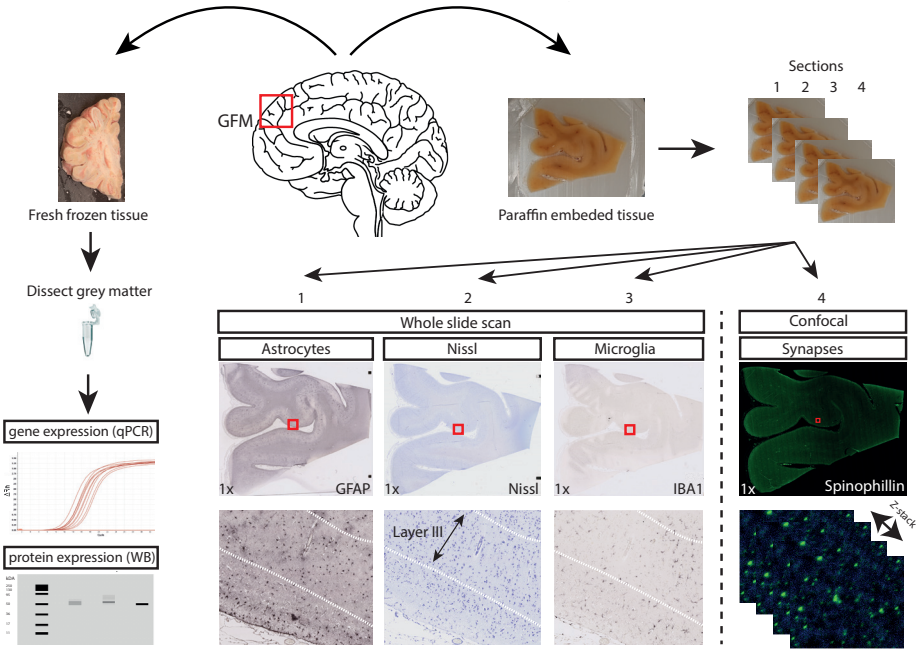


Figure 1. Experimental set-up to assess synapses, glia (microglia and astrocytes) and their interaction in SCZ postmortem cortical grey matter and layer III specifically.
Both frozen and paraffin embedded tissue were used from the *gyrus frontalis medialis* (GFM) from SCZ and CTR donors. Grey matter was dissected from the frozen tissue and processed for gene and protein expression analyses using qPCR and western blot. Paraffin tissue was sectioned in series and stained with a Nissl stain to determine cortical layer III and immunohistochemistry to determine the number of glia using GFAP for astrocytes and IBA1 for microglia and Spinophilin to visualize post-synapses. The cortical layer III determined by Nissl stain was super-imposed on the whole slide scans of the GFAP and IBA1 stainings to determine the number of glia in layer III specifically. Confocal z-stack imaging was used for imaging of the synapses in cortical layer III.

3.1 Demographics

The demographic data of donors included in this study (frozen tissue: SCZ N = 12; CTR N = 11, paraffinized tissue: SCZ N = 13; CTR N = 12) are summarized in Table 1 and details are provided in Supplementary Table 1. Neither age, nor sex, PMD or brain pH differed between SCZ and CTR donors on group level.

Paraffin	% Male	Age (years)	PMD (hours)	pH
CTR (N=12)	58.33	73 (13)	9 (6.1)	6.6 (0.3)
SCZ (N=13)	66.66	75 (13)	26.4 (29.2)	6.5 (0.4)
Frozen	% Male	Age (years)	PMD (hours)	pH
CTR (N=11)	33.33	77 (14)	7.6 (3.5)	6.6 (0.3)
SCZ (N=12)	33.33	69 (15)	9.3 (4.2)	6.7 (0.5)

Table 1. Summary of demographic data of donors within the study.
Summary of main clinical and postmortem variables of SCZ and CTR donors within this study (sex, age, PMD and pH). Information separated for frozen and paraffin tissue donors. Values represent mean and standard deviation. Sex is represented as number of male and female donors. Age in years and PMD in minutes. PMD = postmortem delay, pH = brain pH. None of the measures were found to be different in paraffin or frozen tissue between SCZ patients and CTR donors ($p > 0.05$).

3.2 Synaptic markers

Synaptic changes between SCZ and CTR were measured using both post- and pre-synaptic markers. Gene expression analysis of Spinophilin (*PPP1R9B*/SPIN), Postsynaptic-density protein 95 (PSD95), Synapsin 1 (SYN1) and Synaptophysin (SYP) (Figure 2a) showed no difference between SCZ and CTR donors in cortical grey matter (Figure 2b; Supplementary table2). Protein levels of three of these makers (SPIN, PSD95, and SYP) were assessed by western blot, also revealing no difference between SCZ and CTR donors (Figure2c; Supplementary figure 3; Supplementary table 2).

It is suggested that synaptic changes in the cortex are more specifically localized to cortical layer III[12,51,52]. Therefore, we used immunohistochemistry with the postsynaptic maker SPIN as a proxy for synapse density (Figure 2d). Quantification of the number of immunofluorescent puncta did not show a difference in the postsynaptic $SPIN^+$ puncta per $10\mu m^2$ (SCZ: median = 35.23, IQR = 31.49; CTR: median = 21.09, IQR = 41.53; $p = 0.61$) or area percentage covered by $SPIN^+$ (SCZ: median = 1.71, IQR = 1.79; CTR: median = 0.86, IQR = 1.18; $p = 0.19$) in layer III between the SCZ and CTR group (Figure 2e).

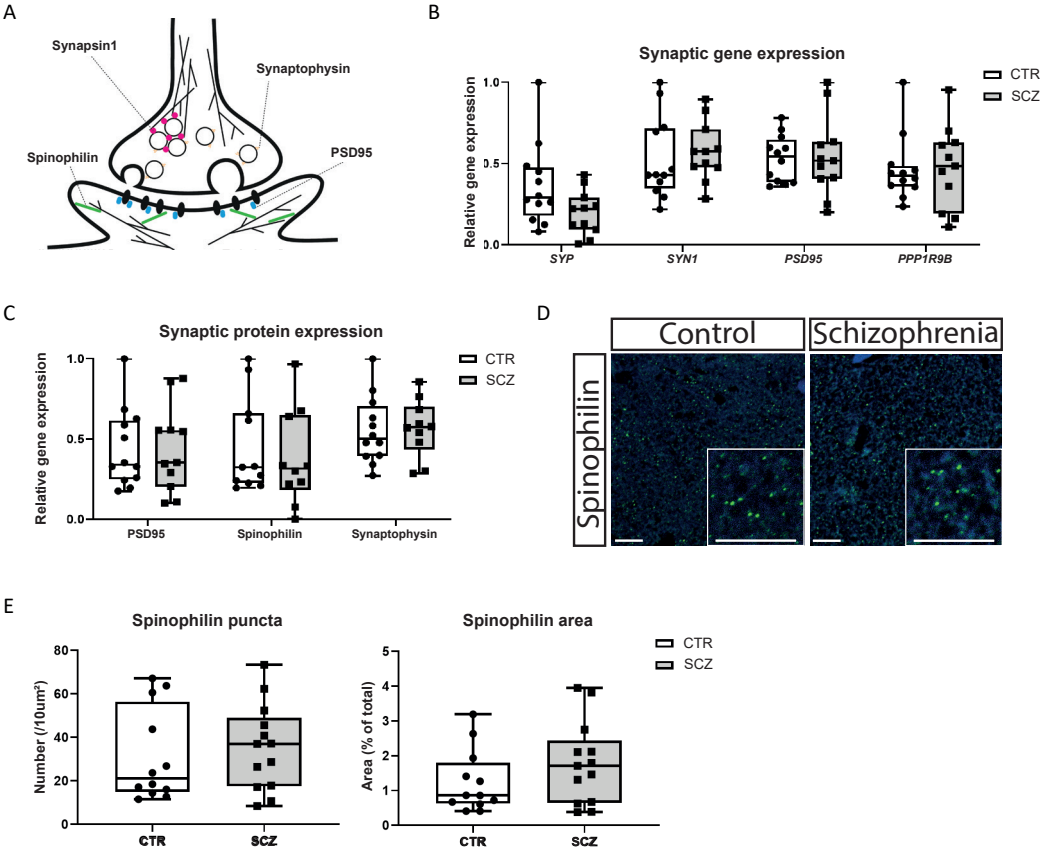


Figure 2. Synaptic measures in cortical grey matter and layer III specifically.

A Markers used within the study for the assessment of synapses in cortical grey matter and layer III. Pre-synaptic markers Synapsin 1 and Synaptophysin and postsynaptic markers Spinophilin and PSD95. B-C mRNA and protein expression of these synaptic markers were determined by qPCR and western blot in the gyrus frontalis medialis of SCZ patients (N = 12) and CTR donors (N = 11). Gene expression was normalized against reference genes 18S ribosomal RNA (18S) and glyceraldehyde 3-phosphate dehydrogenease (GAPDH). Protein expression was normalized against reference proteins GAPDH or beta-actin. Data were transformed to a relative value to show relative gene or protein expression. D Spinophilin immunohistochemistry with HOECHST staining was used to visualize synapses in CTR (D') and SCZ (D") donors at 63x magnification (scale bar 10um). E Spinophilin immunoreactivity was quantified by counting the number of SPIN+ puncta (E') and the area covered by SPIN+ staining (E"). Graphs show boxplots (white for CTR and grey for SCZ) containing individual datapoints for CTR (circles) and SCZ (squares) indicating the median, 25th and 75th percentile, with the whiskers showing the maximum and minimum values.

3.3 Microglia and astrocytes

To assess microglia and astrocytes in cortical grey matter of SCZ patients on a molecular level, we measured gene and protein expression from a panel of markers for microglia, astrocytes, and glia-synapse interacting factors (Supplementary table 2). We found a decreased expression of two microglia markers: *ITGAM* (SCZ: median = 0.07, IQR = 0.04; CTR: median = 0.03, IQR = 0.09; p = 0.048) and *CX3CR1* (SCZ: median = 7.08, IQR = 8.73; CTR: median = 2.85, IQR = 3.63; p = 0.04) in SCZ patients(Figure 3a). There was no difference in expression of the ligand of *CX3CR1, CX3CL1 (*SCZ: median = 3.34, IQR = 3.07; CTR: median = 3.03, IQR = 1.52; p = 0.79), or in any other glial gene or protein from the analysed panel (Supplementary figure 2, Supplementary table 2, Supplementary figure 3).

Subsequently, we assessed whether we could identify changes in number of microglia and astrocytes specifically in cortical layer III using Nissl, GFAP and IBA1 immunohistochemistry (Figure 3c). Whole slide scanning was performed, enabling us to quantify the glia in a large proportion of the cortical layer III opposed to only selected image regions. Based on the Nissl staining we found no difference between SCZ and CTR donors in the relative width of layer III (SCZ: median = 34.08, IQR = 6.78; CTR: median = 36.47, IQR = 05.79 ; p = 0.32) (Supplementary figure 1a), the number of glial nuclei per mm^2 in layer III (SCZ: median = 771.8, IQR = 107.7; CTR: median = 844.9, IQR = 248.2; p = 0.16) (Figure 3d) and the percentage of glial nuclei in layer III (SCZ: median =78.79, IQR = 10.85; CTR: median = 80.65, IQR = 9.47; p = 0.73) (Supplementary figure 1b). Also the number of GFAP-immunoreactive astrocytic cell bodies per mm^2 (SCZ: median = 197.7, IQR = 178.4; CTR: median = 221.8, IQR = 117.6; p = 0.27) and IBA1-immunoreactive microglial cell bodies per mm^2 (SCZ: median = 34.11, IQR = 54.9; CTR: median = 54.67, IQR = 35.68; p = 0.14) in layer III were the same between SCZ and CTR groups (Figure 3d).

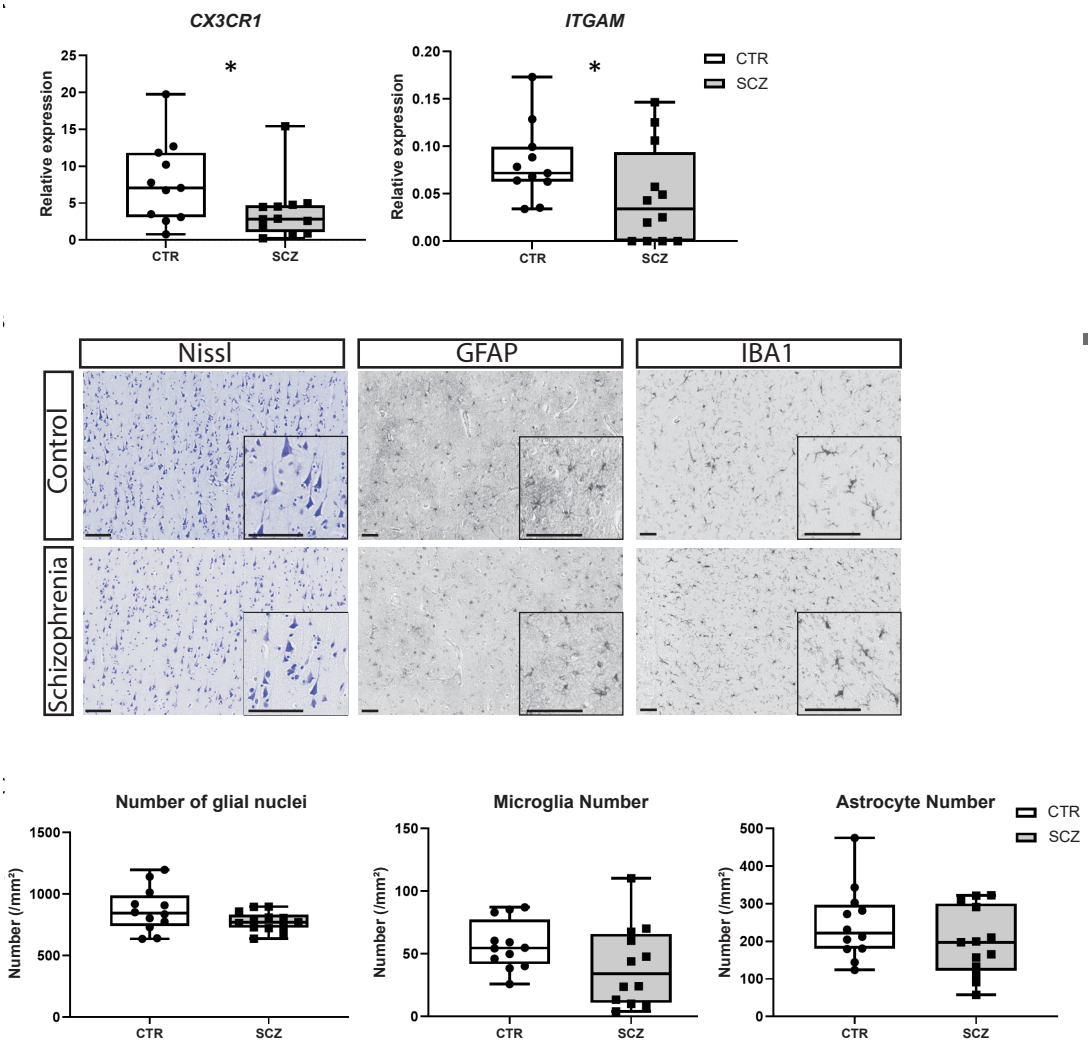


Figure 3. Microglia and astrocytes in grey matter and cortical layer III in SCZ.

A mRNA expression of microglia and astrocyte genes was measured using qPCR in the *gyrus frontalis medialis* of SCZ patients (N = 12) and CTR donors (N = 11). Gene expression was normalized against reference genes 18S ribosomal RNA (*18S*) and glyceraldehyde 3-phosphate dehydrogenease (*GAPDH*). (A) Significant downregulation ($p < 0.05$) was found in SCZ donors for the microglial genes *CX3CR1* (A') and *ITGAM* (A''). **B** The *gyrus frontalis medialis* of CTR donors (top, N = 12) and SCZ patients (bottom, N = 13) was stained using Nissl staining and DAB-immunohistochemistry for GFA and IBA1 to visualize cortical layer III, astrocytes and microglia respectively at 40x magnification (scale bar 100µm). **C** The number of glia was quantified in layer III using the Nissl staining by counting the number of glial nuclei in the Nissl staining and the number of $GFAP^+$ or IBA^+ cell bodies. Boxplot graphs (white for CTR and grey for SCZ) contain individual datapoints for CTR (circles) and SCZ (squares) indicating the median, 25th and 75th percentile, with the whiskers showing the maximum and minimum values. * $p < 0.05$

3.4 Association between synapse- and glial markers

After analysing the synaptic and glial markers in cortical grey matter and layer III specifically, the next goal of this study was to assess the relationship between these different entities. To this end, we performed association studies for both the gene expression and histological data.

Regarding the gene expression data, we created a correlation matrix assessing the association of the average expression of genes between the three clusters: astrocyte, microglia, and synapse for CTR donors and SCZ patients separately (synapse-astrocyte: SCZ= -0.04, p = 0.92; CTR ρ = -0.35, p = 0.30 ; synapse-microglia SCZ ρ = 0.18, p = 0.59; CTR ρ = 0.1, p = 0.78; microglia-astrocyte: SCZ ρ = 0.61, p = 0.04; CTR ρ = 0.33, p = 0.33) (Figure 4a). We did not find a difference in the strength of the interaction of these clusters between SCZ and CTR (synapse-astrocyte: Z = -0.65, p = 0.52; synapse-microglia: Z = -0.25, p = 0.80; microglia-astrocyte: Z = -0.21, p = 0.83).

Further, we assessed the association between the number of synapses and the number of glia in cortical layer III for SCZ and CTR donors (synapse-astrocyte: SCZ ρ = 0.07, p = 0.82; CTR ρ = -0.06 p = 0.87; synapse-microglia SCZ ρ = -0,02 p = 0.96; CTR ρ = 0.13 p = 0.68) (figure 4b). We found no different interaction in SCZ compared to CTR (synapse-astrocyte: Z = -0.24, p = 0.81; synapse-microglia: Z = 1.16, p = 0.25) or an interaction between these measures in general (synapse-astrocyte: ρ = 0.04, p = 0.86; synapse-microglia: ρ = 0.08, p = 0.70) (Supplementary figure 4a).

3.5 Confounder analysis

Spearman correlation analysis and Kruskal Wallis tests with the pre-selected confounders (sex, age, PMD, pH, braak-score and amyloid-score) revealed a significant correlation between microglia number and PMD (r = -0.63, p = <0.001), *CX3CL1* gene expression and age (r = -0.51 , p = 0.005) and *C4* gene expression and amyloid pathology (H = 8.32, p = 0.005) (Supplementary table 6). After adjustment for these potential confounders, using logistic regression, the interpretation of the data remained the same (microglia number: p = 0.44; *CX3CL1*: p = 0.06; *C4*: p = 0.77).

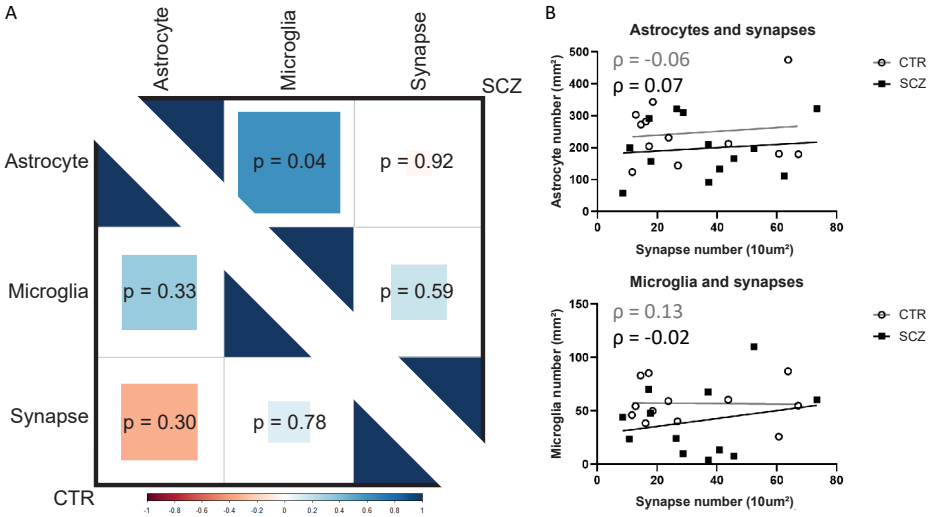


Figure 4. Association between synapse- and glial markers in grey matter and cortical layer III in SCZ. **A** A correlation matrix was generated from the average expression of genes within the three clusters: microglia, astrocyte, and synapse, in the *gyrus frontalis medialis* for SCZ patients (N = 12, right-top) and CTR donors (N = 11, left-bottom) separately. Negative associations are indicated with a red colour and positive associations in blue. The size of the square corresponds to the Spearman's rho and the number represents the p-value. **B** Linear regression was performed to assess the association between the number of synapses and astrocytes (**B'**) and microglia (**B''**) in the *gyrus frontalis medialis* of SCZ patients (N = 13) and CTR donors (N = 12). Spearman's rho correlation is given separately for CTR donors (circles, grey) and SCZ patients (squares, black).

4. Discussion

In this study synapses, microglia, astrocytes, and their association in postmortem brain tissue of SCZ patients were assessed, focussing on grey matter and more specifically on cortical layer III. A multi-level approach assessing gene and protein expression, and morphometric analysis did not find differences in synaptic or glial density, gene- and protein expression, except for an altered gene expression of microglia genes *CX3CR1 and ITGAM* in SCZ.

4.1 Synaptic findings

In contrast to previous studies we did not identify differences in pre- or post-synaptic gene or protein expression, nor in the number of Spinophilin positive puncta in cortical layer III[8,9]. Although the cortical layer III synapse deficit has developed into an important hypothesis in SCZ research, only two studies assessed this phenotype in cortical layer III specifically (both finding a decrease)[51,52]. In contrast to our study, these studies used Golgi staining for synapse quantification. However, previous studies in the auditory cortex (BA 41) of SCZ patients did show an effect on synapse number in cortical layer III using immunohistochemistry[53,54]. Furthermore, gene and protein expression data on this topic are notoriously heterogeneous, and most synaptic genes or proteins have not been replicated in enough datasets to perform individual meta-analyses on them[8,9]. This shows the need for additional studies, like ours, to advance our understanding of synaptic density changes in SCZ. Interestingly, a study in layer III of the auditory cortex (BA 41) of SCZ patients showed that only the smallest spines are lost in cortical layer III of the auditory cortex in SCZ[55]. Therefore, a more in-depth specification of smaller synapses in the GFM could be relevant.

4.2 Glia phenotype

Regarding astrocytes and microglia, studies have focussed on gene expression, protein expression, and density in SCZ, with heterogeneous results[40,41]. Only few glia density studies have taken cortical layer specificity into account[56–58], but those reported varying results. Although we did not find differences in glial density or protein expression, we did identify a downregulation of *ITGAM* and *CX3CR1* in SCZ. This is in line with several previous hypothesis-driven studies[59,60]. Both *ITGAM* and *CX3CR1* are receptors involved in microglia-synapse interaction through complement and fractalkine signaling, respectively[61–63]. In mice, knock-out of *CX3CR1* resulted in 31% decrease in spine density in the rostral migratory stream[64] while depletion of the complement receptor 3 (deleting the CD11b/ITGAM subunit) caused a 50% decrease in engulfment of retinal ganglion cell inputs[65].

Moreover, we performed a correlation analysis between the effect-sizes (log2-fold changes) of the current study with the data from the largest RNAseq dataset on postmortem cortical brain tissue in SCZ to date (Gandal et al., 2018). Interestingly, this analysis showed a

similarity in the direction (mostly downregulated) and size of the effect-sizes measured with the microglia markers ($r = 0.58$, $p = 0.04$) (Supplementary figure 5). Together, these data suggest an altered microglial phenotype in SCZ, with genes that are known to play a role in neuron-glia interactions.

4.3 Glia-synapse interaction effect

Pre-clinical research has convincingly shown an important role for glia in the formation, maintenance, and elimination of synapses[29–31,65], which has led to the hypothesis that glial cells are involved in the synapse pathology underlying schizophrenia[16–20]. Recent RNA-sequencing studies and the present study have shown differential expression of microglial genes[6] and the next step is to understand whether and how microglial phenotype could be linked to synapse pathology. In our study, we did not find an association between glia and synapses on gene, protein, or cell number level. If changes in association between synapses and glia in SCZ are very subtle, more refined analyses may be needed to detect these small effects.

4.4 Limitations

This study should be interpreted in the context of its limitations. SCZ is a complex psychiatric disorder and heterogeneity between patients is further increased in postmortem research due to the large number of technical confounding factors that must be considered. In the current study we have analysed the effect of several potential confounding factors (age, PMD, sex, pH, and Braak-score). However other potential confounders were not included, such as the use of antipsychotic medication. Another important limitation is that the limited sample size of our study could have affected the power to determine differences between SCZ and CTR donors. However, a statistical power analysis based on the pooled effect size of previous studies on synapse density in cortical layer III (Hedges's $g = -1.39$) showed that our sample size ($N = 26$, alpha = 0.05, power = 0.92) would be sufficient to determine the effect on synapse density in a between group comparison. In addition, the size of our cohort ($N = 11$-13 per group) is in the range of samples sizes of previous studies varying from $N = 6$ to $N = 20$ per group[12,51–54,66]. Furthermore, the marker Spinophilin, quantifying postsynaptic elements, is only a proxy of the amount of synapses. Although numerous studies have used these type of measures for synaptic density (Golgi staining or synaptic protein levels), only few studies have quantified actual synapses (pre and post synaptic connections) in SCZ[13–15,67–70]. In the same line, a single antibody marker was utilized for the identification of astrocytes (GFAP) and microglia (IBA1) in the morphometric study of cortical layer III. Although these markers are broadly used for the identification of these glia, we cannot rule out that the results are specific for the proteins we choose to analyse.

Overall, in this study we were able to analyse synapses and glia on multiple levels (gene and protein expression, and morphometric) in the same tissue from a well-matched cohort of SCZ and CTR donors, with a relatively low PMD. However, our data underscore the challenge of detecting convincing preclinical identified mechanisms about the role of glia in synapse formation and pruning in postmortem brain tissue of patients. At present, it is unclear how changes in glia-synapse interaction that are dynamic, dependent on developmental stage and brain region, can best be investigated in postmortem human brain tissue of older patients with psychiatric symptoms. A potential way forward would be to incorporate findings from human postmortem brain tissue into preclinical models (like rodent models or *in vitro* models like cerebral organoids) and explore the impact of these moderate gene-expression changes, like a lower expression of *CX3CR1* or *ITGAM*, in living cells. This may be essential in the pursuit to gain a better understanding about the interaction between synapses and glia in patients with SCZ.

Acknowledgements

The authors thank the team of the Netherlands Brain Bank for their excellent services (www.hersenbank.nl) and Domenico Castigliego from the department of Pathology at the UMC Utrecht for his support with the whole slide scan imaging.

Autor contributions

L.D.W. and A.B.B. designed the project. A.B.B., M.A.M.S., and G.J.L.S. performed matching of the donors and extracted demographic data. G.J.L.S. and L.D.W. performed medical chart review for SCZ diagnosis. A.B.B. and J.E.J. performed tissue sectioning, staining, and qPCR experiments. J.E.J. performed pre-processing of whole-slide scan images. A.B.B. performed western blots, confocal imaging, statistical analyses and figure design. A.B.B. wrote the manuscript with supervision of L.D.W. and M.P.B. Critical feedback was provided by J.M. and E.M.H. over the duration of the project and in reviewing the manuscript. Funding was obtained by L.D.W., M.P.B., and R.S.K.

Supplementary material

Supplementary material is available via Surfdrive:
https://surfdrive.surf.nl/files/index.php/s/lndBdjHUjbH7Rnl

References

1. van den Heuvel, M. P. *et al.* Abnormal Rich Club Organization and Functional Brain Dynamics in Schizophrenia. *JAMA Psychiatry* **70**, 783 (2013).
2. Pettersson-Yeo, W. *et al.* Using genetic, cognitive and multi-modal neuroimaging data to identify ultra-high-risk and first-episode psychosis at the individual level. *Psychological Medicine* **43**, 2547–2562 (2013).
3. Anticevic, A. *et al.* Connectivity, pharmacology, and computation: toward a mechanistic understanding of neural system dysfunction in schizophrenia. *Frontiers in psychiatry* **4**, 169 (2013).
4. Friston, K. J. & Frith, C. D. Schizophrenia: a disconnection syndrome? *Clinical neuroscience (New York, N.Y.)* **3**, 89–97 (1995).
5. Stephan, K. E., Friston, K. J. & Frith, C. D. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. *Schizophrenia bulletin* **35**, 509–27 (2009).
6. Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science (New York, N.Y.)* **362**, eaat8127 (2018).
7. Ripke, S. *et al.* Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421–427 (2014).
8. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular Psychiatry* (2018) doi:10.1038/s41380-018-0041-5.
9. Berdenis Van Berlekom, A. *et al.* Synapse Pathology in Schizophrenia: A Meta-analysis of Postsynaptic Elements in Postmortem Brain Studies. *Schizophrenia Bulletin* **46**, 374–386 (2020).
10. Moyer, C. E., Shelton, M. A. & Sweet, R. A. Dendritic spine alterations in schizophrenia. *Neuroscience letters* **601**, 46–53 (2015).
11. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular psychiatry* (2018) doi:10.1038/s41380-018-0041-5.
12. Kolluri, N., Sun, Z., Sampson, A. R. & Lewis, D. A. Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. *The American journal of psychiatry* **162**, 1200–2 (2005).
13. McCollum, L. A., Walker, C. K., Roche, J. K. & Roberts, R. C. Elevated Excitatory Input to the Nucleus Accumbens in Schizophrenia: A Postmortem Ultrastructural Study. *Schizophrenia bulletin* **41**, 1123–32 (2015).
14. Roberts, R. C., Roche, J. K. & Conley, R. R. Synaptic differences in the postmortem striatum of subjects with schizophrenia: a stereological ultrastructural analysis. *Synapse (New York, N.Y.)* **56**, 185–97 (2005).
15. Kung, L., Conley, R., Chute, D. J., Smialek, J. & Roberts, R. C. Synaptic changes in the striatum of schizophrenic cases: A controlled postmortem ultrastructural study. *Synapse* **28**, 125–139 (1998).
16. Dietz, A. G., Goldman, S. A. & Nedergaard, M. Glial cells in schizophrenia: a unified hypothesis. *The Lancet Psychiatry* vol. 7 272–281 (2020).
17. Roman, C., Egert, L. & Di Benedetto, B. Astrocytic-neuronal crosstalk gets jammed: Alternative perspectives on the onset of neuropsychiatric disorders. *European Journal of Neuroscience* (2020) doi:10.1111/ejn.14900.
18. Mallya, A. P. & Deutch, A. Y. (Micro)Glia as Effectors of Cortical Volume Loss in Schizophrenia. *Schizophrenia Bulletin* **44**, 948–957 (2018).

19. Neniskyte, U. & Gross, C. T. Errant gardeners: Glial-cell-dependent synaptic pruning and neurodevelopmental disorders. *Nature Reviews Neuroscience* vol. 18 658–670 (2017).

20. Druart, M. & Le Magueresse, C. Emerging Roles of Complement in Psychiatric Disorders. *Frontiers in Psychiatry* vol. 10 (2019).

21. Barres, B. A. The Mystery and Magic of Glia: A Perspective on Their Roles in Health and Disease. *Neuron* vol. 60 430–440 (2008).

22. Verkhratsky, A. & Nedergaard, M. Physiology of astroglia. *Physiological Reviews* **98**, 239–389 (2018).

23. Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481–487 (2017).

24. Iadecola, C. & Nedergaard, M. Glial regulation of the cerebral microvasculature. *Nature Neuroscience* vol. 10 1369–1376 (2007).

25. Sofroniew, M. V. & Vinters, H. V. Astrocytes: Biology and pathology. *Acta Neuropathologica* vol. 119 7–35 (2010).

26. Colonna, M. & Butovsky, O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annual Review of Immunology* **35**, 441–468 (2017).

27. Cunningham, C. L., Martínez-Cerdeño, V. & Noctor, S. C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *Journal of Neuroscience* **33**, 4216–4233 (2013).

28. Helmut, K., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiological Reviews* **91**, 461–553 (2011).

29. Perea, G., Navarrete, M. & Araque, A. Tripartite synapses: astrocytes process and control synaptic information. *Trends in Neurosciences* vol. 32 421–431 (2009).

30. Schafer, D. P., Lehrman, E. K. & Stevens, B. The "quad-partite" synapse: Microglia-synapse interactions in the developing and mature CNS. *GLIA* **61**, 24–36 (2013).

31. Chung, W. S. *et al.* Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* **504**, 394–400 (2013).

32. Schafer, D. P. *et al.* Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74**, 691–705 (2012).

33. Stevens, B. *et al.* The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131**, 1164–1178 (2007).

34. Kamitaki, N. *et al.* Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature* **582**, 577–581 (2020).

35. Sellgren, C. M. *et al.* Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nature Neuroscience* **22**, 374–385 (2019).

36. Parkin, G. M., Udawela, M., Gibbons, A. & Dean, B. Glutamate transporters, EAAT1 and EAAT2, are potentially important in the pathophysiology and treatment of schizophrenia and affective disorders. *World Journal of Psychiatry* **8**, 51–63 (2018).

37. McCullumsmith, R. E. *et al.* Cell-specific abnormalities of glutamate transporters in schizophrenia: Sick astrocytes and compensating relay neurons? *Molecular Psychiatry* **21**, 823–830 (2016).

38. Windrem, M. S. *et al.* Human Glial Progenitor Cells Effectively Remyelinate the Demyelinated Adult Brain. *Cell reports* **31**, 107658 (2020).

39. Feinberg, I. Schizophrenia: Caused by a fault in programmed synaptic elimination during adolescence? *Journal of Psychiatric Research* **17**, 319–334 (1982).

40. van Kesteren, C. F. M. G. *et al.* Immune involvement in the pathogenesis of schizophrenia: A meta-analysis on postmortem brain studies. *Translational Psychiatry* **7**, (2017).

41. Trépanier, M. O., Hopperton, K. E., Mizrahi, R., Mechawar, N. & Bazinet, R. P. Postmortem evidence of cerebral inflammation in schizophrenia: A systematic review. *Molecular Psychiatry* vol. 21 1009–1026 (2016).

42. Silvin, A. & Ginhoux, F. Microglia heterogeneity along a spatio–temporal axis: More questions than answers. *GLIA* vol. 66 2045–2057 (2018).

43. Böttcher, C. *et al.* Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry. *Nature Neuroscience* **22**, 78–90 (2019).

44. Lanjakornsiripan, D. *et al.* Layer-specific morphological and molecular differences in neocortical astrocytes and their dependence on neuronal layers. *Nature Communications* **9**, (2018).

45. Bayraktar, O. A. *et al.* Astrocyte layers in the mammalian cerebral cortex revealed by a single-cell in situ transcriptomic map. *Nature Neuroscience* **23**, 500–509 (2020).

46. de Lange, G. M., Rademaker, M., Boks, M. P. & Palmen, S. J. M. C. Brain donation in psychiatry: Results of a Dutch prospective donor program among psychiatric cohort participants. *BMC Psychiatry* **17**, (2017).

47. Zhang, Y. *et al.* Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron* **89**, 37–53 (2016).

48. Butovsky, O. *et al.* Identification of a unique TGF-β-dependent molecular and functional signature in microglia. *Nature Neuroscience* **17**, 131–143 (2014).

49. Melief, J. *et al.* Characterizing primary human microglia: A comparative study with myeloid subsets and culture models. *GLIA* **64**, 1857–1868 (2016).

50. Sneeboer, M. A. M. *et al.* Microglia in post-mortem brain tissue of patients with bipolar disorder are not immune activated. *Translational Psychiatry* **9**, (2019).

51. Konopaske, G. T., Lange, N., Coyle, J. T. & Benes, F. M. Prefrontal cortical dendritic spine pathology in schizophrenia and bipolar disorder. *JAMA psychiatry* **71**, 1323–31 (2014).

52. Glantz, L. A. & Lewis, D. A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of general psychiatry* **57**, 65–73 (2000).

53. Shelton, M. A. *et al.* Loss of Microtubule-Associated Protein 2 Immunoreactivity Linked to Dendritic Spine Loss in Schizophrenia. *Biological psychiatry* **78**, 374–85 (2015).

54. Sweet, R. A., Henteleff, R. A., Zhang, W., Sampson, A. R. & Lewis, D. A. Reduced dendritic spine density in auditory cortex of subjects with schizophrenia. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **34**, 374–89 (2009).

55. MacDonald, M. L. *et al.* Selective Loss of Smaller Spines in Schizophrenia. *The American journal of psychiatry* **174**, 586–594 (2017).

56. Cotter, D. *et al.* Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cerebral Cortex* vol. 12 386–394 (2002).

57. Radewicz, K., Garey, L. J., Gentleman, S. M. & Reynolds, R. Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. *Journal of neuropathology and experimental neurology* **59**, 137–50 (2000).

58. Rajkowska, G. *et al.* Layer-specific reductions in GFAP-reactive astroglia in the dorsolateral prefrontal cortex in schizophrenia. *Schizophrenia Research* **57**, 127–138 (2002).

59. Bergon, A. *et al.* CX3CR1 is dysregulated in blood and brain from schizophrenia patients. *Schizophrenia Research* **168**, 434–443 (2015).

60. Seredenina, T. *et al.* Decreased NOX2 expression in the brain of patients with bipolar disorder: Association with valproic acid prescription and substance abuse. *Translational Psychiatry* **7**, e1206 (2017).

61. Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* **333**, 1456–1458 (2011).

62. Arnoux, I. & Audinat, E. Fractalkine Signaling and Microglia Functions in the Developing Brain. (2015) doi:10.1155/2015/689404.

63. Stephan, A. H., Barres, B. A. & Stevens, B. The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease. *Annual Review of Neuroscience* **35**, 369–389 (2012).

64. Reshef, R. *et al.* The role of microglia and their CX3CR1 signaling in adult neurogenesis in the olfactory bulb. *eLife* **6**, (2017).

65. Schafer, D. P. *et al.* Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74**, 691–705 (2012).

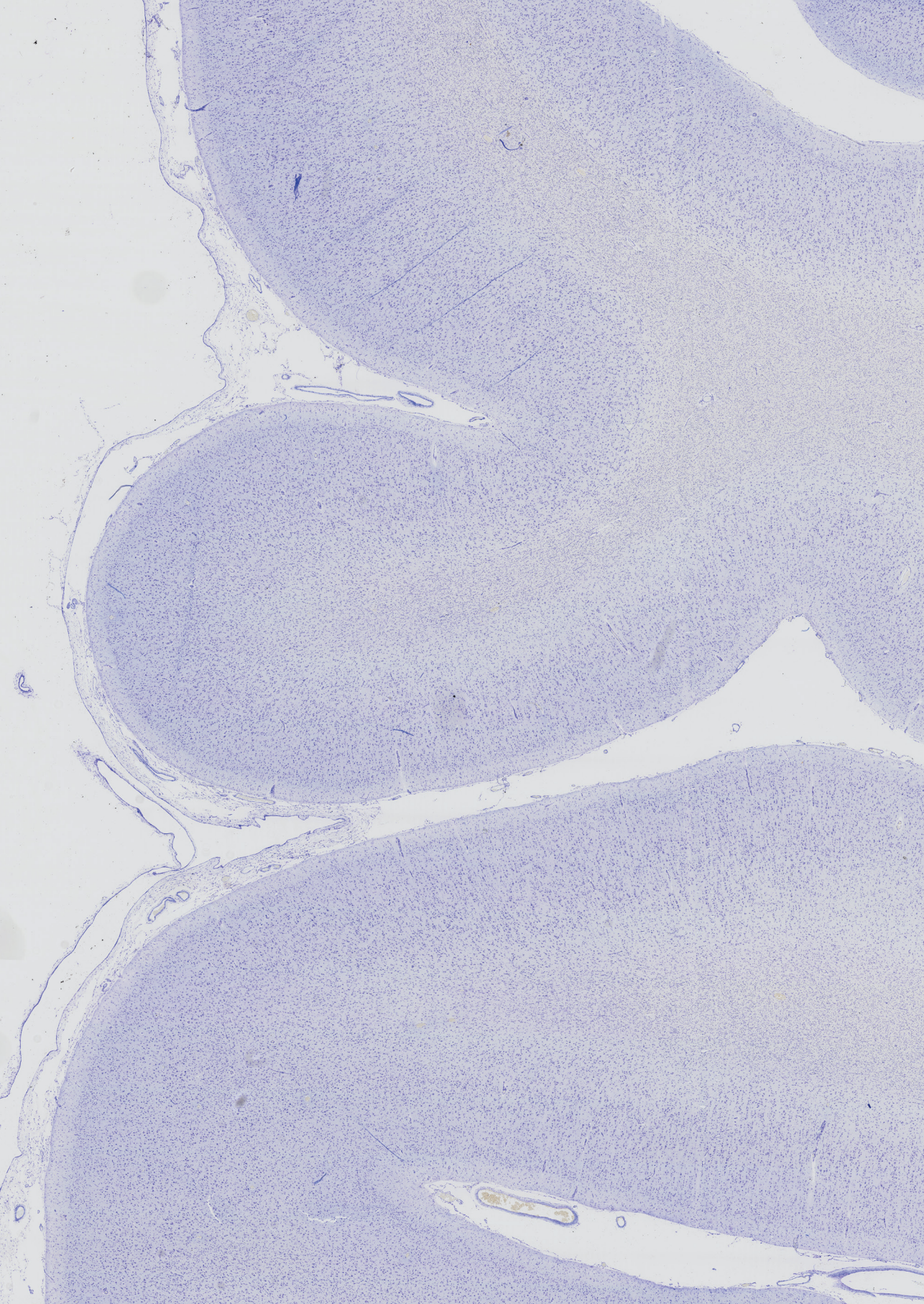
66. Garey, L. J. *et al.* Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *Journal of Neurology, Neurosurgery & Psychiatry* **65**, 446–453 (1998).

67. Roberts, R. C., Roche, J. K. & Conley, R. R. Synaptic differences in the patch matrix compartments of subjects with schizophrenia: A postmortem ultrastructural study of the striatum. *Neurobiology of Disease* **20**, 324–335 (2005).

68. Roberts, Rosalinda C.; Roche, Joy; Somerville, Shahza; Conley, R. Ultrastructural Distinctions Between Treatment Responders and Non-Responders in Schizophrenia: Postmortem Studies of the Striatum. in *Mental Illnesses - Evaluation, Treatments and Implications* (InTech, 2012). doi:10.5772/31396.

69. Roberts, R. C., Roche, J. K. & Conley, R. R. Differential synaptic changes in the striatum of subjects with undifferentiated versus paranoid schizophrenia. *Synapse* **62**, 616–627 (2008).

70. Aganova, E. A. & Uranova, N. A. Morphometric analysis of synaptic contacts in the anterior limbic cortex in the endogenous psychoses. *Neuroscience and behavioral physiology* **22**, 59–65.



Chapter 4

DNA methylation differences in cortical grey and white matter in schizophrenia

Amber Berdenis van Berlekom[a,b], Gijsje J.I.J. Snijders[d,e], Nina Notman[a], Marjolein A.M. Sneeboer[a,b], Lotte C. Houtepen[a], Danny M. Nispeling[a], Yujie He[a,b], Psychiatric Donor Program of the Netherlands Brain Bank (NBB-PSY)[c], Stella Dracheva[d,e], Elly M. Hol[b], René S. Kahn[a,d,e], Lot D. de Witte[a,d,e], and Marco P. Boks[a]

[a] Department of Psychiatry, Brain Center University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[b] Department of translational neuroscience, Brain Center University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[c] Psychiatric donor program of the Netherlands Brain Bank (NBB-PSY), Amsterdam, the Netherlands

[d] Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY, USA and

[e] Mental Illness Research, Education, and Clinical Center (VISN 2 South), James J Peters VA Medical Center, Bronx, NY, USA

Epigenomics 2021

Abstract

Aim: Identify grey- and white-matter-specific DNA methylation differences between schizophrenia patients and controls in postmortem brain cortical tissue.

Methods: Grey and white matter were separated from postmortem brain tissue of the superior temporal and medial frontal gyrus from schizophrenia (N=10) and control (N=11) cases. Genome-wide DNA methylation analysis was performed using the Infinium EPIC Methylation Array.

Results: Four differentially methylated regions associated with schizophrenia status and tissue type (grey vs. white matter) were identified within or near *KLF9*, *SFXN1*, *SPRED2* and *ALS2CL* genes. Gene-expression analysis showed differential expression of *KLF9* and *SFXN1* in SCZ.

Conclusion: Our data show distinct differences in DNA methylation between grey and white matter that are unique to schizophrenia, providing new leads to unravel the pathogenesis of schizophrenia.

1. Introduction

Schizophrenia (SCZ) is a complex chronic psychiatric disorder characterized by a heterogeneous spectrum of symptoms like hallucinations, lack of motivation, and cognitive impairment. Neuro-imaging and postmortem brain studies have identified multiple cortical abnormalities in SCZ patients, such as a decrease in grey and white matter volume and density, disruption of white matter connectivity, and changes in gyrification and cortical thickness[1–10].

Epigenetic regulation that acts as a mediator of both genetic differences[11] and environmental risk factors that are involved in SCZ pathology[12–16], is hypothesized to underlie these cortical differences in SCZ. One of the best-characterized epigenetic modifications is DNA methylation, the process of adding a methyl group (CH3) to a cytosine-guanine (CpG) dinucleotide. Methylated cytosines impact gene transcription with varying directionality[17].

Much remains unknown about the possible role of DNA methylation in SCZ. There have been a large number of epigenetic studies that analyzed DNA methylation differences in peripheral tissue in SCZ (recently reviewed by Smigielski *et al*., (2020))[18]. However, the number of studies on brain tissue of SCZ patients and control donors using an epigenome-wide approach remains limited[19–29]. Recent studies have explored the role of tissue-specific DNA methylation. Although compelling evidence suggests that individual differences exceed differences in tissue type, substantial epigenetic variation has been detected between functionally distinct regions of the brain and between different brain cell types[30,31]. Therefore, an effort is made to identify epigenetic differences specific to the primary tissue type that is relevant for SCZ, the brain. In addition, significant differences have been detected between cortical grey and white matter in function, representation of cell populations, developmental trajectories, and epigenetic marks, including DNA methylation[32–35].

This study aims to identify tissue-specific DNA methylation differences between SCZ and control (CTR) postmortem brain tissue from two cortical areas (*gyrus frontalis medialis* and *gyrus temporalis superior*). To this end, we separated grey and white matter from the brain specimens, followed by epigenome-wide methylation analysis using the Infinium Methylation EPIC Bead Chip. The study focuses on differences in grey and white matter between SCZ and CTR donors. Differences between patients and controls, independent from the tissue type (grey vs. white matter), have previously been identified. Therefore, in this study, we aimed to identify differences between SCZ and CTR that are distinct between grey and white matter tissues. The strongest DNA methylation findings were validated by gene expression analyses. The resultant data contribute to our understanding of grey-white matter DNA methylation differences in SCZ and provide valuable targets for follow-up research of the pathogenesis of SCZ.

2. Materials and Methods

2.1 Donors

Postmortem brain tissue of SCZ patients and CTR donors was obtained from the Netherlands Brain Bank (NBB, www.brainbank.nl) and the Edinburgh Brain and Tissue Bank (Edinburgh). We obtained fresh frozen (SCZ N = 10, CTR N = 11) tissue from the rostral side of the *gyrus frontalis medialis* (area 1 and 2) (GFM), and the middle of the *gyrus temporalis superior* (area 2 to 4) (GTS) (Figure 1A). A detailed description of the anatomical dissection boundaries of these regions used during autopsy can be found on the website of the NBB (www.brainbank.nl/brain-tissue/autopsy/). These brain regions have previously been associated with the pathogenesis of SCZ [36–38]. At the NBB, exclusively Dutch, donors are registered ante-mortem, giving permission for brain autopsy and the use of these materials and medical records for research purposes. The NBB-psychiatry program focusses especially on the establishment of a resource of brain tissue of major psychiatric disorders, together with five Dutch university medical centers. They actively approach extensively phenotyped participants of fifteen large psychiatric research cohorts to register as prospective brain donor at the NBB [39]. At the Edinburgh Brain and Tissue Bank, permission for brain autopsy is obtained (verbally and written) from the next of kin upon sudden deaths, occurring in the general population, reported by police [40]. The Edinburgh Brain and Tissue Bank gains access to general practice and hospital records, including psychiatric records, and confirms the absence of illness with the next of kin [40]. CTR donors did not have a history of major neurological or psychiatric disorders and SCZ donors were diagnosed according to the DSM-III/IV criteria. At the Edinburgh Brain and Tissue Bank two psychiatrists assess the clinical information to confirm a psychiatric diagnosis [40]. At the NBB, the diagnoses of participants were confirmed by trained research assistants using standardized instruments (like the Structured Clinical Interview for DSM-IV (SCID-I), Mini International Neuropsychiatric Interview (M.I.N.I.) plus, Comprehensive Assessment of Symptoms and History (CASH), or Composite International Diagnostic Interview (CIDI)) [39]. Furthermore, the clinicopathological information from all donors of the NBB was reviewed by two individual psychiatrists in our group (LdW and GS) to confirm a SCZ diagnosis. SCZ and CTR donors were matched by age and sex. The tissues from all donors were checked for neuropathology by a pathologist and excluded in case of neurodegenerative pathology in the cortex. For each donor, we obtained information on PMD, pH, age, sex, onset of disease, cause of death, Braak- and amyloid score, substance dependency (smoking, alcohol and drugs), somatic and DSM comorbidities, when available (Table 1 and Supplementary table 1). Four main possible confounding factors (sex, age, pH and PMD) were assessed for difference on group level with Mann-Whitney and Chi-square tests where appropriate (significance level $p < 0.05$).

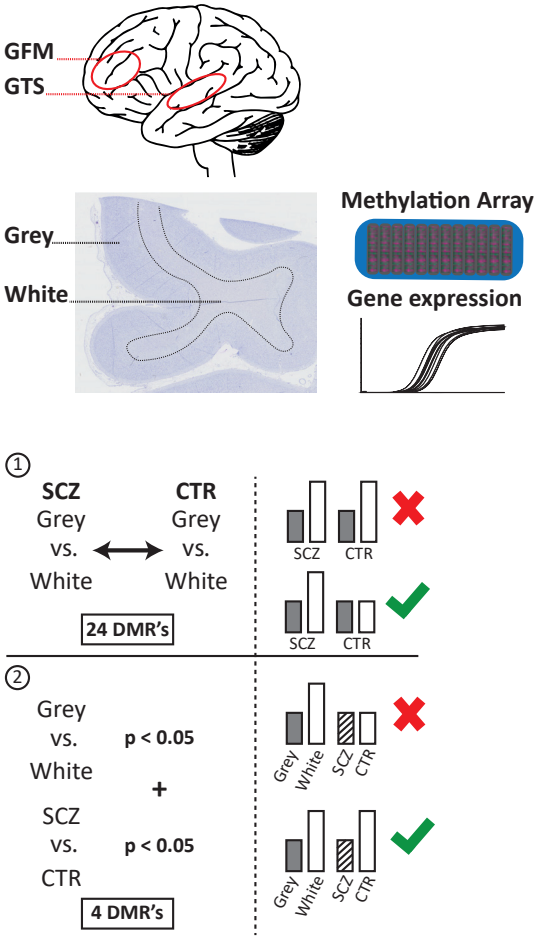


Figure 1: Schematic overview of the study approach.
A Tissue from the *gyrus frontalis medialis* area 1 and 2 and *gyrus temporalis superior* area 2 to 4 were obtained from SCZ (N = 12) and CTR (N = 14) donors, as indicated by the red circles. The Nissl staining shows grey and white matter, which were separated by hand and processed for DNA isolation and RNA isolation for DNA methylation analysis by the Infinium EPIC Methylation Array and gene- expression analyses by qPCR, respectively. **B** Results from the DNA methylation array were analysed in two steps. Step 1: The differences of CpG methylation between grey and white matter were assessed for SCZ and CTR donors separately and then compared. CPGs that differently regulated between grey and white matter in SCZ compared to CTR donors were selected. Examples are shown in the right panel. This analysis resulted in 24 DMRs. Step 2: For further gene expression analysis we selected DMRs that were significantly different when comparing grey and white matter and SCZ and CTR donors. Examples are shown in the right panel. There were 4 DMRs that met these criteria.

Methylation	SCZ (N=9)	CTRN (N=11)
Sex (% Male)	45	55
Age (years)	74 (11)	78 (15)
PMD (hours)	24 (31)	8 (4)
Brain pH	6.4 (0.4)	6.6 (0.3)

Gene-expression	SCZ (N=11)	CTRN (N=14)
Sex (% Male)	30	45
Age (years)	67 (13)	74 (17)
PMD (hours)	34 (31)	24 (33)
Brain pH	6.4 (0.3)	6.5 (0.4)

Table 1. Summary of characteristics of donors from the Netherlands brain bank that were used in the study. Summary of characteristics of donors from the Netherlands Brain Bank that were used the study. Summary of main clinical and postmortem variables of SCZ and CTR donors within this study (sex, age, PMD and pH). Values represent mean and standard deviation. Sex is represented as the % of male donors. PMD = postmortem delay. None of these possible confounders differed between SCZ patients and CTR donors on group level as analysed with Mann-Whitney and Chi-square test where appropriate ($p > 0.05$).

2.2 Ethics statement

The Netherlands Brain Bank and Edinburgh Brain and Tissue Bank received permission to perform autopsies, and to use donor tissue and medical records, from the VU medical center Ethical Committee (VUMC, Amsterdam, The Netherlands) and The East of Scotland Research Ethics Service REC1 (Edinburgh, Scotland) respectively. Written informed consent was obtained from brain donors ante-mortem (The Netherlands Brain Bank) or from relatives postmortem (Edinburgh Brain and Tissue Bank).

2.3 DNA isolation and bisulfite conversion

For DNA isolation grey and white matter from the GFM and the GTS from 9 SCZ and 11 CTR donors were included. Fresh frozen tissue was sectioned (50µm) using a cryostat (Leica Biosystems, Germany) after which grey and white matter was dissected manually. This resulted in the inclusion of 18 grey mater GFM samples (9 SCZ and 9 CTR), 18 white matter GFM samples (9 SCZ and 9 CTR), 17 grey matter GTS samples (7 SCZ and 10 CTR) and 14 white matter GTS samples (6 SCZ and 8 CTR). The grey and white matter tissues were dissociated separately using the Ultra Turrax Homogenizer (IKA) and stored at -80°C. DNA was isolated using the QIAamp DNA mini kit following manufacturer instructions (Qiagen, Germany) and the DNA concentration measured with the Nanodrop Spectrometer (Thermo Fisher Scientific, Massachusetts, USA). Subsequently, DNA was sodium bisulfite converted using the EZ DNA Methylation kit (Zymo Research, CA, USA) according to manufacturer instructions.

2.4 Methylation determination and quantification

Genome-wide DNA methylation was quantified using the Infinium MethylationEPIC BeadChip Kit (Illumina, Inc., San Diego, CA). Data was pre-processed and analyzed using the R program for statistical computation (version 3.3.1, R core team). The level (proportion) of methylation is expressed as a β-value, ranging from 0 (unmethylated cytosine) to 1 (completely methylated cytosine), the subsequent analyses were performed using M-values (log2 of beta values) for a better statistical validity[41]. The dataset was pre-processed using the *meffil* package[42] and functional normalization[43] on the entire dataset (n= 68), including DNA samples from both brain regions. One sample was excluded because the reported sex did not match the methylation- predicted sex. The absence of sample mismatch was verified using the Genotype concordance estimate function in *meffil*. No samples had to be excluded due to high proportion of undetected probes (>10% of the probes in the sample had a detection *p*-value >0.01) or for a low number of beads (>10% of the probes in the sample had a bead count less than three). Ultimately, 67 out of 68 DNA samples survived quality control. Quality control of probes resulted in the removal of 4610 probes (0,54 of total probes) because >10% of the samples had a detection *p*-value > 0.01. 68 probes had to be removed due to a low number of beads per CpG (< 3) in a high proportion (>10%) of the samples. SNP-related probes were removed [44,45] as were probes with potential cross-hybridization [45,46]. Independent surrogate variables (ISVA) were derived to capture technical variation and potential confounding variables[47] as implemented in *meffil*.

2.5 Epigenome-wide association study (EWAS)

To identify distinct methylation patterns associated with SCZ, a linear mixed-effects model was used. The model was run with CpG methylation as outcome, group (SCZ/CTR) and GW (grey-white matter) as main determinants, gender, age, genetic principal components as measure of ancestry (PC), and independent surrogate variables (ISVA) as covariates, and the brain region (GTS/GFM) as random factor. The analysis included an interaction term of grey-white and group. The full model was: *Meth ~ group + GW + GW*group + age + gender + PCs + ISVA, random=brain region*. Multiple testing was accounted for by applying a false discovery rate (FDR) at 5% [48].

2.6 Identification of differentially methylated regions (DMR)

Based on the *p*-value for the interaction coefficient of the CpGs, differentially methylated regions (DMRs) were determined. A DMR consists of highly significant CpGs (*p*-value < 0.0001) within proximity of 1000 base pairs. DMRs were defined as implemented *DMRcate* [49]. *DMRcate* uses a Stouffer procedure to derive a type I error percentage (*p* value) for each DMR [50]. The results were inspected by rerunning the same linear model with mean methylation across each DMR using the *rlmer* package in R (Koller, 2016). To investigate whether there were regional differences in grey-white matter methylation within a DMR,

an additional linear regression was conducted, with DMR methylation as outcome and brain region (GTS, GFM) as determinant. These models were run for each DMR individually. To identify DMRs with the strongest association to SCZ pathology, we prioritized DMRs that showed a significant main effect for DNA methylation between SCZ and CTR, grey and white matter, as well as a significant interaction effect. The significance threshold was set at $p < 0.05$. Figure 1 presents a schematic overview of the study approach.

2.7 Quantification of gene expression using qPCR

For RNA isolation, fresh frozen tissue was sectioned (50µm) using a cryostat after which grey and white matter was dissected manually. Tissue was dissociated in TRIzol reagent (Thermo Life Technologies, USA) using the Ultra Turrax Homogenizer and stored at -80°C. RNA extraction, cDNA preparation and quantitative polymerase chain reaction (qPCR) were performed as described before [51], using the chloroform/isopropanol method, the Quantitect Reverse Transcription Kit (QIAGEN, Germany) and the QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies Corporation, USA). Primers for *KLF9, SFXN1,* and *SPRED2* were designed to be intron spanning, using the NCBI primer-BLAST tool (Supplementary table 2). It was not possible to develop a primer pair to detect *ALS2CL* gene expression in our postmortem brain tissue (previous studies have also been unsuccessful in this regard [52,53]). *KLF9*, *SFXN1,* and *SPRED2* gene expression levels were normalized to the geometric mean of the three stable reference genes beta-actin (*βactin),* Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and Succinate Dehydrogenase Complex Flavoprotein Subunit A (*SDHA*) [54]. The geometric means of the raw CT values from these three reference genes are included for each donor and each tissue in supplementary table 3. Statistical analyses and figure design were performed with Graphpad software (version 8.4). Outliers were defined when values were +/- three times the standard deviation from the mean for each gene, leading to the exclusion of three values (GFM-white 99/131 SPRED2, GFM-grey 12/001 SFXN1, and GTS white SD019-15 SPRED2). Unpaired Mann-Whitney U tests were used to compare gene expression between SCZ and CTR donors in GTS and GFM tissues, because data were not normally distributed. We assessed the effect of five main potential confounding factors, i.e. age, sex, brain pH, PMD and Braak-score for each datapoint with Spearman Rho correlation or the Kruskal Wallis test as appropriate (Supplementary table 4). When a significant association was found (Bonferroni corrected $p < 0.001$), the variable was entered as a covariate in post-hoc analyses using logistic regression.

2.8 Methylation – gene expression correlation analyses

Spearman Rho correlation analyses were applied to determine the main correlation effect between methylation- and gene expression level.

2.9 Confounder analysis

To assess potential confounding, a Pearson correlation was computed to assess the relationship of DMR methylation and brain pH, and Braak-score. No association was detected between these measures. Age and sex were included in the mixed linear regression model and, therefore, were not assessed separately for association with DNA methylation. Also, spearman correlation and Kruskal Wallis tests with the pre-selected potential confounding variables (sex, age, PMD, pH and Braak-score) revealed no significant correlations with gene-expression (Supplementary table 5).

2.10 Data availability statement

Relevant data supporting the discussed findings are included in the paper and its supplementary information files. Data are available at request.

3. Results

3.1 Discovery

Epigenome-wide association analysis revealed 104 CpGs with coefficients below our significant threshold for interaction between grey-white matter differences and SCZ-CTR status ($p < 0.0001$). Inspection of the QQ-plots showed no indication of inflation with a lambda of 0.91 (Supplementary figure 1). Of the significant CpGs, 69 were within a 1000 base pair range of each other, resulting in the identification of 24 differentially methylated regions (DMRs) (Figure 1 and Supplementary table 5).

3.2 DMR analysis

Among the 24 DMRs, we identified four that showed a significant main effect for DNA methylation between SCZ and CTR, grey and white matter, as well as a significant interaction effect (Table 2). All DMRs were located either within or close to the annotated gene. The first DMR (chr9: 73034238 – 73034460) is located 4.6kb outside the *KLF9* gene, within a CpG island of a promoter flanking region. The *KLF9* DMR showed higher methylation in grey vs. white matter and in SCZ vs. CTR subjects. The second DMR (chr5: 174944792 – 174944864) was found in the 8th intron of the *SFXN1* gene, associated with epigenetic regulation. The third DMR (chr2: 65540924 - 65541060) overlaps with a CpG island in the last protein-coding exon of the *SPRED2* gene. Both the *SFXN1* and the *SPRED2* DMRs showed higher methylation in white vs. grey matter and in SCZ vs. CTR subjects. The last DMR (chr3: 46734995 - 46735009) is located at a CpG island in the first exon and promoter region of the *ALS2CL* gene. This DMR showed higher methylation in grey vs. white matter and in CTR vs. SCZ subjects (Supplementary figure 2 and Supplementary table 6).

DMR	DNAm ~ SCZ/Control					DNAm ~ Grey/White				
	DNAm SCZ	*DNAm CTR*	*ß (Beta)*	*t-value*	*p-value*	*DNAm Grey*	*DNAm white*	*ß (Beta)*	*t-value*	*p-value*
KLF9	0.048	0.044	0.009	3.26	0.001	0.053	0.038	0.006	2.02	0.042
SFXN1	0.811	0.806	0.014	2.05	0.04	0.732	0.891	0.025	3.59	0.0003
ALS2CL	0.035	0.0465	0.037	-3.32	0.0009	0.06	0.021	-0.026	-2.07	0.038
SPRED2	0.942	0.929	0.02	4.03	0.00005	0.927	0.944	0.01	1.96	0.05

Table 2. Statistical information significant DMRs.
Four DMRs showed a significant main effect of DNA methylation between SCZ and CTR as well as between grey and white matter. The table shows the methylation difference of the DMRs between SCZ and CTR and between grey and white matter. The β-value represents the log2 of the methylation percentage (0 = unmethylated, 1 = completely methylated). The significance level was set to $p < 0.05$.

3.3 Gene Expression analyses

Following the DMR analyses, we assessed gene expression differences between SCZ and CTR donors for the three of the identified genes (*KLF9*, *SFXN1*, and *SPRED2*) using qPCR. For this analysis, we separated grey and white matter for the two different brain regions (GTS and GFM) in SCZ and CTR subjects. Outliers were removed, and significance corrected for multiple testing using Bonferroni correction ($p < 0.0042$). *KLF9* gene expression was downregulated in SCZ in GFM grey matter (SCZ: median = 391.8, IQR = 438.6; CTR: median = 977.6, IQR = 421.2; $p = 0.0021$) (Figure 2A). For *SFXN1*, we also found a downregulation in GFM grey matter in SCZ (SCZ: median = 139.8, IQR = 162.72; CTR: median = 488.9, IQR = 208.5; $p = 0.0022$) (Figure 2B). The *SPRED2* gene was downregulated in GFM white matter in SCZ (SCZ: median = 98.6, IQR = 44.9; CTR: median = 193.8, IQR = 70.3; $p = 0.0023$ (Figure 2C). An overview of the outcome values and the N for each test can be found in Supplementary table 7. We did not find a correlation between gene expression and methylation level for these genes (Supplementary figure 3).

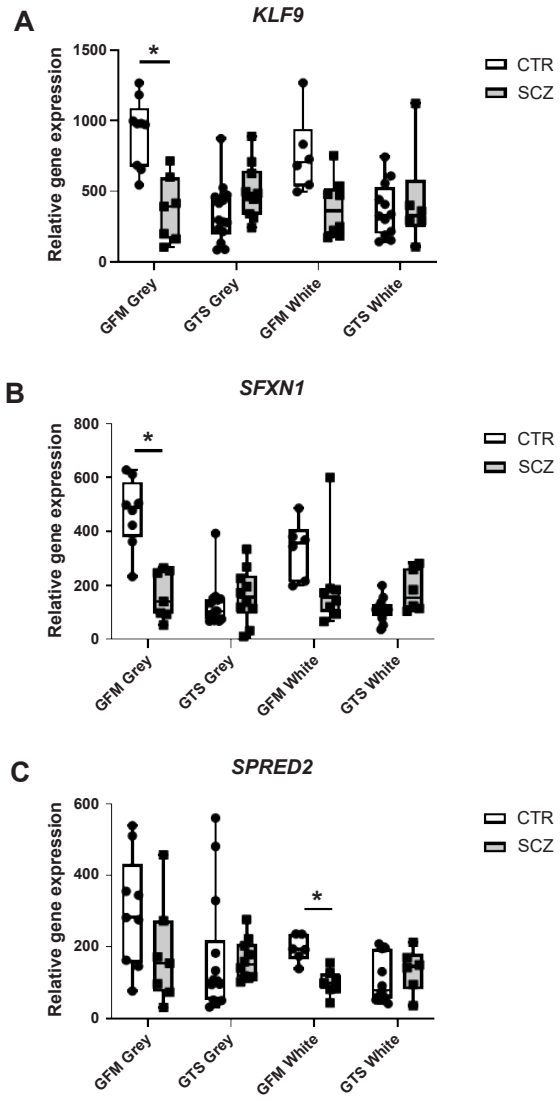


Figure 2. Gene expression in grey and white matter of the GFM and GTS in SCZ and CTR.
mRNA expression of genes was determined by qPCR in the *gyrus frontalis medialis* and *gyrus temporalis superior* of SCZ patients (N = 11) and CTR donors (N = 14). Gene expression was normalized against reference genes beta-actin (*βactin),* Succinate Dehydrogenase Complex Flavoprotein Subunit A (*SDHA*) and glyceraldehyde 3-phosphate dehydrogenease (*GAPDH*). Graphs show boxplots (white for CTR and grey for SCZ) containing individual datapoints for CTR (circles) and SCZ (squares) indicating the median, 25th and 75th percentile, with the whiskers showing the maximum and minimum values. Significance level was Bonferroni corrected and set to $p < 0.0042$.

4. Discussion

This study identified grey vs. white matter DNA methylation differences between SCZ and CTR postmortem cortical tissue. Four DMRs (within or close to *KLF9*, *SFXN1*, *SPRED2,* and *ALS2CL*) were differentially methylated both in grey vs. white matter and in SCZ vs. CTR. The observation of differential gene expression of these genes supported their role in SCZ. These findings provide novel targets for follow-up research and contribute to our understanding of the importance of the cortical grey and white matter differences in general, and in studying SCZ pathogenesis in particular.

The *KLF9* DMR*,* is located in an active promoter of the *KLF9* gene, which is involved in neuronal maturation [55], axon-growth suppression [56,57], and part of the thyroid hormone 3 (T3) signaling cascade [58,59]. In the central nervous system T3 is associated with oligodendrocyte differentiation [60], and therefore the development and regeneration of myelin. In mice, increased T3 levels are associated with decreased white matter volume [61]. The *KLF9* gene, a transcriptional target of T3, transduces these promyelinating effects of T3 [58,59].

The *SFXN1* gene is a mitochondrial serine transporter, important for one-carbon metabolism [61,62]. In general, mitochondrial dynamics are important for neuronal development and synaptic transmission, and impaired mitochondrial function has been associated with an increased risk for developing SCZ [63–65]. Interestingly, mice lacking the SFXN1 gene show striking phenotypic similarities to mice deprived of pyridoxine (a form of vitamin B6) [66], although pyridoxine does not seem to be a substrate of SFXN1 [62]. Low levels of vitamin B6 are associated to SCZ pathology [67] and vitamin B6 is often added to antipsychotic medication, reducing symptoms [68].

The third DMR is located in the *SPRED2* gene, which is expressed in the nervous system and functions as an inhibitor of the Ras/ERK-MAPK pathway [68,69]. The family of SPRED proteins are involved in neurogenesis, cell proliferation, migration, and synaptic vesicle transport [69–71]. The loss of *SPRED2* in a knock-out mouse model has been associated to OCD-like behaviour [69].

Lastly, *ALS2CL* is a homologue of *ALS2*, a guanine-nucleotide exchange factor for Rab5 involved in endosome dynamics, can alter ALS2 function [72,73]. Rab5 mediated endocytosis is essential for proper cortical neurodevelopment, neuronal migration, and synaptic functioning [74,75]. Previously, mutations in this gene have been associated to juvenile recessive amyotrophic lateral sclerosis [76,77], however, a nonsense de-novo mutation of *ALS2CL* has also been associated with SCZ pathology [78,79].

Our results show that the methylation rate of these four DMRs have a different relationship between SCZ and CTR subjects in grey matter compared to white matter. When we assessed the direction of these effects between SCZ and CTR subjects, we found that the DMRs show an opposite direction in methylation rate in grey matter compared to white matter, except for *SPRED2*. Comparing SCZ to CTR subjects, both *KLF9* and *SFXN1* show hypomethylation in white matter, and hypermethylation in grey matter, while *ALSCL2* shows hypermethylation in white matter, and hypomethylation in grey matter. *SPRED2* is hypermethylated in SCZ in both grey and white matter.

To assess the biological relevance of the four identified DMRs, we performed gene expression analyses and comparisons to previously published RNA-sequencing, GWAS, and methylation studies. Our analysis showed a downregulation of *KLF9* in grey matter of the GFM, *SFXN1* in GFM grey matter and *SPRED2* in GTS white matter.

These results reflect the differences in grey/white methylation between SCZ and CTR subject for the GFM, but not for the GTS. In line with our data, *SFXN1* gene expression was also downregulated in SCZ patients in the largest psychiatric RNA-sequencing study to date [80]. In this context, it is noteworthy that the genes identified in our study were not previously identified in genetic studies for SCZ [80–82], but some were highlighted in the other genome-wide methylation studies (Supplementary table 8) [18]).

Interestingly, all four identified genes are involved in functions related to brain connectivity (oligodendrocyte function and synaptic transmission) either directly or through their downstream interactions. Large numbers of imaging and postmortem brain studies have provided evidence for altered brain connectivity in SCZ (the dysconnectivity hypothesis) [83–89]. Several pathological mechanisms have been proposed to be the source of this dysconnectivity, affecting the wiring (white matter) and/or synaptic function (grey matter) in SCZ [85,87,90–95]. Our study might provide new leads to understanding this phenotype and points to a contribution of altered DNA methylation of the four identified genes.

Not much is known about grey and white matter DNA methylation differences in the cortex [32,33]. And these differences have not been considered in SCZ methylation studies until now. Considerable amount of the variance in our data is explained by grey-white matter differences, as is shown by the large correlation between the first principal component of the DNA methylation data and the tissue-type (Supplementary figure 4). This emphasizes the advantages to investigate grey and white matter separately to pinpoint relatively small tissue-specific alterations associated with SCZ pathology.

A limitation of this study is that SCZ is one of the most complex psychiatric disorders, with patients experiencing a pleiotropy of symptoms, differences in disease severity, progression, and treatment. Also, the nature of DNA methylation is very dynamic, which makes epigenetic studies susceptible to biological, clinical, and lifestyle confounding factors [96]. In the current study we have adjusted for five main confounding factors (age, PMD, sex, pH, and Braak-score). However, other potential confounders have not been included, such as smoking, antipsychotic use and disease severity. The heterogeneity of the disease and epigenetic modifications, together with the limited availability of brain tissue, pose a challenge for this type of postmortem brain studies. Therefore, replication studies are imperative.

Overall, in a methodically unique way, dissecting grey and white matter before running EWAS analysis, this study informs about epigenetic variation in the cortical grey and white matter specific to SCZ pathology, resulting in the identification of four genes that are likely involved in SCZ: *KLF9*, *SFXN1*, *SPRED2,* and *ALS2CL*. Furthermore, the data set we generated provides a valuable resource for the identification grey-white matter DNA methylation differences in the cortex in general. In the future similar approaches could be utilized to identify more grey and white matter-specific changes, thus contributing to the elucidation of SCZ pathogenesis.

Acknowledgements

The authors thank the team of the Netherlands Brain Bank for their excellent services (www.hersenbank.nl).

Autor contributions

M.P.B. conceived and designed the project. A.B.B., M.A.M.S performed matching of the donors and extracted demographic data. L.D.W. and G.S. performed medical chart review for SCZ diagnosis. M.A.M.S. and Y.H. performed tissue sectioning, RNA isolation, DNA isolation, bisulfite conversion and qPCR experiments. M.P.B., N.N. and D.M.N performed methylation analyses. A.B.B. performed qPCR analyses. A.B.B. and N.N. wrote the manuscript, with supervision of L.D.W. and M.P.B. Critical feedback was provided by S.D., L.C.H., and E.M.H. over the duration of the project and in writing the manuscript. All authors critically reviewed and approved the manuscript. Funding was obtained by L.D.W., M.P.B., and R.S.K.

Supplementary material

Supplementary material is available at the online version of the paper:
http://doi.org/10.2217/epi-2021-0077

References

1. Olabi, B. *et al.* Are there progressive brain changes in schizophrenia? *A meta-analysis of structural magnetic resonance imaging studies* **70**, 88–96 (2011).
2. Wright, I. *et al.* Meta-analysis of regional brain volumes in schizophrenia. *Am J Psychiatry* **157**, 16–25 (2000).
3. Cahn, W. *et al.* Brain volume changes in the first year of illness and 5-year outcome of schizophrenia. *British Journal of Psychiatry* **189**, 381–382 (2006).
4. Haijma, S. V. *et al.* Brain Volumes in Schizophrenia: A Meta-Analysis in Over 18 000 Subjects. *Schizophrenia Bulletin* **39**, 1129–1138 (2013).
5. Kyriakopoulos, M., Vyas, N. S., Barker, G. J., Chitnis, X. A. & Frangou, S. A Diffusion Tensor Imaging Study of White Matter in Early-Onset Schizophrenia. *Biological Psychiatry* **63**, 519–523 (2008).
6. Mandl, R. C. W. *et al.* Altered white matter connectivity in never-medicated patients with schizophrenia. *Human Brain Mapping* **34**, 2353–2365 (2013).
7. Harrison, P. J. Postmortem studies in schizophrenia. *Dialogues in clinical neuroscience* **2**, 349–57 (2000).
8. Brown, R. *et al.* Postmortem Evidence of Structural Brain Changes in Schizophrenia: Differences in Brain Weight, Temporal Horn Area, and Parahippocampal Gyrus Compared With Affective Disorder. *Archives of General Psychiatry* **43**, 36–42 (1986).
9. Flynn, S. W. *et al.* Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. *Molecular Psychiatry* **8**, 811–820 (2003).
10. Lin, D. *et al.* Network modules linking expression and methylation in prefrontal cortex of schizophrenia. *Epigenetics* 1–18 (2020) doi:10.1080/15592294.2020.1827718.
11. Rajarajan, P. & Akbarian, S. Use of the epigenetic toolbox to contextualize common variants associated with schizophrenia risk. *Dialogues in Clinical Neuroscience* **21**, 407–416 (2019).
12. Boks, M. P. *et al.* The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* **4**, 6767 (2009).
13. Kofink, D., Boks, M. P. M., Timmers, H. T. M. & Kas, M. J. *Epigenetic dynamics in psychiatric disorders: Environmental programming of neurodevelopmental processes*. (Neuroscience and Biobehavioral Reviews, 2013).
14. Labrie, V., Pai, S. & Petronis, A. Epigenetics of major psychosis: Progress, problems and perspectives. *Trends in Genetics* vol. 28 427–435 (2012).
15. Pidsley, R. *et al.* Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology* **15**, (2014).
16. Rutten, B. P. F. & Mill, J. Epigenetic mediation of environmental influences in major psychotic disorders. *Schizophrenia Bulletin* vol. 35 1045–1056 (2009).
17. van Eijk, K. R. *et al. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects*. *BMC Genomics* vol. 13 636- (2012).
18. Smigielski, L., Jagannath, V., Rössler, W., Walitza, S. & Grünblatt, E. Epigenetic mechanisms in schizophrenia and other psychotic disorders: a systematic review of empirical human findings. *Molecular Psychiatry* vol. 25 1718–1748 (2020).
19. Alelú-Paz, R. *et al.* Epigenetics in schizophrenia: A pilot study of global dna methylation in different brain regions associated with higher cognitive functions. *Frontiers in Psychology* **7**, (2016).
20. Chen, C. *et al.* Correlation between DNA methylation and gene expression in the brains of patients with bipolar disorder and schizophrenia. *Bipolar Disorders* **16**, 790–799 (2014).

21. Jaffe, A. E. *et al.* Mapping DNA methylation across development, genotype and schizophrenia in the human frontal cortex. *Nat.Neurosci.* **19**, 40–47 (2016).

22. Mill, J. *et al.* Epigenomic Profiling Reveals DNA-Methylation Changes Associated with Major Psychosis. *American Journal of Human Genetics* **82**, 696–711 (2008).

23. Numata, S., Ye, T., Herman, M. & Lipska, B. K. DNA methylation changes in the postmortem dorsolateral prefrontal cortex of patients with schizophrenia. *Frontiers in Genetics* **5**, (2014).

24. Pidsley, R. *et al.* Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology* **15**, 483 (2014).

25. Van Den Oord, E. J. C. G. *et al.* A whole methylome CpG-SNP association study of psychosis in blood and brain tissue. *Schizophrenia Bulletin* **42**, 1018–1026 (2016).

26. Viana, J. *et al.* Schizophrenia-associated methylomic variation: molecular signatures of disease and polygenic risk burden across multiple brain regions. *Human molecular genetics* **26**, 210–225 (2017).

27. Wockner, L. F. *et al.* Genome-wide DNA methylation analysis of human brain tissue from schizophrenia patients. *Translational Psychiatry* **4**, 1 (2014).

28. Wockner, L. F. *et al.* Brain-specific epigenetic markers of schizophrenia. *Translational Psychiatry* **5**, (2015).

29. Zhao, H. *et al.* Genome-wide DNA methylome reveals the dysfunction of intronic microRNAs in major psychosis. *BMC Medical Genomics* **8**, 62 (2015).

30. Davies, M. N. *et al.* Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biol.* vol. 13 R43-.

31. Guintivano, J., Aryee, M. J. & Kaminsky, Z. A. A cell epigenotype specific model for the correction of brain cellular heterogeneity bias and its application to age, brain region and major depression. *Epigenetics* **8**, (2013).

32. Ghosh, S. *et al.* Tissue specific DNA methylation of CpG islands in normal human adult somatic tissues distinguishes neural from non-neural tissues. *Epigenetics* **5**, 527–538 (2010).

33. Sanchez-Mut, J. v, Heyn, H., Vidal, E. & others. Whole genome grey and white matter DNA methylation profiles in dorsolateral prefrontal cortex. *Synapse00:e* **21959**, (2017).

34. Bohland, J. W. *et al.* Clustering of spatial gene expression patterns in the mouse brain and comparison with classical neuroanatomy. *Methods* **50**, 105–112 (2010).

35. Ko, Y. *et al.* Cell type-specific genes show striking and distinct patterns of spatial expression in the mouse brain. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 3095–3100 (2013).

36. Vita, A., De Peri, L., Deste, G. & Sacchetti, E. Progressive loss of cortical gray matter in schizophrenia: A meta-analysis and meta-regression of longitudinal MRI studies. *Translational Psychiatry* vol. 2 e190 (2012).

37. Glahn, D. C. *et al.* Meta-Analysis of Gray Matter Anomalies in Schizophrenia: Application of Anatomic Likelihood Estimation and Network Analysis. *Biological Psychiatry* **64**, 774–781 (2008).

38. Ellison-Wright, I., Glahn, D. C., Laird, A. R., Thelen, S. M. & Bullmore, E. The anatomy of first-episode and chronic schizophrenia: An anatomical likelihood estimation meta-analysis. *American Journal of Psychiatry* **165**, 1015–1023 (2008).

39. de Lange, G. M., Rademaker, M., Boks, M. P. & Palmen, S. J. M. C. Brain donation in psychiatry: Results of a Dutch prospective donor program among psychiatric cohort participants. *BMC Psychiatry* **17**, (2017).

40. Millar, T. *et al.* Tissue and organ donation for research in forensic pathology: The MRC Sudden Death Brain and Tissue Bank. *Journal of Pathology* **213**, 369–375 (2007).

41. Du, P. *et al.* Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* **11**, 587 (2010).

42. Min, J., Hemani, G., Smith, G. D., Relton, C. L. & Suderman, M. Meffil: efficient normalisation and analysis of very large DNA methylation samples. bioRxiv. **125963**, (2017).

43. Fortin, J.-P. *et al.* Functional normalization of 450k methylation array data improves replication in large cancer studies. *Genome Biology* **15**, 503 (2014).

44. Zhou, W., Laird, P. W. & Shen, H. Comprehensive characterization, annotation and innovative use of Infinium DNA methylation BeadChip probes. *Nucleic acids research* **45**, e22 (2017).

45. Pidsley, R. *et al.* Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biology* **17**, (2016).

46. McCartney, D. L. *et al.* Identification of polymorphic and off-target probe binding sites on the Illumina Infinium MethylationEPIC BeadChip. *Genomics Data* **9**, 22–24 (2016).

47. Teschendorff, A. E., Zhuang, J. & Widschwendter, M. Independent surrogate variable analysis to deconvolve confounding factors in large-scale microarray profiling studies. *Bioinformatics* **27**, 1496–1505 (2011).

48. Hochberg, Y. & Benjamini, Y. More powerful procedures for multiple significance testing. *Statistics in Medicine* **9**, 811–818 (1990).

49. Peters, T. J. *et al.* De novo identification of differentially methylated regions in the human genome. *Epigenetics and Chromatin* **8**, 6 (2015).

50. De Meeûs, T. Statistical decision from k test series with particular focus on population genetics tools: A DIY notice. (2014) doi:10.1016/j.meegid.2014.01.005.

51. Melief, J. *et al.* Characterizing primary human microglia: A comparative study with myeloid subsets and culture models. *GLIA* **64**, 1857–1868 (2016).

52. Devon, R. S. *et al.* Cross-species characterization of the ALS2 gene and analysis of its pattern of expression in development and adulthood. *Neurobiology of disease* **18**, 243–257 (2005).

53. Uhlen, M. *et al.* Tissue-based map of the human proteome. *Science* **347**, 1260419–1260419 (2015).

54. Sneeboer, M. A. M. *et al.* Microglia in post-mortem brain tissue of patients with bipolar disorder are not immune activated. *Translational Psychiatry* **9**, (2019).

55. Scobie, K. N. *et al.* Krüppel-like factor 9 is necessary for late-phase neuronal maturation in the developing dentate gyrus and during adult hippocampal neurogenesis. *Journal of Neuroscience* **29**, 9875–9887 (2009).

56. Apara, A. *et al.* KLF9 and JNK3 interact to suppress axon regeneration in the adult CNS. *Journal of Neuroscience* **37**, 9632–9644 (2017).

57. Trakhtenberg, E. F. *et al.* Zinc chelation and Klf9 knockdown cooperatively promote axon regeneration after optic nerve injury. *Experimental neurology* **300**, 22–29 (2018).

58. Avci, H. X. *et al.* Thyroid hormone triggers the developmental loss of axonal regenerative capacity via thyroid hormone receptor α1 and krüppel-like factor 9 in Purkinje cells. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 14206–14211 (2012).

59. Dugas, J. C., Ibrahim, A. & Barres, B. A. The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. *Molecular and Cellular Neuroscience* **50**, 64–71 (2012).

60. Powell, M. H. *et al.* Magnetic resonance imaging and volumetric analysis: Novel tools to study the effects of thyroid hormone disruption on white matter development. *NeuroToxicology* **33**, 1322–1329 (2012).

61. Fleming, M. D., Campagna, D. R., Haslett, J. N., Trenor, C. C. & Andrews, N. C. A mutation in a mitochondrial transmembrane protein is responsible for the pleiotropic hematological and skeletal phenotype of flexed-tail (f/f) mice. *Genes and Development* **15**, 652–657 (2001).

62. Kory, N. *et al.* SFXN1 is a mitochondrial serine transporter required for one-carbon metabolism. *Science* **362**, (2018).

63. Park, C. & Park, S. K. Molecular links between mitochondrial dysfunctions and schizophrenia. *Molecules and Cells* vol. 33 105–110 (2012).

64. Rajasekaran, A., Venkatasubramanian, G., Berk, M. & Debnath, M. Mitochondrial dysfunction in schizophrenia: Pathways, mechanisms and implications. *Neuroscience and Biobehavioral Reviews* vol. 48 10–21 (2015).

65. Flippo, K. H. & Strack, S. An emerging role for mitochondrial dynamics in schizophrenia. *Schizophrenia Research* vol. 187 26–32 (2017).

66. Roy, C. N. & Andrews, N. C. Recent advances in disorders of iron metabolism: Mutations, mechanisms and modifiers. *Human Molecular Genetics* vol. 10 2181–2186 (2001).

67. Cao, B. *et al.* Association between B vitamins and schizophrenia: A population-based case-control study. *Psychiatry Research* **259**, 501–505 (2018).

68. Firth, J. *et al.* The effects of vitamin and mineral supplementation on symptoms of schizophrenia: A systematic review and meta-analysis. *Psychological Medicine* vol. 47 1515–1527 (2017).

69. Ullrich, M. *et al.* OCD-like behavior is caused by dysfunction of thalamo-amygdala circuits and upregulated TrkB/ERK-MAPK signaling as a result of SPRED2 deficiency. *Molecular Psychiatry* **23**, 444–458 (2018).

70. Engelhardt, C. M. *et al.* Expression and subcellular localization of Spred proteins in mouse and human tissues. *Histochemistry and Cell Biology* **122**, 527–538 (2004).

71. Lim, F. T., Ogawa, S. & Parhar, I. S. Spred-2 expression is associated with neural repair of injured adult zebrafish brain. *Journal of Chemical Neuroanatomy* **77**, 176–186 (2016).

72. Suzuki-Utsunomiya, K. *et al.* ALS2CL, a novel ALS2-interactor, modulates ALS2-mediated endosome dynamics. *Biochemical and biophysical research communications* **354**, 491–497 (2007).

73. Hadano, S. *et al.* ALS2CL, the novel protein highly homologous to the carboxy-terminal half of ALS2, binds to Rab5 and modulates endosome dynamics. *FEBS Letters* **575**, 64–70 (2004).

74. Kawauchi, T. *et al.* Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron* **67**, 588–602 (2010).

75. Wucherpfennig, T., Wilsch-Bräuninger, M. & González-Gaitán, M. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *Journal of Cell Biology* **161**, 609–624 (2003).

76. Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science (New York, N.Y.)* **362**, eaat8127 (2018).

77. Yang, Y. *et al.* The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nature Genetics* **29**, 160–165 (2001).

78. Jouan, L. *et al.* Investigation of rare variants in LRP1, KPNA1, ALS2CL and ZNF480 genes in schizophrenia patients reflects genetic heterogeneity of the disease. *Behavioral and Brain Functions* **9**, 9–9 (2013).

79. Girard, S. L. *et al.* Increased exonic de novo mutation rate in individuals with schizophrenia. *Nature Genetics* **43**, 860–863 (2011).

80. Pardiñas, A. F. *et al.* Common schizophrenia alleles are enriched in mutation-intolerant genes and in regions under strong background selection. *Nature Genetics* **50**, 381–389 (2018).

81. Schizophrenia Working Group of the Psychiatric Genomics Consortium. Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421–7 (2014).

82. Hannon, E. *et al.* DNA methylation meta-analysis reveals cellular alterations in psychosis and markers of treatment-resistant schizophrenia. *eLife* **10**, 1–53 (2021).

83. Hadano, S. *et al.* A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nature Genetics* **29**, 166–173 (2001).

84. Schmitt, A., Hasan, A., Gruber, O. & Falkai, P. Schizophrenia as a disorder of disconnectivity. *European Archives of Psychiatry and Clinical Neuroscience* vol. 261 150 (2011).

85. Stephan, K. E., Friston, K. J. & Frith, C. D. Dysconnection in Schizophrenia: From abnormal synaptic plasticity to failures of self-monitoring. *Schizophrenia Bulletin* vol. 35 509–527 (2009).

86. van den Heuvel, M. P. *et al.* Abnormal Rich Club Organization and Functional Brain Dynamics in Schizophrenia. *JAMA Psychiatry* **70**, 783 (2013).

87. Pettersson-Yeo, W. *et al.* Using genetic, cognitive and multi-modal neuroimaging data to identify ultra-high-risk and first-episode psychosis at the individual level. *Psychological Medicine* **43**, 2547–2562 (2013).

88. Anticevic, A. *et al.* Connectivity, pharmacology, and computation: toward a mechanistic understanding of neural system dysfunction in schizophrenia. *Frontiers in psychiatry* **4**, 169 (2013).

89. Friston, K. J. & Frith, C. D. Schizophrenia: a disconnection syndrome? *Clinical neuroscience (New York, N.Y.)* **3**, 89–97 (1995).

90. Takahashi, N., Sakurai, T., Davis, K. L. & Buxbaum, J. D. Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. *Progress in Neurobiology* vol. 93 13–24 (2011).

91. Berdenis Van Berlekom, A. *et al.* Synapse Pathology in Schizophrenia: A Meta-analysis of Postsynaptic Elements in Postmortem Brain Studies. *Schizophrenia Bulletin* **46**, 374–386 (2020).

92. Glantz, L. A. & Lewis, D. A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry* **57**, 65–73 (2000).

93. Glausier, J. R. & Lewis, D. A. Dendritic spine pathology in schizophrenia. *Neuroscience* **251**, 90–107 (2013).

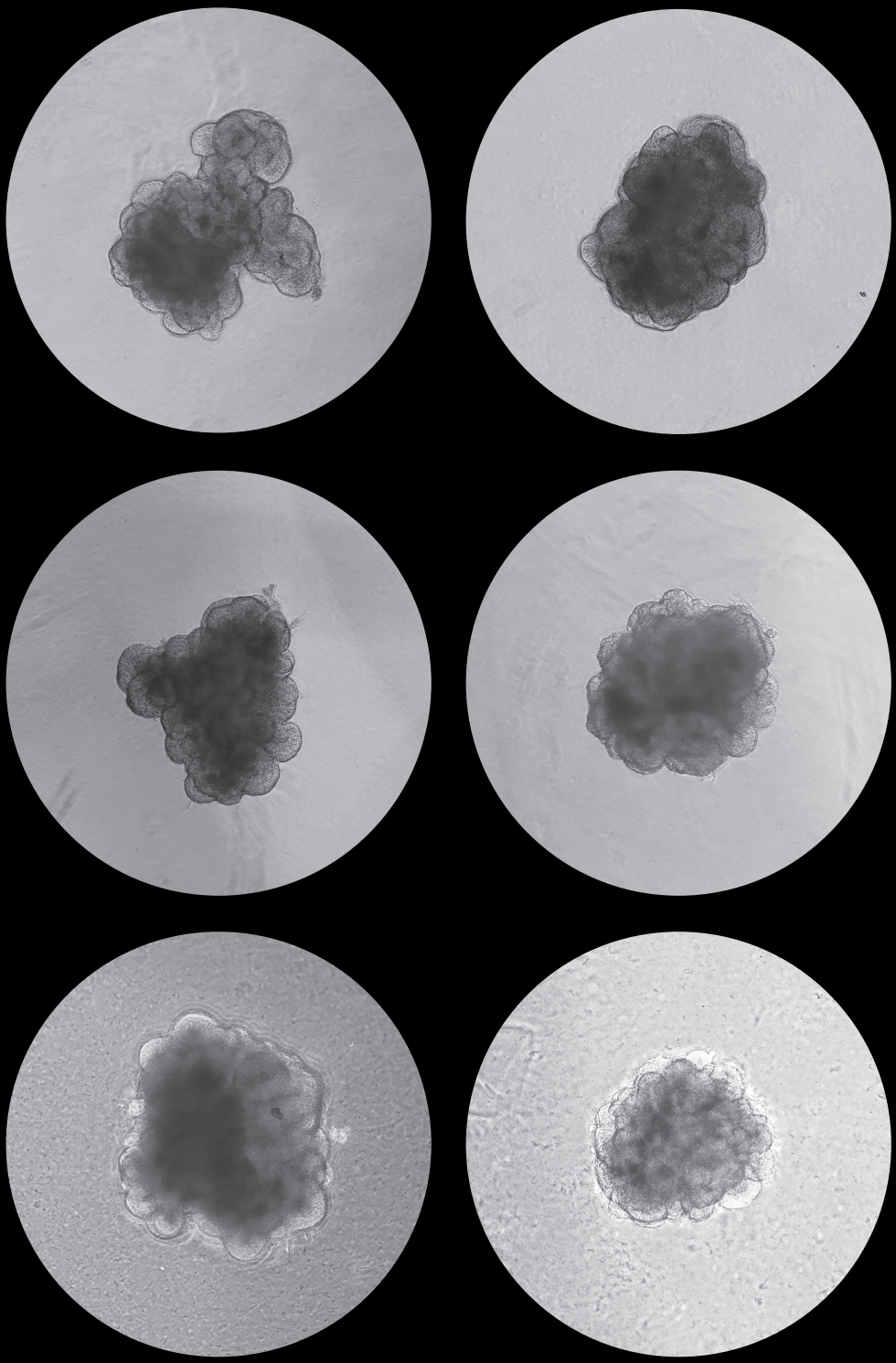
94. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular psychiatry* (2018) doi:10.1038/s41380-018-0041-5.

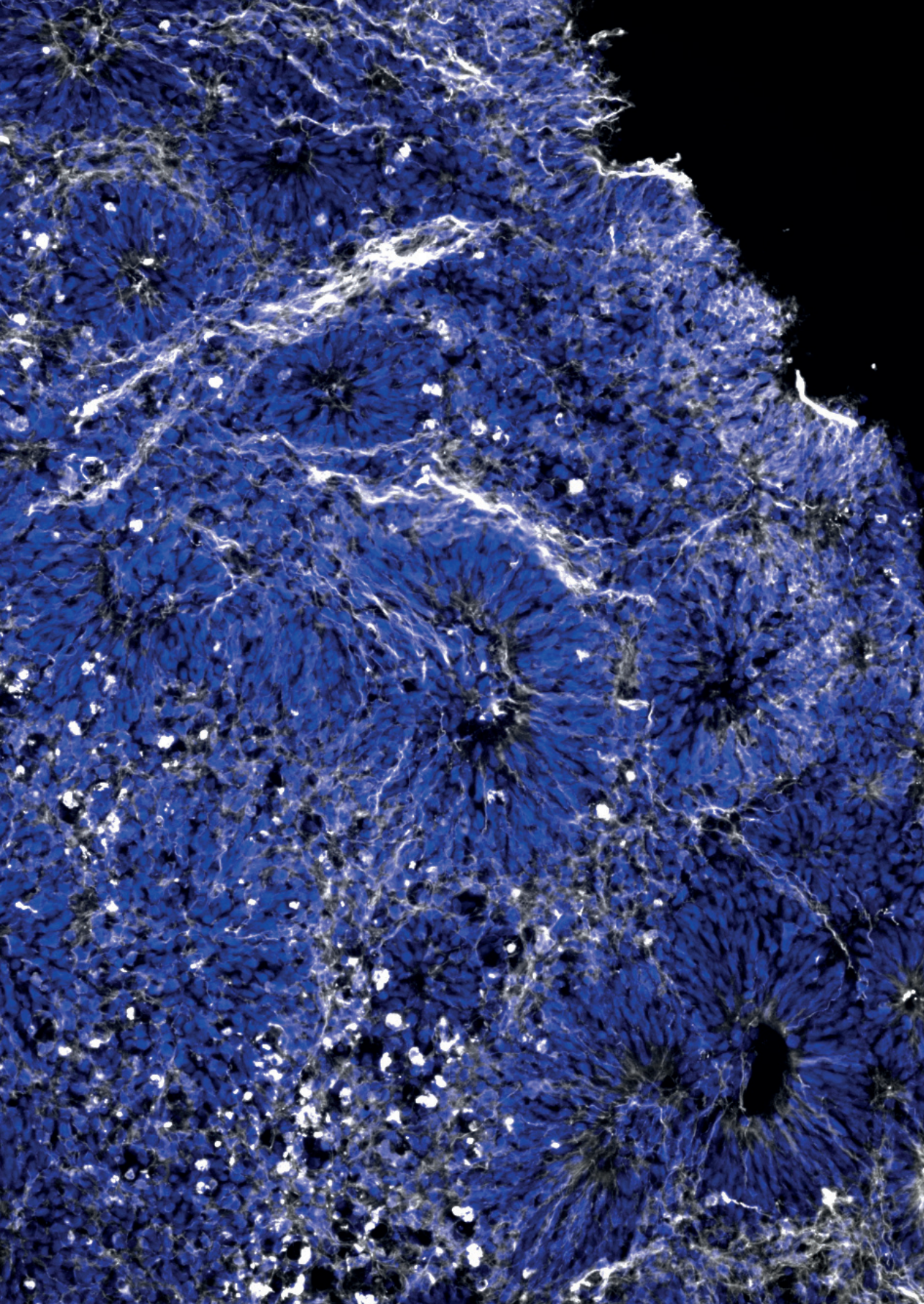
95. Dietz, A. G., Goldman, S. A. & Nedergaard, M. Glial cells in schizophrenia: a unified hypothesis. *The Lancet Psychiatry* vol. 7 272–281 (2020).

96. Mill, J. & BT., H. From promises to practical strategies in epigenetic epidemiology. *Nat Rev Genet* **14**, 585–594 (2013).

Part II

The effect of potential prenatal environmental risk factors for developing schizophrenia on neurodevelopment in cerebral organoids





Chapter 5

Prenatal inflammation evaluated in human cerebral organoids

Amber Berdenis van Berlekom*[a,b], Paul R. Ormel*[a,b], Renata Vieira de Sá[b], Marco P. Boks[a], René S. Kahn[a,c,d], Elly M. Hol[b], and Lot D. de Witte[a,c,d]

*Authors contributed equally

[a] Department of Psychiatry, Brain Center University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[b] Department of translational neuroscience, Brain Center University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[c] Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY, USA and

[d] Mental Illness Research, Education, and Clinical Center (VISN 2 South), James J Peters VA Medical Center, Bronx, NY, USA

Manuscript in preparation

Abstract

Maternal immune activation (MIA) during pregnancy is a risk factor for developing schizophrenia and autism spectrum disorder in the offspring. In rodents, MIA-progeny has been shown to develop changes in behaviour and cognition. However, it is also clear that MIA in humans most often does not induce a neurodevelopment disorder. To investigate possible resilience to prenatal inflammation in brain development of non-psychiatric individuals, in the current study induced pluripotent stem cells derived cerebral organoids from non-psychiatric individuals were challenged with lipopolysaccharide (LPS) during an early developmental stage reflecting the end of the 1st trimester. Acute and long term effects of LPS on immune-activation, microglia and astrocytic phenotypes, together with alterations in neuronal development were assessed. Acute effects included increased gene expression of immune-activation markers (*IL6, IL1B, TNF*), and decreased expression of neuronal markers important in neuronal development (*RELN* and *EOMES/TBR2*) after LPS stimulation. Also an increase in microglia and astrocytic protein expression was detected (IBA1, GFAP). Long term effects solely consisted of a decrease in immune activation markers (*IL1B*, *TNF*). The acute effects replicate findings observed in MIA studies, although long term effects were not replicated. These preliminary results suggest that immune activation leads to transient changes, but also to compensatory mechanisms in immune pathways, which may protect the developing brain from long term structural changes. Since most maternal infections do indeed not result in neurodevelopmental disorders, we hypothesize that compensatory mechanisms that make the human brain resilient against prenatal inflammation may be affected in individuals genetically at risk for developing psychiatric illnesses.

1. Introduction

Epidemiological studies have shown that prenatal inflammation is a risk factor for schizophrenia, autism spectrum disorder, and bipolar disorder[1]. Infections and increased levels of pro-inflammatory cytokines during the first or second trimester of pregnancy increase the risk for developing schizophrenia and autism in offspring 2- to 5-fold and 1.1-fold, respectively[2–5]. A 4-fold increase has been described for bipolar disorder in which the timing of inflammation was not an identified determining factor[6]. It remains difficult to comprehend how an incident during early-development unfolds into a psychiatric illness years later[7]. It is hypothesized that increased levels of cytokines, chemokines, and other inflammatory factors during an infection in the mother, are partly transferred across the placenta. These factors are hypothesized to either directly affect brain development in the fetus[7], or lead to long term dysfunctional changes in the microglia, the immune cells of the brain. IL6 and IL17 have gotten extra attention for a potential detrimental role in the cascade initiated by the infection being able to directly affect brain development[7,8]. The pathogenic trajectory from MIA to neurodevelopmental disorders is difficult to investigate in human subjects. Animal models have therefore been used to study the impact of prenatal inflammation on the brain of the offspring.

The rodent maternal immune activation (MIA) model has been used most extensively to study the induction of long term abnormalities in the brain due to prenatal infection[9]. Generally two procedures are used to evoke MIA: an intraperitoneal injection of the pregnant mouse with a bacterial lipopolysaccharide/LPS) or viral (polyinosinic:polycytidylic acid/PIC) agent. The timing differs per study, some studies induce MIA at embryonic day 9 (E9) before the fetal blood brain barrier closes, but most induce MIA at E12. Hereby stimulating an infection during a gestational stage that correlates to the late first trimester in humans, that is most highly linked to increased risk of schizophrenia and autism, but not necessarily bipolar disorder[2,5,6,10]. Importantly, this time-point is just after microglia settle in the brain parenchyma (E8.5)[11].

Several important biological concepts were deduced from the MIA rodent studies. First an infection in the mother is able to cross the placenta-fetus barrier where it evokes an inflammation wave that travels through the fetal bloodstream and then crosses the blood-brain-barrier of the fetus in the case of MIA induction at E12. Elevated cytokine levels (such as IL 6) and STAT3 phosphorylation can be detected in the fetal brain within three hours and twenty-four after stimulation with PIC or LPS, respectively[9,12,13]. Second, Mia induces long term changes in cytokine levels in the brain of the MIA offspring in rodents[9]. Thrid, prenatal inflammation can induce long term changes in behaviour and cognition in offspring[14]. This includes impaired sensorimotor gating, social, and exploratory behaviour. Fourth, MIA affects neurodevelopment, as shown as shown by

reductions in brain volume, defects in neuronal migration and maturation[15,16]. Intervention in the immune wave in the fetal brain was effective in preventing the MIA phenotype, as shown by inhibiting IL17a and IL1 receptor type 1 knock out studies[8,16]. Corradini et al. (2018) further correlated the stat of inflammation in the fetal brain to delayed neurodevelopment[16], that might underlie the behavioural changes.

Importantly, although most MIA studies show both short- and long term effects, the observed changes in behaviour, the immune system, and neuronal development are highly variable. This disparity can be largely explained by the choice of immunogen, timing of MIA induction, animal strain, and the use of single or multiple immune challenges[9,14]. It also remains unclear how all these findings translate to humans. Especially considering that the MIA animal model has a high penetrant effect inducing a severe phenotype in the offspring, whereas only a small percentage of pregnant women with prenatal infection deliver children that develop schizophrenia or another major psychiatric illness[3,17]. We therefore hypothesize that the human brain has a level of resilience to immune-stressors. Until recently, models that mimic human brain development were absent.

Advances in iPSC technology have unfolded new opportunities to investigate the translation of findings from rodent MIA models to humans. In 2013 there was a breakthrough in iPSC-research due to the publication of a protocol for human cerebral organoids[18]. The protocol is widely used to study the effects of, for example, Zika infection on brain development[19–21]. The trajectory of neuronal development in these organoids closely mimics fetal human brain development, including apical and basal neuronal progenitors, and cortical plate neurons[22,23]. An adaptation the cerebral organoid protocol showed innate development of microglia in the organoids[24]. This is an important addition as these cells, together with the astrocytes, are presumed to play an important role in proliferation of the immune effects in the brain in case of MIA. Furthermore, they are intertwined with the development and maintenance of other brain cells[25–28] through which they can affect developing neurons. In MIA models, inflammation is induced just after microglia enter the brain, and their involvement is confirmed by long term changes in the phenotype end density of microglia after MIA[29,30].

The aim of this study was to delineate acute and long term consequences of an infection during early stages of human brain development, in analogy of the MIA rodent model. By using non-psychiatric donors we were able to investigate resilience of the human brain development against environmental stressors. We grew iPSC-derived organoids and triggered immune-activation with a two-step LPS challenge just after microglia developed within the model. We determined acute and long term effects on glial phenotype and changes in neurodevelopment by assessing gene expression changes that characterize different steps in neurodevelopment that could explain rise of psychiatric problems later in life.

2. Methods

2.1 Research subjects

iPSC line generation was approved by the Medical Ethical Committee of the University Medical Center Utrecht. Written consent was provided by the non-psychiatric subjects.

2.2 iPSC lines generation and maintenance

Generation and characterization, including karyotyping and pluripotency testing, of both iPSC-line 1 (male, 65 years old) and 2 (male, 66 years old) have been described before[31]. iPSC-lines were feeder-free cultured on Geltrex-coated (Thermofisher, A1413202) dishes in mTeSR1 medium at 37°C with 5% CO2. Cells were passaged once a week by first washing the cells with DPBS followed by 2 minutes incubation with EDTA (5 μM). EDTA was removed and cell aggregates were loosened from the plate by rinsing the plate with mTeSR1 which were transferred to a fresh plate. The first 24h after passage they were cultured with ROCK-inhibitor Y-27632 (4.82 μM, Axon 1683). Both lines were frequently tested for mycoplasma infection (Lonza, LT07-318) and kept in culture up to 60 passages.

2.3 Organoid differentiation

Organoids were differentiated as described previously[24]. In short, iPSC were grown till they reached ~90% confluency. They were detached with Accutase (Thermofisher, A11105-01), counted and plated 3.5*10^6 cells per well in an aggrewell800 microwell plate (StemCell Technologies, 27865 in 2 mL hESC4 (DMEM-F12 (ThermoFisher, 11320-074), 20% KOSR (ThermoFisher, 10828028), 1% NEAA (ThermoFisher, 11140-035), 1% L-Glutamine (ThermoFisher, 25030-024), 3% FBS (SigmaAldrich, F7524), 496 μM ß-mercaptoethanol (Merck-Schuchardt, 805740), and 4 ng/mL bFGF (ThermoFisher, AA10-155)) supplemented with ROCK-inhibitor Y-27632 (48.2 μM). Medium was refreshed on day 1 and on day 2 healthy looking embryoid bodies were selected and transferred one organoid per well to an ultra-low attachment 96-well plate (Corning, 3474). The hES4 was replaced by hES0 (without ROCK-inhibitor and bFGF) on day 4. On day 6, 8, 10, and 12 of the protocol, medium was replaced by neural induction medium (DMEM-F12, 1% N2 (ThermoFisher, 17502048), 1% L-Glutamine, 1% NEAA, 0.1ug/mL heparin (Sigma Aldrich, H3149-10KU)). Organoids were embedded in 30 μL Matrigel (Corning, 356234) on day 13 and cultured in cerebral organoid differentiation medium without vitamin A for four days, refreshing the medium on day 15 (DMEM-F12 1:1 with neurobasal medium (ThermoFisher, 21103049), 1% L-Glutamine, 1% P/S, 0.025% insulin (Sigma Aldrich, I9278), 3.5 μL/L 2-mercaptoethanol, 1% NEAA, 1:100 B27 supplement without vitamin A (Sigma Aldrich, 12587010)) sixteen organoids per 60 mm dish. On day 17 of the protocol the medium was replaced by cerebral organoid differentiation medium with vitamin A (DMEM-F12 1:1 with neurobasal medium, 1% L-Glutamine, 1% P/S, 0.025% insulin, 3.5 μL/L 2-mercaptoethanol, 1% NEAA, 1:100 B27 supplement with vitamin A

(Sigma Aldrich, 17504044)) and the organoids were transferred to a belly dancer set to speed 3. Organoids where removed from the culture when they not fulfilled the criteria postulated by Lancaster & Knoblich (2013)[18]. Two batches were grown from iPSC1 (iPSC1a and iPSC1b), and one from iPSC2. Each batch had a minimum of 96 organoids.

2.4 Lipopolysaccharide stimulation

On day 38 in culture, organoids that were cultured sixteen organoids per dish were pooled together and divided over new dishes to prevent a confounding effect of inter-dish variation that is described in organoid research[32]. Two 60 mm dishes were subsequently stimulated with 100 ng/mL LPS from *Escherichia coli* (Sigma-Aldrich, 0111:B4. On day DIV39 (acute effect: timepoint 1) and DIV66 (long term effect: timepoint 2) organoids from one dish per condition were harvested. Three organoids were separately collected in Trizol reagent (Life Technologies, 15596018) for RNA isolation. Three organoids were washed with PBS, fixated in 4% PFA for 24h, washed again with PBS and transferred to a sucrose solution (30% sucrose in PBS) over night at 4°C. They were snap-frozen in isopentane on dry ice after embedment in tissue-tek (CellPath, KMA0100-00A). Frozen organoids were stored at -80°C for subsequent sectioning. For protein enrichment, three organoids were separately collected, washed with PBS, mechanically lysed in protease & phosphatase inhibitor, SDS, 2H-DTT and suspension buffer and stored at -80°C.

2.5 Immunofluorescent stainings

Organoids were sectioned (20 μM per section) with a cryostat and stored at -80°C. Sections were thawed and washed with PBS-0.05% tween20. Sections were immersed in blocking solution for 1h at RT (0.05% Tween 20, 3% BSA, 1% Triton-X, and 10% donkey serum) followed by the primary antibody incubation in the blocking solution over night at 4°C. After washing twice with PBS, the sections were incubated with secondary antibody solution and nuclear staining (hoechst, ThermoFisher, H3569) for 2h at RT. Samples were mounted using Fluorsave reagent (Merck Millipore, 345789) and imaged with Zeiss Axio-Scope A1. See table 1 for the antibodies used.

Antigen/target	*Experiment*	*Host species*	*Dilution*	*Provider*	*Article number*
GFAP-pan	IHC/WB	Rabbit	1:1000	DAKO	Z0334
IBA1	IHC/WB	Goat	1:500	Abcam	AB5076
NEUN	IHC/WB	Mouse	1:500	Abcam	AB104224
TUJ1	IHC	Mouse	1:1000	Covance	MMS-435P
S100b	IHC	Rabbit	1:300	Dako	Z0311
HLA-DR	IHC/WB	Mouse	1:500	eBioscience	14-9956-80
STAT3	WB	Rabbit		Cell signalling	9145
PU.1	IHC	Rabbit	1:300	Invitrogen	A13971
Mouse 555	IHC	Donkey	1:1000	Thermo Fisher	A31570
Mouse 488	IHC	Donkey	1:1000	Thermo Fisher	A21202
Rabbit 555	IHC	Donkey	1:1000	Abcam	150074
Rabbit 488	IHC	Donkey	1:1000	Thermo Fisher	A21206
Rat 488	IHC	Donkey	1:1000	Thermo Fisher	A21208
Goat 555	IHC	Donkey	1:1000	Thermo Fisher	
GAPDH	WB	Mouse	1:1000	Chemicon	MAB374
Rabbit IR800	WB	Donkey	1:1000	Li-Cor biosciences	926-32211
Mouse IR800	WB	Donkey	1:1000	Li-Cor biosciences	926-32210
Rabbit 647	WB	Donkey	1:1000	Jackson immuno research	711-606-152
Mouse 647	WB	Donkey	1:1000	Jackson immuno research	715-606-150

Table 1. Antibodies used in this study for immunohistochemistry and western blot/

2.6 Western blot

For protein isolation, cerebral organoids were collected individually in suspension buffer (0.1 M NaCl, 0.01 M Tris-HCL pH7.6, 0.001 M EDTA, complete EDTA-free protease inhibitor cocktail (Roche, 11697498001), phosphatase inhibitor cocktail (Sigma Aldrich, P5726) and lysed using the Ultra Turrax Homogenizer (IKA, 0003737000). 2x SDS loading buffer (100 mM Tris pH6.8, 4% SDS, 20% glycerol, 2M DTT (Cleland's reagent, 10708984001)) was added and samples were pre-heated at 95°C for 5 minutes. DNA fractions were

broken up by running the lysates through a 25-gauge needle. Proteins were separated by SDS-PAGE gel electrophoresis on 7.5%, 10% or 15% gels, and blotted on a 0.45 μM pore size nitrocellulose membrane (GE Healthcare, A20485269) using wet blotting. Blots were incubated in blocking buffer (50 mM Tris pH7.4, 150 mM NaCl, 0,25 (w/v) gelatin, and 0.5% triton X-100) and incubated in primary antibody in blocking buffer over night at 4°C (Table 1). Blots were washed in TBS-T (100 mM Tris-HCL pH7.4, 150 mM NaCl and 0.2% Tween-20) and incubated in secondary antibody in blocking buffer for 1h at RT (Table 1). Blots were washed in TBS-T and rinsed with DEMI-H_2O before scanning. Both scanning and analysis were performed using the Odyssey CLx Western Blot Detection System (LI-COR biosciences, Lincoln, NE, USA).

2.7 RNA isolation and RT-PCR

RNA was isolated using the miRNeasy mini kit (Qiagen, 217004) according to manufacturer's protocol. Genomic DNA was removed by including the supplementary DNAse step (Qiagen, 79254). The VarioSkan Flash microplate reader (ThermoScientific, MA) was used to measure the RNA concentration. Conversion to cDNA was achieved by using the Quantitect transcriptase kit (Qiagen, 205311) according to manufacturer's protocol and using the biokrom thermalcycler for the PCR step. Primers for RT-PCR were intron-spanning as designed with PrimerBLAST (NCBI). Absolute levels were calculated ($2^{\Delta CT}$) and subsequently normalized with the geomean of reference genes *GAPDH* and *ACTB* (see supplementary Table 2 for primer sequences).

2.8 Primer panel

The primer panel used in this study consisted of 4 categories: Proinflammatory cytokine genes related to immune activation in MIA studies (*IL6, IL1b,* and *TNF*) [29], microglial (*AIF1, SPI1, ITGAM, PTPRC, HLADRA,* and *C1QA*), astrocytic (*GLT1, S100b,* and *Nestin* (which is also a neuronal stem cell marker)), and neuronal (progenitor) markers (*RELN, PAX6, EOMES/TBR2, TBR1, MAP2, TUBB3,* and *RBFOX3/NEUN*). The neuronal panel was composed to reflect different maturation stages in neuronal development, including early markers *PAX6*, *RELN* important for the orchestration of development, intermediate neuronal progenitor (*EOMES/TBR2*) and postmitotic neuronal markers (*TBR1*, *RBFOX3/NEUN/MAP2*).

2.9 Statistics

R (version 3.5.3) software was used for statistical analyses (CRAN:https://www.r-project.org) in the Rstudio environment. Type three (unbalanced) repeated measures two-way ANOVA was used to compare the LPS-stimulated with the control values per timepoint. The unbalanced method was used to deal with unbalanced number of values per group. Plots were generated using R packages pheatmap (version 1.0.12), ggplot2 (version 3.1.1.), and RColorBrewer (version 1.1.2).

Gene	*5'-Forward primer-3'*	*5'-Reverse primer-3'*
GAPDH	TGTTCGACAGTCAGCCGCATCTTC	CAGAGTTAAAAGCAGCCCTGGTGA
ACTB	GCTCCTCCTGAGCGCAAG	CATCTGCTGGAAGGTGGACA
IL6	TGCAATAACCACCCCTGACC	TGCGCAGAATGAGATGAGTTG
TNF	TGGAGAAGGGTGACCGACTC	TCACAGGGCAATGATCCCAA
IL1B	TTTGAGTCTGCCCAGTTCCC	TCAGTTATATCCTGGCCGCC
C1QA	GAGCACCAGACGGGAAGAAA	TAAGGCCTTGGATGCCTGTC
HLADRA	CCCAGGGAAGACCACCTTT	CACCCTGCAGTCGTAAACGT
AIF1	AGACGTTCAGCTACCCTGACTT	GGCCTGTTGGCTTTTCCTTTTCTC
SPI1	GTGCAAAATGGAAGGGTTTCCC	TACTCGTGCGTTTGGCGTTG
ITGAM	TGCTTCCTGTTTGGATCCAACCTA	AGAAGGCAATGTCACTATCCTCTTGA
PTPRC	GCAGCTAGCAAGTGGTTTGTTC	AAACAGCATGCGTCCTTTCTC
GLT1	GCCAACAGAGGACATCAGCC	ATCCCAGCCCCAAAAGAGTC
S100B	TGGAAAAAGCAACTCCATCAGAA	GAATCGCATGGGTCACGG
NESTIN	GGTGTCTGCAAGCGAGAGTT	TCCCTTAGTCTGGAAGTGGCTA
PAX6	CCTATGCCCAGCTTCACCAT	GGCAGCATGCAGGAGTATGA
EOMES	CGGCCTCTGTGGCTCAAAT	TAGTGGGCAGTGGGATTGAGT
TBR1	GTCACCGCCTACCAGAACAC	GCCGGTGTAGATCGTGTCATA
MAP2	CTCAGCACCGCTAACAGAGG	CATTGGCGCTTCGGACAAG
TUBB3	GGCCTTTGGACATCTCTTC	CTCCGTGTAGTGACCCTTG
RBFOX3	TTACGGAGCGGTCGTGTATC	CGGGCTGAGCGTATCTGTAG
RELN	GCTTTGGACCATGTGGAGGT	TGTCTGAGCCCATGTTGTCG

Table 2. Primer sequences used for RT-PCR.

3. Results

3.1 Study design and organoid characterization

Organoids were grown from two different donors with two batches from one of the donors (iPSC1a, iPSC2a, and iPSC2). After 38 days in culture (days in vitro (DIV)) organoids were split and one of the two groups was stimulated twice with LPS (once at DIV38, and again at DIV45). Half of the organoids were harvested one day at the first stimulation (DIV39) and the remainder was harvested at DIV66 (Figure 1a). To predict responsiveness of organoids to LPS, we used RT-PCR data from Ormel et al. (2018)[24]. In that study, organoid -grown microglia (oMG) were enriched from whole organoids using $CD11b^+$ magnetic cell sorting[24]. RT-PCR was performed to validate enrichment of the oMG. Using this data, statistical analysis showed a typical microglia profile comprising significantly enriched expression of *AIF1*/*IBA1* and *PTPRC*/*CD45* compared to the other cells (flow through/FT) performing a paired T-test using data from three different donors and several batches from one donor (Figure 1b) (paired T-test: *AIF1*: t(8.2), $p < 0.001$; *PTPRC*: t(6.93(, $p < 0.001$). In the brain toll-like receptor 4 (TLR4) and CD14 are expressed that can bind LPS particles[33]. Similar to human brain tissue[30,34], *CD14* and *TLR4* expression in organoids was highly enriched in microglia compared to other cells (Figure 1b) (paired T-test: *CD14*: t(7.9), $p = 0.02$; *TLR4*: t(6.07), $p < 0.001$). Immunostainings confirmed presence of IBA1 and HLADR protein expressing in oMG and DIV39 with an amoeboid morphology, as previously described in Ormel et al. (2018)[24] (Figure 1c). oMG had a ramified appearance at DIV66 as shown with a HLADR immunostaining and they all expressed the nuclear microglia-transcription factor PU.1 (Figure 1d). Proliferative zones were visible in the organoids as shown by neuronal progenitor marker PAX6 and general neuronal marker TUJ1 (Figure 1e-f). Mature neurons expressing neuronal-markers SATB2 and nuclear-NEUN were detected (Figure 1g-h). S100B and GFAP staining also showed the presence of astrocytes at DIV66 (Figure 1i-j). Thus, organoids showed characteristic expression of microglial, astrocytic, and neuronal proteins.

3.2 Acute response to LPS stimulation

To study the acute response to LPS, we analysed organoids one day after exposure and compared them to non-stimulated controls (Figure 2a). We used a panel of immune-activation, microglia, astrocyte, and neuronal markers to investigate the effect induced by LPS (Figure 2b). In line with other cerebral organoid studies[32] we observed heterogeneity inter- and intra-donor organoids. Still we observed several acute effects of LPS stimulation, including significant upregulation of *IL6*, *TNF*, and *IL1B* (repeated measures (RM) two-way ANOVA: *IL6*: $F(1, 14) = 8.9$, $p < 0.01$; *TNF*: $F(1, 14) = 15.8$, $p< 0.01$; *IL1B*: $F(1, 14) = 10.2$, $p < 0.01$) (Figure 2b-c). *HLADRA* was also significantly upregulated in LPS-organoid (RM two-way ANOVA: $F(1, 14) = 6.4$, $p = 0.02$). The other microglial and astrocyte markers were all unaffected (Figure 2b,d-e). Neuronal markers *RELN* and *EOMES* were significantly

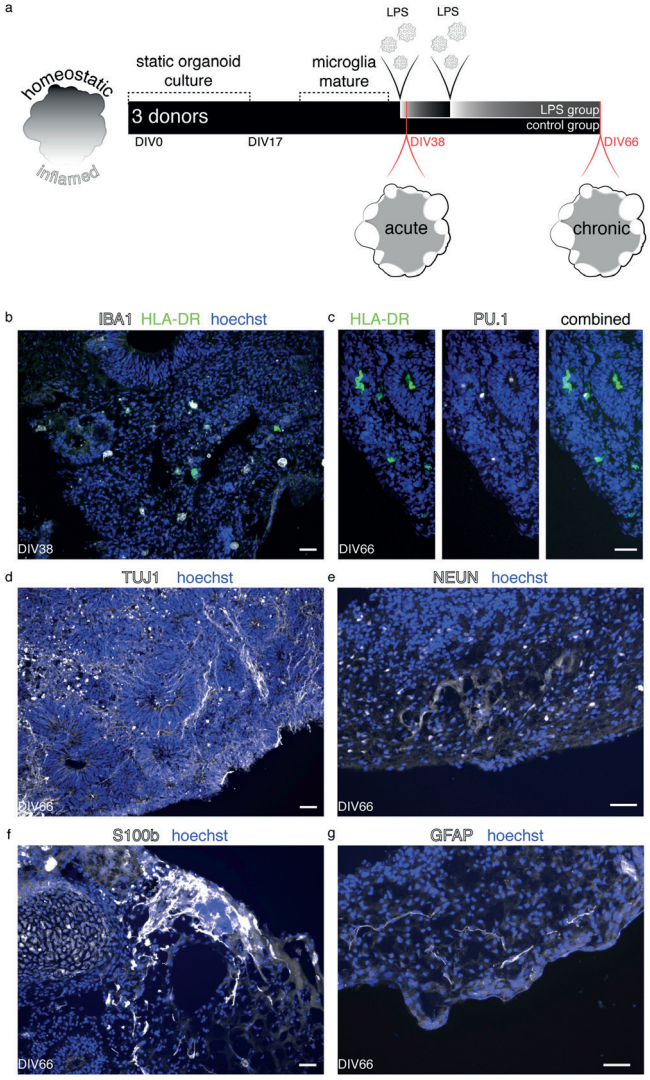


Figure 1. Study design and confirmation of cerebral organoid phenotype.
A Illustration of study design indicating timing of LPS stimulation (DIV37, DIV44) and moments of harvest to compare the acute (DIV39) and chronic (DIV66) effects of LPS versus non-stimulated organoids. **B** Boxplots derived from previously published dataset (Ormel et al. 2018) showing significant increased expression of mRNA of classical microglia markers (*AIF1* and *PTPRC*) and LPS receptors (*TLR4* and *CD14*) in $CD11b^{+}$ magnetic cell sorted organoid-grown microglia versus the $CD11b^{-}$ Flow Through (FT) fraction of the organoids (paired T-test: *AIF1*: t(8.2), $p < 0.001$; *PTPRC*: t(6.93), $p < 0.001$; *CD14*: t(7.9), $p = 0.02$; *TLR4*: t(6.07), $p < 0.001$). Colours of dots correspond to distinct donors. For *CD14* only data from three different batches of one donor was available. **C** Immunostaining of microglia-markers IBA1 and HLA-DR showing amoeboid organoid-grown microglia (oMG) within the organoid at DIV39. **D** Colocaliation of HLA-DR and microglia nuclear-marker PU.1 as assessed with immunostaining in oMG that displayed a ramified morphology at DIV66. **E-F** Immunostainings with neuronal progenitor marker PAX6 (e) and general neuronal marker TUJ1 (f) show presence of neuronal progenitor zones at DIV66. **G-H** Post-mitotic marker SATB2 (g) and mature neuronal marker NEUN (h) expressing neurons were present within organoids at DIV66. **I-J** Astrocytic markers S100b and GFAP were present in the organoid at DIV66. Scale bars in all panels indicate 40 μm. Representative images of iPSC1a, iPSC1b, or iPSC2 are shown.

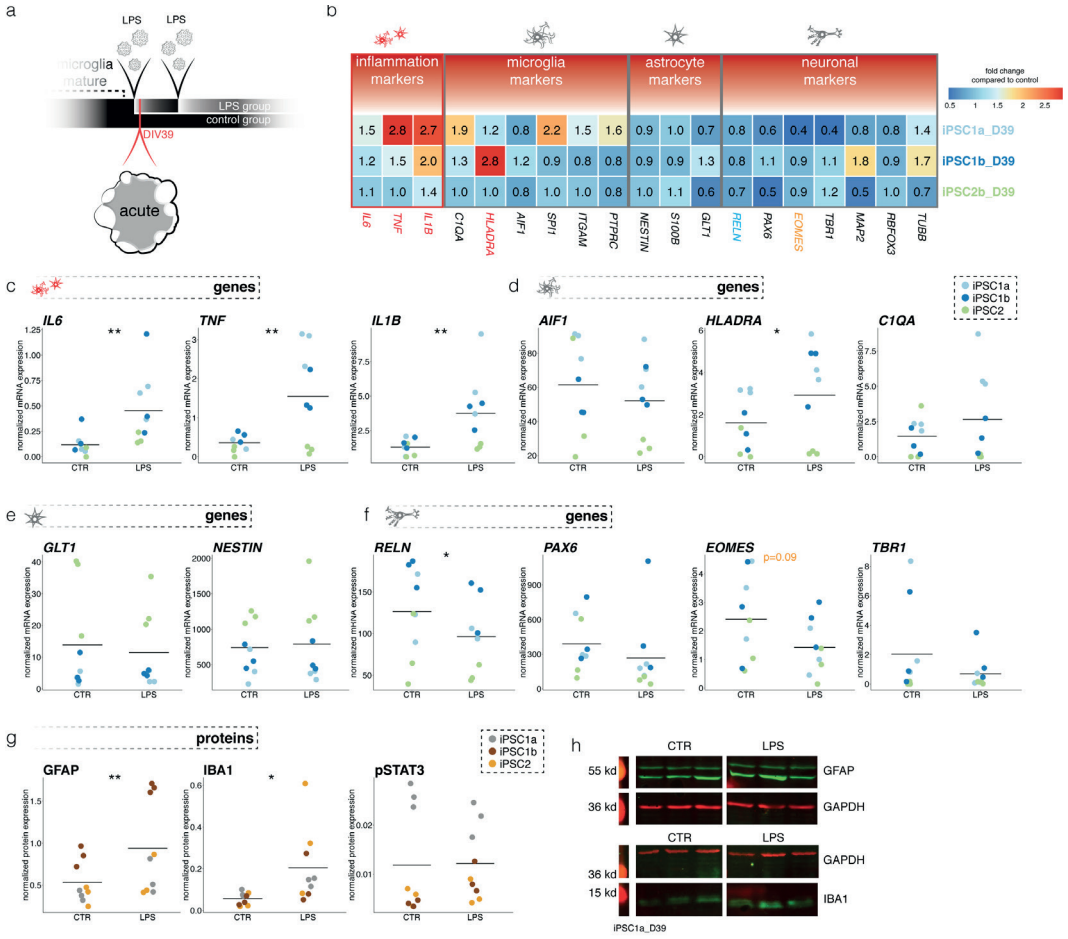


Figure 2. Acute effect of LPS on inflammation status, microglia, astrocyte, and neuron phenotype.
A Illustration depicting a zoom in on the acute part the study design that is elaborated on in this figure. **B** Heatmap showing fold changes of the median expression values of a gene panel of LPS-stimulated organoids versus its non-stimulated controls. The gene panel is comprised of inflammation, microglia, astrocyte, and neuronal markers. Prior to fold change calculation, mRNA levels were first normalized to the geomean of reference genes *ACTB* and *GAPDH*. Gene names are depicted in red or blue when they are significantly up or downregulated after LPS stimulation, respectively. If a trend was visible, the respective gene name is depicted in orange. **C** Dotplots visualizing the significant increased mRNA expression of inflammation markers *IL6, TNF,* and *IL1B* in stimulated versus non-stimulated organoids at DIV39 (repeated measures (RM) two-way ANOVA: *IL6*: $F(1, 14) = 8.9$, $p < 0.01$; *TNF*: $F(1, 14) = 15.8$, $p < 0.01$; *IL1B*: $F(1, 14) = 10.2$, $p < 0.01$). **D** Dotplots visualizing mRNA expression upregulation of *HLADRA*, but not *AIF1* or *C1QA* in LPS-stimulated versus non-stimulated organoids (RM two-way ANOVA: *HLADRA*: $F(1, 14) = 6.4$, $p = 0.02$). **E-F** Dotplots showing astrocytic markers (*GLT1* and *NESTIN*) and neuronal markers (*RELN, PAX6, EOMES,* and *TBR1*) mRNA levels of which *RELN* was significantly decreased and *EOMES* showed a trend towards downregulation (RM two-way ANOVA: *RELN*: $F(1 , 14) = 5.5$, $p = 0.03$; *EOMES*: $F(1, 14) = 3.3$, $p = 0.09$). **G-H**- Protein analyses (visualized in a dotplot (g)) with western blot (h) showed a significant increased expression of GFAP and IBA1, but not pSTAT3, in LPS-stimulated versus non-stimulated organoids (RM two-way ANOVA: GFAP: $F(1 , 14) = 15.4$, $p < 0.01$; IBA1: $F(1, 14) = 6.1$, $p = 0.03$).

downregulated or showed a trend of downregulation, respectively (RM tow-way ANOVA: *RELN*: $F(1, 14) = 5.5$, $p = 0.03$; *EOMES*: $F(1, 14) = 3.3$, $p = 0.09$). Other neuronal markers such as *PAX6* and *TBR1*, remained unaffected (Figure 2b,f). Both the proteins GFAP and IBA1, markers for astrocytes and microglia respectively, were significantly higher expressed after LPS stimulation (RM two-way ANOVA: GFAP: $F(1, 14) = 15.4$, $p < 0.01$; IBA1: $F(1, 14) = 6.1$, $p = 0.03$), whereas phosphorylated STAT3 was unaffected (Figure 2g- h). In sum, LPS exposure of organoids induced expression of microglia- and astrocyte-specific genes and proteins. Neuronal markers *RELN* and *EOMES*, both important for neurodevelopment, were acutely decreased or showed a trend towards a decrease after stimulation with LPS, respectively.

3.3 Long term effects of LPS stimulation

Organoids harvested on DIV66 were analysed for long term effects of inflammation on microglial, astrocytic, and neuronal markers (Figure 3a). We used a similar gene panel as used to assess the acute effects of LPS on inflammation, microglia, astrocyte, and neuronal markers (Figure 3b). The effect of inflammation on the developing organoid was illustrated by displaying the fold changes of stimulated and non-stimulated organoids at DIV66 versus the expression in non-stimulated organoids at DIV39 in a heatmap (Figure 3b). In general, as expected, complement 3 receptor gene (*ITGAM/CD11b*) increased during development of the organoid and mature astrocyte marker *GLT1* was also more expressed at DIV66. Similarly, neurons were maturing as *RELN*, a marker important for early neuronal development was decreased, whereas more mature marker *TBR1* increased in expression at DIV66, although some variation between donors was visible. When comparing stimulated versus unstimulated organoids at DIV66, we observed that both *TNF* and *IL1B* showed a reduction in expression in the LPS group (RM two-way ANOVA: *TNF*: $F(1, 14) = 5.4$, $p = 0.04$; *IL1B*: $F(1, 14) = 5.6$, $p < 0.03$), whereas no effect could be detected on *IL6* expression (Figure 3c). Microglia genes were unaffected, similar to the astrocytic genes except for *GLT1* where the LPS group showed a trend towards upregulation (RM two-way ANOVA: $F(1, 14) = 3.5$, $p = 0.08$) (Figure 3d-e). No long term alterations were detected in neuronal marker gene expression nor alterations in expression of the proteins IBA1, GFAP, or STAT3 (Figure 3f-g). Thus, wo immune activation markers were decreased in expression long term, but the other markers were unaffected.

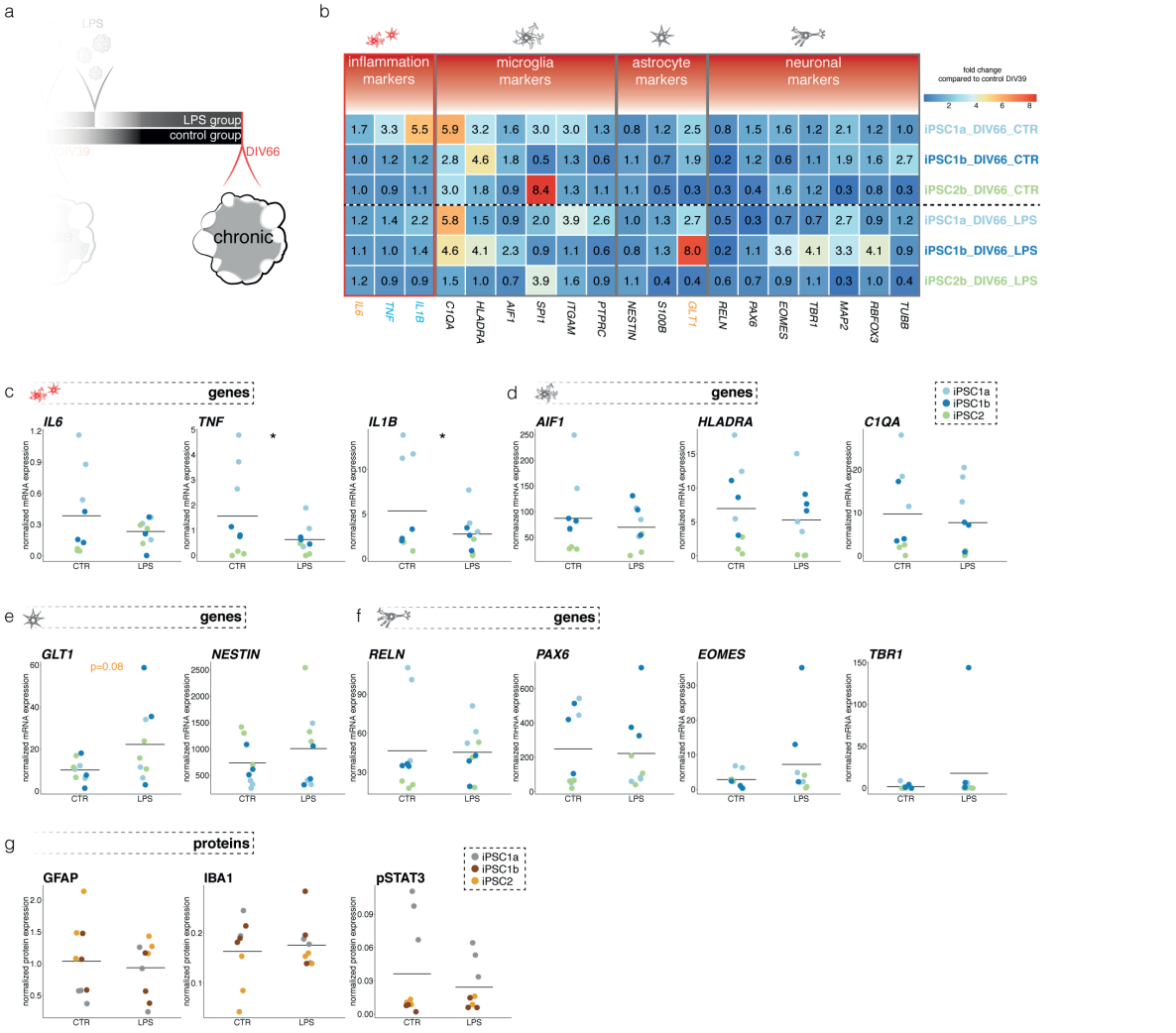


Figure 3. Long term effect of LPS on immune-activation status, and development of microglia, astrocytes, and neurons.

A Illustration depicting a zoom in on the chronic part the study design that is elaborated on in this figure. **B** Heatmap showing fold changes of the median expression values of a gene panel of LPS-stimulated and non-stimulated controls at DIV66 versus non-stimulated controls at DIV39 illustrating deviations in maturation. The gene panel comprised of inflammation, microglia, astrocyte, and neuronal markers. Prior to fold change calculation, mRNA levels were first normalized to the geomean of reference genes *ACTB* and *GAPDH*. Gene names are depicted in blue if they were significantly downregulated in the LPS-stimulated organoids versus non-stimulated organoids at DIV66. If a trend was visible the respective gene name is depicted in orange. **C** Dotplots visualizing the significant decreased mRNA expression of inflammation markers *TNF, IL1B*, but not *IL6* in stimulated versus non-stimulated organoids at DIV66 (repeated measures (RM) two-way ANOVA: *TNF*: $F(1, 14) = 5.4$, $p = 0.04$; *IL1B*: $F(1, 14) = 5.6$, $p < 0.03$). **D-F** Dotplots showing microglial (*AIF1, HLADRA,* and *C1QA*) astrocytic (*GLT1* and *NESTIN*) and neuronal markers (*RELN, PAX6, EOMES,* and *TBR1*) mRNA expression of which only *GLT1* showed a trend towards deviance (RM two-way ANOVA: *GLT1*: $F(1, 14) = 3.5$, $p = 0.08$). **G** Protein analyses of IBA1, GFAP, and pSTAT3 visualized in a dotplot did not show differences in expression between LPS-stimulated and non-stimulated organoids at DIV66.

4. Discussion

In this study we mimicked the MIA rodent model in cerebral organoids thereby investigating the effect of prenatal inflammation on brain development in a human in vitro model. Immune activation was induced as confirmed by the expression of immune-activation markers IL6, TNF, and IL1B, and increased protein expression of glial markers IBA1 and GFAP. LPS exposure also acutely affected neurons as expression of neuronal markers RELN and EOMES was reduced one day after stimulation. On the long term this effect vanished. Exposure to LPS reduced expression of immune- activation markers at DIV66

The acute effects detected in our study showed similarities with the acute effect initiated in LPS-MIA models[29,35]. In the study of O'Loughlin et al. (2017) they used similar read-out parameters in the fetal mouse brain just after MIA induction with LPS[29]. They detected increased IBA1 expression and 1.5-, 2.5-, and 5-fold acutely increased mRNA levels of IL6, TNF, and IL1b, respectively[29], which corresponds with our results. Ghiani et al. (2011), performed a similar experiment in rats and described besides similar effects, also acute increased GFAP expression, as also described in our study[35]. Some, however, reported a more transient effect in gene expression alleviating already 6h post MIA, but still inducing long term effects[12]. They also describe acute increased pSTAT3 expression, which we could not replicate. Possibly the levels of IL6 reached in our study do not meet the levels of this study that were needed to induce the phosphorylation of STAT3. They do note that pSTAT3 expression was regional-specific and it is possible that the variation in brain regions inter-organoids have filtered out this effect.

The reduced expression of RELN and EOMES measured one day after LPS- challenge of the organoids indicate an immediate effect on neuronal development. Interestingly, specifically acute effects on Reelin have been described before in MIA models[35,36]. Both Fatemi et al. (1999) and Ghiani et al. (2011) showed a reduction in RELN expression with similar effect sizes as we had within days after viral or bacterial challenge[35,36]. Nouel et al. (2012) also described a reduction in Reelin-positive neurons in several brain regions in the offspring of MIA rats postnatally[37]. They assessed Reelin neurons at two time-points, detected a reduction at the earlier time-point (postnatal day 14) which disappeared at the later time-point (postnatal day 28). As Reelin is important for proper development of cortical neurons[38], it is possible that changes in expression of Reelin induce long term effects.

MIA in rodents has shown to lead to several long term biological aberrations in the offspring such as prolonged elevated cytokine levels, and deviations in neuronal structure and functioning. These include a decrease in brain volume, delayed interneuron maturation, reduced synaptic organization, and migration deficits of pyramidal neurons (reviewed by Bergdolt and Dunaevsky, 2018[9]). As neuronal deficits could be caused by a

defect in neuronal development prohibiting or limiting the migration and generation of mature neurons, LPS exposure of organoids could also lead to similar long term changes in neurons. However, even though we stimulated organoids twice with LPS, the challenges did not result in prolonged immune activation that therefore might explain the absence of long term neuronal defects in our organoids. In the rodent model, the mother is exposed to LPS which leads to an inflammatory response that partly passes on to the fetus. It is not known which factors are driving neuronal abnormalities in the offspring. It is however thought that LPS is not transferred over the placenta, but induces other factors that do. IL6 has been shown to correlate between the mother' circulation and the fetal circulation as well as brain and abrogation of specifically the IL6 flux removed the long term MIA phenotype[9,12]. We hypothesize that the induction of IL6 in the LPS-exposed organoids may be lower than in the MIA model and therefore less detrimental. This is supported by the lack of phosphorylation of STAT3 after the challenge in organoids. Zuiki et al. (2017) showed that administering an IL6 challenge to human iPSC-derived neural aggregates that were composed of neurons and astrocytes resulted in astrogliosis after nine days in culture[39]. This affected several neuronal subtypes including reduction of TBR1 and CTIP2 expression[39]. Opposed to our study where we used LPS that induced IL6 production, they exposed the neural aggregates directly with high concentrations of IL6 that induced a strong(er) effect. Possibly the timing of assessment also contributed to the disparity in results as we assessed acute and long term effects one day and 29 days after stimulation, respectively, whereas they assessed the response after nine days. Effects might have alleviated in their model after 29 days of culture. An important disadvantage of their model is the absence of microglia, that probably play an important role in the immune response and might also explain the deviance in results in our studies. Especially since they can also counter immune-activity and therefore might be involved in restoring the immune- balance.

Interestingly, on the long term, inflammation markers TNF and IL1B were significantly lower expressed in the LPS-stimulated organoids versus the control organoids. Possibly indicating an immune-restoring mechanism. In some MIA studies a similar effect was detected. In a MIA study in mice they detected significant lower levels of TNFα and IL6 in blood of MIA offspring[40]. In another study they investigated the inflammatory capacity later in life, and reported a reduced TNFα and IL6 response upon LPS-challenge compared to control rats[41]. Other studies however detected a normal immune response upon a re-challenge in MIA offspring[13]. Clark et al. (2019) attributed the blunted immune response to the MIA that induced long term dysfunction of microglia[41]. In our model we did not re-challenge the organoids at DIV66. Further studies are necessary to decipher the exact immune-profile.

The cerebral organoid protocol opened up a lot of research possibilities. A model that can be used to study human brain development as shown by Camp et al. (2015) and even evolutionary differences with other species[22,42]. In only a few years it became a well-es-

tablished model to study brain development in detail. Therefore, it also has a lot of potential for research into prenatal infection and its effect on brain development. An important limitation of its use however is that the organoid only models the fetal brain stadia[43], and therefore mainly susceptibility for neurodevelopmental psychiatric illnesses such as schizophrenia can be investigated. Another limitation is the variability between donors and between organoids. In our study design in which we challenged organoids with LPS, which is known to induce a strong phenotype, organoids were a safe choice. When expecting more subtle differences, large sample sizes will be necessary as also in our study design substantial variation intra- and inter donors was visible. The young research field of cerebral organoids requires more studies in order to do proper assessment of experiment sizes by using power calculations.

Although a status of immune-activation was achieved and an acute effect in neurons was also detected replicating MIA studies, on the long run the neuronal population was not affected. There are two main explanations not discussed yet that could be responsible for this lack of effect and could be studied in the future: 1. We needed a higher resolution to assess composition changes in neuronal development for which single cell RNA sequencing could have been useful[22,42]. 2. We used iPSC-lines from non-psychiatric individuals. Especially considering that not all offspring of pregnant women that have an infection during pregnancy develop psychiatric diseases[3], as expected it is possible that the human brain is resilient for prenatal inflammation except in case of a genetic predisposition for psychiatric disease. This is corroborated by a possible immune-restoring effect indicated by the reduced expression at DIV66 of immune-activation markers in the LPS group. This advocates the use of lines from individuals with established genetic susceptibility to study a link with schizophrenia. For example, the 1.5-3 million basepair hemizygous deletion on the 22nd chromosome that induces the 22q11.2 deletion syndrome that is known to increase risk for schizophrenia with 25-fold[44].

In conclusion, in this study we set out to investigate acute and long term effects of immune-activation on human brain development using an in vitro model. Thereby investigating comparisons with findings in MIA rodent models and trying to understand the disparity in MIA induced effects in rodents versus often lack of long term effects in humans. Acute MIA observed in rodents could be replicated in which not only the glial population, but also neurons were affected. Our first explorative analyses did not show long term neuronal abnormalities, suggesting a level of resilience of the human brain to immune activation. Further research is necessary using lines from individuals with genetic susceptibility, exploring other options for immune activation such as IL6, and using single-cell technologies to increase resolution on altered developmental processes.

Author contribution

P.R.O. and A.B.B. designed and performed experiments. L.D.W, E.M.H. and M.B. critically reviewed the study design. P.R.O. performed RNA processing and RT-PCR. A.B.B. processed protein samples, made organoid sections, performed western blot and immunostainings. P.R.O. did statistical analyses and figure design. P.R.O. wrote the manuscript with supervision of L.D.W. R.V.S helped with the organoid culture. A.B.B. and E.M.H. provided critical feedback in reviewing and editing. Funding of the project was obtained by L.D.W., M.B., and R.S.K.

References

1. Scola, G. & Duong, A. Prenatal maternal immune activation and brain development with relevance to psychiatric disorders. *Neuroscience* **346**, 403–408 (2017).
2. Brown, A. S. Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. *Developmental neurobiology* **72**, 1272–6 (2012).
3. Khandaker, G. M., Zimbron, J., Lewis, G. & Jones, P. B. Prenatal maternal infection, neurodevelopment and adult schizophrenia: a systematic review of population-based studies. *Psychological medicine* **43**, 239–57 (2013).
4. Sørensen, H. J., Mortensen, E. L., Reinisch, J. M. & Mednick, S. A. Association between prenatal exposure to bacterial infection and risk of schizophrenia. *Schizophrenia bulletin* **35**, 631–7 (2009).
5. Atladóttir, H. O. *et al.* Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. *Journal of autism and developmental disorders* **40**, 1423–30 (2010).
6. Parboosing, R., Bao, Y., Shen, L., Schaefer, C. A. & Brown, A. S. Gestational influenza and bipolar disorder in adult offspring. *JAMA psychiatry* **70**, 677–85 (2013).
7. Allswede, D. M. & Cannon, T. D. Prenatal inflammation and risk for schizophrenia: A role for immune proteins in neurodevelopment. *Development and psychopathology* **30**, 1157–1178 (2018).
8. Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science (New York, N.Y.)* **351**, 933–9 (2016).
9. Bergdolt, L. & Dunaevsky, A. Brain changes in a maternal immune activation model of neurodevelopmental brain disorders. *Progress in neurobiology* **175**, 1–19 (2019).
10. Clancy, B., Finlay, B. L., Darlington, R. B. & Anand, K. J. S. Extrapolating brain development from experimental species to humans. *Neurotoxicology* **28**, 931–7 (2007).
11. Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)* **330**, 841–5 (2010).
12. Wu, W.-L., Hsiao, E. Y., Yan, Z., Mazmanian, S. K. & Patterson, P. H. The placental interleukin-6 signaling controls fetal brain development and behavior. *Brain, behavior, and immunity* **62**, 11–23 (2017).
13. Hsueh, P.-T. *et al.* Immune imbalance of global gene expression, and cytokine, chemokine and selectin levels in the brains of offspring with social deficits via maternal immune activation. *Genes, brain, and behavior* **17**, e12479 (2018).
14. Meyer, U. Prenatal poly(i:C) exposure and other developmental immune activation models in rodent systems. *Biological psychiatry* **75**, 307–15 (2014).
15. Pendyala, G. *et al.* Maternal Immune Activation Causes Behavioral Impairments and Altered Cerebellar Cytokine and Synaptic Protein Expression. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **42**, 1435–1446 (2017).
16. Corradini, I. *et al.* Maternal Immune Activation Delays Excitatory-to-Inhibitory Gamma-Aminobutyric Acid Switch in Offspring. *Biological psychiatry* **83**, 680–691 (2018).
17. Cannon, T. D. How Schizophrenia Develops: Cognitive and Brain Mechanisms Underlying Onset of Psychosis. *Trends in Cognitive Sciences* **19**, 744–756 (2015).
18. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).
19. Janssens, S. *et al.* Zika Virus Alters DNA Methylation of Neural Genes in an Organoid Model of the Developing Human Brain. *mSystems* **3**, 1–12.

20. Qian, X., Nguyen, H. N., Jacob, F., Song, H. & Ming, G. L. Using brain organoids to understand Zika virus-induced microcephaly. *Development (Cambridge, England)* **144**, 952 (2017).

21. Dang, J. *et al.* Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate Immune Receptor TLR3. *Cell stem cell* **19**, 258–265 (2016).

22. Camp, J. G. *et al.* Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 15672–7 (2015).

23. di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. *Nature reviews. Neuroscience* **18**, 573–584 (2017).

24. Ormel, P. R. *et al.* Microglia innately develop within cerebral organoids. *Nature Communications* **9**, (2018).

25. Tremblay, M.-È., Lowery, R. L. & Majewska, A. K. Microglial interactions with synapses are modulated by visual experience. *PLoS biology* **8**, e1000527 (2010).

26. Stevens, B. *et al.* The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131**, 1164–1178 (2007).

27. Miyamoto, A. *et al.* Microglia contact induces synapse formation in developing somatosensory cortex. *Nature communications* **7**, 12540 (2016).

28. Cunningham, C. L., Martínez-Cerdeño, V. & Noctor, S. C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**, 4216–33 (2013).

29. O'Loughlin, E., Pakan, J. M. P., Yilmazer-Hanke, D. & McDermott, K. W. Acute in utero exposure to lipopolysaccharide induces inflammation in the pre- and postnatal brain and alters the glial cytoarchitecture in the developing amygdala. *Journal of neuroinflammation* **14**, 212 (2017).

30. Zhang, J., Jing, Y., Zhang, H., Bilkey, D. K. & Liu, P. Maternal immune activation altered microglial immunoreactivity in the brain of postnatal day 2 rat offspring. *Synapse (New York, N.Y.)* **73**, e22072 (2018).

31. Harschnitz, O. *et al.* Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. *Annals of neurology* **80**, 71–88 (2016).

32. Quadrato, G. *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* **545**, 48–53 (2017).

33. Zanoni, I. *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**, 868–80 (2011).

34. Darmanis, S. *et al.* A survey of human brain transcriptome diversity at the single cell level. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 7285–90 (2015).

35. Ghiani, C. A. *et al.* Early effects of lipopolysaccharide-induced inflammation on foetal brain development in rat. *ASN neuro* **3**, 233–245 (2011).

36. Fatemi, S. H. *et al.* Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. *Molecular psychiatry* **4**, 145–54 (1999).

37. Nouel, D., Burt, M., Zhang, Y., Harvey, L. & Boksa, P. Prenatal exposure to bacterial endotoxin reduces the number of GAD67- and reelin-immunoreactive neurons in the hippocampus of rat offspring. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **22**, 300–7 (2012).

38. Lui, J. H., Hansen, D. v & Kriegstein, A. R. Development and evolution of the human neocortex. *Cell* **146**, 18–36 (2011).

39. Zuiki, M. *et al.* Luteolin attenuates interleukin-6-mediated astrogliosis in human iPSC-derived neural aggregates: A candidate preventive substance for maternal immune activation-induced abnormalities. *Neuroscience letters* **653**, 296–301 (2017).

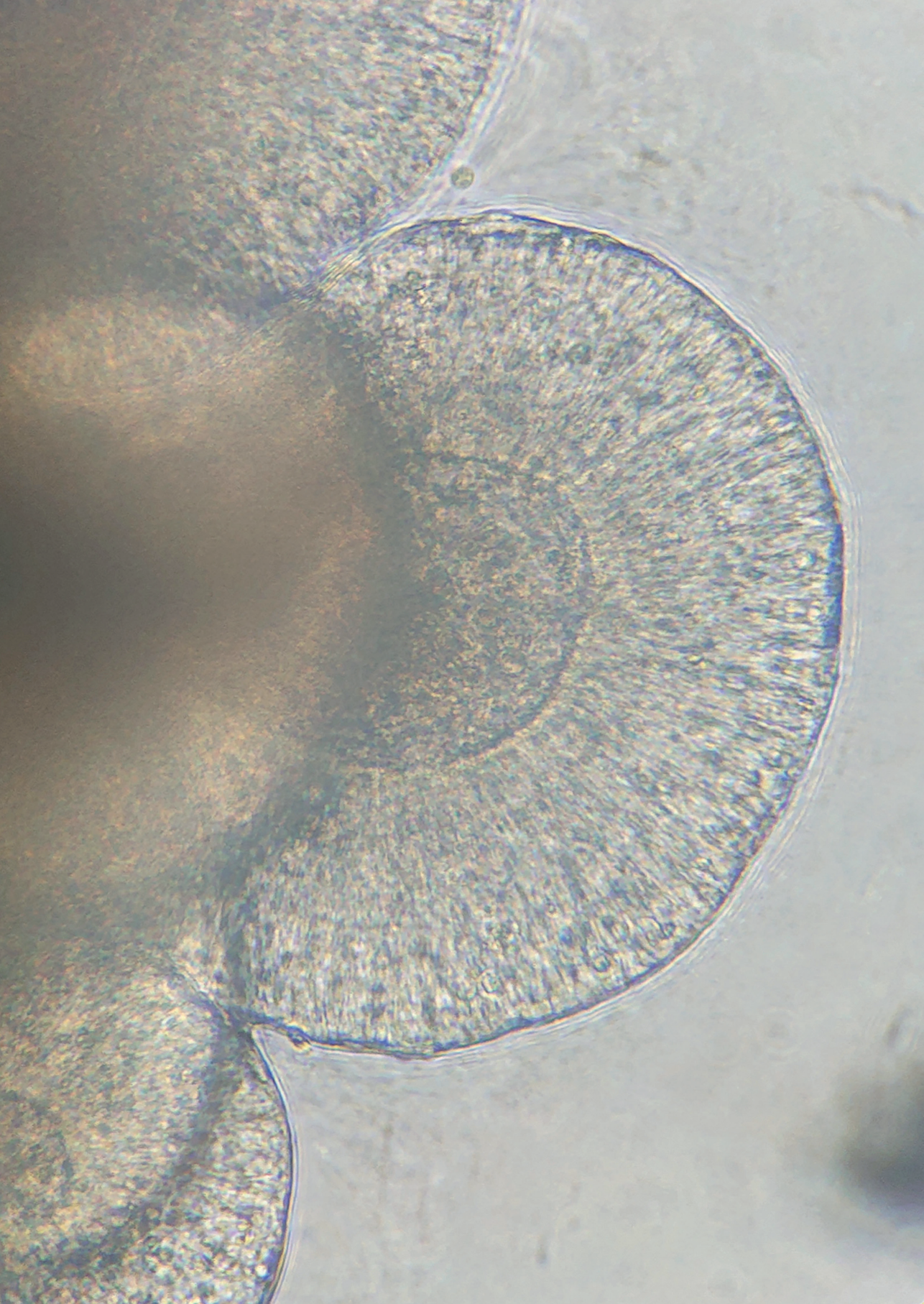
40. Pacheco-López, G., Giovanoli, S., Langhans, W. & Meyer, U. Priming of metabolic dysfunctions by prenatal immune activation in mice: relevance to schizophrenia. *Schizophrenia bulletin* **39**, 319–29 (2013).

41. Clark, S. M. *et al.* Maternal immune activation in rats blunts brain cytokine and kynurenine pathway responses to a second immune challenge in early adulthood. *Progress in neuro-psychopharmacology & biological psychiatry* **89**, 286–294 (2019).

42. Pollen, A. A. *et al.* Establishing Cerebral Organoids as Models of Human-Specific Brain Evolution. *Cell* **176**, 743-756.e17 (2019).

43. Kelava, I. & Lancaster, M. A. Dishing out mini-brains: Current progress and future prospects in brain organoid research. *Developmental biology* **420**, 199–209 (2016).

44. Jonas, R. K., Montojo, C. A. & Bearden, C. E. The 22q11.2 deletion syndrome as a window into complex neuropsychiatric disorders over the lifespan. *Biological psychiatry* **75**, 351–60 (2014).



Chapter 6

Nutrition shaping brain development: exposure to the amino acids histidine, lysine, and threonine reduces mTOR activity and affects neurodevelopment in a human cerebral organoid model

Amber Berdenis van Berlekom[a,b], Raphael Kubler[c], Jeske W. Hoogeboom[b], Daniëlle Vonk[b], Jacqueline A. Sluijs[b], R. Jeroen Pasterkamp[b], Jinte Middeldorp[b,d], Aletta D. Kraneveld[e], Johan Garssen[e,f], René S. Kahn[a,c,g], Elly M. Hol[b], Lot D. de Witte[a,c,g]*, and Marco P. Boks[a]*

[a] Department of Psychiatry, University Medical Center Utrecht, Brain Center, Utrecht University, Utrecht, The Netherlands

[b] Department of Translational Neuroscience, University Medical Center Utrecht, Brain Center, Utrecht University, Utrecht, The Netherlands

[c] Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY, USA and

[d] Department Neurobiology & Aging, Biomedical Primate Research Centre, Rijswijk, The Netherlands

[e] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

[f] Danone Nutricia Research, Utrecht, The Netherlands

[g] Mental Illness Research, Education, and Clinical Center (VISN 2 South), James J Peters VA Medical Center, Bronx, NY, USA

* Contributed equally

Nutrients 2022

Abstract

Background: Evidence of the impact of nutrition on human brain development is compelling, but methods to study the underlying mechanisms are limited. *In vitro* approaches with human cells may be very informative and can potentially reduce or replace animal studies. Here we pioneer the use of human cerebral organoids to investigate the impact on neurodevelopment of specific amino acid supplementation, which was previously shown to affect the mTOR pathway and rodent behaviour.

Method: In this study cerebral organoids were exposed to 10 mM and 50 mM of the amino acids threonine, histidine and lysine. The impact was determined by measuring mTOR activity using western blot, general cerebral organoid size, and gene expression by RNA sequencing.

Results: Exposure to threonine, histidine, and lysine led to decreased mTOR activity and markedly reduced organoid size, supporting findings in rodent studies. RNA sequencing identified comprehensive changes in gene expression, with enrichment in genes related to specific biological processes (among which mTOR signalling and immune function), and to specific cell types including proliferative precursor cells, microglia, and astrocytes.

Conclusion: Cerebral organoids are responsive to nutritional exposure by increasing specific amino acid concentrations and reflect findings from previous rodent studies. Threonine, histidine, and lysine exposure impacts early development of human cerebral organoids, illustrated by inhibition of mTOR activity, reduced size, and altered gene expression.

1. Introduction

Compelling evidence from epidemiological studies suggests that maternal diet during pregnancy is a key modifier of neurodevelopment and impacts later life intelligence, social function, and the risk for acquiring a range of neuropsychiatric conditions such as autism spectrum disorders and schizophrenia[1–6]. Particularly during early gestation, brain development is vulnerable to nutritional deviations with effects persisting later in life. Pre-clinical studies on prenatal nutrition, availability of micronutrients, and composition of the diet, have shown a broad range of effects in offspring, such as decreased neurogenesis, changes in neuronal dendritic arborization, and increased astrocytic GFAP expression[7–10].

Amino acids are a key component of nutrition and some essential amino acids need to be provided through the diet. Dietary intake influences plasma amino acid concentrations and ratios[11] and supplementation of specific amino acids is used to improve health, metabolism, and athletic performance[12]. Amino acids are best known as the building blocks of proteins, but also have an important regulatory function in the cell[12]. The role of specific amino acids during development was recently highlighted in a mouse study enriching the postnatal diet of 5 week old mice with 3 essential amino acids: Histidine (His), Lysine (Lys), and Threonine (Thr), and reporting affected autism related behaviours[13]. Previously, an *in vitro* amino acid immunoblot screen showed that supplementation of these 3 specific amino acids potently inhibits signalling downstream of the mammalian target of rapamycin (mTOR), which was also shown in the mice brains after dietary enrichment[13,14].

Nutrients, like glucose, insulin, but also amino acids, are known to be important for the regulation of mTOR activity[15–17]. mTOR is involved in processes like cell growth, metabolism, and autophagy, but also in neurodevelopment, regulating cortical structure formation through outer radial glia, timing of the gliogenic switch, and axon formation and dendritic arborization[18–22]. Deregulation of mTOR function, due to genetic mutations or altered protein expression is involved in brain diseases, particularly developmental neuropsychiatric disorders such as autism, schizophrenia, and tuberous sclerosis[18,23–26]. Therefore, amino acid availability and especially of His, Lys, and Thr at early developmental stages, is important for healthy brain development and dietary changes during pregnancy may have consequences for risk of neurodevelopmental disorders.

To this date, studies on the role of amino acids on mTOR signalling have been performed in mouse models and 2D *in vitro* studies in rodent mammary epithelial cells[13,14]. Because of the vast differences between human and rodent neurodevelopment and cell function[27–29], translation to a human model is important as it may be more accurate in

reporting transcriptional, functional, and neurodevelopmental changes. The introduction of the induced pluripotent stem cell (iPSC)derived cerebral organoid model by Lancaster et al. (2013) has provided an opportunity to study human neurodevelopment *in vitro*[30,31]. Cerebral organoids show structural properties which are specific for the human developing cortex, such as the presence of an outer subventricular zone containing outer radial glia[30,32]. Furthermore, as cerebral organoids mature, they contain a multitude of different cell types, which form an integrated network, opposed to single cell type 2D *in vitro* approaches[30,33,34]. Interestingly, with slight modifications to the cerebral organoid protocol, the presence of microglia was established[34]. This is unique compared to other brain organoid models and allows for the study of immune related phenotypes[34]. Altogether, cerebral organoids provide a useful representation of early human cortical neurodevelopment at a transcriptomic, epigenetic, and structural level[30,34–36]. Also, this *in vitro* model is a more versatile and accessible resource for sample material then human primary early gestational brain tissue.

Currently it remains to be elucidated if and how specific amino acid modifications impact mTOR activity and neurodevelopment in humans. This study pioneers in researching the effect of His, Lys, and Thr alterations on mTOR activity, size, and transcriptomics in a human neurodevelopmental 3D *in vitro* model. The developing cerebral organoids are exposed to increased concentrations of the 3 amino acids His, Lys, and Thr (AA exposure) and assessed for mTOR activity by western blot, general organoid size, and transcriptomic alterations using RNA-sequencing. The data are cross-referenced with gene-lists on biological processes and cell types to determine leads for future research.

2. Material and methods

2.1 iPSC lines generation and maintenance

The generation and characterization of Induced pluripotent stem-cell (iPSC) lines OH1.5 (male, 62 years old), OH2.6 (male, 61 years old) and OH4.6 (female, 60 years old) was performed in the MIND facility of the UMC Utrecht, described previously[34,37]. iPSC lines were maintained feeder-free on Geltrex (Thermofisher, A1413202) in StemFlex8 medium (ThermoFisher, A3349401) at 37°C with 5% CO2. Medium was changed 3 times a week. Cells were passaged once a week at 80-90% confluency, by incubating the cells in 5 μM EDTA (ThermoFisher, 15575020) for 2 minutes at 37°C and transferring cell aggregates to a new culture dish with StemFlex8 medium, supplemented with 5 μM ROCK-inhibitor (Axon, 1683) for the first 24 hours. All lines were kept in culture to a maximum of 60 passages and were regularly tested for mycoplasma infections (Lonza, LT07-318).

2.2 Ethical approval

For iPSC line generation, written informed consent was provided by volunteers (without neurodevelopmental, psychiatric, neurologic or genetic disorders), and approval granted by the Medical Ethical Committee of the University Medical Center Utrecht. This stud has been conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3 Organoid differentiation

The human iPSCs were differentiated towards cerebral organoids as described previously[34]. In short, iPSCs were grown until ~90% confluency and dissociated to single cells using 5 μM EDTA followed by Accutase (Thermofisher, A11105-01). Cells were counted with the Countess™ II FL Automated Cell Counter (ThermoFisher) and plated at a concentration of 3.5*10^6 cells per well in an aggrewell800 microwell plate (StemCell Technologies, 27865) in 2 mL hES0 medium (DMEM-F12 (ThermoFisher, 11320-074), 20% KOSR (ThermoFisher, 10828028), 1% NEAA (ThermoFisher, 11140-035), 1% L-Glutamine (ThermoFisher, 25030-024), 3% FBS (SigmaAldrich, F7524), 496 μM ß-mercaptoethanol (Merck-Schuchardt, 805740)) supplemented with 4 ng/mL bFGF (ThermoFisher, AA10-155) and 50 μM ROCK-inhibitor (Axon Medchem, Y-27632). Medium was refreshed on day 1. On day 2, embryoid bodies were transferred to an ultra-low attachment 96-well plate (Corning, 3474). Medium was replaced by hES0 without ROCK-inhibitor and bFGF on day 4. On day 6 medium was replaced by neural induction medium (DMEM-F12, 1% N2 (ThermoFisher, 17502048), 1% L-Glutamine, 1% NEAA, 0.1 μg/mL heparin (Sigma Aldrich, H3149-10KU)) which was refreshed on day 8, 10 and 12. Organoids were embedded in 30 μL Matrigel (Corning, 356234) on day 13 and cultured in cerebral organoid differentiation medium without vitamin A (DMEM-F12 1:1 with neurobasal medium (ThermoFisher, 21103049), 1% L-Glutamine, 1% P/S, 0.025% insulin (Sigma Aldrich, I9278), 3.5 μL/L 2-mercaptoethanol, 1% NEAA, 1:100 B27 supplement without vitamin A (Sigma Aldrich, 12587010)) for 4 days, refreshing the medium on day 15 with 16 organoids per 60 mm dish. Medium was replaced by cerebral organoid differentiation medium with vitamin A (DMEM-F12 1:1 with neurobasal medium, 1% L-Glutamine, 1% P/S, 0.025% insulin, 3.5 μL/L 2-mercaptoethanol, 1% NEAA, 1:100 B27 supplement with vitamin A (Sigma Aldrich, 17504044)) at day 17. The organoids were kept on a belly dancer (IBI Scientific, BDRAA115S) at speed 3 and maintained in cerebral organoid differentiation medium with vitamin A for the duration of the experiment (Figure 1A).

2.4 Exposures

Before exposure, cerebral organoids grown in separate culture dishes were combined and randomly re-divided over separate 60 mm culture dishes for the different conditions to reduce intra-dish variation[38]. Cerebral organoids were exposed to 100 nM rapamycin in DMSO (LC laboratories, 5312-88-9), or medium with an additional 10 mM or 50 mM

of the amino acids Thr (Sigma Aldrich, 1084110010), His (Sigma Aldrich, 1043500025), and Lys (Sigma Aldrich, 1057000100) in an 1:1:1 ratio (AA exposure) in differentiation medium with vitamin A. After supplementation with amino acids, the pH of the medium was adjusted to pH 7.4 with NaOH. Rapamycin exposure was performed at week 4 for 1 hour. Short term AA exposure was performed for 1 hour at the start of week 4 to test the acute response to the 3 amino acids. AA exposure continued to week 15, changing the medium 3 times per week with fresh differentiation medium with amino acids. All experiments included a control condition which received standard differentiation medium with vitamin A. To observe the possible inhibitory effect of rapamycin or the AA exposure, cerebral organoids were always harvested 1 hour after medium change (with either control medium, or medium supplemented with rapamycin or the 3 amino acids) for protein and RNA isolation.

2.5 Size measurements

Bright field pictures (2.5x) were obtained (EVOS M5000 microscope, Thermo Fisher) from organoids that exposed to chronic AA exposure from the start of week 4 (day 21). Pictures were taken at the end of week 4, week 5, week 6, week 7, and week 10. Organoid size was determined by creating a mask of the images and measuring the area using a macro in FIJI[39]. The masks were all verified manually.

2.6 Western blot

For protein isolation, cerebral organoids were collected individually in 100 µL suspension buffer (0.1 M NaCl, 0.01 M Tris-HCL pH7.6, 0.001 M EDTA, complete EDTA-free protease inhibitor cocktail (Roche, 11697498001), phosphatase inhibitor cocktail (Sigma Aldrich, P5726)) and lysed using the Ultra Turrax Homogenizer (IKA, 0003737000). 2x SDS loading buffer (100 mM Tris pH6.8, 4% SDS, 20% glycerol, 0.2M DTT (Cleland's reagent, 10708984001)) was added 1:1, and samples were heated at 95°C for 5 minutes. DNA was sheared with a 25-gauge needle. Bromophenol Blue (MERCK, 34725-61-6) was added to each sample for visibility. Samples were stored at -80°C until further use. Proteins were separated by SDS-PAGE gel electrophoresis on 7.5%, 10% or 15% gels, and blotted on a 0.45 µM pore size nitrocellulose membrane (GE Healthcare, A20485269) using wet blotting. Blots were incubated in blocking buffer (50 mM Tris pH7.4, 150 mM NaCl, 0,25 (w/v) gelatin and 0.5% triton X-100)) and incubated with fluorescent primary antibody (Supplementary Table 11) in blocking buffer over night at 4°C. Blots were washed in TBS-T (100 mM Tris-HCL pH7.4, 150 mM NaCl and 0.05% Tween-20) and incubated with secondary antibody in blocking buffer for 1 hour at RT. Blots were washed in TBS-T and rinsed with DEMI-H_2O before fluorescence scanning. Both fluorescence scanning and quantification were performed using the Odyssey CLx Western Blot Detection System (LI-COR biosciences, Lincoln, NE, USA). Each protein of interest was normalized against a reference protein on the same blot (GAPDH).

2.7 RNA isolation

For RNA isolation, cerebral organoids were collected individually in 500 µL of TRIzol Reagent (Thermo Life Technologies, 15596018) and lysed using the Ultra Turrax Homogenizer (IKA, 0003737000). Samples were stored at -80°C until further use. RNA was isolated using the miRNeasy mini kit (Qiagen, 217004) according to manufacturer's instructions. Genomic DNA was removed by including a supplementary DNAse step (Qiagen, 79254). RNA concentrations were measured using the Qubit RNA HS assay kit (Invitrogen, Q32852) on the Qubit 4 (Invitrogen, Q33238).

2.8 RNA-sequencing

2.8.1 Bulk RNAseq analysis

Preparation of the cDNA libraries was performed by Single Cell Discoveries (Utrecht,[40] according to the CelSeq2 protocol[41]. As a quality control pre-sequencing, the quality of the amplified RNA and the final cDNA libraries were determined with RNA 6000 Pico Kit (Agilent, 5067-1513) or the RNA High Sensitivity Kit (Agilent, 5067-4626) respectively, using the Bioanalyzer 2100 (Agilent, G2939BA). Samples were processed and sequenced in 2 separate batches. Libraries were sequenced using the Illumina NextSeq 5000 platform with paired end sequencing (75 bp) at a sequencing depth of 10 million reads per sample. After de-multiplexing of the sample libraries, raw RNAseq reads were aligned along the hg38 human RefSeq transcriptome via Burrows-Wheeler Aligner[42] using MapAndGo.

2.8.2 Quality control

Quality control of all the sequenced samples together was performed based on the following metrics: ERCC spike-ins, library size, and mitochondrial RNA (mtRNA). ERCC spike ins were low (< 1%) in all samples, suggesting high endogenous purity of the RNA content. Samples with a library size below $5x10^5$ counts were excluded. Reads mapped to mitochondrial genes were included as expression effects associated with metabolism (mTOR-related activity) were a potential relevant outcome measure. However, samples identified as distribution-based outliers were excluded. Altogether this led to the exclusion of 6 samples in the long term exposure group (3 50mM AA exposed organoids and 3 CTR organoids, of which 3 from iPSC line OH2.6, 2 from OH1.5, and 1 from OH4.6). To reduce noise within the gene expression data, genes that were lower expressed than the 15th percentile of the count distribution were removed. This accounted for removal of approximately 20% of the genes[43].

2.8.3 Pre-processing and confounders

Inter-sample distances were assessed for each timepoint separately (week 4, week 5, and week 15). We applied unsupervised hierarchical clustering with the pheatmap v1.0.2 package using euclidean distances and principal component analysis (PCA). To dissect the sources of variance in our data, we calculated Pearson *r* correlation between

covariates and principal components. The variance within each gene explained by each single covariate (R^2) was obtained by fitting a variance partition model using variance partition v1.2.5 package [44] (Supplementary figure 4). These analyses suggested that batch and cell line accounted for a significant amount of the variance in our gene expression data. We therefore created a nested variable encompassing both batch and cell line which we implemented in the model for our differential expression analysis, referred to as "experiment". For plotting purposes, we transformed the raw counts with the variance stabilisation function from the DESeq2 v.1.12.3 package[45]. To account for confounding effects during visualization post-normalization, we then took our final model (~log-transformed library size + percentage ERCC + experiment + exposure) and removed variance associated with these confounding variables while retaining variance stemming from exposure (unexposed vs exposed) using the *removeBatchEffect* function from the limma v3.28.14 package. After correction for these covariates there were no sample outliers as assessed with PCA, intra-sample correlation heatmap, and interquartile range (Supplementary figure 5).

2.8.4 Differential gene expression analysis

Differential gene expression between control samples and AA exposed samples (both 10 mM and 50 mM) was analysed for each timepoint separately (Week 4, Week 5, and Week 15) using the DESeq2 package function with the nested variable for batch and cell line as a covariate. Differentially expressed genes (DEG) were visualized using volcano plots from the EnhancedVolcano package v1.7.16[46]. Heatmaps and custom plots were generated with ggplot2 v3.3.2 [47].

2.8.5 Ingenuity pathway analysis

Canonical pathway analyses were performed on the differentially expressed genes (Adj. $p < 0.05$) using the Ingenuity Pathway Analysis (IPA) software. The Ingenuity gene knowledgebase was used as the reference, using Fisher's exact test with Benjamini-Hochberg (BH) correction to determine significant enrichment ($P < 0.05$). Significant pathways with at least 5 overlapping genes were selected.

2.8.6 Comparison with previously published datasets

Differentially regulated genes were compared with previously published datasets on specific cell types in cerebral organoids[38] and human microglia[48] (Supplementary Table 10) significant overlap between gene lists was determined using Fisher's Exact test with Benjamini-Hochberg correction.

2.8.7 Data availability

Relevant data supporting the discussed findings are included in the paper and its supplementary information files. From the RNA sequencing analysis the raw count matrices and the R-code is available through our GitHub repository.

2.9 Weighted gene co-expression network analysis

We generated signed co-expression networks from the gene expression data using the package CoExpNets v0.1.0[49]. Briefly, a topological overlap matrix (TOM) was created based on Pearson *r*-derived gene adjacencies with the WGCNA package v1.70.30[50]. As input, we used a filtered and covariate-corrected gene-matrix (see DGE analysis) containing all samples and genes. Iterative k-means was then applied to an unmodified TOM to generate centroid-based gene-gene clusters (instead of the conventional WGCNA hierarchical clustering based on Pearson *r*-derived 'module membership'). To summarize each cluster's expression pattern by calculating its first principal component (module eigengene; ME). The MEs were then used to a. Calculate each gene's module membership (MM; ME-gene Pearson correlations), b. Calculate module-module similarities (ME-ME Pearson correlations), c. Calculate module expression differences between AA exposure conditions (*t*-test of ME expression and ME-concentration Spearman correlations). MM values were leveraged to select the top hub genes of each module (highest R^2). Hub genes have been shown to be biologically true drivers of their respective gene clusters[50]. All plots were generated in R using ggplot2 v3.3.5. Network graphs were generated using the GGally v2.1.2 and igraph v1.2.7 packages.

2.10 Statistics

Statistical analysis and figure design were performed with Graphpad software (version 8.4) and R studio (version 4.0.0, R core team (2020)). Results are expressed in median and interquartile range (IQR) in boxplots or violin-plots with independent data points. For western blot experiments unpaired Kruskal Wallis tests or Mann-Whitney U test, followed by Dunn's tests for multiple comparisons, were used to compare differences between conditions. Two-way ANOVA with Dunnett's multiple comparisons test was performed to test the differences in size of the organoids over time. The effect of outliers was explored by identifying values +/- 1.5 the interquartile range from the upper or lower quantile. Fisher's exact test was used to test the overlap between the different gene-sets using Benjamini-Hochberg correction. The significance level was set to $p < 0.05$ (two-sided).

3. Results

3.1 mTOR and P70S6K in human cerebral organoids

Cerebral organoids were generated following a previously described protocol[34] (Figure 1A). Baseline expression of mTOR and the downstream ribosomal P70-S6 kinase 1 (P70S6K) is present throughout organoid development (at week 4, week 5, and week 15) at the protein and mRNA level (Figure 1B-C; Supplementary Figure 1; Supplementary Table 1). The figures show both total and phosphorylated protein expression of mTOR, and P70S6K to determine phosphorylation states (Phospho-mTOR/mTOR ratio and Phospho-P70S6K/P70S6K ratio) as a proxy for mTOR activity. Over-time, the phosphorylation state of mTOR remained stable ($H(2) = 2.70$; $p = 0.27$), but the Phospho-P70S6K/P70S6K ratio was downregulated ($H(2) = 9.26$; $p = 0.0097$) at week 15 (mean rank week 4 = 16.50; mean rank week 15 = 6.25; $p = 0.01$). *mTOR* and *RPS6KB1* mRNA expression are increased over time (*mTOR*: $H(2) = 28{,}98$; $p < 0.001$; mean rank week 4 = 10.24; mean rank week 5 = 35.00; mean rank week 15 = 22.77 - *RPS6KB1*: $H(2) = 28.82$; $p < 0.0001$; mean rank week 4 = 11.12; mean rank week 5 = 36.00 ; mean rank week 15 = 21.23). An acute effect of rapamycin (a potent inhibitor of mTOR) on mTOR signaling in cerebral organoids was identified in 4-week-old organoids. 1 hour exposure to rapamycin strongly inhibited the phosphorylation state of P70S6K (CTR: median = 0.94, IQR = 0.87; Rapamycin: median = 0.23, IQR = 0.17; $p = 0.0022$) (Figure 1D; Supplementary Figure 2; Supplementary Table 1).

3.2 Acute effects of AA exposure on mTOR activity in human cerebral organoids

Since mTOR and its downstream target P70S6K are expressed in the cerebral organoid, and mTOR activity can be downregulated with rapamycin, we next investigated the acute effect of AA exposure on mTOR signaling in cerebral organoids. 4-week-old organoids were exposed to medium enriched with 10 mM or 50 mM of the amino acids His, Lysine Lys, and Thr for 1 hour (Figure 1A). The acute AA exposure with 50 mM of the amino acids showed a potent downregulation of the phosphorylation of P70S6K ($H(2) = 18.20$; $p = 0.0001$; mean rank CTR = 16.33; mean rank 50 mM = 5.111; $p = 0.0054$) (Figure 1E-F; Supplementary Figure 3; Supplementary Table 1).

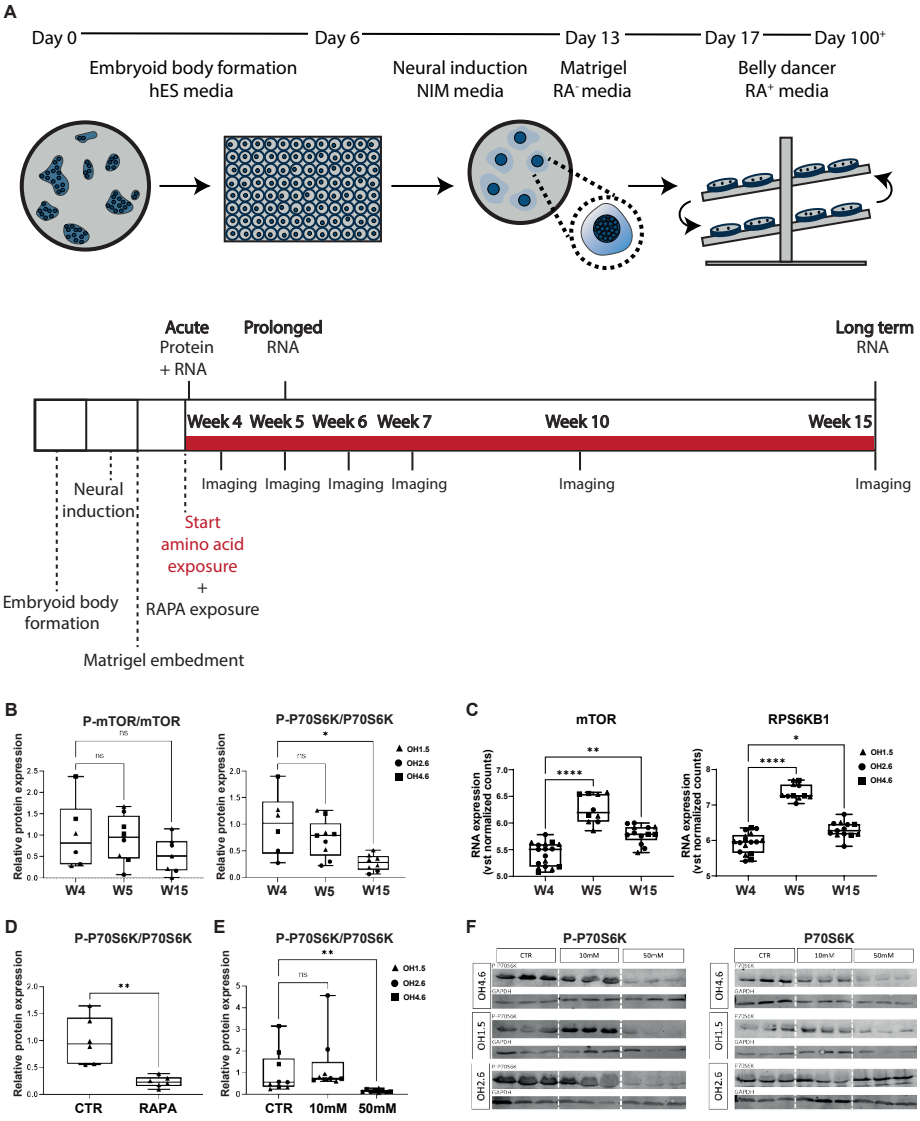


Figure 1. Baseline mTOR pathway expression and response to rapamycin and AA exposure in cerebral organoids.

A Schematic representation of the cerebral organoid protocol (based on Lancaster et al., 2013, with adaptations from Ormel et al., 2018) and experimental set-up. From the start of week 4, organoids were exposed rapamycin, the AA exposure with His, Lys, and Thr (10 mM or 50 mM), or control medium and harvested either acutely (after 1 hour) for protein and RNA isolation or kept in culture for chronic exposure. Organoid size was followed through bright field imaging at the end of week 4, week 5, week 6, week 7, and week 10. Organoids were harvested for RNA isolation after prolonged (week 5), and long term (week 15) AA exposure. Harvesting always occurred 1 hour after medium change. B Quantification of western blots for mTOR - phospho-mTOR (P-mTOR/mTOR) ratio, and the P70S6K - phospho-P70S6K (P-P70S6K/P70S6K) ratio in CTR organoids at different timepoints (week 4, week 5, week 15). C mTOR and P70S6K gene expression in CTR organoids showing differences in DESeq2 vst log transformed raw counts at

different timepoints (week 4, week 5, and week 15). D-E Quantification of western blots for (D) P-P70S6K/P70S6k ratio in rapamycin exposed organoids, and (E) P-P70S6K/P70S6k ratio in acutely AA exposed (10 mM/50 mM) organoids. F Western blots of acutely AA exposed cerebral organoids lysates quantified in E. 3 samples per condition, for 3 cell lines were used. Western blots were cropped to show the relevant bands (P70S6K, P-P70S6K, and GAPDH). Boxplots display median and IQR, with whiskers from minimum to maximum value. Data points represent individual organoids from different cell-lines (circles = OH2.6; triangles = OH1.5; squares = OH4.6). Kruskall-Wallis tests with Dunn test for multiple comparisons was used to compare AA exposed conditions to CTR condition (* $p < 0.05$; ** $p < 0.01$). Mann-Whiteny test was used to compare rapamycin treatment to CTR condition (** $p < 0.01$).

3.3 Chronic amino acid exposure causes size deficits in cerebral organoids

Size (measured by the 2D area) of cerebral organoids which were exposed to chronic AA exposure from the start of week 4 onwards was significantly reduced confirmed by a significant interaction between the effects of time and AA exposure on the size of the organoids ($F(8, 352) = 22.47$; $p < 0.0001$) (Figure 2A). AA exposed cerebral organoids exposed are smaller after several days of exposure (end of week 4) in the 50 mM condition, and from week 7 in the 10 mM condition (Dunnett's multiple comparison; P-adj. < 0.01) (Figure 2A; Supplementary Table 2). Representative images (Figure 2B), and the quantification of all the individual datapoints (Figure 2C-D) at week 5 and 10 illustrate the size differences.

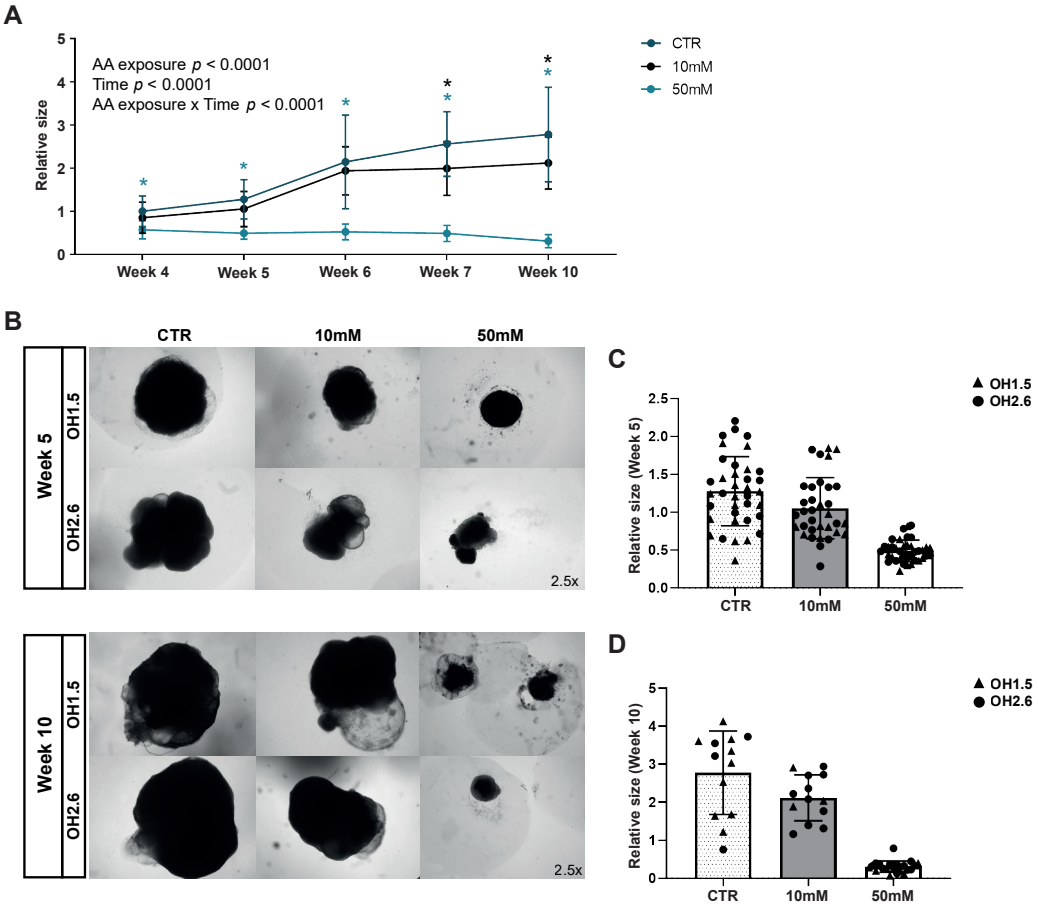


Figure 2. Growth responses to AA exposure in cerebral organoids.

A Quantification of size measurements across 10 weeks of cerebral organoid development (mean ± SEM) for CTR, 10 mM AA exposed, and 50 mM AA exposed conditions. Data are expressed relative to the average of week 4 CTR cerebral organoid sizes within each cell line. N = 5-25 organoids per cell line (OH1.5 and OH2.6) per time-point. Two-way ANOVA p-values are shown in the graph. Significant p values from post-hoc Dunnett's test for multiple comparisons are indicated in blue for difference between CTR and 50 mM AA exposure (week 4-10 $p < 0.0001$), and black for difference between CTR and 10 mM AA exposure (week 7 $p = 0.0041$; week 10 $p = 0.0008$). B Representative bright field microscopy images (2.x magnification) of cerebral organoids at week 5 and week 10 of differentiation, from 2 different cell lines (OH1.5 and OH2.6), for the differently AA exposed conditions (10 mM and 50 mM AA exposure and control). C-D Quantification of size measurements at week 5 (C) and week 10 (D) of cerebral organoid differentiation for CTR, 10 mM AA exposed, and 50 mM AA exposed conditions. Data are expressed relative to the average of week 4 CTR cerebral organoid sizes within each cell line. Bar graphs show mean ± SEM and individual data points (circles = OH2.6, triangles = OH1.5).

3.4 Transcriptomic differences after amino acid exposure in cerebral organoids

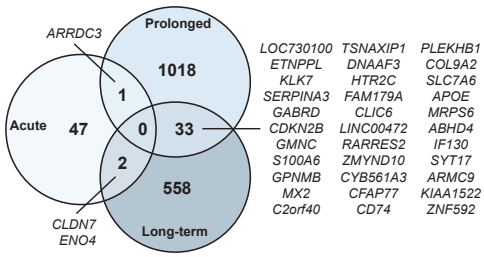
To assess if AA exposure induced changes on transcription, RNA sequencing was performed. Transcriptomic differences were studied between AA exposed (10 mM and 50 mM exposure together) and CTR cerebral organoids (a total of N = 95), for acute (1 hour at week 4 N = 40), prolonged (chronic exposure until week 5 N = 31), and long term (chronic exposure until week 15 N = 24) exposure separately. The whole transcriptome analyses between AA exposed and CTR cerebral organoids at the 3 different timepoints identified a total of 1659 DEG (FDR corrected p value < 0.05) of which 1009 genes were up-regulated and 686 genes down-regulated (Figure 3A-C; Supplementary Figure 5-6; Supplementary Table 3). DEG were identified at each timepoint, with the largest number of DEG represented at the prolonged (week 5) timepoint. Most of the DEG were timepoint specific (Figure 3B).

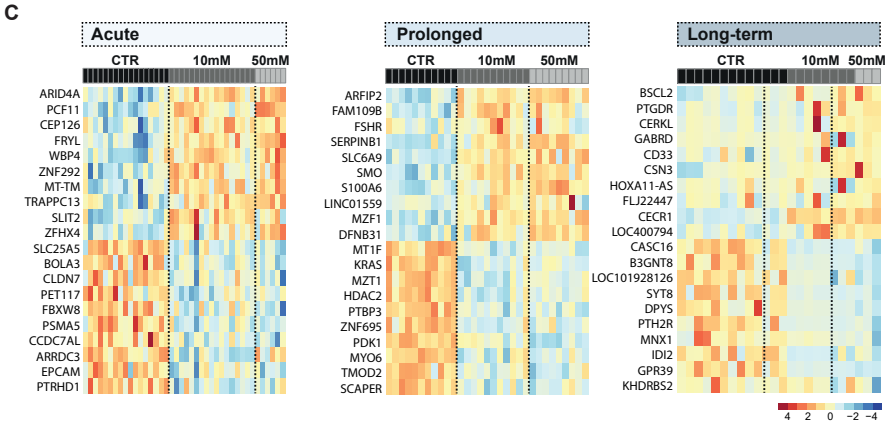
3.5 Cell type and pathway analyses of DEG

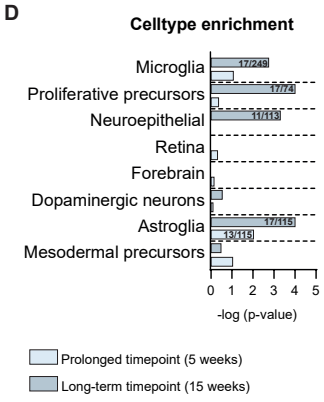
To determine potential cell type specific effects related to the AA exposure we compared our DEG with 2 independent datasets on cerebral organoid cell types and core microglia[38,48]. Fisher's exact test revealed significant enrichment of the DEG of microglial genes (long term; 17/249; p = 0.0018), astroglia genes (prolonged; 13/115; p = 0.0094 and long term; 17/115; p < 0.0001), neuroepithelial genes (long term; 11/113; p = 0.0005), and proliferative precursors (long term; 17/74; p < 0.0001) (Figure 3D, Supplementary Table 4). To gain further insight in the biological processes affected by the AA exposure, we performed pathway analysis using IPA on the significant DEG for each timepoint. In line with the protein expression data, several regulated pathways relate to mTOR signaling ("mTOR signaling", "Regulation of eIF4 and P70S6K signaling", and "EIF2 signaling"). Among the other pathways are processes related to development ("Regulation of the epithelial mesenchymal transition in development pathway"), and immune related pathways ("Autophagy", "IL8 signaling", "Complement System", and "Chondroitin Sulfate Degradation") (Figure 3E, Supplementary Table 5).

A

Stimulation	Adj. p < 0.05
Acute Down	21
Acute Up	29
Prolonged Down	281
Prolonged Up	771
Long-term Down	384
Long-term Up	209







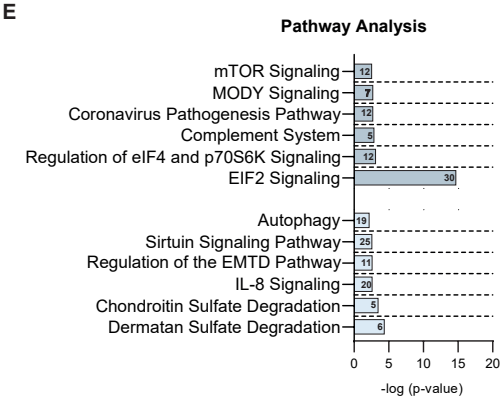


Figure 3. Altered gene transcription in response to AA exposure in cerebral organoids.
A Table providing an overview of the amount of significantly differentially expressed genes after acute, prolonged, and long term AA exposure. B Venn diagram showing the overlap between significantly differentially expressed genes after acute, prolonged, and long term AA exposure. C Heatmap representations of row scaled DESeq2 vst transformed and adjusted z-scores of the most significantly (p-adj) differentially expressed genes (10 up/down regulated) for acute, prolonged, and long term AA exposure. D Cell type analysis of differentially expressed genes after AA exposure at the 3 different timepoints, showing the overlap between cell type specific gene lists and the differentially regulated genes determined by Fisher's exact test. At the acute timepoint, no significant cell type enrichment was found. E Ingenuity pathway analysis (IPA) of differentially expressed genes after AA exposure of organoids at the different timepoints, showing only significant terms (the x-axis shows the significance (-log (p-value)). No significant enrichment of canonical pathways was found for differentially expressed genes of the acute timepoint.

3.6 Weighted gene co-expression network analysis

To further increase insight in the transcriptomic differences between AA exposed and control cerebral organoids we performed a weighted gene co-expression network analysis (WGCNA) on all time points together. This resulted in the identification of 27 modules with intercorrelating transcripts, each containing more than 380 genes (Figure 4A-B; Supplementary Table 6). We used the module eigengene to determine correlations between AA exposure concentration, and module expression patterns (Figure 4C). We identified significant module eigengene association with AA exposure for the Tan and Cyan module (Figure 4C-D). Using IPA we allocated biological pathways to these modules, "inositol signaling" and "EIF2 & mTOR signaling" respectively, again underscoring involvement of the mTOR pathway on a genetic level (Figure 4E, Supplementary Figure 7; Supplementary Table 9). The top hub genes from both modules were mapped to illustrate the interaction networks (Figure 4F, Supplementary Table 7). The central Hub-gene in the "inositol signaling" (Tan) module, HACD3, is involved in metabolism through enzymatic activity creating long-chain fatty acids which function as precursors of membrane lipids and lipid mediators [51]. The "EIF2 & mTOR signaling" (Cyan) module hub gene RACK1, is involved in mTOR signaling and regulated by nutrient starvation induced mTOR inhibition[52]. Next, overlaying the DEG between AA exposed and control organoids from the RNAseq analysis with the WGCNA modules revealed that several modules harbored the majority of these DEG (Figure 4G, Table 1). The blue, cyan, light cyan, and white module are enriched for downregulated DEG, and the dark green, dark grey, dark orange, orange, tan, and yellow module are enriched for upregulated DEG (Table 1). Cell type enrichment analyses and IPA were used to annotate these DEG modules (Figure 4G, Table 1, Supplementary Figure 7; Supplementary table 8; Supplementary table 9). Genes with decreased expression in AA exposed organoids were significantly enriched in modules related to cell-growth ("EIF2 & mTOR signaling", and "DNA replication"), and the "Microglia" module. Furthermore, the "EIF2 & mTOR signaling" module was significantly enriched for proliferative progenitor genes. Genes with increased expression after AA exposure were represented in modules associated to inflammation ("Interleukin signaling", "Neuroinflammation", and "Astrocyte"), the "inositol signaling" module, and the "mitochondrial (dys)function" module.

Module	Annotation	Increased expression		Decreased expression	
		Enrichment	q-value	Enrichment	q-value
Blue	DNA replication			100/883	$1.03 \cdot 10^{-21}$
Cyan	EIF2 & mTOR signaling + proliferative precursors			46/382	$6.44 \cdot 10^{-11}$
Darkgreen	Interleukin signaling	54/689	$1.62 \cdot 10^{-10}$		
Darkgrey	Neuroinflammation	77/620	$5.04 \cdot 10^{-16}$		
Darkorange	Undetermined	85/571	$1.09 \cdot 10^{-18}$		
Lightcyan	Microglia			114/827	$2.88 \cdot 10^{-6}$
Orange	Astrocyte	152/801	$3.5 \cdot 10^{-41}$		
Tan	Inositol signalling	89/545	$3.29 \cdot 10^{-19}$		
White	Undetermined			72/890	$1.04 \cdot 10^{-8}$
Yellow	Mitochondrial (dys) function	171/853	$7.2 \cdot 10^{-50}$		

Table 1. Annotation of the DEG enriched WGCNA modules.
Annotation of DEG enriched WGCNA modules. The ten WGCNA modules which are significantly enriched for increased or decreased differentially expressed genes after AA exposure from the RNA sequencing, using Fisher's exact test with BH correction $p < 0.05$. Showing the annotation of each module based on cell type determination and IPA analysis, the enrichment of differentially expressed genes, module length, and the q-value from the Fisher's exact test.

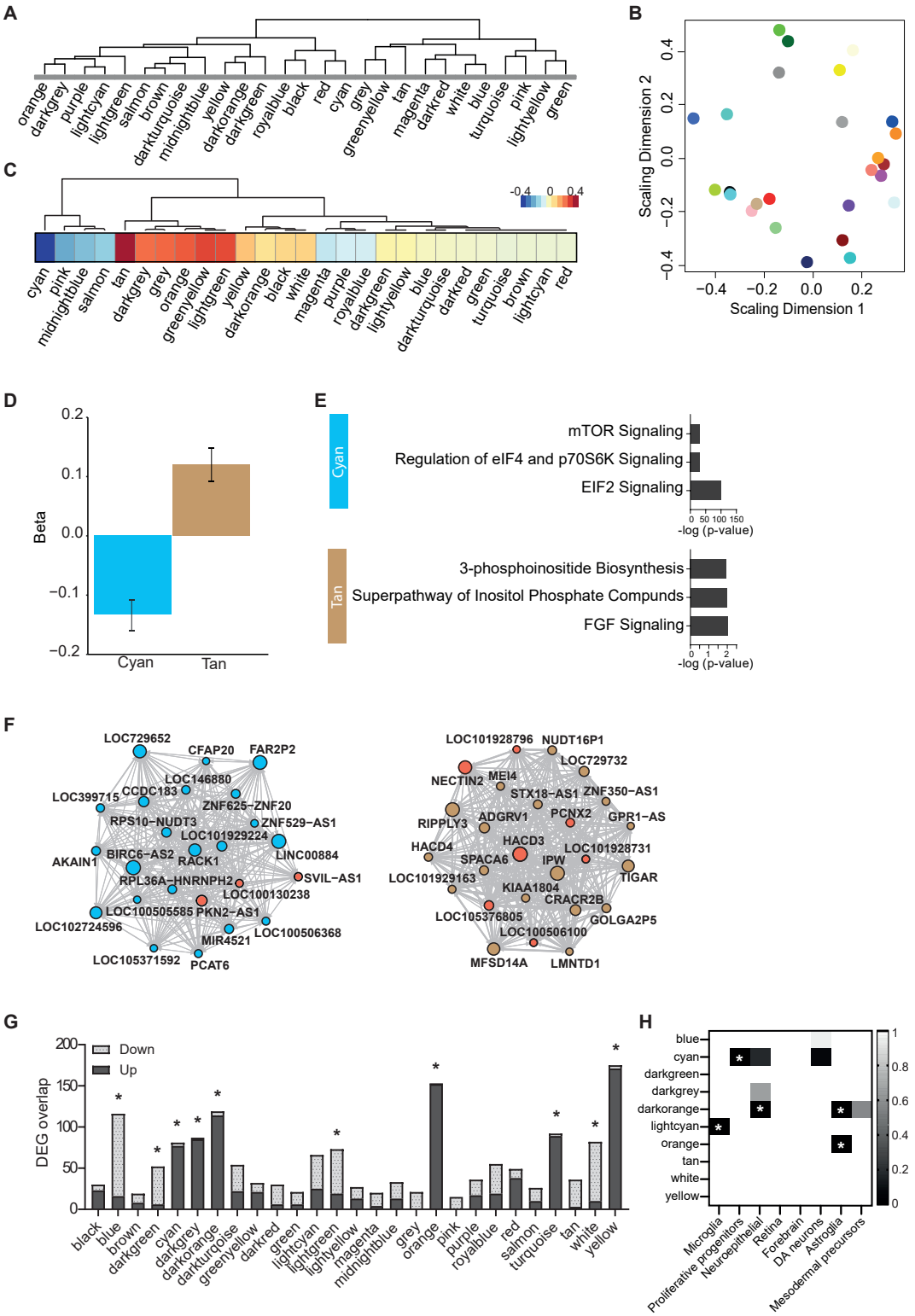

A
orange
darkgrey
purple
lightcyan
lightgreen
salmon
brown
darkturquoise
midnightblue
yellow
darkorange
darkgreen
royalblue
black
red
cyan
grey
greenyellow
tan
magenta
darkred
white
blue
turquoise
pink
lightyellow
green
B
Scaling Dimension 2
Scaling Dimension 1
C
-0.4
0
0.4
cyan
pink
midnightblue
salmon
tan
darkgrey
grey
orange
greenyellow
lightgreen
yellow
darkorange
black
white
magenta
purple
royalblue
darkgreen
lightyellow
blue
darkturquoise
darkred
green
turquoise
brown
lightcyan
red
D
Beta
Cyan
Tan
E
Cyan
Tan
mTOR Signaling
Regulation of eIF4 and p70S6K Signaling
EIF2 Signaling
-log (p-value)
3-phosphoinositide Biosynthesis
Superpathway of Inositol Phosphate Compunds
FGF Signaling
-log (p-value)
F
LOC729652
CFAP20
FAR2P2
LOC146880
LOC399715
CCDC183
ZNF625−ZNF20
RPS10−NUDT3
ZNF529−AS1
LOC101929224
AKAIN1
BIRC6−AS2
RACK1
LINC00884
RPL36A−HNRNPH2
SVIL−AS1
LOC100130238
LOC100505585
PKN2−AS1
LOC102724596
LOC100506368
MIR4521
LOC105371592
PCAT6
LOC101928796
NUDT16P1
LOC729732
NECTIN2
MEI4
STX18−AS1
ZNF350−AS1
RIPPLY3
ADGRV1
PCNX2
GPR1−AS
HACD4
HACD3
LOC101928731
SPACA6
IPW
LOC101929163
TIGAR
KIAA1804
CRACR2B
LOC105376805
GOLGA2P5
LOC100506100
MFSD14A
LMNTD1
G
DEG overlap
Down
Up
black
blue
brown
darkgreen
cyan
darkgrey
darkorange
darkturquoise
greenyellow
darkred
green
lightcyan
lightgreen
lightyellow
magenta
midnightblue
grey
orange
pink
purple
royalblue
red
salmon
turquoise
tan
white
yellow
H
blue
cyan
darkgreen
darkgrey
darkorange
lightcyan
orange
tan
white
yellow
Microglia
Proliferative progenitors
Neuroepithelial
Retina
Forebrain
DA neurons
Astroglia
Mesodermal precursors

Figure 4. Gene network analysis of responses to AA exposure in cerebral organoids.
A Tree cluster structure of WGCNA module generation. B Principle component analysis plot with ME distances, representing module similarity. C Module eigengene correlation with the factor "amino acid concentration". D Significant module-eigengene associations with amino acid concentration are observed for the Cyan (downregulated) and Tan (upregulated) module. E Top 3 enriched canonical pathways determined by Ingenuity pathway analysis (IPA) for the Cyan and Tan module (the x-axis shows the significance (-log (p- value)). F Plots show top hub-genes and their interaction network for the Cyan and Tan module. G Overlap of down- and up-regulated differential expressed genes for each module as determined by Fisher's exact test. Significant enrichment is indicated with *. H Heatmap showing cell type enrichment of each module as determined by Fisher's exact test. Significant enrichment is indicated with *, scale bar indicates q-value.

4. Discussion

In an endeavour to translate the effect of dietary enrichment of specific amino acids in rodent models[13], this study reports on the effects of exposure to Thr, His, and Lys on cerebral organoids as a model for nutritional effects on human neurodevelopment. In cerebral organoids AA exposure inhibited mTOR activity, caused a retention in size, and affected gene expression in the developing cerebral organoids

In cerebral organoids the mTOR signalling cascade was affected by exposure to rapamycin, similar to previous studies[13,14]. In response to AA exposure with 50 mM of Thr, His, and Lys, mTOR activity was potently inhibited (illustrated by a strong decrease of P70S6K phosphorylation) in agreement with *in vitro* models and rodent studies[13,14]. Exposure to lower concentrations of the specific amino acids (10 mM) was related to size changes, but had no effect on the P70S6K phosphorylation in the cerebral organoids, potentially due to the sensitivity of the assay (western blot).

The find of substantially inhibited size of the cerebral organoids after AA exposure is consistent with the fact that mTOR is involved in cell proliferation, metabolism, and growth and underscores the impact of amino acids on the early stages of human brain development[18,19,53,54]. After 1 week of AA exposure cerebral organoid size was impacted, and at 10 weeks the cerebral organoid size was substantially smaller. Although the large size difference can indicate a compromised state of the cerebral organoids after AA exposure, gene expression data do not point to an increase in apoptosis or necrosis. Instead, gene expression data was consistent with mTOR changes and therefore the size deficit is likely to be related to inhibited cell growth and proliferation, as this pathway, trough P70S6K, plays a crucial role in protein translation at the ribosome, and its activity is associated with protein synthesis and cell proliferation[55,56].

Elaborate analysis of gene expression using RNA sequencing and WGCNA consistently pointed to the involvement of mTOR and the immune system. Evidence for the involvement of mTOR was present at several levels. Several identified pathways enriched in the DEG at both week 5 and week 15 are directly and indirectly associated to mTOR signaling. Furthermore, WGCNA identified 2 modules which were regulated by the AA exposure and enriched for DEG, the Cyan and Tan module. The Cyan module is enriched for genes involved in mTOR and EIF2 signaling. The Tan module is defined as the Inositol phosphate pathway, involved in processes such as membrane transport, cytoskeletal function, and plasma membrane signaling, which has also been associated with mTOR signaling, and even more interesting, with amino acid mTOR sensing[57,58]. Moreover, the Cyan module is enriched for genes related to proliferative precursors. These RNA sequencing results combined with the fact that the mTOR pathway is involved in proliferation[53,54], and the

finding that cerebral organoids remain smaller after AA exposure, together points to a mTOR regulated defect in proliferation during neurodevelopment in the AA exposed cerebral organoids. This perspective is consistent with the fact that outer radial glia, (a class of precursor cells predominant in human cortical development), express high mTOR activity, and are therefore expected to be extra vulnerable for decreases in mTOR activity, affecting their proliferative capacity[20,32]. Furthermore, the DEG and the WGCNA results also point to involvement of the immune system and identified enrichment for microglial and astrocytic genes after AA exposure, both glial cell types are involved in inflammation and are highly responsive to external stimuli[59,60]. Previous research showed that mTOR plays a role in regulation of the gliogenic switch, and changes in mTOR activity during neurodevelopment can lead to a changed ratio between the number of neurons and astrocytes[21,22]. Also, DEG at week 15 are enriched for genes from the microglia associated complement system, which has previously been associated with increased mTOR activity in tuberous sclerosis patient post-mortem brain material [61]. Finally, results of the pathway analysis of the DEG and the WGCNA modules were consistent with a role of microglial and astrocytic immune function after AA exposure and identified processes related to inflammation: "Autophagy"[62], "IL8 signaling", "Chondroitin Sulfate Degradation"[63], "Interleukin signaling", and "Neuroinflammation".

Future perspectives

The fact that the results from this study are consistent with the finding from animal research that mTOR activity is decreased in the brain after dietary AA supplementation[13] opens new avenues of nutritional research into neurodevelopment, while reducing the need for animal studies. The cerebral organoid proves a valuable translational model between animal studies and humans, and because of its versatility could be employed in many different paradigms of nutritional intervention.

This study contributes to the body of evidence relating dietary amino acid changes to mTOR inhibition and neurodevelopment. The results suggest that cerebral organoid models can be employed to identify human neurodevelopmental processes that are vulnerable to environmental impacts or can be targeted by (nutritional) interventions. Assessing cell type specific changes using immunohistochemistry and single cell RNA sequencing can further advance this emerging field.

In the future, investigating the effect of amino acid mediated mTOR inhibition on neurodevelopment can contribute to our understanding of dietary modifiers of neurodevelopment and potentially, later life cognition and risk for acquiring neuropsychiatric conditions[1–6]. The results from this study suggest that prenatal dietary amino acid exposure of His, Lys, and Thr is detrimental for neurodevelopment, however they also strengthen the idea that dietary amino acids may have potential as therapeutic

applications for neurodevelopmental disorders related to mTOR function, such as autism spectrum disorder, schizophrenia, and tuberous sclerosis[64–66].

Altogether, the current study warrants further research in this direction and may provide leverage for new applications of cerebral organoid *in vitro* models in studies of neurodevelopmental disorders, as well as to pioneer application of nutritional interventions.

Limitations

This study should be interpreted in the context of its limitations. In our study we increased concentrations of the amino acids Thr, His, and Lys to non-physiological levels (10 mM and 50 mM). However, the concentrations we applied, are in a similar millimolar range as previous *in vitro* studies targeting mTOR with these amino acids[13,14]. Another important limitation is the heterogeneity between cerebral organoids[38]. The cell type composition is known to be variable. Due to extensive filtering of the RNAseq data, cell types with low frequencies (such as microglia) will have a smaller impact on the analysis. Furthermore, we cannot rule out that differences in cell composition between conditions may have had impact. Differences in cell type composition between time points are expected and by analysing each time point separately these differences will have not contributed to differentially expressed genes. Also, studying 3 cell lines, from 3 separate donors, at 3 time points, at 3 concentrations, does involve a substantial number of organoids. Nevertheless, heterogeneity between organoids may require larger sample sizes to detect smaller differences.

Conclusion

In conclusion, our study shows for the first time that exposure to increased levels of the amino acids histidine, lysine and threonine inhibits mTOR activity, decreases size, and affects gene expression pathways related to mTOR, proliferation, and immune function in a human model for neurodevelopment. The results echo findings in animal studies that His, Lys, and Thr inhibit mTOR activity in brain cells and support the potential of cerebral organoid models as additional model system for nutritional studies of brain development, thus enabling the reduction and replacement of animal studies. It confirms the impact of early nutritional availability of amino acids as a powerful moderator of mTOR function. Considering the role of mTOR in neurodevelopment and particularly in neurodevelopmental disorders such as autism, schizophrenia, and tuberous sclerosis, this study has the potential to facilitate further progress in the understanding of the etiology of these disorders and the development of early nutritional interventions.

Acknowledgements

The authors thank the team of Single Cell Discoveries for their excellent services (https://www.scdiscoveries.com/).

This work was supported by the ZonMw Meer Kennis met Minder Dieren grant. Materials for this study were funded by Danone Nutricia Research.

Author contribution

M.P.B, A.B.B., and L.D.W. designed the study. E.M.H, R.J.P., A.D.K, and J.G. critically reviewed the study design. A.B.B. performed experiments. R.K. analysed RNA sequencing. J.W.H. performed organoid size measurements. D.V. assisted with iPSC culture. J.A.S. and A.B.B. performed western blots. A.B.B. did statistical analysis and figure design. A.B.B. wrote the manuscript with supervision of M.P.B. and L.D.W.. J.M., R.J.P., A.D.K., J.G., E.M.H., and R.S.K provided critical feedback in reviewing and editing of the manuscript. Funding for the project was obtained by M.P.B.

Supplementary material

Supplementary material is available at the online version of the paper:
https://www.mdpi.com/2072-6643/14/10/2175

References

1. Kang, Y. *et al.* Nutritional Deficiency in Early Life Facilitates Aging-Associated Cognitive Decline. *Current Alzheimer Research* 14, (2017).
2. de Rooij, S. R., Wouters, H., Yonker, J. E., Painter, R. C. & Roseboom, T. J. Prenatal undernutrition and cognitive function in late adulthood. *Proceedings of the National Academy of Sciences of the United States of America* 107, 16881–16886 (2010).
3. Li, M., Francis, E., Hinkle, S. N., Ajjarapu, A. S. & Zhang, C. Preconception and prenatal nutrition and neurodevelopmental disorders: A systematic review and meta-analysis. *Nutrients* vol. 11 (2019).
4. Peretti, S. *et al.* Diet: the keystone of autism spectrum disorder? *Nutritional Neuroscience* vol. 22 825–839 (2019).
5. St Clair, D. *et al.* Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. *Journal of the American Medical Association* 294, 557–562 (2005).
6. Hoek, H. W., Brown, A. S. & Susser, E. The Dutch Famine and schizophrenia spectrum disorders. in *Social Psychiatry and Psychiatric Epidemiology* vol. 33 373–379 (Soc Psychiatry Psychiatr Epidemiol, 1998).
7. Salas, M., Torrero, C., Rubio, L. & Regalado, M. Effects of perinatal undernutrition on the development of neurons in the rat insular cortex. *Nutritional Neuroscience* 15, 20–25 (2012).
8. Watanabe, K., Furumizo, Y., Usui, T., Hattori, Y. & Uemura, T. Nutrient-dependent increased dendritic arborization of somatosensory neurons. *Genes to Cells* 22, 105–114 (2017).
9. Marques, A. H., Bjørke-Monsen, A. L., Teixeira, A. L. & Silverman, M. N. Maternal stress, nutrition and physical activity: Impact on immune function, CNS development and psychopathology. *Brain Research* 1617, 28–46 (2015).
10. Abbink, M. R., van Deijk, A. L. F., Heine, V. M., Verheijen, M. H. & Korosi, A. The involvement of astrocytes in early-life adversity induced programming of the brain. *GLIA* vol. 67 1637–1653 (2019).
11. Schmidt, J. A. *et al.* Plasma concentrations and intakes of amino acids in male meat-eaters, fish-eaters, vegetarians and vegans: a cross-sectional analysis in the EPIC-Oxford cohort. *European Journal of Clinical Nutrition 2016 70:3* 70, 306–312 (2015).
12. Wu, G. Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37, 1–17 (2009).
13. Wu, J. *et al.* Dietary interventions that reduce mTOR activity rescue autistic-like behavioral deficits in mice. *Brain, Behavior, and Immunity* 59, 273–287 (2017).
14. Prizant, R. L. & Barash, I. Negative effects of the amino acids Lys, His, and Thr on S6K1 phosphorylation in mammary epithelial cells. *Journal of Cellular Biochemistry* 105, 1038–1047 (2008).
15. Sengupta, S., Peterson, T. R. & Sabatini, D. M. Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress. *Molecular Cell* vol. 40 310–322 (2010).
16. Bar-Peled, L. & Sabatini, D. M. Regulation of mTORC1 by amino acids. *Trends in Cell Biology* vol. 24 400–406 (2014).
17. Efeyan, A., Zoncu, R. & Sabatini, D. M. Amino acids and mTORC1: From lysosomes to disease. *Trends in Molecular Medicine* vol. 18 524–533 (2012).
18. Bockaert, J. & Marin, P. mTOR in Brain Physiology and Pathologies. *Physiological Reviews* 95, 1157–1187 (2015).
19. Swiech, L., Perycz, M., Malik, A. & Jaworski, J. Role of mTOR in physiology and pathology of the nervous system. *Biochimica et Biophysica Acta - Proteins and Proteomics* vol. 1784 116–132 (2008).

20. Andrews, M. G., Subramanian, L. & Kriegstein, A. R. Mtor signaling regulates the morphology and migration of outer radial glia in developing human cortex. *eLife* 9, 1–21 (2020).

21. Cloëtta, D. *et al.* Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis. *Journal of Neuroscience* 33, 7799–7810 (2013).

22. Blair, J. D., Hockemeyer, D. & Bateup, H. S. Genetically engineered human cortical spheroid models of tuberous sclerosis. *Nature Medicine* 24, 1568–1578 (2018).

23. Winden, K. D., Ebrahimi-Fakhari, D. & Sahin, M. Abnormal mTOR Activation in Autism. *Annual Review of Neuroscience* vol. 41 1–23 (2018).

24. Howell, K. R. & Law, A. J. Neurodevelopmental concepts of schizophrenia in the genome-wide association era: AKT/mTOR signaling as a pathological mediator of genetic and environmental programming during development. *Schizophrenia Research* 217, 95–104 (2020).

25. Curatolo, P., Moavero, R. & de Vries, P. J. Neurological and neuropsychiatric aspects of tuberous sclerosis complex. *The Lancet Neurology* vol. 14 733–745 (2015).

26. Zimmer, T. S. *et al.* Tuberous Sclerosis Complex as Disease Model for Investigating mTOR-Related Gliopathy During Epileptogenesis. *Frontiers in Neurology* 0, 1028 (2020).

27. Lui, J. H., Hansen, D. V. & Kriegstein, A. R. Development and evolution of the human neocortex. *Cell* vol. 146 18–36 (2011).

28. Patir, A., Shih, B., McColl, B. W. & Freeman, T. C. A core transcriptional signature of human microglia: Derivation and utility in describing region-dependent alterations associated with Alzheimer's disease. *GLIA* 67, 1240–1253 (2019).

29. Oberheim, N. A. *et al.* Uniquely hominid features of adult human astrocytes. *Journal of Neuroscience* 29, 3276–3287 (2009).

30. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373–379 (2013).

31. di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. *Nature Reviews Neuroscience* 18, 573–584 (2017).

32. Pollen, A. A. *et al.* Molecular Identity of Human Outer Radial Glia during Cortical Development. *Cell* 163, 55–67 (2015).

33. Renner, M. *et al.* Self-organized developmental patterning and differentiation in cerebral organoids. *The EMBO Journal* 36, 1316–1329 (2017).

34. Ormel, P. R. *et al.* Microglia innately develop within cerebral organoids. *Nature Communications* 9, (2018).

35. Kelava, I. & Lancaster, M. A. Dishing out mini-brains: Current progress and future prospects in brain organoid research. *Developmental Biology* vol. 420 199–209 (2016).

36. Luo, C. *et al.* Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. *Cell Reports* 17, 3369–3384 (2016).

37. Harschnitz, O. *et al.* Autoantibody pathogenicity in a multifocal motor neuropathy induced pluripotent stem cell-derived model. *Annals of neurology* 80, 71–88 (2016).

38. Quadrato, G. *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* 545, 48–53 (2017).

39. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9, 676–682 (2012).

40. Muraro, M. J. *et al.* A Single-Cell Transcriptome Atlas of the Human Pancreas. *Cell systems* 3, 385-394.e3 (2016).

41. Hashimshony, T., Wagner, F., Sher, N. & Yanai, I. CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. *Cell reports* 2, 666–673 (2012).

42. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)* 26, 589–95 (2010).

43. Sha, Y., Phan, J. H. & Wang, M. D. Effect of low-expression gene filtering on detection of differentially expressed genes in RNA-seq data. *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual Conference* 2015, 6461 (2015).

44. Hoffman, G. E. & Roussos, P. Dream: powerful differential expression analysis for repeated measures designs. *Bioinformatics (Oxford, England)* 37, 192–201 (2021).

45. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15, (2014).

46. Blighe K, R. S. L. M. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. . *R package version 1.12.0, https://github.com/kevinblighe/EnhancedVolcano.* (2021).

47. H. Wickham. *ggplot2: Elegant Graphics for Data Analysis.* (Springer-Verlag, 2016).

48. Patir, A., Shih, B., McColl, B. W. & Freeman, T. C. A core transcriptional signature of human microglia: Derivation and utility in describing region-dependent alterations associated with Alzheimer's disease. *Glia* 67, 1240–1253 (2019).

49. Botía, J. A. *et al.* An additional k-means clustering step improves the biological features of WGCNA gene co-expression networks. *BMC systems biology* 11, (2017).

50. Langfelder, P. & Horvath, S. WGCNA: An R package for weighted correlation network analysis. *BMC Bioinformatics* 9, 1–13 (2008).

51. Ikeda, M. *et al.* Characterization of four mammalian 3-hydroxyacyl-CoA dehydratases involved in very long-chain fatty acid synthesis. *FEBS letters* 582, 2435–2440 (2008).

52. Erbil, S. *et al.* RACK1 Is an Interaction Partner of ATG5 and a Novel Regulator of Autophagy. *The Journal of biological chemistry* 291, 16753–16765 (2016).

53. Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* 168, 960 (2017).

54. Morita, M. *et al.* mTOR coordinates protein synthesis, mitochondrial activity and proliferation. *Cell cycle (Georgetown, Tex.)* 14, 473–480 (2015).

55. Tavares, M. R. *et al.* The S6K protein family in health and disease. *Life sciences* 131, 1–10 (2015).

56. Magnuson, B., Ekim, B. & Fingar, D. C. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *The Biochemical journal* 441, 1–21 (2012).

57. Kim, S. *et al.* Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. *Cell metabolism* 13, 215–221 (2011).

58. Raghu, P., Joseph, A., Krishnan, H., Singh, P. & Saha, S. Phosphoinositides: Regulators of Nervous System Function in Health and Disease. *Frontiers in Molecular Neuroscience* 12, 208 (2019).

59. Verkhratsky, A., Parpura, V., Vardjan, N. & Zorec, R. Physiology of Astroglia. *Advances in experimental medicine and biology* 1175, 45–91 (2019).

60. Kettenmann, H., Hanisch, U.-K., Noda, M. & Verkhratsky, A. Physiology of Microglia. *https://doi.org/10.1152/physrev.00011.2010* 91, 461–553 (2011).

61. Mills, J. D. *et al.* Coding and small non-coding transcriptional landscape of tuberous sclerosis complex cortical tubers: implications for pathophysiology and treatment. *Scientific Reports 2017 7:1* 7, 1–16 (2017).

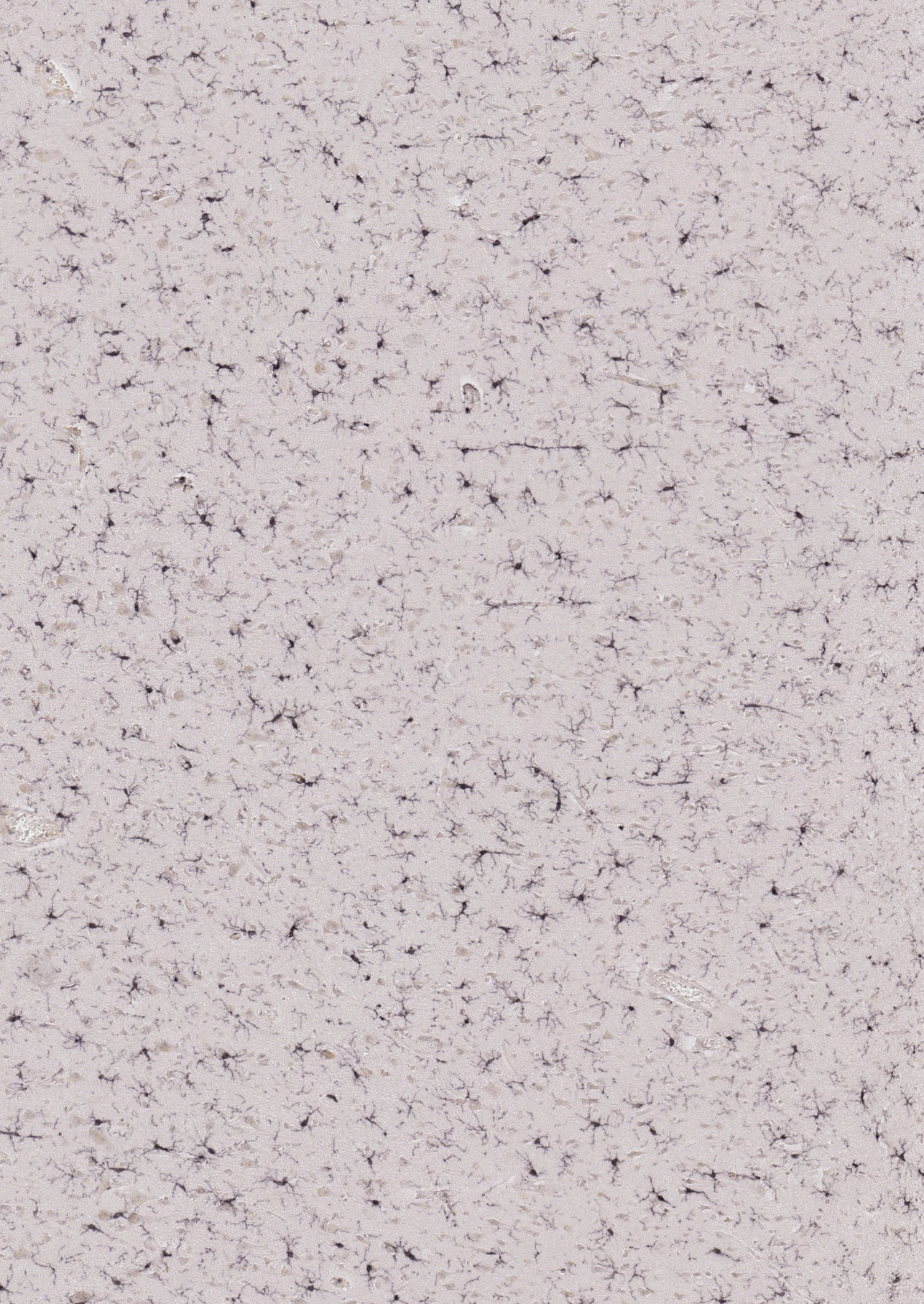
62. Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature 2011 469:7330* 469, 323–335 (2011).

63. Stephenson, E. L. & Yong, V. W. Pro-inflammatory roles of chondroitin sulfate proteoglycans in disorders of the central nervous system. *Matrix biology : journal of the International Society for Matrix Biology* 71–72, 432–442 (2018).

64. Chadha, R., Alganem, K., Mccullumsmith, R. E. & Meador-Woodruff, J. H. mTOR kinase activity disrupts a phosphorylation signaling network in schizophrenia brain. *Molecular psychiatry* (2021) doi:10.1038/S41380-021-01135-9.

65. Henske, E. P., Józwiak, S., Kingswood, J. C., Sampson, J. R. & Thiele, E. A. Tuberous sclerosis complex. *Nature reviews. Disease primers* 2, (2016).

66. Winden, K. D., Ebrahimi-Fakhari, D. & Sahin, M. Abnormal mTOR Activation in Autism. *Annual review of neuroscience* 41, 1–23 (2018).



Chapter 7

Summary and General Discussion

Neurodevelopment has been implicated in the etiology of schizophrenia by epidemiological, imaging, and genomic studies. However, pinpointing which biological processes are affected has been a challenge to the field.

We hypothesized that specific neurodevelopmental molecular and/or cellular processes are affected in schizophrenia pathogenesis.

Therefore, the aim of this research was to study neurodevelopmental phenotypes in schizophrenia from different perspectives, studying the dysconnectivity phenotype related to postnatal neurodevelopment, and the effect of prenatal environmental schizophrenia risk factors on early neurodevelopment, with a specific focus on tissue specificity, and glial cells (astrocytes and microglia), using both established and novel translational approaches.

This chapter provides an overview of the main findings of this thesis. First, a summary of each chapter is given (7.1). We discuss and evaluate the studies from part I (7.2) and part II (7.3) and give a perspective on the biological insights we gained. Lastly, we provide an overview of methodological considerations (7.4), suggestions for future directions (7.5), and some concluding remarks (7.6).

7.1 Summary of results

Part I 1 Tissue specific cortical abnormalities in schizophrenia using postmortem human brain tissue

Chapter 2: Synapse pathology in schizophrenia: A meta-analysis of postsynaptic elements in postmortem brain studies.

Synaptic density changes are hypothesized to underly the altered brain connectivity observed in schizophrenia pathology[1–10]. To study if synaptic density changes are a robust phenotype in postmortem human brain tissue of schizophrenia patients, we performed a meta-analysis of the current literature on this topic. We included three outcome measurements for postsynaptic elements: dendritic spine density, postsynaptic density number, and postsynaptic density protein expression. Our meta-analysis showed region specific postsynaptic density changes in schizophrenia. We found a decrease of postsynaptic elements in the prefrontal cortex and cortical layer III specifically. Importantly, high heterogeneity was present in all analyses. These findings show decreased synaptic density is an important brain region-specific cellular hallmark in schizophrenia.

Chapter 3: A postmortem study on cortical synapses, microglia, and astrocytes in schizophrenia.

Altered astrocyte and microglia function has been suggested to impact synapse number in schizophrenia[11–20]. In our study we assessed, for the first time, the amount synaptic protein in combination with glial cell number and phenotype in schizophrenia postmortem tissue. Importantly, based on the results from chapter 2, we took brain region specificity into account, focussing on frontal cortical grey matter, and layer III specifically. We found that the numbers of Spinophillin^{+} postsynaptic elements, GFAP^{+} astrocytes, and IBA1^{+} microglia are not affected in cortical layer III of the prefrontal cortex in our schizophrenia cohort. Furthermore, synaptic and astrocyte markers are not affected in cortical grey matter, but two homeostatic microglia genes (*CX3CR1* and *ITGAM*) are lower expressed in cortical grey matter in our schizophrenia cohort. Altogether, despite our rigorous efforts, we did not find evidence that synapses, or the association between synapses and glia are different in schizophrenia patients.

Chapter 4: DNA methylation differences in cortical grey and white matter in schizophrenia.

Epigenetic regulation, such as DNA methylation, affects gene transcription[21]. Epigenetic differences are caused by genetic differences, but also through environmental factors involved in schizophrenia pathology[22–28]. We wanted to identify if there are grey- and white-matter specific DNA methylation differences between schizophrenia patients and controls in postmortem brain cortical tissues. We identified 24 differentially methylated regions of which the difference in DNA methylation between grey and white matter tissue is different between schizophrenia and controls. Four of these differentially methylated regions, located near the genes *KLF9*, *SFXN1*, *SPRED2*, and *ALS2CL*, are differentially methylated comparing schizophrenia to controls, and grey to white matter tissue. *KLF9* and *SFXN1* gene expression were decreased in *gyrus frontalis medialis* grey matter, and *SPRED2* in *gyrus frontalis medialis* white matter of schizophrenia patients. Interestingly *SFXN1*, *SPRED2*, and *ALS2CL* are all involved in neurodevelopment and synaptic functioning[29–43].These data show that there are distinct DNA methylation patterns between grey and white matter which are unique to schizophrenia, and are related to neurodevelopment and synaptic functioning.

Part I main findings:

We identified specific phenotypes related to neurodevelopment in schizophrenia postmortem brain tissue, but also inconsistencies.

- Meta-analysis of previous literature shows the number of postsynaptic elements is affected in specific brain regions (the cortex and cortical layer III) in schizophrenia.

- The number of synapses and glia, and synapse and astrocyte marker expression are not affected in prefrontal cortical grey matter in our schizophrenia cohort, but homeostatic microglial gene expression is downregulated.

- Specific regions of the DNA are differentially methylated between prefrontal cortical grey and white matter in schizophrenia versus control donors, accompanied by differential gene expression in genes related to neurodevelopment and synaptic functioning.

Part II The effect of prenatal environmental risk factors for developing schizophrenia on neurodevelopment in cerebral organoids

Chapter 5: Prenatal inflammation evaluated in human cerebral organoids.

Maternal infection during pregnancy is one of the most extensively investigated prenatal risk factors for developing schizophrenia to date[44–54]. Cerebral organoids provide an opportunity to study the effect of maternal immune activation on early human neurodevelopment. We investigated the effect of LPS exposure on inflammatory and neurodevelopmental pathways in the cerebral organoid. One day after the LPS challenge, immune related gene expression was upregulated (*IL6*, *HLADR*, *IL1B*, *TNFa*), together with an upregulation of microglial IBA1 and astrocytic GFAP protein expression. Gene expression levels of important neurodevelopmental genes *EOMES* and *RELN* showed acute downregulation 24 hours after LPS stimulation. However, long term (28 days after LPS stimulation) only a downregulation of several immune genes was observed (*TNFa* and *IL1B*). Altogether, a state of inflammation was induced in cerebral organoids, also affecting neurons, although chronic effects as described in maternal immune activation rodent models were not replicated in our study.

Chapter 6: Nutrition shaping brain development: exposure to the amino acids histidine, lysine, and threonine reduces mTOR activity and affects neurodevelopment in a human cerebral organoid model.

Maternal diet during pregnancy is a key modifier of neurodevelopment and associated with the risk of developing neuropsychiatric disorders such as schizophrenia in the offspring[55–60]. In our study we pioneered the use of the human cerebral organoid model

to investigate the impact of specific amino acid supplementation on neurodevelopment. We found that supplementation of the amino acids lysine, threonine, and histidine in the culture medium caused a decrease in mTOR signaling and a strong retention in size in the cerebral organoids. RNA sequencing revealed vast changes in gene expression with enrichment of genes related to mTOR, proliferation, and immune function. Our results show, for the first time, that nutritional studies with exposure to the amino acids can be performed using cerebral organoids to study the effect on neurodevelopment.

Part II main findings:

Environmental risk factors for schizophrenia, maternal immune activation, and prenatal nutrition, defined by epidemiological research, affect early human brain development in cerebral organoids.

- Maternal immune activation, modelled by LPS exposure in cerebral organoids, causes an acute phenotype in immune related microglial and astrocytic markers, and neuronal-related gene expression, which is absent on the long term.

- A dietary risk factor, stimulating cerebral organoids with specific amino acids, leads to decreased mTOR signaling, growth retention, and altered gene expression related to mTOR, proliferation, and immune function.

7.2 Synapses and glia in schizophrenia postmortem brain tissue

In part one of this thesis, we used postmortem human brain tissue to determine if there are specific cellular/molecular phenotypes in schizophrenia that are related to postnatal neurodevelopment. Synapse formation and elimination, through pruning by glia (astrocytes and microglia), are postnatal neurodevelopmental processes. Synapse density changes have long been suggested to underly symptoms of schizophrenia and the role of glia (astrocytes and microglia) in excessive synaptic pruning has been subject of investigation[1–20]. In chapter 2 and 3 we focussed on synaptic density changes, and how these could be related to glia. We applied a multi modal approach using a broad spectrum of techniques, thoroughly investigating changes in gene expression, protein expression, and DNA methylation in schizophrenia postmortem brain tissue.

Importance of tissue specificity

In part one of this thesis, we set out to include tissue specificity as one of the key focus points in our studies. The results from our studies have shown that this is important. Data from the meta-analysis in chapter 2 showed great variation of synapse density changes depending on the brain region we analysed. Furthermore, our study defining differences between grey and white matter DNA methylation in schizophrenia and controls in chapter 4, also illustrated the importance to study these tissues separately. We utilised various methods to reach this goal, firstly we manually dissected grey and with matter for RNA, DNA, and protein measurements, and secondly, we studied cortical layer III specifically with immuno-histochemistry by overlaying images with adjacent nissl stained sections. In the future, tissue specificity could be more increased by nuclei sorting of individual cell populations and microdissection of cells in specific cortical layers for RNA/DNA/protein analyses[61,62].

Perspective of the biological effects

Evidence for synaptic density changes in schizophrenia

Although our meta-analysis in chapter 2 showed region specific synaptic density reductions in the prefrontal cortex, and layer III especially, in our own postmortem study this finding did not replicate. Updating our meta-analysis on cortical layer III postsynaptic elements with the results from our study, we still find an overall lower number in schizophrenia patients (Figure 1). Meta-analysis is one of the most powerful research tools available, cumulating and summarizing knowledge within the field. Our data on the postsynaptic elements together with the meta-analysis showing a decrease of pre-synaptic synaptophysin in the frontal cortex[10], provide strong evidence that cortical synaptic density is decreased.

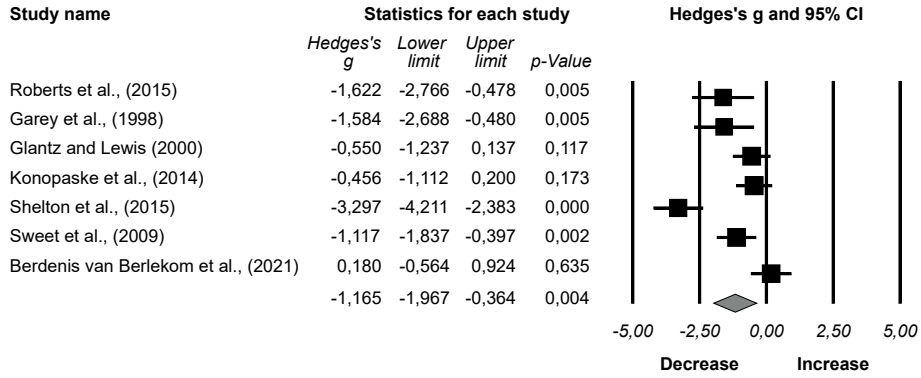


Figure 1. Updated meta-analysis of postsynaptic elements in cortical layer III.
Forest plot of cortical layer III subgroup meta-analysis for 7 studies on postsynaptic elements (spine density determined with Golgi staining, or immunohistochemistry of postsynaptic marker Spinophilin) in schizophrenia. The pooled effect size on the density of postsynaptic elements is significantly decreased ($p < 0.05$).

It was rather unexpected that the decrease in synapse density not replicate in our study. However, layer III of the *prefrontal cortex* has only been researched in 3 separate studies[63–65], from which only one study found a significant decrease of dendritic spines[63], which is also the oldest study with a smaller sample size compared to the other studies (schizophrenia 8, control 7). This emphasizes the need for replication studies.

On the other hand, we could explain the absence of this phenotype in our schizophrenia postmortem cohort in other ways. Firstly, there might be substantial subtype specificity considering the decrease in synapses depending on clinical factors, such as the severity of symptoms or the age of onset of the disease, which remains largely unexplored. As an example, previous studies have shown differences in cortical gene expression that were specific only for schizophrenia patients who completed suicide versus healthy controls[66,67]. Secondly, technical differences in, for example, brain tissue processing or assay type, might affect reproducibility[68].

Recent studies focussing on brain changes in schizophrenia using, MRI, DTI, or RNA sequencing include large sample sizes (of 100 patients or more) to better explore patient subtype specific phenotypes and correct for technical confounders, while enabling the detection of small effect sizes[69–71]. These techniques allow for high throughput experiments and/or analyses. Labour intensive studies, quantifying the number of synapses using imaging or other molecular techniques, are not yet performed on this scale in schizophrenia research. Until we are able do that, meta-analysis is the tool to bring together the data of these smaller studies on this topic.

Altogether, our data from chapter 2, 3 and the updated meta-analysis in this chapter (Figure 1) show that there is a decrease in synapse density in schizophrenia postmortem tissue, which is specific for brain regions, and might be specific for patient subtypes.

Glial contributions

From our study in chapter 3, we did not find strong evidence that glia number or phenotype is altered in the prefrontal cortical grey matter and layer III specifically in schizophrenia. However, the interpretation of these data was difficult: in absence of a synapse phenotype in our schizophrenia cohort, would we still expect a glial phenotype? Although we performed various analyses to assess the interaction between glial number or phenotype and synapse number or gene expression, we did not find any relation. Therefore, it would be important to repeat the study, in a cohort where synapse density changes previously have been shown.

As described in the introduction of this thesis and chapter 3, studies assessing microglial and astrocytic phenotype in schizophrenia have shown heterogeneous results[72–76]. For example, a recent study, very similar to ours, assessing the expression of a broad panel of astrocytic and microglial genes in samples from the Stanley Medical Research Institute, identified an increase in expression of only one astrocytic gene (*ALDH1L1*) in the DLPFC comparing schizophrenia patients to control subjects[66]. This underlines the heterogeneity and thereby the difficulty that is inherent to schizophrenia postmortem research. However, in our study in chapter 3 we did identify a downregulation of two homeostatic microglia genes, *ITGAM* and *CX3CR1*. These data are in line with data from a recent publication of our group[77]. In this study, meta-analysis showed no microglia density changes or an immune activated profile, instead they identified a schizophrenia related microglia profile characterized by the loss of mature microglia genes, among which *CX3CR1* and *ITGAM*. Downregulation of these genes in schizophrenia could have a profound effect on neuron microglia signaling at the synapse because of their function in complement and fractalkine signaling[78–80], and thereby contribute to symptoms observed in patients with schizophrenia.

Altogether, we did not find strong involvement of glia. However, our data, together with recent published literature, suggest it would be valuable if future research would pursue microglia homeostatic profile changes in schizophrenia.

Designing *in vivo* patient studies targeting synaptic changes and glia

To answer the question if (and how) astrocytes or microglia might be involved in synapse pathology in schizophrenia, we need to take the data from postmortem studies and study them from different perspectives. Opposed to postmortem studies, *in vivo* techniques such as MRI, fMRI, EEG, or DTI have the advantage that they can be applied in research

on patients with schizophrenia during specific phases of disease manifestation. However, these technologies do not provide the degree of resolution to study cell type specific changes. PET tracing studies have the potential to address this challenge, as it allows for specific protein labelling in the brain. PET ligands targeting synaptic density marker SV2A have recently been used to show synaptic terminal decrease in the frontal and cingulate cortex of patients with schizophrenia[81,82]. In a similar way tracers are developed to study glial involvement. However, currently these ligands are primarily targeted to study microglial and astrocytic activation[83]. Importantly, although TSPO labelling was previously used to study microglial activation in schizophrenia with PET, this is not recommended anymore because novel insights show that the protein is not specifically expressed in microglia[84]. The development of PET tracers targeting proteins involved in glia-synapse interaction could prove to be a valuable tool in elucidating glial involvement in synapse pathology in schizophrenia.

Gap between postmortem studies and preclinical models

There are multiple examples of studies using preclinical models (rodent or cell culture) to study schizophrenia related synaptic density changes and glial synaptic pruning. For example, the use of knock-out mice, excluding genes such as *CX3CR1*, to study the effect on spine density in the olfactory bulb[85]. Or iPSC derived microglia models studying phagocytic activity[86]. Especially the mouse retinogeniculate system has been employed to study astrocytic and microglial involvement in (aberrant) synapse elimination through complement signaling[87]. This model provides a great opportunity to study fundamental processes involved in synaptic pruning as there is a very specific time window when the excess retinal ganglion cell inputs to the dorsal lateral geniculate nucleus neurons are eliminated. However, it is evident that there is a gap between these models and the phenotypes observed in postmortem human brain tissue of schizophrenia patients. This is especially important in context of the brain region (and even cortical layer) specificity of the synapse phenotype in schizophrenia. It remains very difficult to design a model to tackle this issue.

The use of cerebral organoids could prove a valuable alternative as a model of the human cortex. Although brain organoids have not yet been utilized to investigate synaptic pruning, as they contain functional synapses, astrocytes, ánd microglia, they are expected to become a powerful tool for analysing the molecular mechanisms underlying glia-mediated synaptic pruning[89]. There are many possibilities to utilize the model as such, for example: introduce fluorescent reporters for glial cells and neurons for live cell imaging of interactions at the synapse, or use genetic manipulation using SiRNA's or CRISPR-Cas9 technology[90,91] to reduce the expression of genes of interest (such as *CX3CR1* and *ITGAM*).

7.3 Cerebral organoids to study early environmental schizophrenia risk factors

Epidemiological research has identified several prenatal environmental risk factors, increasing the risk of schizophrenia, such as maternal immune activation and altered nutrition during pregnancy [44,46,55,56,58,92,93]. Unravelling the biological mechanisms causative of this relation are of great importance for our understanding of the pathogenesis of schizophrenia. It remains challenging to study early brain development using human samples, and therefore mechanistic studies are performed using rodents. However, rodent and human brains differ from each other, not only in size and form, but also on a cellular level, such as the representation and organization of cell types during development and the transcriptomic profile of various cell types[94–98]. In chapter 5 and 6 we exploited the cerebral organoid model to study the effect of environmental risk factors for schizophrenia on early human brain development.

Applying the model

Cerebral organoids have been shown to resemble human cortical development on a transcriptomic, proteomic, and morphological level[99–102]. They provide the possibility to study human neurodevelopment *in vitro* in alive cells, in a co-culture environment comprising of different cell types similar as the *in vivo* circumstances. Previous studies have employed the cerebral organoid model to study the effects of both genetic and environmental factors on neurodevelopment[103–108]. Therefore, we considered the cerebral organoid model a tool to study the effects of environmental risk factors for schizophrenia on neurodevelopment.

The studies from chapter 5 and 6 show that cerebral organoids can be used to study environmental risk factors for schizophrenia during early human brain development. Our data show that cerebral organoids are responsive to the environmental stimulations applied in our studies (LPS stimulation and specific amino acid exposure). We were able to study neurodevelopment at multiple levels using this model: protein expression, gene expression, and general organoid growth. However, in both studies it remained difficult to pinpoint specific neurobiological processes affected based on these data. Perhaps because our approach on 'neurodevelopment' was broad and general. More specific follow up analyses will be valuable. These could include investigation of specific cell types, their composition, and organization, using immunostaining of the progenitor regions and single cell RNA sequencing (example from our own data in Figure 2). The model provides many more possibilities for indepth analyses which were not (yet) explored in these projects, including functional studies, such as live-cell imaging, calcium imaging, and electrophysiology. Furthermore, in relation to environmental manipulations, studying epigenetic differences (through ATAC sequencing[109]) would be an interesting avenue to pursue, as a possible link between environment and gene expression in the cerebral organoid.

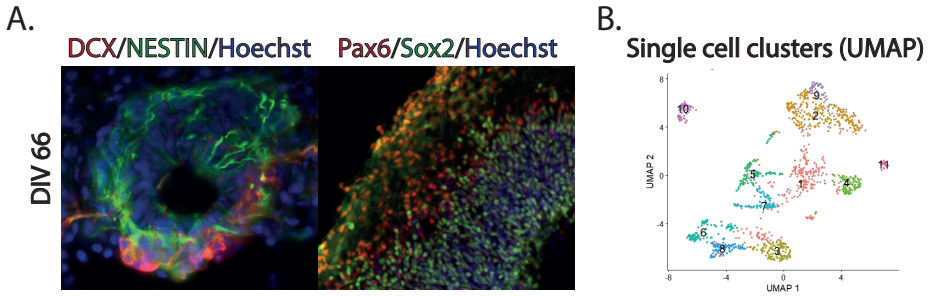


Figure 2. Cell type specific analyses using immunohistochemistry and single cell RNA-seq.
A. Example of immunohistochemistry of various proteins in progenitor regions of the cerebral organoid, enabling the investigation of cellular organization. B. Colour coded cell type clusters by UMAP of a single cell RNA sequencing experiment with LPS stimulated and control cerebral organoids, which allows the study of cell type specific transcriptomic changes.

Altogether, we successfully used cerebral organoids to study the effect of environmental risk factors for schizophrenia on early neurodevelopment, and the cerebral organoid offers great potential for more in-depth analyses of neurodevelopment on different levels. Therefore, the cerebral organoid model is a promising model to study neurodevelopmental effects of environmental schizophrenia risk factors.

Physiological validity of the stimuli – a critical evaluation

One of the most important points of critique on our stimulation models is the physiological validity of the used stimuli. In chapter 5 we used lipopolysaccharide to evoke an immune response in the cerebral organoid to model maternal immune activation. LPS is present in the outer membranes of bacteria and is very potent in eliciting an immune response in human and animal cells[110–113]. LPS is commonly used in rodent studies of maternal immune activation[110,111]. In our study, we exposed LPS directly to the cerebral organoids. In physiological situations, maternal immune activation in humans is more often the result from a viral infection and bacterial infections are not often directly exposed to the developing foetus[114]. However, literature on maternal immune activation suggest that not necessarily the pathogen, but the inflammation that follows is detrimental[112]. LPS causes a broad response in our cerebral organoid model (increased gene expression of cytokines *IL6*, *TNFa*, and *IL1b*), emphasizing the validity of the use of LPS to study the effects of immune activation in our study. Furthermore, other brain organoid studies used exposure to single cytokines such as *TNFa* or *IL6,* identifying neurodevelopmental deficits related to FGFR1 dysfunction and altered neuron-astrocyte ratio respectively[103,115]. However, in the context of maternal immune activation, we suggest that a broader immune response is more physiologically relevant, as a maternal infection causes a broad spectrum of cytokines to increase, which could influence the developing brain[116,117].

In chapter 6 we exposed cerebral organoids to an increased concentration of the amino acids threonine, histidine, and lysine. The physiological levels of these amino acids in human blood are approximately 100-300µmol/L[118] and are present in culture media at approximately 0.2-0.9mM. In our study, we drastically increased the concentration of these amino acids above these physiological conditions. However, the concentrations we applied, are in a similar range as previous *in vitro* studies targeting mTOR using the same amino acids[119,120]. We showed that mTOR was acutely downregulated when organoids were exposed to 50mM and not to 10mM of the amino acids. However, this might be influenced by the sensitivity of the assay (western blot) as we also identified size defects and transcriptional changes when the organoids were exposed long term to the lower amino acids concentration of 10mM. Lastly, in the introduction of this thesis we stated that prenatal nutrition is linked to schizophrenia risk. The most well-known example is that maternal famine is related to a two-fold increase in schizophrenia incidence in the offspring[60]. Our model does not simulate maternal famine but does model how nutritional components can influence early brain development.

In both studies, we argue it is necessary to use the 'environmental stimuli' in a strong way to 'evoke the phenotype' and to be able to measure any effects amidst the prominent heterogeneity present in the cerebral organoid model (discussed further in section 7.4). Therefore, even though our approach using cerebral organoids provides a novel and elegant way of studying these types of questions, we must emphasize that it remains a model and that the conditions in this model are still far from the physiological situation. Our studies from chapter 5 and 6 can therefore be considered as proof-of-concept studies and provide leads for future research on which neurodevelopmental mechanisms should be further studied in relation to environmental schizophrenia risk factors.

Perspective of the biological effects

Maternal immune activation immune resilience

In chapter 5 we showed that acute effects of maternal immune activation were replicated in the cerebral organoid model, although we did not detect long term immune activation or neuronal deficits.

Despite the relatively high prevalence of maternal infections, only a small portion of the exposed offspring develops long lasting neurodevelopmental disorders[121]. This emphasizes that there is a certain resilience to maternal immune activation. There are many factors that affect maternal immune activation susceptibility, such as maternal stress, and resilience, such as high maternal iron, zinc, and vitamin D status[121]. The intensity of the infection is also a key determinant for neurodevelopmental effects. Therefore, in future experiments we should experiment with the stimuli intensities exposed to cerebral organoids.

Glial cells, microglia and astrocytes, are the first responders to environmental stimuli and upon activation they secrete cytokines, which was replicated in our model. The decrease of *TNFa* and *IL1b* in aged LPS-stimulated organoids suggests an altered immune phenotype. This adaptation of immune cells upon LPS stimulation could be related to the phenomenon 'immune tolerance'[122,123]. Previously it has been described that exposure to low doses of LPS can lead to a lower inflammatory response to a second stimulation[123]. This persistent state of immunological tolerance could be attributed to long term disfunction of microglia and astrocytes. An altered phenotype of glia could impact neuronal development and function at different stages, as they do not fulfil their appropriate function. The glia might respond differently upon a new inflammatory stimulus (similarly as described in Clark et al. 2018[124]) or might not perform their homeostatic functions at the synapse appropriately. Further dissemination of the glial phenotype though transcriptional profiling of (fluorescent or magnetically) sorted microglia and astrocyte populations will provide further insight into this matter.

Amino acid mediated mTOR inhibition effects on cortical growth

In our study of chapter 6 we confirmed that mTOR was downregulated upon exposure to specific amino acids, affecting neurodevelopment. We hypothesize that alterations in the maternal diet could affect the risk of psychiatric disorders in the offspring through inhibition of mTOR, causing neurodevelopmental effects. In this context, a particular interesting cell-population to investigate are the outer radial glia. These cells are proliferative progenitors involved in expansion and growth of the cortex, are human specific, and have high mTOR expression[98,125]. Therefore, this specific population in the human cerebral organoids could be extra vulnerable for a decrease in mTOR activation, resulting in growth deficits. To study this, a BrdU assay, tracking proliferating cells, and HOPX/PAX6 immuno-staining, targeting the outer radial glia population will enable to investigate the effect on proliferation in outer radial glia in cortical progenitor regions of the cerebral organoid.

Previously, increased mTOR activation has been associated with neurodevelopmental disorders such as tuberous sclerosis and autism spectrum disorder[126–129]. Interestingly, a recent study in brain organoids with a genetic risk factor for autism showed a drastic increase in organoid size, opposite to our model[130]. Dietary interventions, using the three amino acids from our study, could potentially be used to downregulate mTOR in these diseases.

mTOR as a common regulator in schizophrenia?

Interestingly, mTOR inhibition is related to schizophrenia pathology in multiple ways. Firstly, mTOR is involved in biological processes associated with schizophrenia pathology, such as synaptic signaling[131]. Furthermore, maternal immune activation (well-known as a risk factor for schizophrenia) induced by both Poly(I:C) and LPS in rodents has been found

to decrease mTOR activation in offspring[132,133]. And lastly, recent postmortem brain studies have shown that mTOR activity is downregulated in the prefrontal cortex of schizophrenia patients[134,135]. Altogether, this suggests that mTOR has a role as a common regulator in schizophrenia pathology.

Connection between maternal diet and maternal inflammation

Maternal diet and maternal inflammation are connected to each other. Dietary components, such as the type of carbohydrate intake, a ketogenic diet, and high fat, have been associated with anti- or pro- inflammatory effects and microglial phenotype, also in relation to neuropsychiatric disorders[93,136–138]. In rodent studies, a high fat-diet was associated with microglia mediated connectivity changes, affecting microglia morphology and phagocytic activity, and causing reduced prefrontal spine density[138–140]. Furthermore, poly(I:C)-mediated maternal immune activation was found to inhibit mTOR activity in rat placenta, affecting membrane localization of amino acid transporters and elevation of maternal cytokines can alter the placental function, reducing the transport of essential nutrients[133,141]. Therefore, it would be of great interest to integrate the two models from chapter 5 and 6 and further explore how maternal diet can impact neurodevelopment through inflammatory effects in our cerebral organoid model.

Combination with genetic background

An important hypothesis for the etiology of schizophrenia is the gene x environment hypothesis, stating that genetic risk factors render certain individuals more susceptible for environmental risk factors leading to disease manifestations. In our studies we assessed the effects of environmental risk factors on cerebral organoids generated from non-psychiatric control donors. It is possible that the absence of the genetic susceptibility the effect of the LPS stimulation or amino acid exposure is not as strong or detrimental. Because cerebral organoids are generated from human blood or skin cells reprogrammed to iPSC, it enables the opportunity to use patient derived cells. Therefore, incorporating a context of genetic susceptibility for schizophrenia in our environmental exposure models would be of interest. The polygenetic background of schizophrenia could be addressed in this paradigm particularly by using cells from specific patient populations. Firstly, using cells from patients with a high schizophrenia polygenic risk score. Schizophrenia polygenic risk scores represent the weighted effect of many single-nucleotide polymorphisms that are more prevalent in schizophrenia patients compared to healthy controls and can explain up to 18% of disease incidence[142]. A second approach could be using cells from individuals with a 22q11.2 deletion syndrome. This 1.5-3 million base pair deletion increases the risk of developing schizophrenia up to 25 times, and thereby poses the largest genetic risk factor for developing schizophrenia[143,144]. In a pilot study, we generated cerebral organoids from iPSC from patients with 22q11.2 deletion syndrome previously generated by Lin et al., (2016)[145]. Although the hallmarks of healthy organoid

development were met, such as the presence of cortical progenitor regions and PAX6 and FOXG1 expression, these organoids indeed showed lower expression of genes which are present in the deleted genetic region, thereby confirming the validity of this approach (unpublished). Future studies could combine both genetic and environmental risk factors for developing schizophrenia using cerebral organoids, to study the synergistic effect.

Reducing animal research

Advanced human cell models like the cerebral organoid have the potential to increase the translation between preclinical studies and patient care for complex brain diseases, such as schizophrenia. However, another important aspect from this technology is that it offers significant potential to reduce the number of animal studies, by replacing rodents. As illustrated by our research in chapter 5 and 6 cerebral organoid technology provides complementary approaches for animal research in neuroscience. The technology provides even further possibilities for the reduction of animals in pharmaceutical drug screening processes. Current legislation and public opinion increasingly push the field to reduce the use of animals in neuroscience. One of our studies (chapter 6) was funded by a grant specifically encouraging innovations that contribute to science without the use of animal studies (ZonMw Meer Kennis met Minder Dieren grant). Furthermore, there is a growing interest to use the cerebral organoid technology for the benefit of other species, such as the study of neuro-infectious diseases in domestic animals[146].

It is fair to point out that cerebral organoids are not an alternative that could completely replace the use of animals in neuroscience. Cerebral organoids are isolated structures, lack interorgan communication, do not contain a blood brain barrier or peripheral immune cells that might infiltrate, are deprived of sensory input (except for their response to light[147]), and cannot be used for behavioural and cognitive measures[148]. Furthermore, the development of cerebral organoids is also dependent on several animal derived components, such as sera and Matrigel. Therefore, for now, cerebral organoids provide a complementary approach to animal research, reducing the number of experimental animals in neuroscience, thereby impacting animal welfare.

7.4 Methodological considerations

For this thesis we used two models to study neurodevelopmental phenotypes in schizophrenia: postmortem human brain tissue and cerebral organoids. It is inherent to research that each model has benefits and challenges. We here discuss the methodological considerations for both models, which can aid the interpretation and dissemination of our results.

Postmortem brain tissue

In part one of this thesis, experimental work was performed using postmortem brain tissue. Postmortem brain tissue has long been the "golden standard" for understanding biological mechanisms of neuropsychiatric disorders. It provides the most direct strategy to study molecular and cellular underpinnings of disorders of the brain.

Heterogeneity and covariates: The clinical heterogeneity of schizophrenia as a disease is undisputed. Patients differ in symptomatology, severity of the symptoms, and responsiveness to treatment. These factors can also change over time within the same patient. Further increase of the heterogeneity between patients, is due to the large number of confounding factors that must be considered using postmortem human brain tissue. Besides technical factors affecting tissue quality, such as processing of samples by brain banks, pH and PMD of the tissue, clinical characteristics, such as substance abuse, antipsychotic medication, and psychiatric comorbidity are known to affect brain physiological processes[68,149,150] .

Tissue availability and patient subtype overrepresentation: Although brain banks world-wide collect brain tissue, the availability of tissue from schizophrenia patients remains limited. To illustrate: during the past 5 years, the Netherlands Brain Bank included 5 schizophrenia donors. This results in multiple studies using samples from the same patient cohort, (as we also observed in the meta-analysis of chapter 1) and therefore could lead to an overrepresentation of specific patient populations. The Netherlands brain bank program uses ante-mortem donor registration, and we suggest that this could interest a specific patient population, such as those without addiction and with higher education.

Endpoint: The result of early neurodevelopmental alterations can lead to persistent neurological changes, which can be observed in postmortem brain tissue. However, using postmortem brain tissue, we only see a snapshot of the pathology in schizophrenia, often in aged individuals. This model does not provide the opportunity to study dynamic processes at multiple disease stages.

Cerebral organoids

In part two of this thesis, cerebral organoids were utilized to answer our questions about potential environmental risk factors for schizophrenia during early brain development. This novel technique has gained a lot of interest and is promising to study neurodevelopmental mechanisms in alive cells.

Heterogeneity: One of the main challenges with cerebral organoids, is the variability of the model. The heterogeneity starts on the level of iPSC culture, as cell lines can differ from each other concerning various experimental properties, such as their differentiation potential or proliferation rate[151,152]. From our experience, there is also some variation in how robustly each iPSC line generates neuroectoderm during early phases of cerebral organoid differentiation. Furthermore, organoid development is subject to batch effects, as organoids grown in the same bioreactor are more alike, compared to organoids from a different batch[147]. Importantly, the model developed by Lancaster in 2013 relies on self-assembling of the organoid after neuroectoderm induction and does not use any factors to inhibit mesoderm and endoderm formation (such as dual SMAD inhibition), which are used in other brain organoid models[153–156]. This approach increases organoid to organoid variability[147]. Although some of these other models are more reproducible, they do not contain microglia, which are present in the cerebral organoid model[157].

Necrotic core: Cerebral organoids are not vascularized. Therefore, when the organoid grows bigger than approximately 5µm, the core will be depleted of fresh nutrients and oxygen because of diffusion limitations. This leads to necrosis in the core of the organoid[158]. It is not yet fully understood how this necrotic core affects the surrounding tissues.

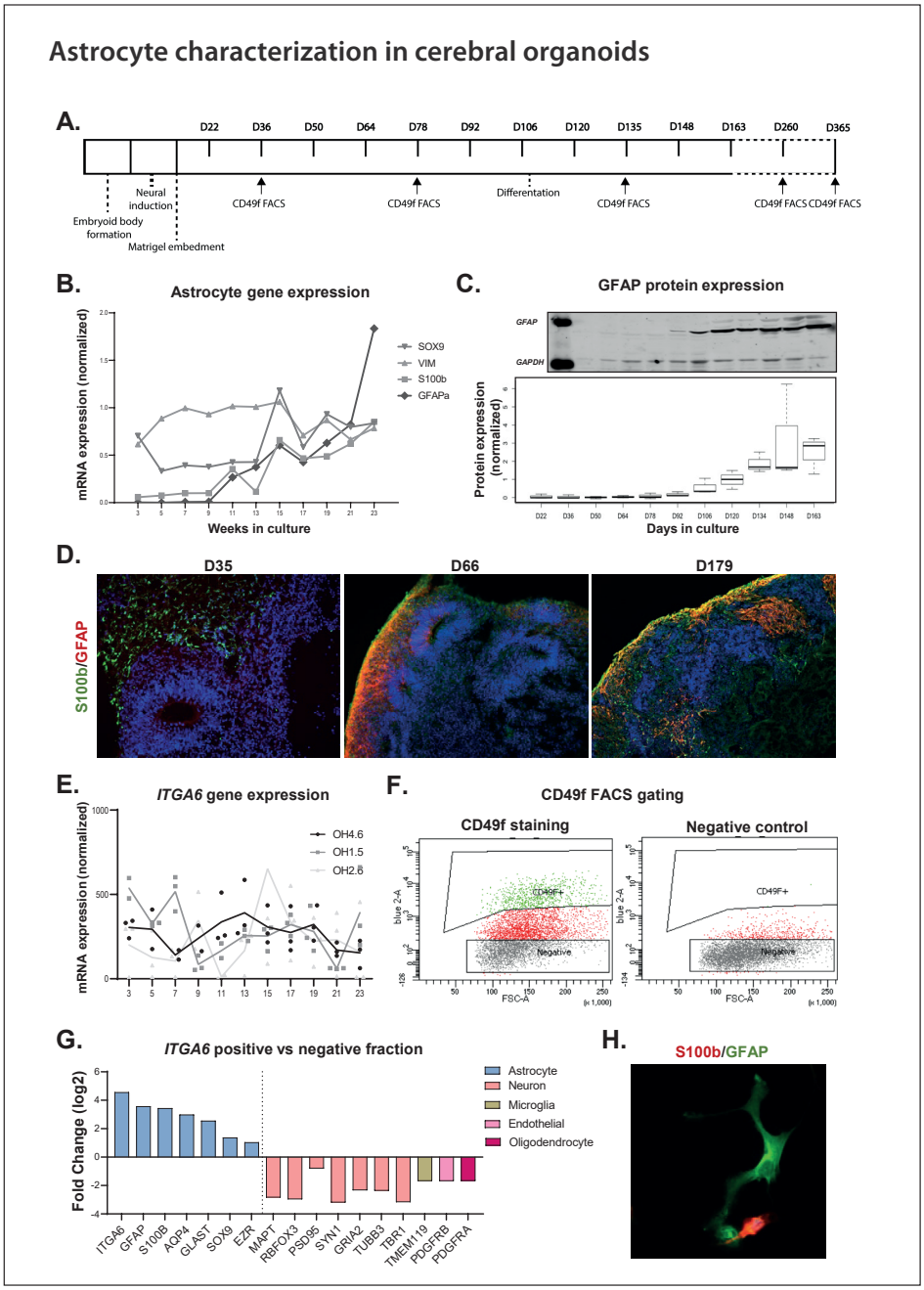
Early human development: As described in the introduction of this thesis, the cerebral organoid model accurately resembles the development of the human brain until about the end of the second trimester[101]. Therefore, the model is less suited to study more mature brain processes. Because schizophrenia often manifests around adolescence, certain phenotypes might not be as well represented.

Astrocytes are not well defined: Neuronal and microglia development, morphology, and function have been characterized within the cerebral organoid model[99,157,159]. However, such characterization has not been performed for astrocytes, besides the expression of GFAP[159]. Therefore, we started thoroughly investigating transcriptomic, proteomic, and morphological development of cerebral organoids within this model (unpublished). A preview of our findings is shown in BOX 1. In short, we made cerebral organoids from four control iPSC lines, and developed an extensive timeline, obtaining RNA, protein,

and samples for staining, from developmental day 30 to 165. Furthermore, we set up a fluorescent activated cell sorting protocol, using the previously identified cell-surface marker CD49f[160], to successfully sort astrocytes from cerebral organoids at different developmental ages up to 365 days. Also, we utilized iDISCO technology[161] to stain astrocytes and visualize them in transparent 3D organoids, enabling reconstruction of the spatial distribution of these cells. Further exploration of this sample set, and these data will give us insight in astrocyte maturation, and their association to other cell types in the cerebral organoid model.

Time and resources: The entire process of generation of iPSC from somatic cells, iPSC quality control, and cerebral organoid generation takes up to six months. Furthermore, the resources necessary for iPSC and organoid culture exceed what is available in an average cell-culture facility and are labour intensive. Therefore, it is of utmost importance to consider if some research questions can be better answered using simpler models (regular cell-lines, 2D cultures of differentiated iPSC, or co-culture systems).

Ethics: The technology surrounding brain organoids is rapidly developing, as it offers possibilities to study the "human brain" as never before and has the potential to contribute to personalized medicine in brain diseases. However, the development of "brains in a dish" also raises ethical questions. Not only regarding the moral status of the cerebral organoids, but also about how we use them[162]. Although some research has been done to define the ethical considerations related to organoid use in general[163,164], only few studies have focussed on brain organoids specifically. Because the brain is more related to our identity than any other organ, the ethical considerations are even more sensitive. In collaboration with us, master students from the Vrije Universiteit Amsterdam have performed a pilot study to explore the perspectives of laypeople on ethical considerations using cerebral organoid technology (BOX 2).

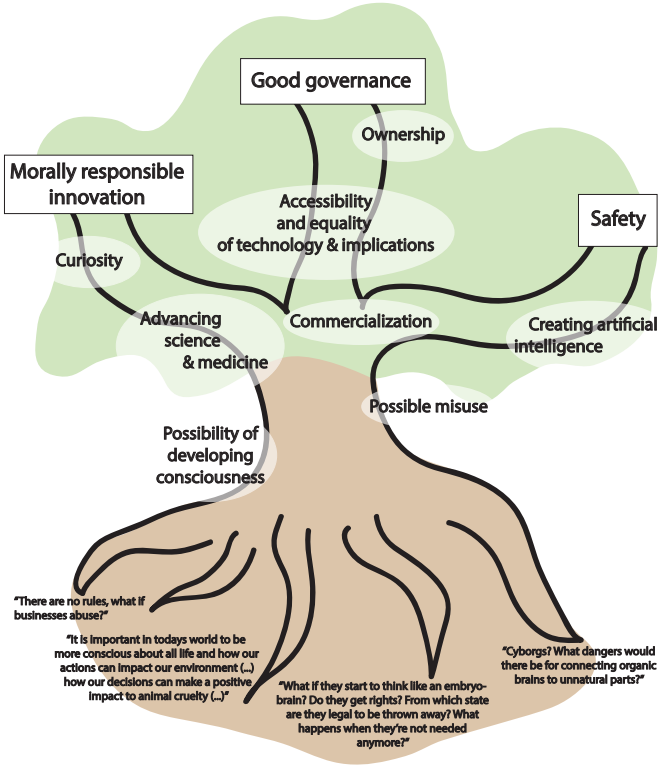


BOX 1. Astrocyte characterization in cerebral organoids

A. Organoids were harvested for RNA, protein, IHC, and FACS at the indicated timepoints. B. Gene expression over time for 4 general astrocyte genes in whole organoid (qPCR). C. Protein expression of GFAP over time in whole organoid (western blot). D. Images of S100b and GFAP at 3 different timepoints. E. CD49f gene expression (*ITGA6)* over time in whole organoid is consistent. F. Gating example for CD49f FACS at day 35. G. RNA sequencing shows the *ITGA6* positive fraction is enriched for astrocytic genes. H. CD49f FACS cells express GFAP and S100b when cultured in 2D. Project in collaboration with M. Verkerke (design/experiments/analysis) and V. Donega (RNA sequencing).

Ethical considerations regarding cerebral organoid use

During the course "science in Dialogue" master students at the Vrije Universiteit designed and executed a dialogue among laypeople (11 participants) to identify opinions and values regarding brain organoid technology, to evaluate ethical challenges in the field. For the dialogue the "World Café" format was employed, in which participants work on three assignments in consecutive rounds in small groups[165]. First, participants were asked to make a selection from multiple statements about brain organoid technology that resonated most with them. Secondly, they assigned values or themes to these statements, and thirdly prioritized and selected the ones most important to them. The overall structure of the dialogue was maintained by adding post-it's on top of a drawing of a tree. The tree does not only provide a playful design, but also gives participants the possibility to work on a concrete product that allows for reflection[166]. Bringing together the results of the multiple dialogues, we dissected three themes that were addressed most: morally responsible innovation, good governance, and safety.



BOX 2. Results from World Café dialogue on ethical considerations regarding cerebral organoids
The image represents a preliminary dissemination of the dialogue data. With some quotes from participants about their selected statements in the roots of the tree, values and themes identified on the trunk and branches, coming together to the central themes at the end of each branch. (Dialogue was designed and performed by Paul Bethlehem, Felicia Roozendaal, Oliver Sandys, and Anouk Spruit).

7.5 Future perspectives

In addition to the specific suggestions for follow up research on our studies we have mentioned in this discussion, we also want to draft a future perspectives for the field at large. As stated in the introduction of this thesis, there is a dire need for translational approaches in schizophrenia research, connecting epidemiological, and genetic findings, to specific molecular and cellular mechanisms in the brain. From our perspective, future studies in the field should focus on robust replication, taking a novel approach towards disease categorization, emphasize brain region/tissue-type/cell type specificity, and use state of the art *in vitro* models to make the translation from genes to function.

Replication-Replication-Replication

Currently, studies designed for the soul purpose of replication of previous findings, are not often performed. For example: although the decrease of synapses in the cortex is considered a robust phenotype in schizophrenia postmortem tissue, there are few studies that focus on replicating results in the same brain region, using the same technique, in a different cohort. Replication studies are even less common among brain organoid studies. For example, the effect of maternal immune activation on neurodevelopment has been explored using different brain organoid models studying multiple different exposures such as TNFa, IL6, or ZIKA virus[103,104,167], all identifying different biological effects. It is of utmost importance for the field that replication of previous studies is encouraged to define if specific molecular/cellular findings are robust.

Symptom dimensions of schizophrenia

In postmortem schizophrenia research, future directions often include the obvious arguments of enlarging cohorts and perform more detailed screening of clinical and technical variables to illuminate confounding factors. However, we might want to more radically change the way we perform this type of research. The heterogeneity within schizophrenia as a disease, and the differential response to treatment among patients raises the question if schizophrenia might constitute of multiple different underlying pathologies, which result in a generally common phenotype. Furthermore, psychiatric disorders are classified as separate diseases by a specific set of behaviours, although research shows that symptoms, genetics, and environmental risk factors overlap[70,168,169]. Therefore, going forward, it would be interesting to include multiple psychiatric disorders, and perform the research based on symptomatology and disease polygenic risk score, instead of the DSMIV disease categorisation alone.

Specificity is key

An important take away from the research performed within this thesis, is that brain region/tissue-type/cell type specificity is important. This is illustrated by the results from our meta-analysis in chapter 2, the differences in grey and white matter methylation in chapter 4, and by the need for further dissection of the phenotypes in chapter 5 and 6. A novel approach to obtain resolution on a cell type specific level is single-cell RNA sequencing[170]. This technology employs cell sorting to isolate individual cells, and asses their transcriptome. This allows for the dissemination of gene expression data on a cell type, even cell-state specific level. We have recently employed this technique to further dissect cell type specific effect of our environmental stimulations on the cerebral organoids (unpublished, Figure 2). Although single cell atlases from healthy control donors have shown before that schizophrenia risk genes (identified by GWAS) are enriched in pyramidal neurons, and specific types of inhibitory neurons[171], a recent publication performed the first single-cell transcriptomic atlas of schizophrenia prefrontal cortex specifically[172]. Schizophrenia related transcriptional alterations were highly enriched in deep layer cortico-cortical projection neurons and PV-expressing inhibitory neurons. Schizophrenia associated transcriptional alterations identified in this study are regulated by a set of upstream transcription factors affecting both early fetal brain development and adult brain synaptic processes. These data provide a promising link between the schizophrenia phenotype and the phases of neurodevelopment discussed in this thesis.

From genes to function – need for advanced *in vitro* models

Large scale GWAS and RNA-sequencing studies give tremendous insight in which genes could be involved in schizophrenia. *In vitro* culture systems from human cells pave the way to take these data and translate them to determine specific alterations that are resulting from these risk genes. With an *in vitro* model there is an opportunity to microscopically study molecular and cellular processes in a controllable context, in living cells. Especially more complex culture systems containing multiple cell types (co-cultures or organoids) offer the possibility to study cell(type) interactions. Other strengths of *in vitro* work include the possibility for gene manipulation and the use of patient cells. Previous studies have identified many schizophrenia risk genes that are involved in neurodevelopment[69,172–174]. Advanced *in vitro* culture systems can help to decipher the molecular and cellular effects of dysfunction of these genes and *how* this affects neurodevelopment.

7.6 Concluding remarks

This thesis contributes to the research unravelling specific cellular and molecular neurodevelopmental processes affected in schizophrenia by exploring different research angles, covering both postnatal neurodevelopment and prenatal environmental risk factors, focussing on tissue specificity and glial cells, using postmortem human brain tissue and cerebral organoids. Research from these different perspectives has resulted in the identification of several specific phenotypes. Throughout this thesis it has been shown that many challenges are faced regarding clinical and experimental factors involved in this type of research, and much work remains for the dissemination of the biological mechanisms behind these phenotypes. Future research, continuing to investigate these questions, will contribute to our understanding of the disease and the development of novel treatment options, to benefit patients with schizophrenia.

References

1. Schijven, D. *et al.* Comprehensive pathway analyses of schizophrenia risk loci point to dysfunctional postsynaptic signaling. *Schizophrenia Research* (2018) doi:10.1016/j.schres.2018.03.032.
2. Soler, J. *et al.* Genetic variability in scaffolding proteins and risk for schizophrenia and autism-spectrum disorders: a systematic review. *Journal of psychiatry & neuroscience : JPN* **43**, 223–244 (2018).
3. Shelton, M. A. *et al.* Loss of Microtubule-Associated Protein 2 Immunoreactivity Linked to Dendritic Spine Loss in Schizophrenia. *Biological psychiatry* **78**, 374–85 (2015).
4. Glausier, J. R. & Lewis, D. A. Dendritic spine pathology in schizophrenia. *Neuroscience* **251**, 90–107 (2013).
5. Coley, A. A. & Gao, W.-J. PSD95: A synaptic protein implicated in schizophrenia or autism? *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **82**, 187–194 (2018).
6. Harrison, PaulJ. The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. *Psychopharmacology* **174**, 151–162 (2004).
7. Moyer, C. E., Shelton, M. A. & Sweet, R. A. Dendritic spine alterations in schizophrenia. *Neuroscience letters* **601**, 46–53 (2015).
8. Fornito, A., Yücel, M., Dean, B., Wood, S. J. & Pantelis, C. Anatomical abnormalities of the anterior cingulate cortex in schizophrenia: bridging the gap between neuroimaging and neuropathology. *Schizophrenia bulletin* **35**, 973–93 (2009).
9. Parker, E. M. & Sweet, R. A. Stereological Assessments of Neuronal Pathology in Auditory Cortex in Schizophrenia. *Frontiers in neuroanatomy* **11**, 131 (2017).
10. Osimo, E. F., Beck, K., Reis Marques, T. & Howes, O. D. Synaptic loss in schizophrenia: a meta-analysis and systematic review of synaptic protein and mRNA measures. *Molecular psychiatry* (2018) doi:10.1038/s41380-018-0041-5.
11. Ripke, S. *et al.* Biological insights from 108 schizophrenia-associated genetic loci. *Nature* **511**, 421–427 (2014).
12. Stevens, B. *et al.* The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell* **131**, 1164–1178 (2007).
13. Kamitaki, N. *et al.* Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature* **582**, 577–581 (2020).
14. Sekar, A. *et al.* Schizophrenia risk from complex variation of complement component 4. *Nature* **530**, 177–83 (2016).
15. Sellgren, C. M. *et al.* Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nature Neuroscience* **22**, 374–385 (2019).
16. Parkin, G. M., Udawela, M., Gibbons, A. & Dean, B. Glutamate transporters, EAAT1 and EAAT2, are potentially important in the pathophysiology and treatment of schizophrenia and affective disorders. *World Journal of Psychiatry* **8**, 51–63 (2018).
17. O'Donovan, S. M., Sullivan, C. R. & McCullumsmith, R. E. The role of glutamate transporters in the pathophysiology of neuropsychiatric disorders. *npj Schizophrenia* **3**, 32 (2017).
18. McCullumsmith, R. E. *et al.* Cell-specific abnormalities of glutamate transporters in schizophrenia: Sick astrocytes and compensating relay neurons? *Molecular Psychiatry* **21**, 823–830 (2016).
19. Windrem, M. S. *et al.* Human Glial Progenitor Cells Effectively Remyelinate the Demyelinated Adult Brain. *Cell reports* **31**, 107658 (2020).

20. Feinberg, I. Schizophrenia: Caused by a fault in programmed synaptic elimination during adolescence? *Journal of Psychiatric Research* **17**, 319–334 (1982).

21. van Eijk, K. R. *et al. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects*. *BMC Genomics* vol. 13 636- (2012).

22. Rajarajan, P. & Akbarian, S. Use of the epigenetic toolbox to contextualize common variants associated with schizophrenia risk. *Dialogues in Clinical Neuroscience* **21**, 407–416 (2019).

23. Boks, M. P. *et al.* The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* **4**, 6767 (2009).

24. Kofink, D., Boks, M. P. M., Timmers, H. T. M. & Kas, M. J. Epigenetic dynamics in psychiatric disorders: Environmental programming of neurodevelopmental processes. *Neuroscience and Biobehavioral Reviews* vol. 37 831–845 (2013).

25. Labrie, V., Pai, S. & Petronis, A. Epigenetics of major psychosis: Progress, problems and perspectives. *Trends in Genetics* vol. 28 427–435 (2012).

26. Pidsley, R. *et al.* Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia. *Genome Biology* **15**, 483 (2014).

27. Dempster, E., Viana, J., Pidsley, R. & Mill, J. Epigenetic studies of schizophrenia: Progress, predicaments, and promises for the future. *Schizophrenia Bulletin* **39**, 11–16 (2013).

28. Rutten, B. P. F. & Mill, J. Epigenetic mediation of environmental influences in major psychotic disorders. *Schizophrenia Bulletin* vol. 35 1045–1056 (2009).

29. Scobie, K. N. *et al.* Krüppel-like factor 9 is necessary for late-phase neuronal maturation in the developing dentate gyrus and during adult hippocampal neurogenesis. *Journal of Neuroscience* **29**, 9875–9887 (2009).

30. Apara, A. *et al.* KLF9 and JNK3 interact to suppress axon regeneration in the adult CNS. *Journal of Neuroscience* **37**, 9632–9644 (2017).

31. Trakhtenberg, E. F. *et al.* Zinc chelation and Klf9 knockdown cooperatively promote axon regeneration after optic nerve injury. *Experimental Neurology* **300**, 22–29 (2018).

32. Avci, H. X. *et al.* Thyroid hormone triggers the developmental loss of axonal regenerative capacity via thyroid hormone receptor α1 and krüppel-like factor 9 in Purkinje cells. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 14206–14211 (2012).

33. Dugas, J. C., Ibrahim, A. & Barres, B. A. The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. *Molecular and Cellular Neuroscience* **50**, 45–57 (2012).

34. Fleming, M. D., Campagna, D. R., Haslett, J. N., Trenor, C. C. & Andrews, N. C. A mutation in a mitochondrial transmembrane protein is responsible for the pleiotropic hematological and skeletal phenotype of flexed-tail (f/f) mice. *Genes and Development* **15**, 652–657 (2001).

35. Kory, N. *et al.* SFXN1 is a mitochondrial serine transporter required for one-carbon metabolism. *Science* **362**, (2018).

36. Park, C. & Park, S. K. Molecular links between mitochondrial dysfunctions and schizophrenia. *Molecules and Cells* vol. 33 105–110 (2012).

37. Rajasekaran, A., Venkatasubramanian, G., Berk, M. & Debnath, M. Mitochondrial dysfunction in schizophrenia: Pathways, mechanisms and implications. *Neuroscience and Biobehavioral Reviews* vol. 48 10–21 (2015).

38. Flippo, K. H. & Strack, S. An emerging role for mitochondrial dynamics in schizophrenia. *Schizophrenia Research* vol. 187 26–32 (2017).

39. Ullrich, M. *et al.* OCD-like behavior is caused by dysfunction of thalamo-amygdala circuits and upregulated TrkB/ERK-MAPK signaling as a result of SPRED2 deficiency. *Molecular Psychiatry* **23**, 444–458 (2018).

40. Engelhardt, C. M. *et al.* Expression and subcellular localization of Spred proteins in mouse and human tissues. *Histochemistry and Cell Biology* **122**, 527–538 (2004).

41. Lim, F. T., Ogawa, S. & Parhar, I. S. Spred-2 expression is associated with neural repair of injured adult zebrafish brain. *Journal of Chemical Neuroanatomy* **77**, 176–186 (2016).

42. Kawauchi, T. *et al.* Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron* **67**, 588–602 (2010).

43. Wucherpfennig, T., Wilsch-Bräuninger, M. & González-Gaitán, M. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *Journal of Cell Biology* **161**, 609–624 (2003).

44. Canetta, S. *et al.* Elevated maternal C-reactive protein and increased risk of schizophrenia in a national birth cohort. *American Journal of Psychiatry* **171**, 960–968 (2014).

45. Brown, A. S. & Conway, F. Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Frontiers in Psychiatry* vol. 10 430 (2019).

46. Debost, J. C. P. G. *et al.* Joint effects of exposure to prenatal infection and peripubertal psychological trauma in schizophrenia. *Schizophrenia Bulletin* **43**, 171–179 (2017).

47. Machado, C. J., Whitaker, A. M., Smith, S. E. P., Patterson, P. H. & Bauman, M. D. Maternal immune activation in nonhuman primates alters social attention in juvenile offspring. *Biological Psychiatry* **77**, 823–832 (2015).

48. Meyer, U. Prenatal Poly(I:C) exposure and other developmental immune activation models in rodent systems. *Biological Psychiatry* vol. 75 307–315 (2014).

49. Meyer, U., Feldon, J. & Fatemi, S. H. In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders. *Neuroscience and Biobehavioral Reviews* vol. 33 1061–1079 (2009).

50. Schwartzer, J. J. *et al.* Behavioral impact of maternal allergic-asthma in two genetically distinct mouse strains. *Brain, Behavior, and Immunity* **63**, 99–107 (2017).

51. Malkova, N. v., Yu, C. Z., Hsiao, E. Y., Moore, M. J. & Patterson, P. H. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, Behavior, and Immunity* **26**, 607–616 (2012).

52. Bauman, M. D. *et al.* Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological Psychiatry* vol. 75 332–341 (2014).

53. Brown, A. S. & Conway, F. Maternal immune activation and related factors in the risk of offspring psychiatric disorders. *Frontiers in Psychiatry* vol. 10 430 (2019).

54. Bergdolt, L. & Dunaevsky, A. Brain changes in a maternal immune activation model of neurodevelopmental brain disorders. *Progress in Neurobiology* vol. 175 1–19 (2019).

55. de Rooij, S. R., Wouters, H., Yonker, J. E., Painter, R. C. & Roseboom, T. J. Prenatal undernutrition and cognitive function in late adulthood. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 16881–16886 (2010).

56. Hoek, H. W., Brown, A. S. & Susser, E. The Dutch Famine and schizophrenia spectrum disorders. in *Social Psychiatry and Psychiatric Epidemiology* vol. 33 373–379 (Soc Psychiatry Psychiatr Epidemiol, 1998).

57. Kang, Y. *et al.* Nutritional Deficiency in Early Life Facilitates Aging-Associated Cognitive Decline. *Current Alzheimer Research* **14**, (2017).

58. Li, M., Francis, E., Hinkle, S. N., Ajjarapu, A. S. & Zhang, C. Preconception and prenatal nutrition and neurodevelopmental disorders: A systematic review and meta-analysis. *Nutrients* vol. 11 (2019).

59. Peretti, S. *et al.* Diet: the keystone of autism spectrum disorder? *Nutritional Neuroscience* vol. 22 825–839 (2019).

60. St Clair, D. *et al.* Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. *Journal of the American Medical Association* **294**, 557–562 (2005).

61. Rocco, B. R., Oh, H., Shukla, R., Mechawar, N. & Sibille, E. Fluorescence-based cell-specific detection for laser-capture microdissection in human brain. *Scientific reports* **7**, (2017).

62. Nott, A., Schlachetzki, J. C. M., Fixsen, B. R. & Glass, C. K. Nuclei isolation of multiple brain cell types for omics interrogation. *Nature protocols* **16**, 1629–1646 (2021).

63. Garey, L. J. *et al.* Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *Journal of Neurology, Neurosurgery & Psychiatry* **65**, 446–453 (1998).

64. Glantz, L. A. & Lewis, D. A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry* **57**, 65–73 (2000).

65. Konopaske, G. T., Lange, N., Coyle, J. T. & Benes, F. M. Prefrontal cortical dendritic spine pathology in schizophrenia and bipolar disorder. *JAMA psychiatry* **71**, 1323–31 (2014).

66. Zhang, L., Verwer, R. W. H., Lucassen, P. J., Huitinga, I. & Swaab, D. F. Prefrontal cortex alterations in glia gene expression in schizophrenia with and without suicide. *Journal of Psychiatric Research* **121**, 31–38 (2020).

67. Garbett, K., Gal-Chis, R., Gaszner, G., Lewis, D. A. & Mirnics, K. Transcriptome alterations in the prefrontal cortex of subjects with schizophrenia who committed suicide. *Neuropsychopharmacologia Hungarica : a Magyar Pszichofarmakologiai Egyesulet lapja = official journal of the Hungarian Association of Psychopharmacology* **10**, 9–14 (2008).

68. Lewis, D. A. The Human Brain Revisited: Opportunities and Challenges in Postmortem Studies of Psychiatric Disorders. *Neuropsychopharmacology 2002 26:2* **26**, 143–154 (2002).

69. Gandal, M. J. *et al.* Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science (New York, N.Y.)* **362**, eaat8127 (2018).

70. Gandal, M. J. *et al.* Shared molecular neuropathology across major psychiatric disorders parallels polygenic overlap. *Science (New York, N.Y.)* **359**, 693–697 (2018).

71. Patel, Y. *et al.* Virtual Histology of Cortical Thickness and Shared Neurobiology in 6 Psychiatric Disorders. *JAMA psychiatry* **78**, 47–63 (2021).

72. van Kesteren, C. F. M. G. *et al.* Immune involvement in the pathogenesis of schizophrenia: A meta-analysis on postmortem brain studies. *Translational Psychiatry* **7**, (2017).

73. Trépanier, M. O., Hopperton, K. E., Mizrahi, R., Mechawar, N. & Bazinet, R. P. Postmortem evidence of cerebral inflammation in schizophrenia: a systematic review. *Molecular Psychiatry* **21**, 1009–1026 (2016).

74. Cotter, D. *et al.* Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cerebral Cortex* vol. 12 386–394 (2002).

75. Radewicz, K., Garey, L. J., Gentleman, S. M. & Reynolds, R. Increase in HLA-DR immunoreactive microglia in frontal and temporal cortex of chronic schizophrenics. *Journal of neuropathology and experimental neurology* **59**, 137–50 (2000).

76. Rajkowska, G. *et al.* Layer-specific reductions in GFAP-reactive astroglia in the dorsolateral prefrontal cortex in schizophrenia. *Schizophrenia Research* **57**, 127–138 (2002).

77. Snijders, G. J. L. J. *et al.* A loss of mature microglial markers without immune activation in schizophrenia. *Glia* **69**, 1251–1267 (2021).

78. Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* **333**, 1456–1458 (2011).

79. Arnoux, I. & Audinat, E. Fractalkine Signaling and Microglia Functions in the Developing Brain. (2015) doi:10.1155/2015/689404.

80. Stephan, A. H., Barres, B. A. & Stevens, B. The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease. *Annual Review of Neuroscience* **35**, 369–389 (2012).

81. Onwordi, E. C. *et al.* Synaptic density marker SV2A is reduced in schizophrenia patients and unaffected by antipsychotics in rats. *Nature communications* **11**, (2020).

82. Cai, Z., Li, S., Matuskey, D., Nabulsi, N. & Huang, Y. PET imaging of synaptic density: A new tool for investigation of neuropsychiatric diseases. *Neuroscience letters* **691**, 44–50 (2019).

83. Cavaliere, C. *et al.* Gliosis and Neurodegenerative Diseases: The Role of PET and MR Imaging. *Frontiers in cellular neuroscience* **14**, (2020).

84. Sneeboer, M. A. M. *et al.* Microglial activation in schizophrenia: Is translocator 18 kDa protein (TSPO) the right marker? *Schizophrenia research* **215**, 167–172 (2020).

85. Reshef, R. *et al.* The role of microglia and their CX3CR1 signaling in adult neurogenesis in the olfactory bulb. *eLife* **6**, (2017).

86. Murai, N., Mitalipova, M. & Jaenisch, R. Functional analysis of CX3CR1 in human induced pluripotent stem (iPS) cell-derived microglia-like cells. *European Journal of Neuroscience* **52**, 3667–3678 (2020).

87. Schafer, D. P. *et al.* Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron* **74**, 691–705 (2012).

88. Hooks, B. M. & Chen, C. Distinct Roles for Spontaneous and Visual Activity in Remodeling of the Retinogeniculate Synapse. *Neuron* **52**, 281–291 (2006).

89. Matsui, T. K., Tsuru, Y. & Kuwako, K. Challenges in Modeling Human Neural Circuit Formation via Brain Organoid Technology. *Frontiers in Cellular Neuroscience* **0**, 418 (2020).

90. Torres-Ruiz, R. & Rodriguez-Perales, S. CRISPR-Cas9 technology: applications and human disease modelling. *Briefings in functional genomics* **16**, 4–12 (2017).

91. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nature Protocols 2013 8:11* **8**, 2281–2308 (2013).

92. Brown, A. S. & Derkits, E. J. Prenatal infection and schizophrenia: A review of epidemiologic and translational studies. *American Journal of Psychiatry* vol. 167 261–280 (2010).

93. Bordeleau, M., Fernández de Cossío, L., Chakravarty, M. M. & Tremblay, M.-È. From Maternal Diet to Neurodevelopmental Disorders: A Story of Neuroinflammation. *Frontiers in Cellular Neuroscience* **0**, 461 (2021).

94. Verkhratsky, A. & Nedergaard, M. The homeostatic astroglia emerges from evolutionary specialization of neural cells. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **371**, (2016).

95. Oberheim, N. A. *et al.* Uniquely hominid features of adult human astrocytes. *Journal of Neuroscience* **29**, 3276–3287 (2009).

96. Geirsdottir, L. *et al.* Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell* **179**, 1609-1622.e16 (2019).

97. Hodge, R. D. *et al.* Conserved cell types with divergent features in human versus mouse cortex. *Nature 2019 573:7772* **573**, 61–68 (2019).

98. Lui, J. H., Hansen, D. v. & Kriegstein, A. R. Development and evolution of the human neocortex. *Cell* vol. 146 18–36 (2011).

99. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013).

100. di Lullo, E. & Kriegstein, A. R. The use of brain organoids to investigate neural development and disease. *Nature Reviews Neuroscience* **18**, 573–584 (2017).

101. Kelava, I. & Lancaster, M. A. Dishing out mini-brains: Current progress and future prospects in brain organoid research. *Developmental Biology* vol. 420 199–209 (2016).

102. Luo, C. *et al.* Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. *Cell Reports* **17**, 3369–3384 (2016).

103. Benson, C. A. *et al.* Immune Factor, TNFα, Disrupts Human Brain Organoid Development Similar to Schizophrenia-Schizophrenia Increases Developmental Vulnerability to TNFα. *Frontiers in cellular neuroscience* **14**, 233 (2020).

104. Zuiki, M. *et al.* Luteolin attenuates interleukin-6-mediated astrogliosis in human iPSC-derived neural aggregates: A candidate preventive substance for maternal immune activation-induced abnormalities. *Neuroscience Letters* **653**, 296–301 (2017).

105. Kathuria, A. *et al.* Transcriptome analysis and functional characterization of cerebral organoids in bipolar disorder. *Genome Medicine 2020 12:1* **12**, 1–16 (2020).

106. Khan, T. A. *et al.* Neuronal defects in a human cellular model of 22q11.2 deletion syndrome. *Nature Medicine* **26**, 1888–1898 (2020).

107. Arzua, T. *et al.* Modeling alcohol-induced neurotoxicity using human induced pluripotent stem cell-derived three-dimensional cerebral organoids. *Translational Psychiatry* **10**, (2020).

108. Dang, J. *et al.* Glial cell diversity and methamphetamine-induced neuroinflammation in human cerebral organoids. *Molecular Psychiatry* (2020) doi:10.1038/s41380-020-0676-x.

109. Buenrostro, J. D., Wu, B., Chang, H. Y. & Greenleaf, W. J. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Current protocols in molecular biology* **109**, 21.29.1-21.29.9 (2015).

110. Harvey, L. & Boksa, P. Prenatal and postnatal animal models of immune activation: relevance to a range of neurodevelopmental disorders. *Developmental neurobiology* **72**, 1335–1348 (2012).

111. Boksa, P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain, behavior, and immunity* **24**, 881–897 (2010).

112. Knuesel, I. *et al.* Maternal immune activation and abnormal brain development across CNS disorders. *Nature reviews. Neurology* **10**, 643–660 (2014).

113. Arsenault, D., St-Amour, I., Cisbani, G., Rousseau, L. S. & Cicchetti, F. The different effects of LPS and poly I:C prenatal immune challenges on the behavior, development and inflammatory responses in pregnant mice and their offspring. *Brain, behavior, and immunity* **38**, 77–90 (2014).

114. Megli, C. J. & Coyne, C. B. Infections at the maternal–fetal interface: an overview of pathogenesis and defence. *Nature Reviews Microbiology 2021 20:2* **20**, 67–82 (2021).

115. Zuiki, M. *et al.* Luteolin attenuates interleukin-6-mediated astrogliosis in human iPSC-derived neural aggregates: A candidate preventive substance for maternal immune activation-induced abnormalities. *Neuroscience Letters* **653**, 296–301 (2017).

116. Pendyala, G. *et al.* Maternal Immune Activation Causes Behavioral Impairments and Altered Cerebellar Cytokine and Synaptic Protein Expression. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **42**, 1435–1446 (2017).

117. Corradini, I. *et al.* Maternal Immune Activation Delays Excitatory-to-Inhibitory Gamma-Aminobutyric Acid Switch in Offspring. *Biological psychiatry* **83**, 680–691 (2018).

118. Schmidt, J. A. *et al.* Plasma concentrations and intakes of amino acids in male meat-eaters, fish-eaters, vegetarians and vegans: a cross-sectional analysis in the EPIC-Oxford cohort. *European Journal of Clinical Nutrition 2016 70:3* **70**, 306–312 (2015).

119. Wu, J. *et al.* Dietary interventions that reduce mTOR activity rescue autistic-like behavioral deficits in mice. *Brain, Behavior, and Immunity* **59**, 273–287 (2017).

120. Prizant, R. L. & Barash, I. Negative effects of the amino acids Lys, His, and Thr on S6K1 phosphorylation in mammary epithelial cells. *Journal of Cellular Biochemistry* **105**, 1038–1047 (2008).

121. Meyer, U. Neurodevelopmental Resilience and Susceptibility to Maternal Immune Activation. *Trends in Neurosciences* **42**, 793–806 (2019).

122. Netea, M. G. *et al.* Defining trained immunity and its role in health and disease. *Nature reviews. Immunology* **20**, 375 (2020).

123. Novakovic, B. *et al.* β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. *Cell* **167**, 1354-1368.e14 (2016).

124. Clark, S. M. *et al.* Maternal immune activation in rats blunts brain cytokine and kynurenine pathway responses to a second immune challenge in early adulthood. *Progress in neuro-psychopharmacology & biological psychiatry* **89**, 286–294 (2019).

125. Pollen, A. A. *et al.* Molecular Identity of Human Outer Radial Glia during Cortical Development. *Cell* **163**, 55–67 (2015).

126. Bockaert, J. & Marin, P. mTOR in Brain Physiology and Pathologies. *Physiological Reviews* **95**, 1157–1187 (2015).

127. Curatolo, P., Moavero, R. & de Vries, P. J. Neurological and neuropsychiatric aspects of tuberous sclerosis complex. *The Lancet Neurology* vol. 14 733–745 (2015).

128. Winden, K. D., Ebrahimi-Fakhari, D. & Sahin, M. Abnormal mTOR Activation in Autism. *Annual review of neuroscience* **41**, 1–23 (2018).

129. Zimmer, T. S. *et al.* Tuberous Sclerosis Complex as Disease Model for Investigating mTOR-Related Gliopathy During Epileptogenesis. *Frontiers in Neurology* **0**, 1028 (2020).

130. de Jong, J. O. *et al.* Cortical overgrowth in a preclinical forebrain organoid model of CNTNAP2-associated autism spectrum disorder. *Nature Communications 2021 12:1* **12**, 1–14 (2021).

131. McCabe, M. P. *et al.* Genetic inactivation of mTORC1 or mTORC2 in neurons reveals distinct functions in glutamatergic synaptic transmission. *eLife* **9**, (2020).

132. Lombardo, M. v *et al.* Maternal immune activation dysregulation of the fetal brain transcriptome and relevance to the pathophysiology of autism spectrum disorder. *Molecular psychiatry* **23**, 1001–1013 (2018).

133. McColl, E. R. & Piquette-Miller, M. Viral model of maternal immune activation alters placental AMPK and mTORC1 signaling in rats. *Placenta* **112**, 36–44 (2021).

134. Chadha, R., Alganem, K., Mccullumsmith, R. E. & Meador-Woodruff, J. H. mTOR kinase activity disrupts a phosphorylation signaling network in schizophrenia brain. *Molecular psychiatry* (2021) doi:10.1038/S41380-021-01135-9.

135. Ibarra-Lecue, I. *et al.* Ribosomal Protein S6 Hypofunction in Postmortem Human Brain Links mTORC1-Dependent Signaling and Schizophrenia. *Frontiers in Pharmacology* **0**, 344 (2020).

136. Buyken, A. E. *et al.* Association between carbohydrate quality and inflammatory markers: systematic review of observational and interventional studies. *The American Journal of Clinical Nutrition* **99**, 813–833 (2014).

137. Ludwig, D. S., Willett, W. C., Volek, J. S. & Neuhouser, M. L. Dietary fat: From foe to friend? *Science* **362**, 764–770 (2018).

138. Vuong, B. *et al.* Exposure to gestational diabetes mellitus induces neuroinflammation, derangement of hippocampal neurons, and cognitive changes in rat offspring. *Journal of Neuroinflammation* **14**, 80 (2017).

139. Bocarsly, M. E. *et al.* Obesity diminishes synaptic markers, alters microglial morphology, and impairs cognitive function. *Proceedings of the National Academy of Sciences* **112**, 15731–15736 (2015).

140. Hao, S., Dey, A., Yu, X. & Stranahan, A. M. Dietary obesity reversibly induces synaptic stripping by microglia and impairs hippocampal plasticity. *Brain, Behavior, and Immunity* **51**, 230–239 (2016).

141. Woods, R. M. *et al.* Maternal immune activation in rodent models: A systematic review of neurodevelopmental changes in gene expression and epigenetic modulation in the offspring brain. *Neuroscience & Biobehavioral Reviews* **129**, 389–421 (2021).

142. Purcell, S. *et al.* A polygenic burden of rare disruptive mutations in schizophrenia. *Nature* **506**, 185–190 (2014).

143. Bassett, A. S., Chow, E. W. & Weksberg, R. Chromosomal abnormalities and schizophrenia. *American journal of medical genetics* **97**, 45–51 (2000).

144. Hodgkinson, K. A., Murphy, J., O'Neill, S., Brzustowicz, L. & Bassett, A. S. Genetic counselling for schizophrenia in the era of molecular genetics. *Canadian journal of psychiatry. Revue canadienne de psychiatrie* **46**, 123–30 (2001).

145. M, L. *et al.* Integrative transcriptome network analysis of iPSC-derived neurons from schizophrenia and schizoaffective disorder patients with 22q11.2 deletion. *BMC systems biology* **10**, 105 (2016).

146. Pain, B., Baquerre, C. & Coulpier, M. Cerebral organoids and their potential for studies of brain diseases in domestic animals. *Veterinary Research* **52**, 65 (2021).

147. Quadrato, G. *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* **545**, 48–53 (2017).

148. Kim, J., Koo, B.-K. & Knoblich, J. A. Human organoids: model systems for human biology and medicine. *Nature Reviews Molecular Cell Biology* **21**, 571–584 (2020).

149. Ferrer, I., Martinez, A., Boluda, S., Parchi, P. & Barrachina, M. Brain banks: benefits, limitations and cautions concerning the use of post-mortem brain tissue for molecular studies. *Cell and tissue banking* **9**, 181–194 (2008).

150. Schmitz, P. Information technology for brain banking. *Handbook of clinical neurology* **150**, 157–165 (2018).

151. Carcamo-Orive, I. *et al.* Analysis of Transcriptional Variability in a Large Human iPSC Library Reveals Genetic and Non-genetic Determinants of Heterogeneity. *Cell Stem Cell* **20**, 518-532.e9 (2017).

152. Hoekstra, S. D., Stringer, S., Heine, V. M. & Posthuma, D. Genetically-Informed Patient Selection for iPSC Studies of Complex Diseases May Aid in Reducing Cellular Heterogeneity. *Frontiers in cellular neuroscience* **11**, (2017).

153. Sloan, S. A. *et al.* Human Astrocyte Maturation Captured in 3D Cerebral Cortical Spheroids Derived from Pluripotent Stem Cells. *Neuron* **95**, 779-790.e6 (2017).

154. Krencik, R. *et al.* Systematic Three-Dimensional Coculture Rapidly Recapitulates Interactions between Human Neurons and Astrocytes. *Stem cell reports* **9**, 1745–1753 (2017).

155. Kadoshima, T. *et al.* Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 20284–20289 (2013).

156. Yamashita, S. *et al.* Mislocalization of syntaxin-1 and impaired neurite growth observed in a human iPSC model for STXBP1-related epileptic encephalopathy. *Epilepsia* **57**, e81–e86 (2016).

157. Ormel, P. R. *et al.* Microglia innately develop within cerebral organoids. *Nature Communications* **9**, (2018).

158. Rothenbücher, T. S. P. *et al.* Next generation human brain models: engineered flat brain organoids featuring gyrification. *Biofabrication* **13**, 011001 (2021).

159. Renner, M. *et al.* Self-organized developmental patterning and differentiation in cerebral organoids. *The EMBO Journal* **36**, 1316–1329 (2017).

160. Barbar, L. *et al.* CD49f Is a Novel Marker of Functional and Reactive Human iPSC-Derived Astrocytes. *Neuron* **107**, 436-453.e12 (2020).

161. Renier, N. *et al.* iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell* **159**, 896–910 (2014).

162. Haselager, D. R. *et al.* Breeding brains? Patients' and laymen's perspectives on cerebral organoids. *https://doi.org/10.2217/rme-2020-0108* **15**, 2351–2360 (2021).

163. Boers, S. N. & Bredenoord, A. L. Consent for governance in the ethical use of organoids. *Nature cell biology* **20**, 642–645 (2018).

164. Boers, S. N., Delden, J. J., Clevers, H. & Bredenoord, A. L. Organoid biobanking: identifying the ethics: Organoids revive old and raise new ethical challenges for basic research and therapeutic use. *EMBO reports* **17**, 938–941 (2016).

165. Fouché, C. & Light, G. An Invitation to Dialogue: 'The World Café' In Social Work Research. *http://dx.doi.org/10.1177/1473325010376016* **10**, 28–48 (2010).

166. van der Meij, M. G., Broerse, J. E. W. & Kupper, F. Conceptualizing playfulness for reflection processes in responsible research and innovation contexts: a narrative literature review. *JOURNAL OF RESPONSIBLE INNOVATION* **4**, 43–63 (2017).

167. Qian, X., Nguyen, H. N., Jacob, F., Song, H. & Ming, G. L. Using brain organoids to understand Zika virus-induced microcephaly. *Development (Cambridge, England)* **144**, 952 (2017).

168. Adam, D. Mental health: On the spectrum. *Nature* **496**, 416–418 (2013).

169. Guloksuz, S. & van Os, J. Renaming schizophrenia: 5 × 5. *Epidemiology and psychiatric sciences* **28**, 254–257 (2019).

170. Muraro, M. J. *et al.* A Single-Cell Transcriptome Atlas of the Human Pancreas. *Cell systems* **3**, 385-394.e3 (2016).

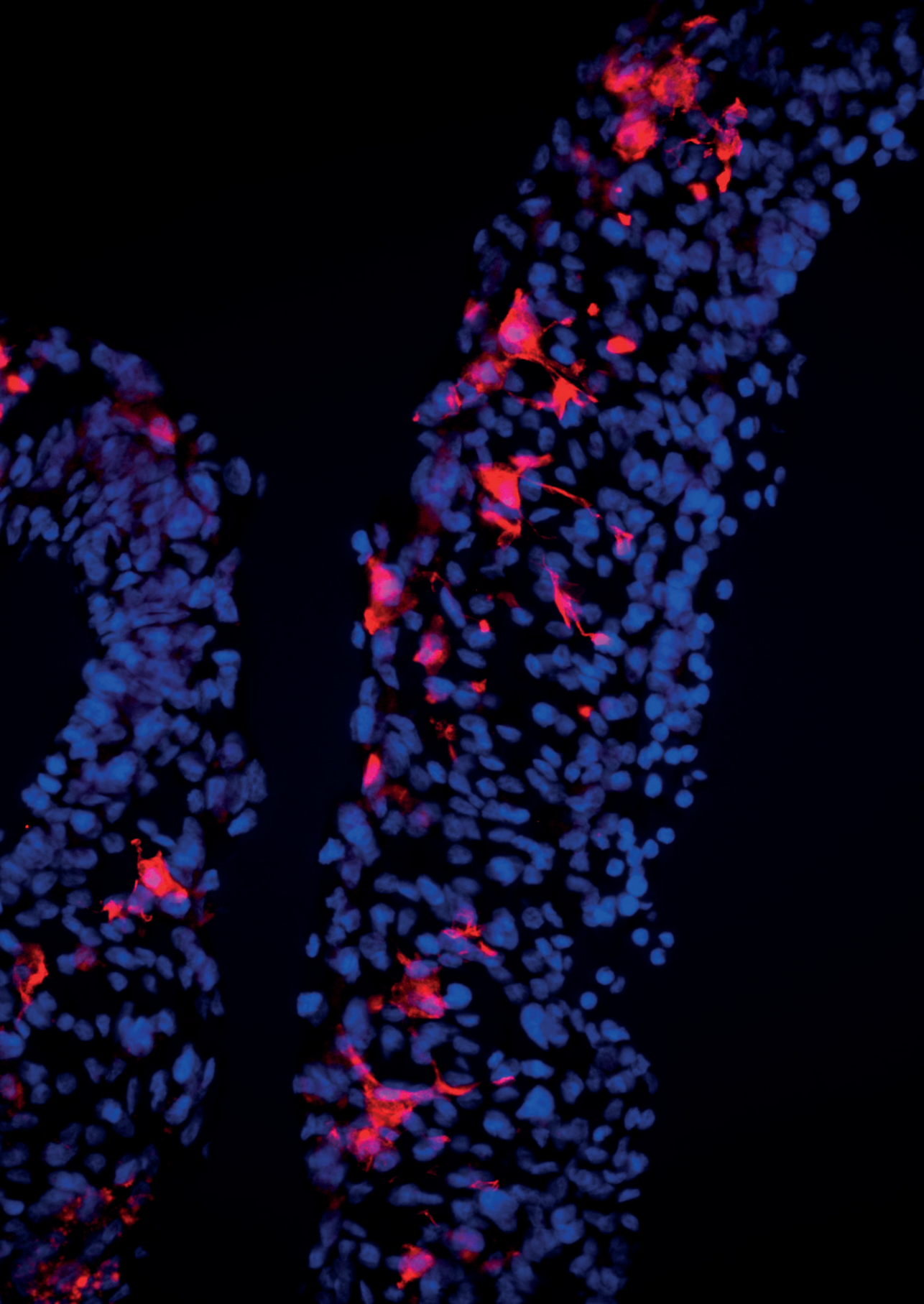
171. Skene, N. G. *et al.* Genetic identification of brain cell types underlying schizophrenia. *Nature genetics* **50**, 825–833 (2018).

172. Ruzicka, B. *et al.* Single-Cell Dissection of Schizophrenia Reveals Neurodevelopmental-Synaptic Link and Transcriptional Resilience Associated Cellular State. *Biological Psychiatry* **89**, S106 (2021).

173. Birnbaum, R. & Weinberger, D. R. Genetic insights into the neurodevelopmental origins of schizophrenia. *Nature Reviews Neuroscience* vol. 18 727–740 (2017).

174. Jaffe, A. E. *et al.* Mapping DNA methylation across development, genotype and schizophrenia in the human frontal cortex. *Nat.Neurosci.* **19**, 40–47 (2016).

7



Appendices

Nederlandse samenvatting

Schizofrenie is een psychiatrische ziekte met een grote morbiditeit en hoge mortaliteit. In de hersenen van patiënten met schizofrenie worden macroscopische en microscopische veranderingen gezien. Steeds meer onderzoek wijst er op dat de hersenontwikkeling betrokken is bij het ontstaan van schizofrenie. Genetische- en omgevingsfactoren beïnvloeden zowel prenatale als postnatale processen in het ontwikkelende brein, die leiden tot een verhoogd risico op het ontwikkelen van schizofrenie. Er is echter nog veel onduidelijk over welke specifieke cellulaire en moleculaire veranderingen hieraan ten grondslag liggen. Het is belangrijk om deze biologische processen te ontrafelen om het ziekteproces beter te begrijpen en nieuwe therapieën te ontwikkelen.

Daarom bestuderen wij in dit proefschrift specifieke moleculaire en cellulaire processen tijdens de hersenontwikkeling die een rol spelen bij schizofrenie. We bekijken de hersenontwikkeling vanuit verschillende perspectieven en maken gebruik van bekende maar ook vernieuwende translationele technieken.

Deel I is gericht op het onderzoeken van corticale veranderingen in schizofrenie patiënten met behulp van postmortem humaan hersen materiaal.

Tijdens gezonde hersenontwikkeling neemt het aantal synapsen in de kindertijd enorm toe. In de adolescentie verdwijnen synaptische verbindingen die minder gebruikt worden. Als in deze fase te veel of te weinig synapsen verdwijnen heeft dit effect op het functioneren van de hersenen. Al lange tijd wordt gesuggereerd dat een afname in het aantal synapsen in de cortex ten grondslag ligt aan veranderingen in de hersen connectiviteit in patiënten met schizofrenie. In **hoofdstuk 2** hebben wij literatuur met kwantitatieve uitkomstmaten van postsynaptische elementen systematisch samengevat en geanalyseerd in een meta-analyse. Hiervoor hebben wij gekeken naar studies in subcorticale en corticale hersengebieden in postmortem hersenweefsel van patiënten met schizofrenie en gezonde controles. Onze meta-analyse liet zien dat er regio specifieke veranderingen zijn in het aantal postsynaptische elementen in patiënten met schizofrenie. We vonden een vermindering van postsynaptische elementen in de prefrontale cortex en laag III van de cortex specifiek. Het valt op dat de heterogeniteit in de analyses groot is. Deze bevindingen laten zien dat verminderde synaptische densiteit een belangrijk, hersen regio specifiek, cellulair kenmerk is in de hersenen van patiënten met schizofrenie.

Eerder onderzoek suggereert dat microglia en astrocyten een rol hebben de veranderde synapsen bij schzofrenie. Microglia en astrocyten zijn gliacellen. Deze gliacellen zijn betrokken bij een grote verscheidenheid aan functies in de hersenen. Een belangrijke

rol voor microglia en astrocyten is het reguleren van de eliminatie van synapsen tijdens de adolescentie. Daarom zouden veranderingen in microglia en/of astrocyten kunnen bijdragen aan het verlaagde aantal synapsen in de cortex van patiënten met schizofrenie. In **hoofdstuk 3** hebben wij microglia, astrocyten, en synapsen tegelijkertijd onderzocht in postmortem hersenweefsel van patiënten met schizofrenie. In ons onderzoek hebben we hersen regio specificiteit mee genomen door ons te richten op de grijze stof van de frontale cortex, en specifiek op laag III. De immunohistochemische kleuringen uit onze studie lieten zien dat het aantal Spinophillin positieve synapsen, GFAP positieve astrocyten, en IBA1 positieve microglia niet is veranderd in laag III van de prefrontale cortex in ons schizofrenie cohort. Ook was er geen effect in gen of eiwit expressie van synaptische of astrocyt markers in de grijze stof van de prefrontale cortex. We vonden wel een verlaging van twee microglia genen (*CX3CR1* en *ITGAM*) die betrokken zijn bij microglia-synaps communicatie. Tegengesteld aan onze verwachting, ondanks een uitgebreide karakterisatie, vonden wij geen bewijs dat synapsen, of de associatie tussen gliacellen en synapsen veranderd is in patiënten met schizofrenie.

Wanneer we de meta-analyse uit hoofdstuk 2 updaten met onze eigen verkregen data uit hoofdstuk 3 vinden we nog steeds een significante verlaging van het aantal synapsen in laag III van de cortex in patiënten met schizofrenie. Redenen die kunnen verklaren waarom wij deze vorige bevindingen niet hebben kunnen repliceren zijn zowel technisch (het gebruik van verschillende assays voor synaps kwantificatie) als inhoudelijk (dat het fenotype wellicht gerelateerd is aan een specifiek subtype van patiënten met schizofrenie). De verlaging van de twee microglia genen *CX3CR1* en *ITGAM* is in lijn met een recente meta-analyse van onze groep, waarin staat beschreven dat hoewel het aantal microglia en het immuun profiel van de microglia onveranderd is, volwassen microglia genen zoals *CX3CR1* en *ITGAM* zijn verlaagd in patiënten met schizofrenie.

Epigenetische verschillen, zoals de mate van DNA methylatie, kunnen worden veroorzaakt door genetische verschillen of omgevingsfactoren die geassocieerd zijn met schizofrenie. Eerder onderzoek naar DNA methylatie is voornamelijk gericht op perifere weefsels zoals bloed, maar weefsel specifieke verschillen tussen grijze en witte stof zijn niet eerder bestudeerd. In **hoofdstuk 4** hebben wij DNA methylatie bestudeerd in grijze en witte stof van de frontale en temporale cortex van patiënten met schizofrenie in postmortem hersenweefsel. We identificeerde vier weefsel specifieke differentieel gemethyleerde regio's tussen schizofrenie patiënten en controle donoren in de buurt van de genen *KLF9*, *SFXN1*, *SPRED2*, en *ALS2CL*. Daarbij vonden we een verlaging van de genexpressie van *KLF9* en *SFXN1* in de grijze stof, en van *SPRED2* in de witte stof van de frontale cortex in patiënten met schizofrenie. Interessant genoeg zijn de genen *SFXN1*, *SPRED2* en *ALS2CL* betrokken bij hersenontwikkeling en synaptische functies.

Deel II is gericht op de effecten van potentiële prenatale risicofactoren voor schizofrenie op de ontwikkeling van cerebrale organoïden.

In 2013 publiceerde Lancaster en haar collega's een revolutionair nieuw model voor het bestuderen van humane hersenontwikkeling in een petrischaaltje: de cerebrale organoïde. Dit zijn kleine 3D hersenstructuren (2 tot 5 mm groot) die een grote verscheidenheid van hersencellen laten zien en zich organiseren zoals in de humane ontwikkelende cortex tijdens de zwangerschap. Dit model zorgt ervoor dat we elementen van de hersenontwikkeling beter kunnen onderzoeken.

Maternale infectie tijdens de zwangerschap is een veel bestudeerde risico factor voor het ontwikkelen van psychiatrische aandoeningen, waaronder schizofrenie. In **hoofdstuk 5** hebben wij onderzocht wat de effecten zijn van prenatale immuun activatie op de hersenontwikkeling door cerebrale organoïden bloot te stellen aan lipopolysaccharide (wat voorkomt in de membraan van bacteriën). We vonden dat de cerebrale organoïden inderdaad acuut reageerden op de stimulus met verhoogde expressie van immuun gerelateerde gen expressie (*IL6*, *HLADR*, *IL1B*, *TNFa*) samen met een verhoogde eiwit expressie van microglia marker *IBA1* en astrocyt marker *GFAP*. Genexpressie van *EOMES* en *RELN*, belangrijke neuronale ontwikkelingsgenen, was ook verlaagd. Echter, een maand na de initiële stimulus vonden we, naast een verlaging van *TNFa* en *IL1B* genexpressie, geen blijvende effecten op de hersenontwikkeling. Dus hoewel de organoïden initieel reageerde met een immuun reactie op de stimulus, waarbij ook effecten in neuronen werden gezien, werden er geen chronische effecten gevonden.

Voeding tijdens de zwangerschap beïnvloedt hersenontwikkeling en het risico op verschillende neuropsychiatrische aandoeningen, zoals schizofrenie. Dieronderzoek heeft laten zien dat drie specifieke aminozuren invloed hebben op het eiwit mTOR, wat een belangrijke rol speelt in de groei en het metabolisme van cellen. Het verhogen van deze aminozuren in het dieet van 5 weken oude muizen had effect op het gedrag op lange termijn. In **hoofdstuk 6** hebben wij onderzocht of het verhogen van deze drie aminozuren (histidine, lysine, en threonine) invloed heeft op de ontwikkeling van humane cerebrale organoïden. We vonden dat suppletie van deze aminozuren ervoor zorgde dat mTOR sterk werd geïnhibeerd en de cerebrale organoïden kleiner bleven. RNA sequencing liet verschillen in genexpressie zien tussen de gestimuleerde en controle cerebrale organoïden die betrokken zijn bij processen als mTOR, proliferatie, en immuun functie.

Concluderend, in dit proefschrift hebben we corticale fenotypen in schizofrenie onderzocht die gerelateerd zijn aan het synaps afwijkingen en DNA methylatie in postmortem humaan hersenweefsel. Daarnaast, hebben we de effecten van twee

potentiële prenatale risicofactoren voor schizofrenie (maternale immuun activatie en voeding) onderzocht met behulp van cerebrale organoïden. Hierbij hebben we inderdaad specifieke moleculaire en cellulaire kenmerken geïdentificeerd die gerelateerd zijn aan hersenontwikkeling in schizofrenie. Echter zijn we ook tegenstrijdigheden en heterogeniteit tegengekomen die laten zien dat het van groot belang is dat bevindingen op een structurele manier worden gerepliceerd en verder uitgediept om de biologische grondslag van schizofrenie te ontrafelen, en daarmee de behandeling voor patiënten te verbeteren.

Dankwoord

Een groot aantal mensen hebben een onmisbare bijdrage geleverd aan de totstandkoming van dit proefschrift. Allereerst wil ik graag mijn promotieteam bedanken voor alle kansen, de supervisie, en de prettige samenwerking.

Elly, de afgelopen jaren ben jij een mentor voor mij geweest. Ondanks je drukke agenda was je altijd bereikbaar en voelde ik me gesteund. Soms liep ik even binnen voor een vraag, en stond ik opeens anderhalf uur later nog steeds in de deuropening te kletsen. Je wetenschappelijke input en onze discussies waren altijd heel waardevol. Bedankt dat je zo betrokken bent, en oog hebt voor de mensen in je groep, niet alleen op wetenschappelijk maar ook op sociaal vlak.

René, bedankt voor de kansen die je mij hebt geboden. Dankzij jou kon ik met dit promotietraject beginnen en mij ontwikkelen als onderzoeker. Mijn tijd in New York was een enorm inspirerende ervaring, bedankt hiervoor.

Lot, samen hebben we een hele zoektocht afgelegd, naar de richting van mijn PhD, maar ook naar het moleculaire schizofrenie onderzoek in het algemeen. Je bent een bron van creativiteit en ideeeën, wat ik altijd heel inspirerend vond tijdens onze brainstormsessies. Ik heb bewondering voor hoe jij uitdagingen aangaat, met lef op het niveau van wetenschappelijke inhoud én carriere gebied. Bedankt dat je me tijdens mijn PhD zoveel vrijheid hebt gegeven, maar dat ik ook bij je terecht kon voor sturing of als ik onzeker was.

Marco, na een jaar of twee was jij een aanwinst voor mijn promotieteam. Het viel me meteen op dat jij mensen verbindt. Je wist mij in onze meetings altijd te motiveren (en gerust te stellen als iets anders liep dan gedacht), en hielp mij met het stellen van prioriteiten. Dit alles altijd met een enorme positiviteit, bedankt daarvoor.

Jinte, hoewel geen "officiele" co-promotor, jouw supervisie en begeleiding van de afgelopen jaren zijn heel waardevol geweest voor mij. Bedankt voor het altijd stellen van kritische vragen, de hulp met praktische lab problemen, en je grote betrokkenheid op persoonlijk vlak. Je bent een voorbeeld op het gebied van doorzettingsvermogen, en ik ben ontzettend blij voor je dat je nu aan het hoofd staat van je eigen groep.

Ten tweede bedank ik graag de leden van de promotiecommissie. **Prof. dr. R. J. Pasterkamp**, **Prof. dr. W. Cahn**, **Dr. C. H. A. Nijboer**, **Prof. dr. A. D. Kraneveld**, en **Prof. dr. I. Huitinga**. Dank dat jullie de tijd hebben genomen om mijn proefschrift te lezen en te beoordelen.

Mijn lieve paranimfen **Jessy** en **Jantien**. **Jessy**, vanaf het eerste moment dat jij je aansloot bij het Hol lab wist ik dat wij het goed met elkaar konden vinden. Je zachtaardige karakter maakt je heel benaderbaar en ik voelde mij snel vertrouwd bij jou. Ik was altijd onder de indruk van hoe innovatief jij bent met je onderzoeksprojecten. Maar achter die wetenschapper schuilt ook een stel pretogen, en was je mijn partner in crime voor een spontane borrel, een intermediate fillament dansje, of een grap. Je bent ambiteus en daadkrachtig, dus ik weet zeker dat je het geweldig gaat doen daar bij de zeebravissen. **Jantien**, lieve emulgator. Wat fijn dat ik mij zo aan jou kan spiegelen, dat we aan een half woord genoeg hebben om elkaar te snappen. Je aanmoediging, afleiding, en onze wandeltjes waren onmisbaar de afgelopen jaren. Je bent op meerdere momenten echt een voorbeeld voor mij geweest, bijzonder wijs, energiek, en liefdevol. Wie had gedacht toen we samen in de soos stonden te dansen dat we paranimf zouden zijn op elkaars promoties? Bedankt dat jullie naast mij willen staan op dit bijzondere moment in mijn leven.

Toen ik begon met mijn PhD kwam ik in een warm bad terecht van een leuke, hechte groep promovendi van het psymuun team en het Hol lab. Hoewel ik het best spannend vond ontvingen jullie mij als nieuwkomer met open armen, dank daarvoor.

Allereerst de psymuun groep, **Paul**, ik leerde je kennen als begeleider van mijn master scriptie en eenmaal begonnen aan mijn PhD nam jij mij op sleeptouw in de wondere wereld van de organoids. We werden een goed team, jij als de doorpakker en ik weloverwogen. Met optimisme en enthousiasme ging jij ieder experiment tegemoet, wat aanstekelijk werkte. Je was de sfeermaker van de groep, altijd in voor een beetje compititie, maar ook heel zorgzaam. Ik heb enorm genoten van onze samenwerking, de uren zij aan zij pipeteren, onze discussies, je grappen en grollen, en krokret-vrijdagen. **Marjolein**, je ontving mij als kersvers PhD studentje met open armen, en ik leerde je al snel kennen als een gedreven, gedisciplineerde, en georganiseerde microglia fanatiekeling. Van je organisatietalent heb ik zelfs nog lang na je vertrek profijt gehad. Jij was degene die altijd een goed groepsgevoel wist te creëren. Bedankt dat ik altijd even op de bank kon komen ploffen om wat goede raad van je te krijgen. **Gijsje**, we hebben in verschilldende fases met elkaar samengewerkt. Uitzoeken hoe je een meta-analyse moet doen, met verschillende post-mortem projecten, en later ook in New York. Je wist me vaak te verrassen, met een ad remme opmerking, of dat je opeens een avond-opleiding tot schoonheidsspecialist deed. Je bent grappig en nuchter en het was heel fijn dat bij frustraties je maar een appje van mij verwijderd was. Daarnaast ben je een ware papermachine, en heb ik het geluk gehad dat ik er ook op een paar mocht staan. **Hans**, een rustige constante in onze groep, en altijd in voor een grapje. **Yujie**, thank you for your sparking personality, and the salsa dancing together.

Het team van Lot had ook een New Yorkse tak, waar ik zowel virtueel als "live" in New York mee heb samengewerkt. **Raphael**, our collaboration was invaluable for the last part of my PhD. Your contribution to chapter 6 was essential and I think we worked well together. I really enjoyed our long virtual meetings in which we coded "together". **Frederieke**, mijn organoid maatje in New York. Wat hebben we het leuk gehad tijdens onze samenwerking. Eén van de hoogtepunten was wel ons bezoek aan het organoid congres in Cold Spring Harbor, maar ook van onze classpass lesjes, naar Ethyl's, en de kerstboom bij Rockefeller heb ik genoten.

Maar al deze jaren heb ik mij altijd evenveel onderdeel gevoeld van het Hol lab. **Emma**, wat begon met heel wat treinreizen tussen Utrecht en Amsterdam groeide over de afgelopen jaren uit tot een fijne vriendschap. Bedankt voor alle goede gesprekken, je adviezen, en het samen lachen. Extra bijzonder dat we nu ook het jonge moederschap met elkaar kunnen delen tijdens onze wandelingetjes in het Flevopark. **Katherine**, samen met **Jessy** begonnen wij ongeveer tegelijkertijd aan ons promotietraject en als snel vormde wij een gezellige drie eenheid. Ik heb zo veel ontzag voor hoe snel jij wegwijs bent geworden op het lab, en allerlei uitdagende projecten wist op te zetten. Regelmatig zaten we samen in het humane kweek/organoid lab, en hoewel jij vaak een druk schema had, mocht ik altijd bij je kletsen of klagen. Maar daarnaast hebben we ook heel veel gelachen, vaak onder het genot van een colaatje light of een Broodje Ben. **Bart**, ik had bedacht hier een stukje code voor je op te schrijven, maar ik kan dat helemaal niet, dus daarom slechts in "gewone" woorden: ik vond het altijd zo gezellig als je even kwam buurten voor een gesprekje, of als we samen een biertje dronken en het over zin en onzin hadden. Dankzij jouw editing skills ligt de lat voor de PhD filmpjes een flink stuk hoger. **Tamar**, ik waardeer jouw lieve, rustige karakter enorm. Fijn dat je je vanuit het Amsterdamse bij ons aansloot voor het laatste deel van je PhD. **Sophietje**, je sprankelende aanwezigheid was ontzettend fijn in de groep. **Marloes**, terwijl ik je een beetje wegwijs maakte in het stamcel/organoid werk ontstond een fijne samenwerking. We zaten op dezelfde golflengte en hadden het ook nog eens heel gezellig. Ookal is ons gezamenlijke project tot 1 pagina gereduceerd in dit proefschrift, het is fijn om te weten dat het nu in jouw capabele handen ligt en ik kijk uit naar het eindresultaat. Bedankt dat je de afgelopen jaren mijn sparringspartner was, en voor je initiatief met het organiseren van sociale dingen binnen het Hol lab. **Christiaan**, ik moet mij inhouden om niet anderhalve pagina te wijden aan jou stukje, want dat verdien je zeker. Een rustige factor in de groep, met enorme humor. Jij lijkt je zaken altijd helemaal op orde te hebben, en produceerde bakken aan data, met mooie resultaten. Daar was ik altijd wel van onder de indruk. **Lianne**, sportief en positief. Voor mij was jij echt hét sociale organisatietalent van het Hol lab, want welk evenement heb jij niet georganiseerd? Met je sterke werk ethiek en goede planning lukte het jou om, zonder te klagen, enorme gedragsexperimenten te doen, dat vind ik heel bewonderenswaardig. **Vanessa** en **Saskia**, de postdocs van de groep, bedankt voor jullie altijd rake vragen bij meetings en het delen van jullie ervaring. **Isadora**, it was so great to get to know you

during your time in our lab. With your kindness and your amazing immunohistochemistry skills you were a great addition. **Claudia**, I always enjoyed when you talked about one of your many interests, such as space, plants, or axolotls. **Anna** en **Tiziana**, jullie vormen een dynamisch duo die als een frisse wind door het Hol lab waait. Heel veel plezier tijdens de rest van jullie PhD.

Er waren twee essensiele factoren die er samen voor zorgden dat het Hol lab georganiseerd bleef en een helpende hand boden bij experimenten. **Roland,** wat hebben we veel uren samen doorgebracht in de humane kweek of de FACS-faciliteit, waarin we altijd goede gesprekken hadden. Je behulpzaamheid en sociale karakter zorgde ervoor dat je een gangmaker was op de afdeling. Als ik iets kwijt was dan kon ik dat altijd wel van jouw bench stelen danzij de next-level labeling van al je lab spulletjes. **Jacqueline,** ik vond het altijd reuzefijn om met je samen te werken. Met je rustige uitstraling en de enorme bak aan kennis dacht je altijd in oplossingen voor problemen en vragen waarmee ik bij je aanklopte. Volgensmij heb jij ongeveer iedere PhD student van onze groep uit de brand geholpen met een belangrijk western-blot experiment in de laatste fase van zijn/haar PhD, en dat was bij mij niet anders, dankjewel.

Daarnaast wil ik ook graag alle studenten bedanken waarvan ik het genoegen heb gehad om de afgelopen jaren mee samen te werken, **Cita**, **Akinde**, **Laurie**, en **Naomi**. En speciaal de master stage studenten: **Dilara** met je zelfstandigheid, ijzeren discipline, en geweldige labskils, **Jesca**, met je enthousiasme, leergierigheid, en proactieve houding, en **Jeske** met je doorzettingsvermogen, flexibiliteit, en motivatie. Ik heb veel van jullie geleerd, en jullie hebben enorm bijgedragen aan de totstandkoming van dit proefschrift, dankjulliewel!

My dear roomies: **Ketharini**, and **Lill Eva**, thank you for creating a comfortable atmosphere for working, random discussions, celebrating and complaining. It was great to have our own personal ICT helpdesk, and although I was a bit wary of them, the axelotls were a source of great entertainment. Many times you pondered with me over some scientific problem, and raised my mood when I was feeling low. Thank you for being great roomies. Lieve **Janna**, wat heb ik altijd bewondering gehad voor je kracht en genoten van je humor. Je inspireerde me. Als ik kauwtjes zie vliegen, denk ik aan je. Maar plotseling bevond ik mijzelf temidden van "the boys room" samen met **Laurens** en **Ioannis** (I hope you guys will enjoy the office during the rest of your PhD) en met **Werner**, we hebben veel over organoids gepraat, waarbij jij mij nog het een en ander wist te leren, je enorme gedrevenheid en positiviteit gaan je vast ver brengen.

Then, I would like to continue to **the department of Translational Neuroscience**. It was such a great environment to work, with so many smart and inspiring people. I really appreciate all the adventures we had together, scientific ones, organizing and attending

seminars together, sportive ones, such as the Amsterdam City Swim, and fun ones, lunches in the sun, lab-outings, and random borrels. **Peter**, het grootste gedeelte van mijn PhD stond jij aan het hoofd van de afdeling, bedankt voor de fijne atmosfeer die je creëerde. Grote dank gaat ook uit naar huidige en vroegere leden van het managementtteam en secretariaat **Joke**, **Remi**, **Roger**, **Krista**, **Vicki**, **Rianne**, **Ria**, en **Sandra**. Wat fijn dat jullie altijd klaarstaan met wijze raad. **Youri**, **Keith**, **Marina**, **Leo**, **Desiree**, **Jos**, **Erwin**, bedankt voor jullie hulp met lab-gerelateerde vragen en voor de gezellige praatjes. A special thanks to the members of the **borrel committee** for organizing some great borrels together! A great deal of time during my PhD was spend at the iPSC/organoid lab. Medium changes or endless Matrigel embedment's were made that much more enjoyable by some great, helpful colleagues: **Paul, Marloes, Lill Eva**, **Svetlana**, **Marta**, **Pavol**, **Astrid**, **Tijana**, **Rianne**, **Lauri**, and **Katherine**. Thank you for sharing in the highs and for being there during the lows inherent to this type of work. I want to especially thank you **Renata** for teaching me so much about stem cell and organoid culture, and always providing a listening ear to my research (struggles) and beyond. **Daniëlle V**, ik zou niet weten hoe het iPSC lab het zou redden zonder jou aan het roer, (hoe deden we dat eigenlijk voordat jij kwam?) bedankt! Also, to all other PI's, PhD students, Postdocs and supporting staff of the department, **Jeroen**, **Roger**, **Frank**, **Onur**, **Geert**, **Andreia**, **Anna**, **Danai**, **Daniëlle**, **Dick**, **Diviya**, **Emma**, **Eljo**, **Evelien**, **Ilia**, **Jacques**, **Jelle**, **Jiska**, **Karlijn**, **Lieke**, **Louisa**, **Mark B**, **Mark**, **Mieneke**, **Mateja**, **Marieke**, **Nefeli**, **Nicky**, **Özge**, **Paul H**, **Ramona**, **Rogier**, **Suzanne**, **Valeria**, **Vamshi**, and **Veronne** thank you for being there the past years and for making them so memorable.

Zo blijkt maar weer, onderzoek doe je nooit alleen. Zo zijn er nog een aantal mensen die ik graag wil bedanken voor hun hulp, input, en tijd: **Domenico** van de afdelng Pathologie van het UMC Utrecht, **Mauro** en **Judith** van Single Cell Discoveries, en **Michiel** van de Nederlanse Hersenbank.

Stephanie, **Pauline**, en **Monique**, bedankt voor de interessante samenwerking op het HIV organoid project.

Ricardo en **Kasper**, bedankt voor alles wat jullie mij hebben geleerd in het lab, maar bovenal voor het laten zien hoe leuk de wetenschap is en het vertrouwen wat jullie mij hebben meegegeven.

Dan mijn lieve vrienden, die onopgemerkt misschien wel een even belangrijke rol hebben gespeeld in de voltooing van dit boekje, maar in ieder geval in mijn levensvreugde.

Te beginnen met m'n lieve **Evie**, wat waardeer ik onze bijzondere vriendschap. Ik kan altijd bij jou terrecht, je geeft de beste knuffels, we delen creatieve uitspattingen, en

zijn een heel goed team. Dansend gaan we door onze vriendschap, op de bar, lowlands, onder de sterren, of in je huiskamer, en ik hoop nog heel veel dansjes met je te maken. Dankje dat jij er bent.

Lieve PM meisjes, en speciaal **Jantien**, **Evie**, **Marie, Anna**, **Anouk**, **Babeth**, **Hester**, **Judith**, **Letty**, **Leylah**, **Lisa**, **Lorijne**, **Maxime**, **Saskia**, **Sophie**, en **Suzanne**, jullie zijn het Roze kasteel waar ik naartoe kan ontstnappen om even helemaal op te laden. We kennen elkaar ondertussen al een poosje, en ik koester al onze mooie momenten samen, vakanties, etentjes, gesprekken tot diep in de nacht, virtueel discobingoborrelen, het delen van een lach en een traan, en recent de wereld van het moederschap. Bedankt voor jullie liefde en bemoedigende woorden.

Toen ik begon aan mijn PhD woonde ik nog op de Amsteldijk, met uitzicht op de Amstel, met mijn lieve huisgenootjes. **Sophie**, altijd vol energie en creative ideeen, zorgzaam en ondernemend, en **Jantine**, ontzettend slim en even grappig, attent en supportive, wat fijn dat ik jullie in mijn leven heb.

Nadat ik een aantal jaar surrograat huisgenoot ben geweest vind ik het ontzettend leuk om deze fase van het leven ook te delen met de setjes van de BV, **Casper** en **Philine**, **Rens** en **Tamara**, en **Wouter** en **Myriam**. Bedankt voor jullie vriendschap.

Dan rest mij nog om mij te richten op het fundament van mijn leven, mijn lieve familie, bedankt voor de interesse, het meeleven en steunen, en het goede gezelschap. Mijn lieve schoonfamilie **Erik**, **Annette**, **Joris**, **Jiska**, **Fabian**, **Nora**, **Louis**, **Lonneke**, en **Han**, het is altijd zo fijn om bij jullie te zijn.

Lieve **Mila**, je bent een prachtig mens, observerend, creatief, gepassioneerd. Wat bijzonder dat ik je vanaf dat je een klein hummeltje was heb mogen zien opgroeien. Ik ben altijd geinspireerd geweest door je vele talenten en bewonder de dapperheid waarmee jij je authentieke leven tegemoet treedt. Lieve **Merlijn**, altijd ben je mijn maatje geweest. Ook al zijn we zo verschillend, we zijn ook zo hetzelfde. Ik ben heel trots op hoe jij je hebt ontwikkeld tot de zelfverzekerde persoon die je nu bent. Ik bewonder je welbespraaktheid en de kunde in je vak, maar daarnaast ben je ook heel erg trouw en liefdevol naar je omgeving, waaronder natuurlijk je lieve vriendin **Marlinde**. Lieve **mama** en **papa**, jullie liefde en onvoorwaardelijke steun is de basis van alles. Jullie zijn het allersterkste team dat ik ken. Samen hebben jullie een liefdevol nest gecreeerd waarin jullie ons de vrijheid en aanmoediging gaven om te worden wie we zijn. Lieve **mama**, met jouw grenzeloze creativiteit en zorgzaamheid en lieve **papa**, ambiteus en met enorme toewijding aan alles wat je doet en de mensen om je heen. Ik heb maar geluk met ouders zoals jullie. Ik hoop dat **opa** een beetje trots is als het zometeen is gelukt om een Dr. titel in de wacht te slepen.

Als laatste, mijn prachtige gezin. Liefste **Wouter**, mijn grote liefde en veilige haven. Wat begon op een feestje met wat ducktape en een roze regenbroek, leidde ons in 10 jaar langs prachtige avonturen die voor altijd in ons hart zitten. Bedankt dat je me altijd weer met twee voeten op de grond weet te krijgen wanneer dat nodig is, en voor je enthousiasme als ik een foto van een mooi gelukte western-blot naar je stuurde ook al had je geen idee wat het was. Wat ongelofelijk bijzonder dat we nu samen zo kunnen genieten van onze prachtige dochter. **Mente**, m'n lieve hartje, met jou is alles mooier.

About the Author

Amber Berdenis van Berlekom is a neuroscientist with a specific interest in molecular biology and cellular disease modelling, eager to learn, empathic, and creative. She grew up in a loving family together with her two younger siblings in Bilthoven. She attended gymnasium at de Werkplaats Kindergemeenschap. Here her interest in the brain was already sparked, as shown by her graduation literature project studying the implications of neglect on the developing brain. Although at a younger age Amber dreamt of being an actress in the theatre, she became to enjoy studying, and was curious to expand her knowledge at university. She started with the bachelor Psychobiology at the University of Amsterdam in 2010. Besides learning about the brain, she was active as a member of the Amsterdam student rowing society Skøll. During her bachelor, Amber had had the opportunity to experience the full cycle of a research project for the first time at her internship at the Developmental Psychology department of the University of Amsterdam under supervision of Dr. Tessa J. P. van Schijndel, studying the influence of pedagogical sampling when teaching young children (age 4-6) in science education. As an extension of her bachelor studies she went on exchange to the University of Birmingham in the U.K. for a semester. After finishing her bachelor degree, she worked as a teaching assistant, supervising psychobiology students during laboratory practicals and study groups. Her interest for molecular biology grew, and in 2014 she applied for the master program Molecular Neuroscience at the University of Amsterdam. During her first research internship she worked on the role of the transcription factor FOXO6 in corpus callosum development in the lab of Prof. dr. Marten P. Smidt, under the supervision of Dr. Ricardo H. Paap at the Swammerdam Institute for Life Sciences at the University of Amsterdam. She continued her studies by writing a master literature thesis describing a novel ontogeny based *in vitro* model for the differentiation of microglia from human iPSC under supervision of Dr. Paul R. Ormel at the department of Translational Neuroscience at the University Medical Center Utrecht. In 2016 she moved to Boston in the U.S. to do a second research internship at the Boston Childrens Hospital and Kirby Neurobiology Center at Harvard Medical School. She researched approaches to study and decrease motor neuron hyperexcitability in ALS by developing and applying new high throughput screens on iPSC generated motor neurons in the lab of Prof. dr. Clifford J. Woolf under supervision of Dr. Kasper C. D. Roet. Her excellent performances during her courses and internships resulted in a Cum Laude graduation from her masters. At the end of 2016 she started with a PhD at the department of Translational Neuroscience and department of Psychiatry at the University Medical Center Utrecht under supervision of promotors Prof. dr. Elly M. Hol and Prof. dr. René S. Kahn and co-promotors Dr. Lot D. de Witte and Dr. Marco P. Boks, with support of Dr. Jinte Middeldorp. As a part of her PhD she had the opportunity to go on an exchange to Mount Sinaï in New York for several months. Furthermore, during her PhD she attended and organised several conferences and symposia, supervised bachelor and master students, and established several collaborations. In the final phase of her PhD, while finishing her thesis, she became a mother to Mente. The years of extensive research focussed on different cellular and molecular neurodevelopmental phenotypes in schizophrenia resulted in this PhD thesis.

List of publications

Berdenis van Berlekom, A., Kübler, R., Hoogeboom, J.W., Vonk, D., Sluijs, J.A., Pasterkamp, R.J., Middeldorp, J., Kraneveld, A.D., Garssen, J., Kahn, R.S., Hol, E.M., de Witte, L.D., Boks, M.P. **Exposure to the Amino Acids Histidine, Lysine, and Threonine Reduces mTOR Activity and Affects Neurodevelopment in a Human Cerebral Organoid Model.** Nutrients. 2022 May;14:2175.

Berdenis van Berlekom A, Notman N, Sneeboer MA, Snijders GJ, Houtepen LC, Nispeling DM, He Y, Dracheva S, Hol EM, Kahn RS, de Witte LD, Boks MP; Psychiatric Donor Program of the Netherlands Brain Bank (NBB-PSY). **DNA methylation differences in cortical grey and white matter in schizophrenia.** Epigenomics. 2021 Aug;13(15):1157-1169.

Berdenis van Berlekom A, Muflihah CH, Snijders GJLJ, MacGillavry HD, Middeldorp J, Hol EM, Kahn RS, de Witte LD. Synapse Pathology in Schizophrenia: **A Meta-analysis of Postsynaptic Elements in Postmortem Brain Studies.** Schizophr Bull. 2020 Feb 26;46(2):374-386.

Gumbs, S. B. H., Berdenis van Berlekom, A., Kübler, R., Schipper, P. J., Gharu, L., Boks, M. P., Ormel, P. R., Wensing, A. M. J., de Witte, L. D., & Nijhuis, M. **Characterization of HIV-1 Infection in Microglia-Containing Human Cerebral Organoids**. Viruses. 2022 Apr;4(4):829.

Gumbs SBH, Kübler R, Gharu L, Schipper PJ, Borst AL, Snijders GJLJ, Ormel PR, Berdenis van Berlekom A, Wensing AMJ, de Witte LD, Nijhuis M. **Human microglial models to study HIV infection and neuropathogenesis: a literature overview and comparative analyses.** Journal of Neurovirology. 2022 Feb;Online ahead of print.

Lopes KP, Snijders GJL, Humphrey J, Allan A, Sneeboer MAM, Navarro E, Schilder BM, Vialle RA, Parks M, Missall R, van Zuiden W, Gigase FAJ, Kübler R, van Berlekom AB, Hicks EM, Böttcher C, Priller J, Kahn RS, de Witte LD, Raj T. **Genetic analysis of the human microglial transcriptome across brain regions, aging and disease pathologies.** Nat Genet. 2022 Jan;54(1):4-17.

Snijders GJLJ, Sneeboer MAM, Fernández-Andreu A, Udine E; Psychiatric donor program of the Netherlands Brain Bank (NBB-Psy), Boks MP, Ormel PR, van Berlekom AB, van Mierlo HC, Böttcher C, Priller J, Raj T, Hol EM, Kahn RS, de Witte LD. **Distinct non-inflammatory signature of microglia in post-mortem brain tissue of patients with major depressive disorder.** Mol Psychiatry. 2021 Jul;26(7):3336-3349.

Snijders GJLJ, van Zuiden W, Sneeboer MAM, Berdenis van Berlekom A, van der Geest AT, Schnieder T, MacIntyre DJ, Hol EM, Kahn RS, de Witte LD. **A loss of mature microglial markers without immune activation in schizophrenia.** Glia. 2021 May;69(5):1251-1267.

Snijders GJLJ, de Witte LD, van den Berk D, van der Laan C, Regeer E, Begemann MJH, Berdenis van Berlekom A, Litjens M, Boks MP, Ophoff RA, Kahn RS, Hillegers MHJ. **No association between anti-thyroidperoxidase antibodies and bipolar disorder: a study in the Dutch Bipolar Cohort and a meta-analysis.** Psychoneuroendocrinology. 2020 Feb;112:104518.

Sneeboer MAM, Snijders GJLJ, Berdowski WM, Fernández-Andreu A; Psychiatric Donor Program of the Netherlands Brain Bank (NBB-Psy), van Mierlo HC, Berdenis van Berlekom A, Litjens M, Kahn RS, Hol EM, de Witte LD. **Microglia in post-mortem brain tissue of patients with bipolar disorder are not immune activated.** Transl Psychiatry. 2019 May 24;9(1):153.

Ormel PR, Vieira de Sá R, van Bodegraven EJ, Karst H, Harschnitz O, Sneeboer MAM, Johansen LE, van Dijk RE, Scheefhals N, Berdenis van Berlekom A, Ribes Martínez E, Kling S, MacGillavry HD, van den Berg LH, Kahn RS, Hol EM, de Witte LD, Pasterkamp RJ. **Microglia innately develop within cerebral organoids.** NatCommun. 2018 Oct 9;9(1):4167.

Samenstelling beoordelingscommissie

Prof. dr. R. J. Pasterkamp
Prof. dr. W. Cahn
Dr. C. H. A. Nijboer
Prof. dr. A. D. Kraneveld
Prof. dr. I. Huitinga

UMC Utrecht

Universiteit Utrecht

ISBN 978-90-393-7490-0