

Inaugural dissertation
for
obtaining the doctoral degree
of the
Combined Faculty of Mathematics, Engineering and Natural Sciences
of the
Ruprecht - Karls -University
Heidelberg

Presented by
M.Sc. Julia Wangemann
Born in: Munich, Germany
Oral examination: 23.05.2023

A human iPSC-based model for synaptic pruning-associated changes in schizophrenia

Referees: PD. Dr. Georg Köhr
Prof. Dr. Philipp Koch

Table of Contents

Table of Contents	I
List of Figures	V
List of Tables	VII
List of Abbreviations	IX
1 Abstract.....	1
2 Zusammenfassung.....	3
3 Introduction	5
3.1 SCZ pathogenesis.....	5
3.2 The complement system and synaptic pruning.....	7
3.2.1 The complement cascade in innate immunity.....	7
3.2.2 Complement expression in the brain	9
3.2.3 Complement-dependent synaptic pruning	10
3.2.4 The role of microglia in synaptic pruning	11
3.3 The complement system and SCZ pathogenesis	13
3.3.1 C4 structural variation in SCZ.....	13
3.3.2 C4 expression as a cerebrospinal fluid (CSF) and serum biomarker in SCZ ...	14
3.3.3 C4 overexpression in mouse models of SCZ.....	15
3.4 The NLRP3 inflammasome: a potential new target for SCZ.....	16
3.4.1 The NLRP3 inflammasome pathway	16
3.4.2 The NFκB pathway.....	20
3.4.3 The NLRP3 inflammasome in neurodegenerative and psychiatric disorders ...	21
3.4.4 NLRP3 Inflammasome inhibitors	23
3.5 Human stem cell technology	25
3.5.1 Human induced pluripotent stem cells (iPSCs).....	25
3.5.2 Generation of different brain cell types from human iPSCs.....	27
3.5.3 Modeling complement-dependent synaptic pruning using human iPSCs.....	29
4 Aims of this study.....	31
5 Materials	33
5.1 Cell culture	33
5.1.1 Cell lines	33
5.1.2 Cell culture base media, solutions and small molecules	33
5.1.3 Composition of self-made cell culture media and solutions.....	36
5.2 Molecular biology and biochemistry.....	38
5.2.1 Bacterial cultures.....	38
5.2.2 Plasmids	39
5.2.3 Enzymes	40

Table of Contents

5.2.4	Primers and PCR components	40
5.2.5	Kits	42
5.2.6	Antibodies and fluorescent probes	43
5.2.7	Buffers and solutions	45
5.2.8	Reagents and chemicals	46
5.3	Cell culture, molecular biology and biochemistry consumables	48
5.4	Technical equipment	49
5.5	Software	50
6	Methods	51
6.1	Cell culture	51
6.1.1	Cultivation of human iPSCs	51
6.1.2	Generation of human iPSC-derived cortical neurons	51
6.1.3	Generation of human iPSC-derived microglia	52
6.1.4	Co-cultures of human iPSC-derived cortical neurons and microglia	53
6.1.5	Treatments of microglia, cortical neurons and co-cultures	53
6.1.6	Phagocytosis assay and <i>in vitro</i> synaptic pruning assay	53
6.1.7	Cultivation of HEK-293T cells	54
6.1.8	Cryopreservation of cells	54
6.2	Molecular biology	54
6.2.1	Genomic DNA (gDNA) isolation	54
6.2.2	RNA Isolation and cDNA synthesis	55
6.2.3	Reverse transcription PCR (RT-PCR)	56
6.2.4	Quantitative PCR (qPCR)	56
6.2.5	Mycoplasma PCR	56
6.2.6	Agarose gel electrophoresis and gel extraction of DNA	57
6.2.7	Quality control for bulk RNAseq	57
6.2.8	Bulk RNAseq	57
6.2.9	Nuclei isolation	58
6.2.10	Single-nucleus RNAseq (snRNAseq)	59
6.2.11	Transformation of chemically competent <i>E. Coli</i>	59
6.2.12	Plasmid DNA isolation	60
6.2.13	Lipofectamine transfection	60
6.3	Generation of a mCherry fluorescent reporter cell line	60
6.3.1	Molecular cloning	60
6.3.2	Sanger sequencing	62
6.3.3	Nucleofection and clonal selection	62
6.3.4	SNP analysis	63
6.4	Generation of a C4A overexpression cell line	63

Table of Contents

6.4.1	Molecular cloning	63
6.4.2	Lentivirus production	65
6.4.3	Infection and selection of human iPSCs	66
6.5	Biochemistry.....	66
6.5.1	Immunocytochemistry	66
6.5.2	Cell lysis and protein quantification	66
6.5.3	SDS-PAGE and Western blotting	67
6.5.4	Extraction of synaptosomes and labeling with pHrodo.....	67
6.6	Microscopy and image analysis.....	68
6.6.1	Fluorescence microscopy.....	68
6.6.2	Live cell imaging.....	68
6.7	Statistical analysis and visualization.....	68
7	Results	71
7.1	Generation and characterization of human iPSC-derived microglia	71
7.2	Generation of human cortical neuronal cultures from iPSCs.....	76
7.3	Overexpression of complement component C4A in human iPSC-derived cortical neurons	78
7.4	Increased engulfment of synaptosomes from C4A overexpressing neurons in an <i>in vitro</i> synaptic pruning assay	81
7.5	Co-cultures of human iPSC-derived microglia with cortical neurons	83
7.6	Overexpression of C4A promotes synaptic loss in co-cultures of iPSC-derived cortical neurons and microglia by increased microglial activation	85
7.7	NLRP3 inflammasome inhibitor oridonin attenuates inflammatory response in iPSC-derived microglia and inhibits synapse engulfment	89
7.8	Anti-inflammatory effects of oridonin and minocycline reduce microglial activation after exposure to C4A in co-cultures with cortical neurons	93
8	Discussion.....	99
8.1	Generation of human iPSC-derived cortical neurons and microglia	99
8.2	Generation of a human iPSC-derived <i>in vitro</i> model to study synaptic pruning-associated changes in SCZ.....	101
8.3	The microglial NLRP3 inflammasome as a potential drug target in SCZ.....	104
8.4	Inhibition of microglia activation in a human iPSC-derived model of synaptic pruning.....	105
9	Summary and outlook	109
10	References	111
11	Contributions and previous work.....	131
12	Acknowledgements.....	133

List of Figures

Figure 1: The complement system.....	8
Figure 2: Glial cells in synaptic pruning.....	12
Figure 3: Structural variation of the complement component 4 (C4) gene.....	14
Figure 4: The NLRP3 multiprotein complex.	17
Figure 5: Schematic representation of mechanisms regulating inflammasome activation.	19
Figure 6: Canonical and non-canonical NFκB pathways.....	20
Figure 7: iPSC and direct conversion technology.	26
Figure 8: Plasmid map of pMK243- EF-1α-mCherry.	62
Figure 9: Plasmid map of pCDH-CuO-C4A-Flag-HA-T2A-Puro.	65
Figure 10: Differentiation protocol for the generation of human iPSC-derived microglia.....	71
Figure 11: iPSC-derived microglia express canonical microglia markers and respond to pro-inflammatory stimuli.....	72
Figure 12: Differentially expressed genes in LPS-treated microglia.	74
Figure 13: iPSC-derived microglia are phagocytically competent.	74
Figure 14: iPSC-derived microglia undergo morphological change after LPS-treatment.....	75
Figure 15: Differentiation protocol for the generation of human iPSC-derived cortical neurons.	76
Figure 16: Differentiation and characterization of human iPSC-derived cortical neurons.	77
Figure 17: Generation of a C4A overexpression cell line.	79
Figure 18: Overexpression of C4A in human iPSC-derived cortical neurons.	80
Figure 19: Overexpression of C4A promotes phagocytosis of synaptosomes by microglia in an <i>in vitro</i> synaptic pruning assay.	81
Figure 20: Establishment of a microglia-neuron co-culture system.	83
Figure 21: Live imaging of microglia-neuron co-cultures.....	84
Figure 22: Overexpression of C4A promotes synaptic pruning in neuron-microglia co- cultures.....	85
Figure 23: Overexpression of C4A in neurons leads to increased NFκB pathway activation in microglia.	86
Figure 24: Single-nucleus RNAseq (snRNAseq) cell type identification analysis.	87
Figure 25: Comparative analysis of single-nucleus microglial transcriptomics from co- cultures with control and C4A overexpressing neurons confirms microglial activation by C4A.	88
Figure 26: Expression of the NLRP3 inflammasome in iPSC-derived microglia.	89
Figure 27: Oridonin attenuates LPS-induced changes in iPSC-derived microglia.	91
Figure 28: Oridonin inhibits synapse engulfment in an <i>in vitro</i> synaptic pruning assay.	92
Figure 29: Effects of oridonin on synapse density and NFκB nuclear translocation in neuron-microglia co-cultures.	93
Figure 30: Effects of minocycline on microglial synaptic pruning and phagocytic activity.	95
Figure 31: Effects of oridonin and minocycline on synapse density in control human iPSC- derived cortical neurons.	96
Figure 32: Proposed model for microglial NLRP3 inflammasome activation by increased neuronal complement expression implicating a potential new therapeutic approach.	110

List of Tables

Table 1: Cell lines.....	33
Table 2: Cell culture base media	33
Table 3: Commercial ready-to-use cell culture solutions.....	33
Table 4: Small molecules and chemicals used for cell culture	34
Table 5: Final composition of cell culture media	36
Table 6: Composition of self-made buffers, supplements and solutions.....	38
Table 7: Media used for cultivation of bacteria.....	38
Table 8: Plasmids used for cloning, nucleofection and lentivirus production	39
Table 9: Enzymes used for cloning and molecular biology	40
Table 10: Primers for RT-PCR and qPCR	40
Table 11: Primers and oligonucleotides used for molecular cloning.....	41
Table 12: RT-PCR reaction mixture.....	41
Table 13: qPCR reaction mixture.....	42
Table 14: Commercial kits	42
Table 15: Primary antibodies	43
Table 16: Secondary antibodies	44
Table 17: Fluorescent probes	44
Table 18: Self-made buffers and solutions.....	45
Table 19: Molecular biology reagents and commercial buffers	46
Table 20: Chemicals.....	47
Table 21: Consumables.....	48
Table 22: Notable laboratory equipment.....	49
Table 23: Computer software	50
Table 24: cDNA synthesis reaction mixture	55
Table 25: Cycling program for cDNA synthesis.....	55
Table 26: RT-PCR cycling program	56
Table 27: qPCR program.....	56
Table 28: Mycoplasma PCR cycling program	57
Table 29: 2x ST buffer	58
Table 30: TST buffer	58
Table 31: Restriction digest of pMK243	61
Table 32: LongAmp reaction mixture	61
Table 33: LongAmp PCR cycling program.....	61
Table 34: Gibson assembly reaction mixture for pMK243- EF-1 α -mCherry	61
Table 35: Restriction digest of pCDH-CuO-MCS-T2A-GFP SparQ [™]	64
Table 36: Reaction mixture for oligonucleotide phosphorylation	64
Table 37: Ligation reaction T2A-NotI-XmaI-Sall oligos with backbone	64
Table 38: Ligation mixture for T4 DNA Ligase	64
Table 39: Gibson assembly reaction mixture	65

List of Abbreviations

Abbreviation	Full name	Abbreviation	Full name
%	percent	CLEM	correlative light and electron microscopy
∞	infinity	CMV	cytomegalovirus
(v/v)	volume per volume	CNS	central nervous system
(w/v)	weight per volume	CR	complement receptor
°C	degree Celsius	CREB	cAMP response element-binding protein
μ	micro-	CRP	C-reactive protein
1x	single concentrated	CSF	cerebrospinal fluid
A	ampere	CSF1R	colony stimulating factor 1 receptor
AD	Alzheimer's disease	CSMD1	CUB and Sushi multiple domains 1
ADP	adenosine diphosphate	Ct	threshold cycle
<i>AIF1</i>	allograft inflammatory factor 1	CTB	cholera toxin subunit B
ALS	amyotrophic lateral sclerosis	Ctrl	control
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	CXCL	C-X-C motif chemokine ligand
AmpR	ampicillin resistance	D	day
ANOVA	analysis of variance	Da	dalton
A-P	anteroposterior	DAMP	damage-associated molecular pattern
APS	ammonium persulfate	DAPI	4',6-diamidino-2-phenylindole
ARC	activity-regulated cytoskeleton-associated protein	DAPT	N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester
Arg-1	arginase 1	DDX3X	dead-box helicase 3 X-linked
ASC	Apoptosis-associated speck-like protein containing a CARD	DEPC	Diethyl pyrocarbonate
ATP	adenosine triphosphate	<i>DLG4</i>	discs large homolog 4
A β	amyloid β	dLGN	dorsolateral geniculate nucleus
BBB	Blood-brain barrier	DMSO	dimethyl sulfoxide
BCA	bicinchoninic acid assay	DNA	deoxyribonucleic acid
<i>BCL11B</i>	B-cell lymphoma/leukemia 11B	dNTPs	deoxynucleoside triphosphate
BD	bipolar disorder	DPBS	Dulbecco's phosphate-buffered saline
BDI	Beck Depression Inventory	D-V	dorsoventral
BDNF	brain-derived neurotrophic factor	<i>E. coli</i>	<i>Escherichia coli</i>
BHB	β -hydroxybutyrate	e.g.	<i>exempli gratia</i> (for example)
BMDM	bone marrow-derived macrophage	EB	embryoid body
BMP	bone morphogenetic protein	EDTA	ethylenediaminetetraacetic acid
bp	base pair	EF-1 α	elongation factor-1 alpha
BP	biological processes	EM	electron microscopy
BSA	bovine serum albumin	ESCs	embryonic stem cells
BSL	laboratory biosafety level	et al.	<i>et alia</i> (and others)
C	complement component	FADD	Fas-associated protein with death domain
CASP	caspase	FEP	first-episode psychosis
Cat.	catalogue		
CC	cellular components		
CCL	C-C motif ligand		
CD	cluster of differentiation		
cDNA	complementary DNA		

List of Abbreviations

FGF-2	fibroblast growth factor 2	M	molar (mol/L)
fMRI	functional magnetic resonance imaging	m	milli-
for	forward	MAC	membrane attack complex
g	gram	MAP2	microtubule-associated protein 2
GABA	γ-aminobutyric acid	MAPK	mitogen-activated protein kinase
GAD1	glutamate decarboxylase 1	MARCO	macrophage receptor with collagenous structure
GAPDH	glyceraldehyd-3-phosphat-dehydrogenase	MASP	mannose binding lectin serine protease
GAS6	Growth Arrest Specific 6	MBL	mannose-binding lectin
gDNA	genomic DNA	MCS	multiple cloning site
GFAP	glial fibrillary acidic protein	M-CSF	macrophage colony-stimulating factor
GFP	green fluorescent protein	MDD	major depressive disorder
GM-CSF	granulocyte macrophage colony stimulating factor	MEGF10	multiple EGF-like domains 10
GO	gene ontology	MERTK	MER proto-oncogene, tyrosine kinase
GPR	G protein-coupled receptor	MF	molecular functions
GRIA	glutamate ionotropic receptor AMPA type subunit	MHC	major histocompatibility complex
GRIN	glutamate ionotropic receptor NMDA type subunit	MIA	maternal immune activation
GSDMD	gasdermin D	min	minute
GWAS	genome-wide association studies	Mino	minocycline
h	hour	mol	mole
HA	hemagglutinin	mPFC	medial prefrontal cortex
HBSS	HEPES buffered saline solution	MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
HEK	human embryonic kidney	MRI	magnetic resonance imaging
HERV	human endogenous retrovirus	mRNA	messenger ribonucleic acid
HLA	human leukocyte antigen	MRT	Magnetresonanztomographie
Iba1	ionized calcium-binding adapter molecule 1	MS	Multiple sclerosis
IGF-1	insulin like growth factor 1	n	nano-
IKK	IκB kinase	n.s.	not significant
IL	interleukin	N/A	not applicable
iPSCs	induced pluripotent stem cells	NACHT	NAIP, CIITA, HET-E, and TP1
IR	infrared	NEAA	non-essential amino acid
ITGAM	integrin alpha M	NEK7	NIMA related kinase 7
IκB	inhibitor of nuclear factor kappa B	NEMO	NFκB essential modulator
k	kilogram	NeuN	neuronal nuclei
KEGG	Kyoto Encyclopedia of Genes and Genomes	NFTs	neurofibrillary tangles
KO	knockout	NFκB	nuclear factor kappa B
l	liter	NIK	NFκB-inducing kinase
LAAP	L-ascorbic acid 2-phosphate	NLR	NOD-like receptor
LB	lysogeny broth	NLRP3	NOD-, LRR-, and pyrin domain-containing 3
LBP	lipopolysaccharide binding protein	NMDA	N-methyl-D-aspartate
LPS	lipopolysaccharide	NPAS4	neuronal PAS domain protein 4
LRR	leucine-rich repeat	NPC	neural progenitor cell
LSD	lysergic acid diethylamide	Ø	diameter
LTA	lymphotoxin alpha	Oct3/4	octamer-binding transcription factor 3/4

List of Abbreviations

Ori	oridonin	S	synaptosomes
<i>P</i>	significance level	<i>S100A8</i>	S100 calcium binding protein A8
p	pico-	SCF	stem cell factor
P	total protein	SCZ	schizophrenia
P2RX7	purinergic receptor P2X 7	SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis	sec	seconds
PAMP	pathogen-associated molecular pattern	SEM	standard error of mean
PAR	protease-activated receptor	shh	sonic hedgehog
Pax6	paired box 6	SIRP α	signal regulatory protein α
PBMC	peripheral blood mononuclear cell	SNAP-25	synaptosome-associated protein 25
PBS	phosphate-buffered saline	SNP	single nucleotide polymorphism
PCR	polymerase chain reaction	snRNAseq	single-nucleus RNA sequencing
PD	Parkinson's disease	SOC	super optimal broth with catabolite repression medium
PEG	polyethylene glycol	SOCS3	suppressor of cytokine signaling 3
PEI	polyethyleneimine	Sox2	sex determining region Y-box 2
Pen/Strep	penicillin/streptomycin	SRPX2	Sushi Repeat Containing Protein X-Linked 2
PET	positron emission tomography	SSEA4	stage specific embryonic antigen 4
PFA	paraformaldehyde	SV2 A	synaptic vesicle protein 2 A
PFC	prefrontal cortex	SYN	synapsin
PGC	Psychiatric Genomics Consortium	Syn-PER	synaptic protein extraction reagent
pH	potential of hydrogen	TBR1	T-box brain transcription factor 1
pH3	phospho-histone H3	TBST	tris-buffered saline with Tween [®] 20
PKC	protein kinase C	TC	tissue culture
PLL	poly-L-lysine hydrobromide	TEMED	N, N, N, N'–tetramethylethylenediamine
<i>PROS1</i>	protein S	TGF- β	transforming growth factor beta
PRR	pattern recognition receptor	TGN	<i>trans</i> -Golgi network
PSD	postsynaptic density	TLR	toll-like receptor
<i>PTPRC</i>	protein tyrosine phosphatase receptor type C	TMEM119	transmembrane protein 119
Puro	puromycin	TNF	tumor necrosis factor
PVDF	polyvinylidene fluoride	TNFR	tumor necrosis factor receptor
PYD	pyrin domain	TPM	transcripts per kilobase million
qPCR	quantitative polymerase chain reaction	TREM2	triggering receptor expressed on myeloid cells 2
RA	retinoic acid	TRIF	TIR-domain-containing adapter-inducing interferon- β
<i>RBFOX3</i>	RNA-binding protein FOX 3	TrkB	tropomyosin receptor kinase B
rcf	relative centrifugal force	U	units
rev	reverse	V	volt
RGC	retinal ganglion cells		
RIPK1	receptor-interacting protein kinase 1		
RNA	ribonucleic acid		
RNAseq	RNA sequencing		
ROI	region of interest		
ROS	reactive oxygen species		
rpm	revolutions per minute		
rRNA	ribosomal ribonucleic acid		
RT	room temperature		
RT-PCR	reverse transcription polymerase chain reaction		

List of Abbreviations

VEGF	vascular endothelial growth factor
VEH	vehicle
w	week
x g	times gravity
α	anti-
αSyn	α-synuclein

1 Abstract

Schizophrenia (SCZ) is a heritable psychiatric disorder which affects approximately 1% of the world population. The disease is characterized by a range of symptoms such as hallucinations, disorganized speech, lack of motivation and depression, as well as cognitive impairments. Genome-wide association studies (GWAS) have identified over 100 genomic regions that associate with SCZ risk and the major histocompatibility complex (MHC) region on chromosome 6, more specifically the complement component 4 (C4) gene, was shown to be the most significantly associated genetic locus within this region. Thereby, alleles leading to a higher C4A expression were correlated to an increased risk for SCZ. The complement cascade has been shown to be involved in microglia-mediated synapse elimination during development. Given the reduced numbers of synapses in brains of individuals with SCZ, it has been suggested that excessive complement activity and synaptic pruning contributes to the development of the disease during late adolescence and early adulthood. In this thesis, a human *in vitro* model to study synaptic pruning in the context of SCZ pathogenesis was established using induced pluripotent stem cell (iPSC)-derived cortical neurons and microglia. A differentiation protocol to generate iPSC-derived microglia was established which allowed the generation of human microglia-like cells in high purity. These cells expressed key microglia markers, showed a high phagocytic capacity and responded to pro-inflammatory stimuli. Using an *in vitro* synaptic pruning assay, it was shown that synaptosomes derived from C4A overexpressing neurons were more efficiently phagocytosed compared to control synaptosomes. Overexpression of C4A in mature iPSC-derived cortical neurons in co-cultures with microglia, led to an activation of the NFκB pathway in microglia and an increased synaptic loss in iPSC-derived cortical neurons. Furthermore, it was shown that oridonin, a specific NLRP3 inflammasome inhibitor, has anti-inflammatory effects on iPSC-derived microglia, can inhibit phagocytosis of synaptosomes and can increase synaptic density in C4A overexpressing iPSC-derived cortical neurons after co-culture with microglia.

Taken together, this study investigates genotype-phenotype relationships with respect to synaptic pruning and C4A expression in the context of SCZ pathogenesis using a human *in vitro* model. The applied experimental setting identified the microglial NLRP3 inflammasome as a potential novel therapeutic option for SCZ as the NLRP3 inhibitor oridonin was able to inhibit C4A-induced microglial activation and rescue synaptic densities in iPSC-derived cortical neurons.

2 Zusammenfassung

Schizophrenie ist eine vererbare psychische Erkrankung, welche etwa 1% der weltweiten Bevölkerung betrifft und durch Halluzinationen, Wahnvorstellungen, einer Abnahme geistiger Funktionen, Antriebsmangel und einer damit einhergehenden Beeinträchtigung im gesellschaftlichen und beruflichen Leben gekennzeichnet ist. Genomweite Assoziationsstudien (GWAS) haben deutliche Verbindungen zwischen einem DNA-Abschnitt, welcher Proteine des Haupthistokompatibilitätskomplexes MHC (major histocompatibility complex) kodiert und der Entstehung von Schizophrenie aufgezeigt. Ein Großteil dieser Assoziation kommt von einem Gen, welches das Komplementprotein C4 kodiert und in diesem Abschnitt liegt. Es wurde gezeigt, dass bestimmte Varianten des C4 Gens, welche zu einer besonders hohen Expression führen, das Risiko an Schizophrenie zu erkranken deutlich erhöhen. Komplementproteine spielen eine zentrale Rolle bei der Eliminierung von überschüssigen Synapsen während der Gehirnentwicklung. Dabei werden weniger aktive Synapsen markiert und von Mikrogliazellen erkannt und phagozytiert. Das häufige Auftreten von Schizophrenie während der Kindheit und Pubertät, einem Zeitpunkt starker synaptischer Plastizität, sowie die auf MRT-Bildern zu beobachtende Verringerung der grauen Substanz bei Schizophreniepatienten, hat die These weiterhin bestärkt, dass eine hohe Expression des Komplementproteins C4 zu einem übermäßigen Abbau von Synapsen führen und damit ursächlich für manche der Krankheitssymptome sein könnte. Das Ziel dieser Arbeit war es, ein humanes Zellkulturmodell zu entwickeln, mit dem die vermehrte Synapseneliminierung durch Mikrogliazellen im Kontext der Entstehung einer Schizophrenie weiter erforscht werden kann. Dazu wurden zunächst Differenzierungsprotokolle für die Generierung von kortikalen Neuronen und Mikrogliazellen aus induzierten pluripotenten Stammzellen (iPS) etabliert. Die aus iPS abgeleiteten Mikroglia zeigten eine hohe Expression von typischen Markerproteinen, hatten die Fähigkeit Erregerpartikel zu phagozytieren und konnten durch pro-inflammatorische Stimuli aktiviert werden. Um die Rolle des C4 Proteins im Kontext der Entstehung von Schizophrenie zu analysieren, wurden reife kortikale Neurone aus einer iPS-Linie mit stabiler Überexpression des C4 Gens differenziert. Nach Extraktion und fluoreszenter Markierung der Synaptosomen von Kontrollzellen und C4-Überexpressionszellen, zeigte sich eine vermehrte Phagozytose der Synaptosomen von C4-Überexpressionszellen durch Mikroglia. Eine direkte Co-Kultur bestehend aus Mikrogliazellen und kortikalen Neuronen zeigte eine Aktivierung des NFkB Signalwegs in den Mikroglia und eine signifikante Verringerung der Synapsendichte in den kortikalen Neuronen. Weiterhin konnte gezeigt werden, dass Oridonin, ein spezifischer Inhibitor des NLRP3 Inflammasom Signalwegs, anti-inflammatorische Effekte auf humane Mikrogliazellen hat, die Phagozytose von Synaptosomen inhibiert und die Anzahl der synaptischen Partikel in Neuronen mit C4-Überexpression in Co-Kulturen mit Mikrogliazellen erhöht. Zusammenfassend wurde in dieser Arbeit gezeigt, dass humane Zellkulturmodelle

einen wertvollen Beitrag zur Erforschung bestimmter Aspekte komplexer psychischer Erkrankungen wie Schizophrenie leisten. Die hier beschriebenen Experimente haben das NLRP3 Inflammasom als potentiell neues Ziel zur Inhibierung überaktiver Mikrogliazellen identifiziert und bilden damit eine Basis für weitere vertiefende Studien.

3 Introduction

3.1 SCZ pathogenesis

SCZ is a heritable psychiatric disorder with a worldwide prevalence of approximately 1% (Messias et al., 2007). The disease is characterized by a range of symptoms, which can be divided into three main categories: positive symptoms, negative symptoms and cognitive impairments. Positive symptoms include hallucinations and delusions, whereas negative symptoms are characterized by lack of motivation, depression, deficits in affect, apathy, alogia (poverty of speech) and neglect. Cognitive impairments include memory, language and attention deficits (Picchioni and Murray, 2007). The pathogenesis of SCZ is considered to be multi-factorial and likely involves gene-environment interactions. A monozygotic twin of an affected individual has a 40-50 times higher risk of developing the disease and the risk is also significantly increased in other first degree relatives due to a high heritability of up to 80% (Gejman et al., 2010). Meta-analyses have shown that very early environmental stressors such as fetal hypoxia, premature birth or low birth weight can further increase the risk to develop the disorder (Cannon et al., 2003; Picchioni and Murray, 2007). SCZ symptoms often manifest during puberty or early adulthood, a phase where the prefrontal cortex (PFC) is still developing, after exposure to environmental stressors such as traumatic experiences, social isolation, drug abuse or infections (Dean and Murray, 2005; Spataro et al., 2004; Spauwen et al., 2006; Whitfield et al., 2005). Disease onset is often preceded by a prodromal phase which is characterized by unspecific sub-diagnostic symptoms such as anxiety, social withdrawal and educational difficulties. Diagnosis is usually made after first-episode psychosis (FEP) and once diagnosed, positive symptoms often follow a fluctuating course where patients recover after acute psychotic crises but commonly suffer from relapse after adverse environmental events (Millan et al., 2016). Negative and cognitive symptoms often remain between acute psychotic episodes, persist over time and increase in severity (Heilbronner et al., 2016).

Neuroimaging studies have widely reported brain structural and functional abnormalities in SCZ. Magnetic resonance imaging (MRI) studies have consistently found reduced grey matter volumes of the medial temporal, superior temporal, and prefrontal areas of the brain, regions especially important for episodic memory, auditory processing, as well as short-term memory and decision making (Karlsgodt et al., 2010). Disruptions of white matter integrity have also been described, supporting the conception of SCZ as a disorder of reduced neural connectivity (Wright et al., 2000). Interestingly, grey and white matter changes are already present by the time of FEP, indicating that they are important contributors to disease onset and are not caused by later disease progression or treatment effects (Karlsgodt et al., 2010). When comparing brain changes in healthy adolescents with FEP patients, there is a similar pattern of prefrontal surface contraction across the groups, with a higher magnitude in individuals suffering from

SCZ. This decline in grey matter volume is associated with normal developmental processes occurring at this time such as synaptic pruning (Sun et al., 2009). Differences in brain function have also been reported in SCZ patients, as measured by functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). Abnormal functional activation was especially found in working memory and episodic memory networks, which are areas of cognition that are known to be disrupted in SCZ (Karlsgodt et al., 2009; Karlsgodt et al., 2010).

Imbalances in neurotransmitter systems and cellular signaling involving dopamine, glutamate, serotonin and γ -aminobutyric acid (GABA) are also thought to play an important part in SCZ pathophysiology (Keshavan et al., 2020b). Dopamine dysfunction is one of the most intensively studied hypotheses and all available treatment options for SCZ predominantly act by antagonism of the dopamine D2 receptor. However, dopamine blockade is only effective against positive symptoms in some patients and does not improve negative or cognitive symptoms, indicating that other factors are involved. After identifying the psychotomimetic effects of N-methyl-D-aspartate (NMDA) receptor antagonists, a potential glutamatergic dysfunction was proposed for SCZ. Genetic findings supporting this idea have identified genes encoding glutamate receptors as potential loci for SCZ risk variants. However, glutamate modulating agents have had no clear clinical efficacy so far (McCutcheon et al., 2020). The involvement of serotonin has been implicated in SCZ pathogenesis after observations of the psychogenic effects of lysergic acid diethylamide (LSD), as well as antipsychotic effects of serotonin-dopamine antagonists. However, direct evidence of serotonergic dysfunction in SCZ pathogenesis is not available (Yang and Tsai, 2017). Parvalbumin-expressing GABAergic interneurons have also been studied in the context of SCZ and alterations in the GABA neurotransmitter system have been proposed to underlie alterations in neural synchrony, abnormal gamma oscillations and working memory impairments (Uhlhaas and Singer, 2010; Yang and Tsai, 2017). The development of novel treatment targets for SCZ has been challenging due to complex network-like interactions between neurotransmitter systems which have not been fully understood. Furthermore, it is not clear if changes in neurotransmitter systems are cause or consequence of the disease and cross-talk with environmental factors such as stress or drug abuse have to be taken into account.

An important role for immune processes in the pathogenesis of SCZ has been suggested by genetic and epidemiological studies. Prenatal infections and autoimmune diseases have been associated with a higher risk of SCZ and meta-analyses have shown increased plasma levels of pro-inflammatory cytokines such as IL-6 or IL-1 β in patients with FEP (Benros et al., 2011; Miller et al., 2011). Cytokines are largely produced by microglia in the brain and increased microglial activation has been visualized by PET imaging in patients with recent onset of SCZ symptoms (Doorduyn et al., 2009; van Berckel et al., 2008). Furthermore, beneficial effects of

anti-inflammatory medications have been described in the context of SCZ. Minocycline, a tetracycline antibiotic that inhibits microglial activation, has been described to have neuroprotective and antipsychotic-like effects in animal models, as well as efficacy in alleviating negative symptoms in patients when used in combination with classical antipsychotics (Chaudhry et al., 2012; Kelly et al., 2015; Levkovitz et al., 2007; Liu et al., 2014).

Extensive research on the role of microglia in the brain has indicated that microglial activation is not only a hallmark of neuroinflammation, but also plays an important role in neurodevelopmental processes such as synaptic pruning and brain plasticity (Schafer et al., 2012). Moreover, GWAS studies have identified a clear link between SCZ risk and the complement component C4A, a protein of the complement cascade and an important regulator of synaptic refinement (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Sekar et al., 2016). These findings have led to the synaptic pruning hypothesis in SCZ which will be further discussed below.

3.2 The complement system and synaptic pruning

3.2.1 The complement cascade in innate immunity

The complement system incorporates numerous soluble and membrane bound proteins which can recognize and mark pathogens, cellular debris and apoptotic cells for rapid elimination, thereby playing an important role in the innate immune system. Complement proteins are mainly produced by the liver and circulate throughout the body until they become activated upon binding to specific particles or membranes. Activation of the complement cascade results in proteolytic cleavage and assembly of active enzyme complexes. The complement system can be activated via three different routes: the classical pathway, the alternative pathway and the lectin pathway, all of which converge on the complement component C3 (Figure 1). C3 then covalently binds to cell surfaces and thereby localizes the innate immune response to specific cellular or tissue sites. The classical pathway is activated when C1q binds to antigen-antibody complexes, C reactive protein (CRP) or other pentraxins, polyanions (DNA, RNA, lipopolysaccharides), viral membranes or apoptotic cells (Stephan et al., 2012). After initiation, the C1 complex is formed by binding of the proteases C1s and C1r to the C1q tail region. After conformational change of C1q, the autocatalytic enzymatic activity of C1r is activated, which then cleaves and activates C1s, a serine protease. C1s cleaves C4 into C4a (anaphylatoxin) and C4b (opsonin) leading to a covalent binding of C4b to cell surfaces. After cleavage of C2 into C2a and C2b by C1s, the C3 convertase C4bC2a is formed which finally cleaves C3 into C3a and C3b. Activated C3b then binds to target surfaces to promote clearance (Presumey et al., 2017).

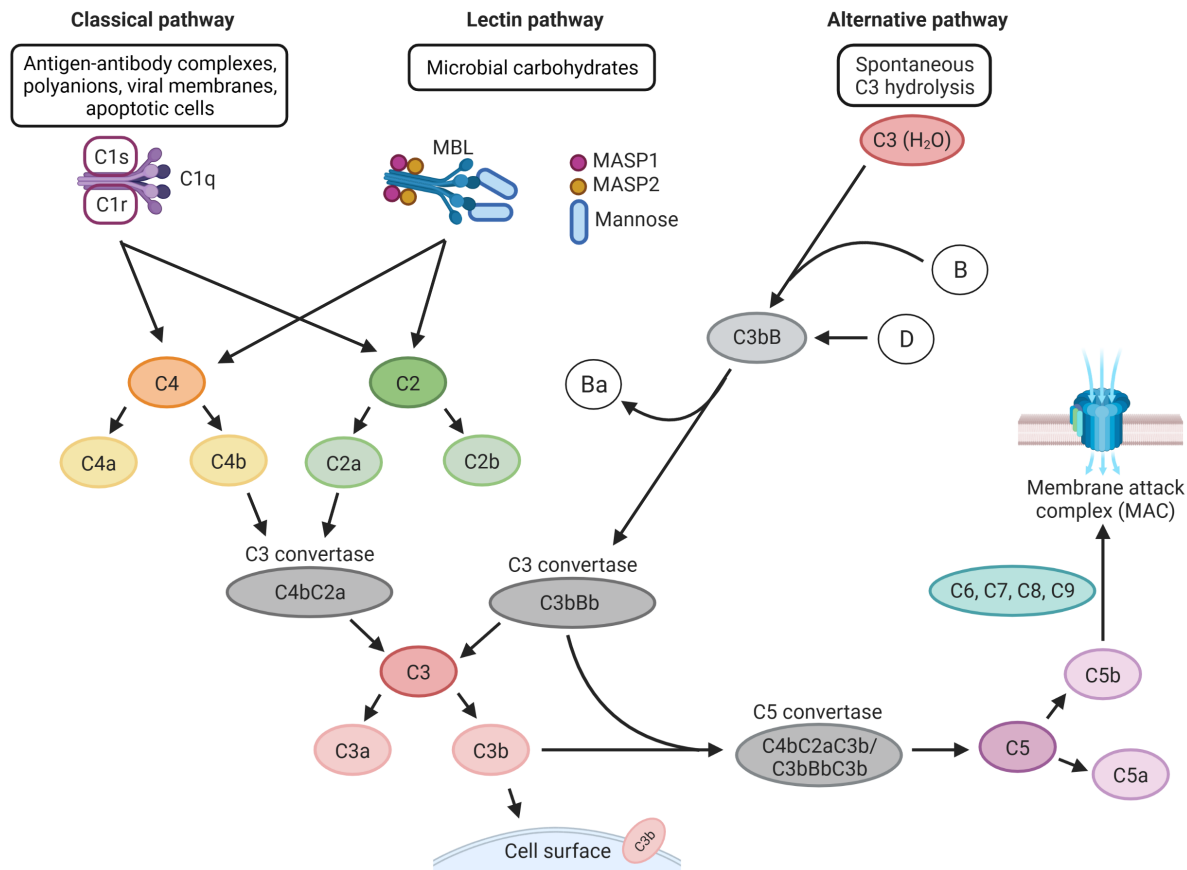


Figure 1: The complement system.

The complement cascade can be activated via three different routes. The classical pathway is initiated when C1q binds to antigen-antibody complexes, polyanions, viral membranes or apoptotic cells. The C1 complex, consisting of C1q, C1r and C1s, forms and cleaves C4 into C4a and C4b, and C2 into C2a and C2b. Together, C4b and C2a form the C3 convertase, which in turn cleaves C3 into C3a and C3b. C3b can either directly bind to target cell surfaces or act together with the C3 convertase to form a C5 convertase. C5 convertase cleaves C5 into C5a and C5b and activates the terminal complement cascade which leads to the assembly of the membrane attack complex (MAC) after engaging the complement proteins C6-C9. The lectin pathway is activated by binding of pattern-recognizing mannose-binding lectins (MBLs) to carbohydrate ligands on the surface of pathogens. Mannose-binding lectin serine proteases 1 and 2 (MASP 1 and 2) are activated and act similar to C1 complex by cleaving C4 and C2. The alternative pathway is initiated by spontaneous hydrolysis of C3. C3b binds to factor B that is subsequently cleaved by factor D. The C3bBb complex then also acts as a C3 convertase.

The lectin pathway is initiated by binding of mannose-binding lectin (MBL) to specific carbohydrate patterns on damaged cell surfaces or invading bacteria, fungi or viruses. This in turn activates the MBL-associated proteases mannose binding lectin serine protease 1 (MASP1) and MASP2 which function similar to C1s and cleave C4 and C2 to generate the C3 convertase. In contrast, the alternative pathway is spontaneously activated by hydrolysis of C3. C3b then binds to factor B which is cleaved by factor D to Bb. C3bBb complex then also acts as a C3 convertase. The alternative pathway mainly serves to amplify the cascade after initiation by the classical or lectin pathway. All three pathways finally converge on the cleavage of complement component C3 and the opsonization of target structures by C3b. Immune cells

like macrophages and microglia express C3-receptors which can bind to C3b leading to the elimination of these target structures by phagocytosis. Activation of the complement cascade can also initiate the assembly of the pore-forming membrane attack complex (MAC), which leads to the lysis of the target cell. Thereby, C3b and the C3 convertase (C4bC2a or C3bBb) act together to form the C5 convertase (C4bC2aC3b or C3bBbC3b) which then generates C5a and C5b and leads to the activation of the terminal complement cascade and the assembly of the C5b-C9 membrane attack complex (Coulthard et al., 2018; Nimgaonkar et al., 2017; Stephan et al., 2012).

3.2.2 Complement expression in the brain

The complement system is well known for its role as an important immune surveillance and defense system in the periphery, however, the blood brain barrier protects the brain from infiltrating immune cells and plasma-derived complement. Therefore, complement proteins are also produced locally in the brain by neurons and glial cells, whereby microglia and astrocytes are the major producers in the central nervous system (CNS) (Stephan et al., 2012). Microglia express especially high levels of C1q, as well as C3- and C5-receptors (CR3/CD11b and CR5), which makes them the most important cells to recognize and phagocytose structures that are marked for removal by complement proteins in the brain. Studies have shown the local synthesis of the most important complement components of the classical and alternative complement pathways by human astrocytes and microglia and their expression can be modulated by pro-inflammatory cytokines or toll-like receptor (TLR) activation. Neurons have also been shown to express the complete set of complement proteins locally in the brain, however the expression is lower compared to glial cells (Veerhuis et al., 2011). Recent work using human induced pluripotent stem cell (iPSC)-derived tri-cultures of microglia, astrocytes and neurons identified a neuroinflammatory axis, where C3 is potentiated by reciprocal signaling of microglia with astrocytes, indicating the involvement of a more complex feed-back loop between the different cell types (Guttikonda et al., 2021). Furthermore, it has been shown that astrocyte-derived transforming growth factor beta (TGF- β) is a key regulator of C1q expression in neurons (Bialas and Stevens, 2013). Complement proteins are known to be upregulated in neural cells after brain injury or in post mortem Alzheimer's disease (AD) brain tissue, however, more recently, novel nonimmune roles of the complement system in brain development have been identified, including neurogenesis, migration, neuronal survival and synaptic pruning (Stephan et al., 2012).

3.2.3 Complement-dependent synaptic pruning

Synaptic pruning is a process of activity-dependent refinement and synaptic plasticity which is essential for normal brain development. Thereby, an excess number of axons, dendrites and synapses are initially produced and are then eliminated over the course of development in order to form mature and precise neural circuits. Synaptic pruning is defined as the elimination of pre-synaptic terminals and post-synaptic membranes in consequence of which the remaining synapses are maintained and strengthened (Faust et al., 2021). There are a number of molecular mechanisms linking neuronal activity with the physical removal of certain synapses and strengthening of others. The retinogeniculate system has been one of the most important circuits to study spontaneous neural activity-driven synaptic pruning. In early postnatal mice, eye-specific territories still overlap, as retinal ganglion cells (RGCs) from both eyes form synapses with the same relay neurons in the dorsolateral geniculate nucleus (dLGN) of the thalamus. During development, synaptic pruning removes these extra connections so that eye-specific territories can be generated. It has been shown that waves of spontaneous activity drive segregation of RGC inputs and that if spontaneous activity is blocked or increased in one eye, the synaptic territory of the less active RGCs is reduced and the territory of the more active RGCs is expanded (Penn et al., 1998; Stellwagen and Shatz, 2002). The retinogeniculate system has also served as a useful tool to unravel potential molecular mechanisms underlying activity-dependent synaptic pruning. First clues for the role of the complement cascade in synapse development came from the identification of the developmental expression and synaptic localization of classical complement proteins in the postnatal brain. C1q was identified as one of the few genes highly upregulated in developing RGCs during the period of active synaptic refinement. Furthermore, downstream complement component C3 was shown to localize to subsets of synapses throughout the developing brain. The role of the classical complement cascade in synaptic pruning was thoroughly studied using the developing retinogeniculate system as a well-established model system. Thereby, C1q and C3 knockout (KO) mice were found to have defects in synaptic elimination, as shown by their failure to segregate into eye-specific territories (Stevens et al., 2007). Later studies using C4 KO mice showed similar defects in synaptic pruning activity and decreased localization of C3 at RGC axon terminals in the dLGN (Sekar et al., 2016). Although C1q and C3 KO mice display defects in synaptic pruning activity, these mice still undergo a certain degree of synapse elimination, indicating the co-existence of other pathways that can regulate synaptic refinement (Stevens et al., 2007). Other neuronal immune-related molecules, such as class I major histocompatibility complex antigens (MHC class I) and neuronal pentraxins, synaptic proteins with homology to pentraxins of the peripheral immune system, have been identified in this context (Stephan et al., 2012). Thereby, mice lacking cell surface MHC class I or neuronal pentraxin 1 (NP1) and neuronal pentraxin 2 (NP2) expression also showed increased numbers

of synapses and deficits in eye-specific segregation in the retinogeniculate system (Bjartmar et al., 2006; Huh et al., 2000; Lee et al., 2014). Recent work has further confirmed the co-localization of neuronal pentraxins with C1q at cortical synapses (Kovacs et al., 2020). A possible role for pentraxins in this context could be to provide a synaptic binding site to which the otherwise highly soluble molecule C1q can bind to initiate complement signaling (Perry and O'Connor, 2008). The exact mechanisms of interaction, however, remain unclear.

3.2.4 The role of microglia in synaptic pruning

Microglia, the immune cells of the brain, fulfill a variety of different tasks mainly related to both immune response and maintaining homeostasis. Microglia provide a first line of defense against invading pathogens and are able to secrete a wide range of chemokines and pro- and anti-inflammatory cytokines. Microglia further play an important role during CNS development by secreting neurotrophic factors and growth factors and by contributing to the maturation of neural circuits (Wolf et al., 2017). As mentioned above, the activated complement component C3 fragment C3b marks the surface of cells or debris for removal by phagocytic macrophages as an important part of the innate immune system in the periphery. C3b is also thought to mark synapses for removal during the synaptic pruning process and interestingly, microglia are the only cells in the brain that express the complement component C3 receptor (CR3) (Figure 2) (Ransohoff and Perry, 2009; Stephan et al., 2012). Microglia are highly motile cells which are constantly sensing their environment and their role in synapse development has been supported by the observation of their close interaction with dendritic spines (Presumey et al., 2017). The internalization of synaptic material by microglia has been visualized in the retinogeniculate system of mice during the main period of synaptic pruning with the use of high-resolution imaging and electron microscopy (EM). Thereby, presynaptic inputs labeled with the anterograde tracer cholera toxin subunit B (CTB) co-localized with the microglia-specific lysosomal marker CD68 (Schafer et al., 2012). Furthermore, the disruption of microglia-mediated synapse engulfment by KO of CR3 in mice or by pharmacological inhibition of microglia activation with minocycline resulted in a defect in eye-specific segregation and synaptic pruning, suggesting the process to be microglia- and complement-dependent (Schafer et al., 2012). Other studies have demonstrated the engulfment of postsynaptic structures in other brain regions, such as the hippocampus and the visual cortex, indicating that complement-dependent engulfment of synapses by microglia could be a global mechanism in the CNS. Thereby, mice lacking the fractalkine receptor CX3CR1, which leads to a delayed infiltration of microglia into the brain, displayed impaired synapse maturation, elevation in spine density and increased synaptic connectivity (Paolicelli et al., 2011; Tremblay et al., 2010; Zhan et al., 2014). Furthermore, it has been shown that microglia eliminate synapses in the adult hippocampus which leads to the dissociation of engrams and forgetting

of previously learned contextual fear memory in mice. Interestingly, by depletion of microglia, general inhibition of microglial phagocytosis or specific inhibition of complement pathways in engram cells, synapse elimination and forgetting can be prevented (Wang et al., 2020).

To date, most research characterizes microglia as phagocytic cells which engulf entire synapses to mediate synaptic pruning, however, the precise mechanisms and synaptic structures involved in the process are still unknown. High resolution correlative light and electron microscopy (CLEM) data suggests a selective partial phagocytosis of only small fragments of presynaptic structures, termed trogocytosis, whereas other studies visualize postsynaptic density (PSD)-like material inside microglia by EM or show a co-localization of PSD-95 immunoreactive post-synaptic puncta with microglia (Paolicelli et al., 2011; Weinhard et al., 2018).

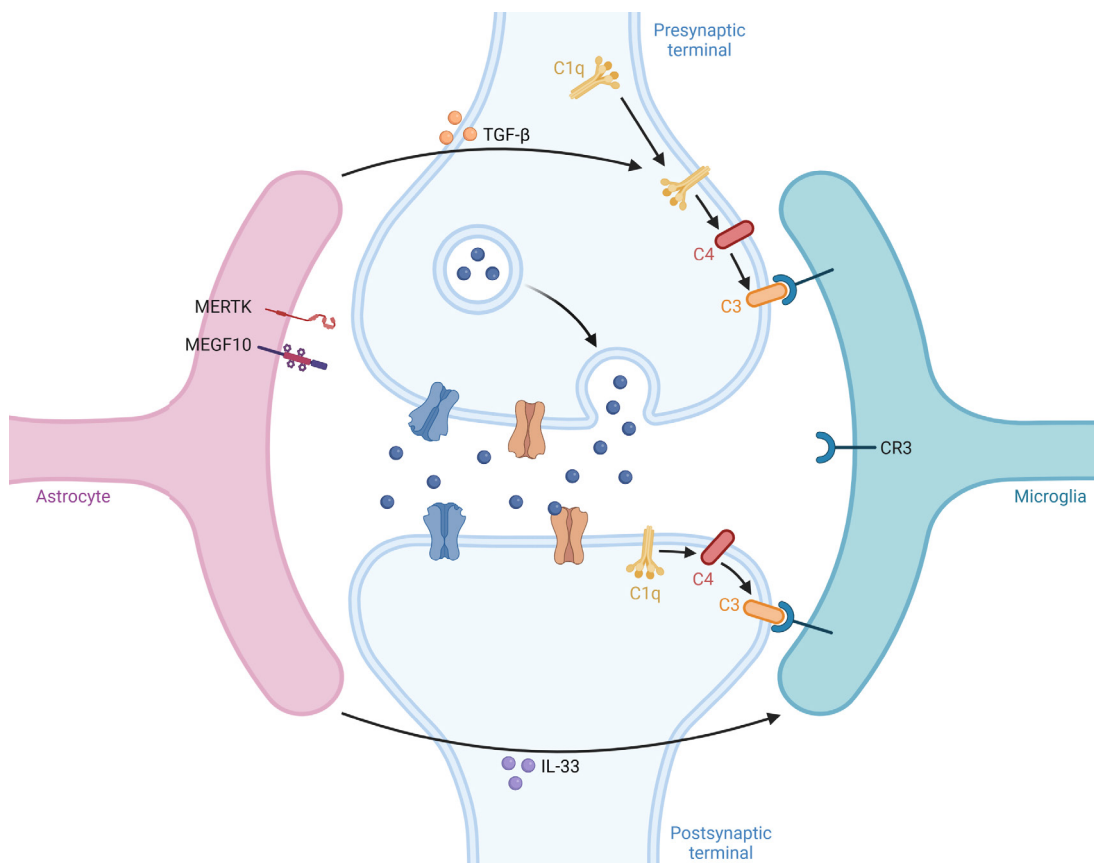


Figure 2: Glial cells in synaptic pruning.

Complement proteins mark less active synapses for removal during normal brain development. Activated complement component C3 binds to the cell surface after activation of the complement cascade and is recognized by microglia through binding of the complement component C3 receptor (CR3). Astrocytes are thought to participate in synapse elimination via the two phagocytic receptors MEGF10 and MERTK and by secreting factors that promote complement expression in neurons and synapse engulfment by microglia such as TGF- β and IL-33.

Although there have been many studies confirming the role of complement proteins in synaptic pruning, it is still unclear how complement leads to the elimination of specific synapses, but not others. Recently, new research has shown that the canonical “don’t eat me” signal CD47 might be involved. CD47 was found to be expressed by RGC and its receptor SIRP α by microglia and the expression patterns of both proteins correlated with peak pruning activity in the retinogeniculate system. Furthermore, CD47- and SIRP α -deficient mice displayed increased pruning and reduced synapse numbers (Lehrman et al., 2018). Another study was able to show that the endogenous C1q inhibitor SRPX2 is expressed by neurons and that SRPX2-deficient mice show increased C3 deposition and microglial synapse engulfment (Cong et al., 2020).

Microglia are the most extensively studied cell type in the context of synaptic pruning, however, other cell types such as astrocytes are likely to be involved in the process. Identification of the now well-defined tripartite synapse, referring to bidirectional communication between astrocytes and neurons, as well as studies reporting that astrocytes can participate in synapse elimination via the two phagocytic receptors MEGF10 and MERTK, further underline this idea (Chung et al., 2013; Halassa et al., 2007; Husemann et al., 2002; Perea et al., 2009). Furthermore, it was shown that interleukin-33 (IL-33), as well as TGF- β are produced by developing astrocytes promoting synapse engulfment by microglia under physiological conditions (Bialas and Stevens, 2013; Vainchtein et al., 2018).

3.3 The complement system and SCZ pathogenesis

3.3.1 C4 structural variation in SCZ

Important evidence suggesting a link between the complement system and SCZ pathogenesis is coming from large-scale GWAS. In these studies, more than 100 regions of the genome have been identified that associate with the risk of SCZ. The most significantly associated locus for SCZ lies within the MHC region on chromosome 6 and involves structurally distinct alleles of the *C4* gene (Schizophrenia Working Group of the Psychiatric Genomics, 2014). The human *C4* locus has complex genetic characteristics (Figure 3). There are two paralogous forms of the gene, *C4A* and *C4B*, which have a sequence homology of over 95%, but nevertheless encode proteins with different substrate affinities and molecular targets (Isenman and Young, 1984; Law et al., 1984). The insertion of a human endogenous retroviral (HERV) sequence into intron 9 gives rise to a long (*C4L*) and a short form of the gene (*C4S*) without changing the protein sequence (Dangel et al., 1994). Therefore, four distinct genomic forms of the *C4* gene exist (*C4AL*, *C4AS*, *C4BL* and *C4BS*) and the number of copies of each of these forms can vary from two to eight in a diploid genome (Belt et al., 1984). However, analysis of a European ancestry population sample of 111 unrelated individuals showed that the four most common *C4* haplotypes AL-BL, AL-BS, AL-AL and BS are present on 90% of the

chromosomes (Sekar et al., 2016). *C4A* and *C4B* expression both increase with increased gene copy number and analysis of genetic data from a large cohort of the Psychiatric Genomics Consortium (PGC) revealed that specific alleles leading to a higher expression of *C4A* correlate with an increased risk for the development of SCZ (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Sekar et al., 2016). Furthermore, *C4A* RNA expression is significantly increased in post-mortem brain tissue from SCZ patients compared to controls (Sekar et al., 2016). The binding sites of C4 on synapses are not known, however, in the peripheral immune system C4A proteins are more important for clearing immune complexes and C4B proteins are involved in the defense against microbes (Carroll et al., 1990). Due to the different chemical binding preferences, it is thought that C4A binds more efficiently to synapses than C4B, thereby providing a link to the hypothesis of increased complement-dependent synaptic pruning in SCZ (Presumey et al., 2017). Immunostainings of post-mortem brain tissue from SCZ patients, as well as human primary cortical neurons, further revealed a co-localization of C4 with synaptic structures (Sekar et al., 2016).

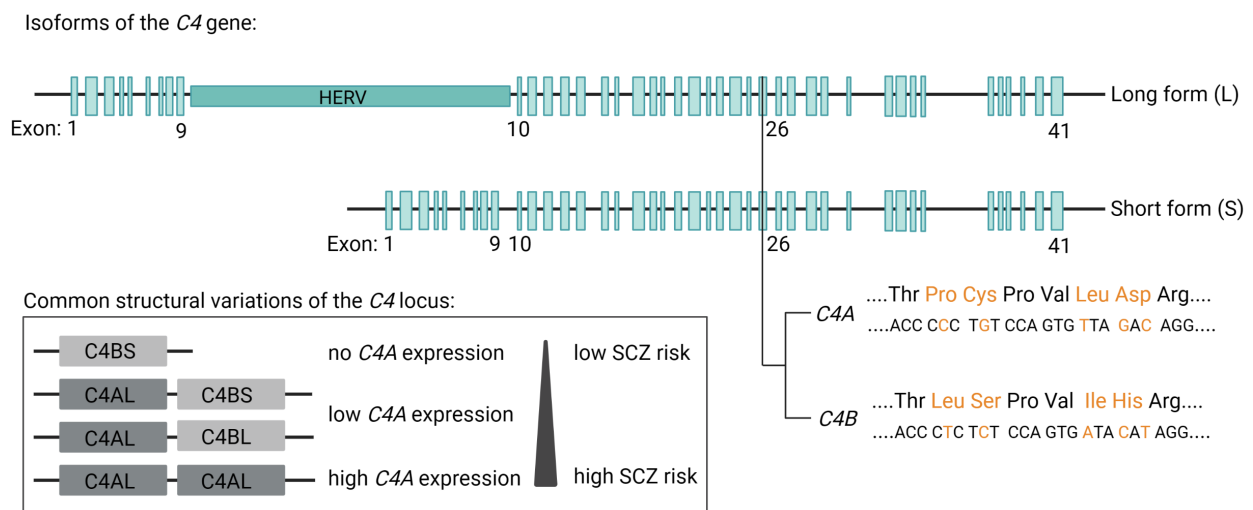


Figure 3: Structural variation of the complement component 4 (*C4*) gene.

The human *C4* gene exists as two isoforms, *C4A* and *C4B*, which differ in a five-nucleotide sequence polymorphism located in exon 26. Long (L) and short (S) genomic forms are distinguished by the presence or absence of a human endogenous retroviral (HERV) sequence in intron 9. Four common structural variations of the *C4* gene exist and increased gene copy number leads to increased gene expression. High copy numbers of C4AL have been correlated with an increased risk for SCZ. Adapted from (Sekar et al., 2016).

3.3.2 *C4* expression as a cerebrospinal fluid (CSF) and serum biomarker in SCZ

Studies comparing *C4* mRNA and protein levels in the peripheral blood or cerebrospinal fluid (CSF) of patients with FEP who develop SCZ and healthy controls further confirm complement pathway perturbations in SCZ pathogenesis, suggesting aberrant complement protein expression as a potential biomarker for SCZ. *C4A* mRNA expression in the peripheral blood

and C4A serum protein levels were investigated and compared to *C4* locus structural alleles. Thereby, the effect of *C4* structural variants on gene expression was confirmed by showing higher *C4A* mRNA levels in FEP cases harboring the previously described SCZ risk alleles. Interestingly, the mRNA expression of the CUB and Sushi multiple domains 1 (*CSMD1*) gene, which encodes a protein inhibitor of C4A activity and has also been implicated in SCZ risk, was decreased in SCZ patients with FEP (Hatzimanolis et al., 2022; Sekar et al., 2016). Furthermore, higher *C4A* mRNA levels and lower *CSMD1* mRNA levels in peripheral blood cells were shown to correlate with more severe SCZ symptoms (Hatzimanolis et al., 2022; Melbourne et al., 2018). C4A protein levels were found to be increased in plasma as well as in CSF from patients with SCZ (Chen et al., 2021; Gallego et al., 2021; Gracias et al., 2022; Kalinowski et al., 2021; Laskaris et al., 2019; Walss-Bass et al., 2019). In line with the previously described effect of high C4A expression on microglial synapse engulfment, CSF C4A protein concentration was shown to inversely correlate with CSF levels of neuronal pentraxins in SCZ patients (Gracias et al., 2022). NP1 and NP2 protein levels are used as an *in vivo* proxy of synapse density and can predict PFC thickness. Thereby, reduced levels of NP1 and NP2 predict synapse loss and PFC thinning (Nilsson et al., 2021; van der Ende et al., 2020). Interestingly, elevation of C4A protein levels in the CSF of SCZ patients with FEP was shown to exceed the expression expected from variance in the *C4* locus. Thereby, a novel mechanism was discovered by which SCZ associated cytokines IL-6 and IL-1 β induce *C4A* mRNA expression providing a link to studies showing increased immune activation in SCZ, including increased levels of pro-inflammatory cytokines such as IL-6 and IL-1 β (Fillman et al., 2016; Gracias et al., 2022; Jacomb et al., 2018; North et al., 2021). In addition to general changes in blood and CSF levels of complement proteins, studies have also found an increased complement activation in peripheral blood of patients with SCZ. C4-anaphylatoxin (C4a) levels, a C4 protein fragment released upon C4 activation after initiation of the classical and lectin complement pathways, were higher in SCZ patients compared to healthy controls. Moreover, initial experiments have shown that C4a binding to protease-activated receptors 1/4 (PAR1/4) on endothelial cells can disrupt the blood-brain barrier (BBB) and thereby further contribute to neurodegeneration in individuals suffering from SCZ (Kalinowski et al., 2021).

3.3.3 C4 overexpression in mouse models of SCZ

Further insights into complement-dependent synaptic pruning in the context of SCZ pathogenesis come from *C4A* overexpression mouse models. In mice, only one *C4* isoform exists, which evolved into *C4A* and *C4B* in primates (Belt et al., 1985; Carroll et al., 1984; Nonaka et al., 1985). Given these limitations, mouse models expressing human *C4A* or *C4B* have been generated. Thereby, quantifications of C4 deposition at the synapses confirmed the finding that C4A binds synapses more efficiently than C4B. Moreover, human *C4A* expression,

but not *C4B*, rescued deficits in synaptic pruning and segregation of RGC projections into nonoverlapping territories in the dLGN of *C4* KO mice. Interestingly, human *C4A* overexpression in mice resulted in an excessive synaptic loss in the adult medial prefrontal cortex (mPFC) due to increased engulfment by microglia. Behavioral studies in these mice further demonstrated altered social behavior, increased anxiety and deficits in spatial working memory, similar to negative symptoms experienced by SCZ patients (Yilmaz et al., 2021).

Another study overexpressing *C4* in mice showed similar effects of mouse and human *C4*. Thereby, effects of the overexpression of the mouse homologue of *C4* were investigated in the mPFC. Increased *C4* expression led to defects in dendritic spine density, preferentially affecting less mature protrusions. Electrophysiological recordings demonstrated reduced functional connectivity in *C4* overexpressing cortical neurons with no changes in overall intrinsic excitability. Confocal imaging revealed enhanced microglia engulfment of postsynaptic PSD-95 in *C4* overexpressing mice and behavioral experiments suggested long-lasting alterations in PFC circuitry leading to a reduction in maternal-pup social interactions. These experiments further confirm that increased *C4* expression drives circuit dysfunction and SCZ-related behavioral changes rather than being a consequence of the disease (Comer et al., 2020).

Taken together, these different lines of evidence support the involvement of complement component *C4A* in the development of SCZ offering a mechanism linking known genetic risk variants to observed structural brain changes during an early phase of disease onset.

3.4 The NLRP3 inflammasome: a potential new target for SCZ

3.4.1 The NLRP3 inflammasome pathway

The NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) inflammasome pathway is an important innate immune cascade which plays an important role in regulating inflammatory signaling. The NLRP3 inflammasome is a multiprotein complex that assembles in the cytosol after being triggered by pathogen-associated stimuli and subsequently activates caspase-1 and the secretion of pro-inflammatory cytokines IL-1 β and IL-18. A variety of different stimuli such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are known to activate the NLRP3 inflammasome, however, the exact cellular events initiating the cascade are not fully understood (Kelley et al., 2019).

For canonical activation of the NLRP3 inflammasome, a two-signal model has been proposed. In a first step, cells are exposed to a priming signal, which is provided by microbial components or endogenous cytokines. These stimuli are ligands for TLRs, cytokine receptors or NOD-like receptors (NLRs), which activate the nuclear factor kappa B (NF κ B) pathway. Activation of the

transcription factor NF κ B leads to a subsequent upregulation of *NLRP3*, *IL1 β* and *IL18* expression (Kelley et al., 2019). NLRP3 is a cytosolic pattern recognition receptor (PRR) protein, which consists of a C-terminal leucine-rich repeat (LRR) domain, an ATPase-containing NACHT (NAIP, CIITA, HET-E, and TP1) domain and an N-terminal pyrin domain (PYD) which recruits proteins for the formation of the inflammasome complex (Figure 4A) (Blevins et al., 2022). Under resting conditions NLRP3 is only expressed at low concentrations, insufficient for initiation of the inflammasome pathway (Kelley et al., 2019).

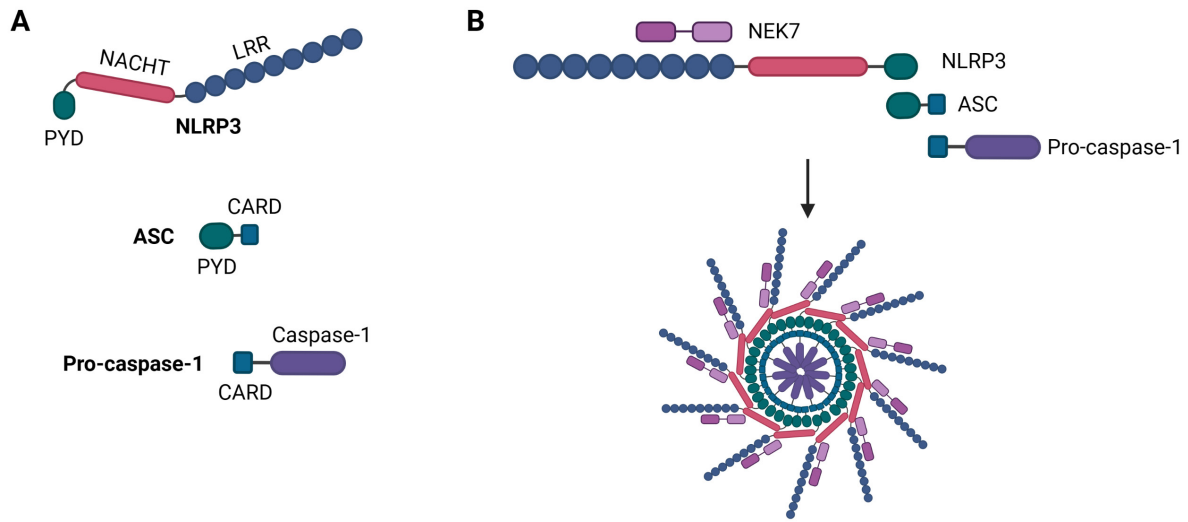


Figure 4: The NLRP3 multiprotein complex.

(A) The NLRP3 protein is comprised of three domains: an N-terminal pyrin domain (PYD), an ATPase-containing NACHT (NAIP, CIITA, HET-E, and TP1) domain and a C-terminal leucine-rich repeat (LRR) domain. The adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) contains PYD and CARD domains and pro-caspase-1 carries a CARD and caspase-1 domain. (B) Upon activation, NIMA-related kinase 7 (NEK7) binds to NLRP3 and causes a structural rearrangement. NLRP3 then binds ASC via their shared PYD domains. ASC then recruits the effector protease pro-caspase-1 through its CARD domain. Activation of the NLRP3 inflammasome pathway results in the formation of the NLRP3 inflammasome multiprotein complex.

The NLRP3 inflammasome complex is assembled upon activation of the sensor protein NLRP3 by a secondary stimulus. NLRP3 can be activated by many different signals including extracellular ATP, toxins or pathogens such as bacteria, fungi or viruses (Jo et al., 2016; Latz et al., 2013). These activators can further induce cellular processes including ion fluxes such as K⁺ efflux, Ca²⁺ signaling or Na⁺ influx, the production of mitochondrial reactive oxygen species (ROS) or lysosomal damage (Blevins et al., 2022; Cruz et al., 2007; Hornung et al., 2008; Katsnelson et al., 2015; Munoz-Planillo et al., 2013). Due to the large variety of activating signals, it has been proposed that NLRP3 senses a common cellular event caused by these stimuli rather than directly binding to them, however the exact mechanism is unknown (Kelley et al., 2019). One proposed mechanism has been the disassembly of the *trans*-Golgi network (TGN) by multiple NLRP3 stimuli (Chen and Chen, 2018). Upon inflammasome activation,

NIMA-related kinase 7 (NEK7) binds to NLRP3 which is hypothesized to cause a structural rearrangement and an exposure of the PYD domain (Andreeva et al., 2021). The PYD domain of NLRP3 then recruits the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) to the multiprotein complex which then binds the effector protease pro-caspase-1 through its CARD domain (Figure 4B). Pro-caspase-1 is converted to its active form via proximity-induced autocatalysis into mature caspase-1. Active caspase-1 then cleaves pro-IL-1 β and pro-IL-18 into their mature forms IL-1 β and IL-18 (Blevins et al., 2022; Lu and Wu, 2015; Malik and Kanneganti, 2017). The secretion of pro-inflammatory cytokines activates signaling pathways resulting in the up-regulation of additional inflammatory reactions and recruitment of immune cells. Alternatively, the inflammasome can induce pyroptosis, an inflammatory form of programmed cell death, through activation of the membrane pore-forming gasdermin D (GSDMD) which is also cleaved by caspase-1 (Kesavardhana and Kanneganti, 2017; Shi et al., 2015). The resulting disruption of the cell leads to release of intracellular contents including pro-inflammatory cytokines such as IL-1 β and IL-18 (Figure 5) (Ding et al., 2016; Evavold et al., 2018).

The NLRP3 inflammasome can also be activated through non-canonical or alternative pathways. The non-canonical inflammasome results in the activation of caspases-4/5 in humans (caspase-11 in mice) and can be activated by Gram-negative bacteria (Baker et al., 2015; Kayagaki et al., 2015). Since caspase-4 is constitutively expressed in human cells, the non-canonical inflammasome can be activated without an additional priming step through binding of cytosolic Lipopolysaccharide (LPS) (Yang et al., 2015). Cytosolic LPS can be directly recognized by caspases-4/5 resulting in their autoproteolysis and activation. Canonical and non-canonical inflammasome activation have a similar outcome leading to pyroptosis and the subsequent release of pro-inflammatory cytokines (Blevins et al., 2022). Furthermore, an alternative activation pathway has been proposed where LPS alone can induce caspase-1-dependent IL-1 β maturation via TLR4 signaling (Netea et al., 2009). Thereby, activation of NLRP3 is mediated by TIR-domain-containing adapter-inducing interferon- β (TRIF) and cleavage of caspase-8 through the TLR4–TRIF–receptor-interacting protein kinase 1 (RIPK1)–Fas-associated protein with death domain (FADD)–caspase-8 signaling pathway (Gaidt et al., 2016). Overall, the activation of the NLRP3 inflammasome is regulated by various different molecular processes, which occur independently or can be closely interconnected (Fusco et al., 2020).

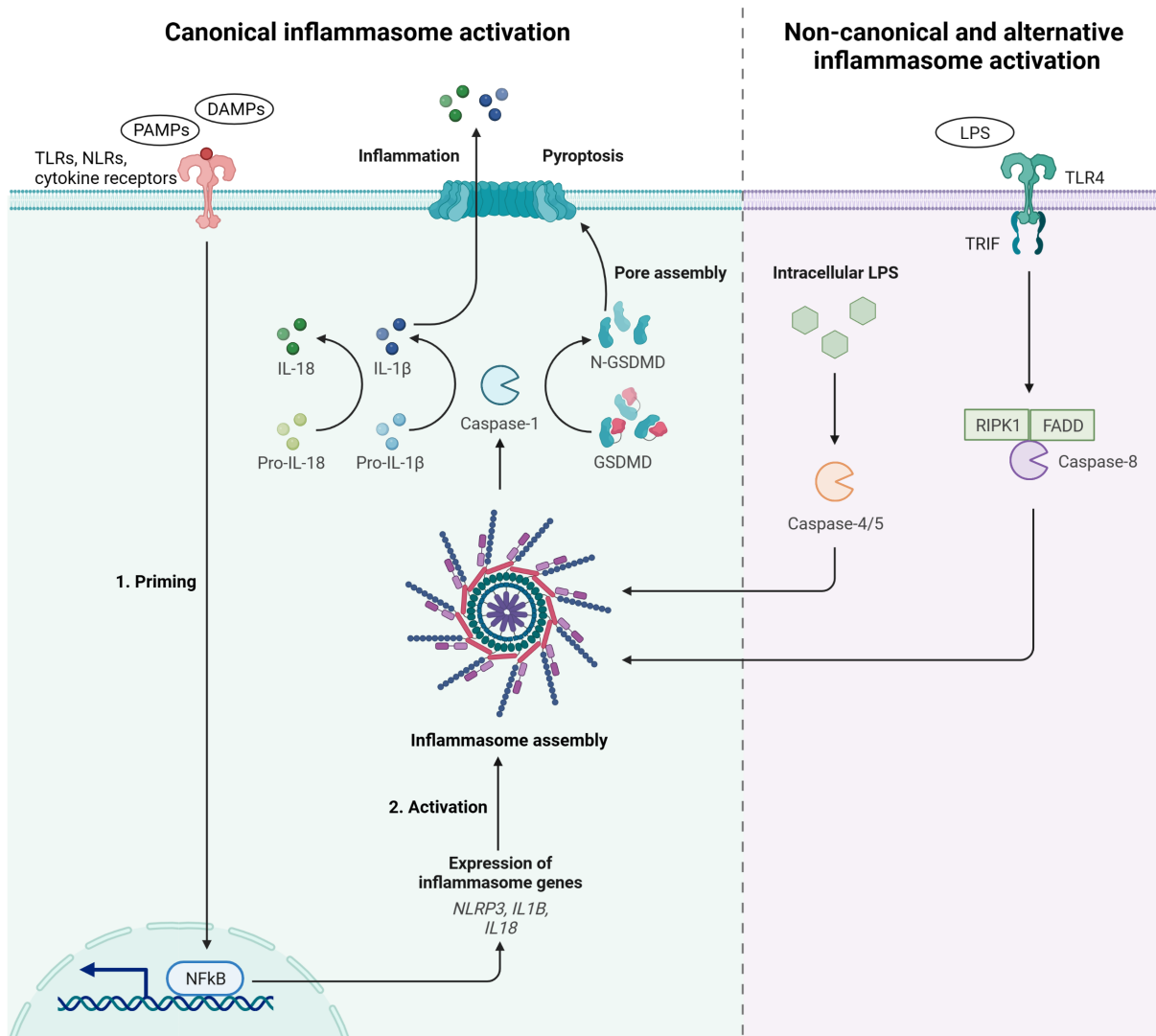


Figure 5: Schematic representation of mechanisms regulating inflammasome activation.

Canonical inflammasome activation is a two-step process. The first step is a priming step, which is triggered by ligands of Toll-like receptors (TLRs), cytokine receptors or NOD-like receptors (NLRs), such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Priming leads to the activation of the nuclear factor kappa B (NFκB) pathway and subsequent upregulation of *NLRP3*, *IL1B* and *IL18* expression. Upon activation, the inflammasome multiprotein complex is assembled which in turn causes caspase-1 activation, leading to the maturation of IL-1β and IL-18 or pyroptosis through activation of the membrane pore-forming gasdermin D (GSDMD). Non-canonical inflammasome activation is mediated by caspases 4 and 5 without a priming step through binding of intracellular Lipopolysaccharide (LPS). An additional alternative activation pathway can be triggered by binding of LPS to TLR4 and the subsequent activation of the TRIF-RIPK1-FADD-caspase-8 signaling pathway.

TRIF: TIR-domain-containing adapter-inducing interferon-β, RIPK1: receptor-interacting protein kinase 1, FADD: Fas-associated protein with death domain.

3.4.2 The NFκB pathway

NFκB is a family of highly conserved dimeric transcription factors which play an important role in innate and adaptive immunity, as well as cellular differentiation, proliferation and survival (Mitchell et al., 2016). In mammals, the NFκB family is composed of five protein monomers, including NFκB-1 (p50), NFκB-2 (p52), RelA (p65), RelB and c-Rel, which can form different homo- or heterodimers with individual DNA binding characteristics (Hoffmann et al., 2003; Leung et al., 2004; Sanjabi et al., 2000). The NFκB proteins are normally sequestered in the cytoplasm by ankyrin repeat-containing IκB proteins, which act as inhibitors. Activation of NFκB is tightly regulated and involves a canonical and noncanonical pathway (Figure 6).

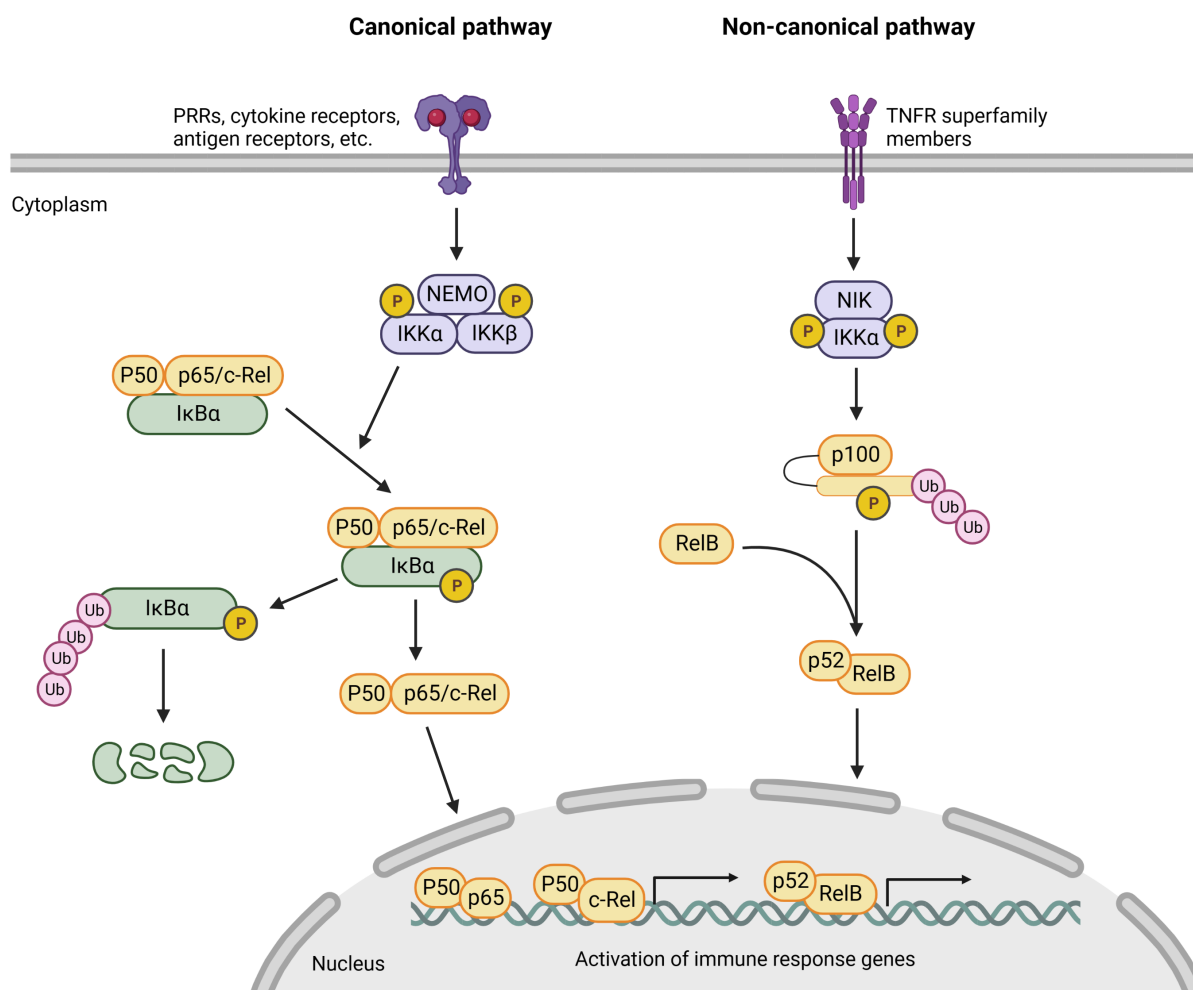


Figure 6: Canonical and non-canonical NFκB pathways.

The canonical NFκB pathway can be activated by ligands of pattern recognition receptors (PRRs), cytokine receptors or antigen receptors. The NFκB heterodimers p50/p65 and p50/c-Rel are kept in an inactive state in the cytoplasm through interaction with IκBα inhibitory proteins. Upon stimulation of NFκB signaling, IκBα proteins are degraded through site-specific phosphorylation by the IκB kinase (IKK) complex, which is composed of the two subunits IKKα and IKKβ, as well as a NFκB essential modulator (NEMO). Phosphorylation marks IκBα proteins for proteasomal degradation, resulting in rapid translocation of canonical NFκB dimers into the nucleus. Non-canonical NFκB signaling is activated by the specific tumor necrosis factor receptor (TNFR) superfamily. Receptor activity induces the activation

of NF κ B-inducing kinase (NIK) and phosphorylation of IKK α . Subsequent phosphorylation and ubiquitination of p100 leads to the degradation of the C-terminal I κ B-like structure and the generation of mature p52. p52/RelB dimers are formed which can then translocate to the nucleus.

The canonical NF κ B pathway responds to a wide variety of stimuli such as ligands of cytokine receptors and PRRs (Liu et al., 2017). The primary mechanism of canonical NF κ B activation is the inducible degradation of I κ B α through site-specific phosphorylation by the I κ B kinase (IKK) complex, which is composed of the two catalytic subunits IKK α and IKK β , as well as a NF κ B essential modulator (NEMO) (Karin and Delhase, 2000; Sun and Ley, 2008). Phosphorylation of I κ B α triggers ubiquitin-dependent degradation by the proteasome, resulting in rapid translocation of canonical NF κ B dimers into the nucleus, predominantly p50/p65 and p50/c-Rel (Liu et al., 2017). Following I κ B release the NF κ B subunits are subject to posttranslational modifications which can fine-tune gene expression control (Chen and Greene, 2004; Mitchell et al., 2016). NF κ B activity is strictly regulated by negative feedback loops to keep the system sensitive for reactivation and to prevent hyperactivity. Among the target genes regulated by κ B sites on the DNA are I κ Bs which translocate to the nucleus after resynthesis, bind to NF κ B dimers and inhibit NF κ B activity. After inhibition, NF κ B proteins are shuttled back to the cytosol (Hoffmann et al., 2002).

The noncanonical NF κ B pathway is activated by specific ligands of tumor necrosis factor receptors (TNFRs) and involves processing of the NF κ B-2 precursor protein p100 instead of I κ B α degradation (Sun, 2012). Thereby, NF κ B-inducing kinase (NIK) functionally cooperates with IKK α to mediate p100 phosphorylation, ubiquitination and processing (Xiao et al., 2001). The C-terminal I κ B-like structure is degraded, resulting in the generation of mature NF κ B-2 (p52) and nuclear translocation of the p52/RelB dimer (Liu et al., 2017). NIK-induced p100 processing and subsequent dimerization with RelB is a slower process compared to the release of NF κ B dimers from I κ B inhibitors. Therefore, the noncanonical NF κ B pathway results in a late-phase and sustained gene-expression response (Mitchell et al., 2016).

3.4.3 The NLRP3 inflammasome in neurodegenerative and psychiatric disorders

The NLRP3 inflammasome is critical for defense against bacterial, viral or fungal infections, however, it has also been associated to a number of neurodegenerative diseases such as AD and Parkinson's disease (PD), as well as psychiatric disorders such as depression, bipolar disorder (BD) and SCZ where it plays an important role in microglia-mediated inflammation (Fusco et al., 2020).

AD is characterized by the presence of amyloid β (A β) plaques and neurofibrillary tangles (NFTs) composed of A β peptides and abnormally hyperphosphorylated tau, respectively (Glenner and Wong, 1984; Grundke-Iqbal et al., 1986). *In vitro* and *in vivo* studies have

suggested an important role for the NLRP3 inflammasome in AD by showing that A β can stimulate NLRP3 activation and IL-1 β secretion by microglia (Halle et al., 2008; Heneka et al., 2013). Interestingly, cross-talk between complement and inflammasome signaling pathways has been described in this context. For canonical NLRP3 inflammasome activation, the NF κ B pathway can be triggered via the C3/C5 mitogen-activated protein kinase (MAPK) pathway which is induced by C3a/C5a binding to their respective receptors (Yang et al., 2020). Complement components C1q, C3, C4 and C5b-C9 have been shown to accumulate in A β plaques and NFTs of AD patients, leading to synapse pruning and loss by microglial phagocytosis (Ishii and Haga, 1984; Rogers et al., 1992; Stoltzner et al., 2000; Yang et al., 2020). In cell culture experiments, stimulation of human monocytes with aggregated A β and C5a has been shown to increase IL-1 β and IL-6 secretion via NF κ B activation (O'Barr and Cooper, 2000). Furthermore, the formation of the complement MAC has been shown to trigger increased cytosolic Ca²⁺ concentrations resulting in NLRP3 inflammasome activation (Triantafilou et al., 2013).

In the context of PD, a neurodegenerative disorder characterized by the aggregation of α -synuclein (α Syn) and the death of dopaminergic neurons in the substantia nigra, studies have also confirmed an increased activation of the NLRP3 inflammasome (Fusco et al., 2020; Shulman et al., 2011). Extracellular α Syn has been shown to induce the activation of microglia and astrocytes and increase the production of IL-1 β (Beraud and Maguire-Zeiss, 2012; Lee, 2008). Furthermore, in human monocytes, treatment with fibrillary α Syn increases pro- IL-1 β levels via TLR2 signaling, as well as activation of caspase-1 and the production of mature IL-1 β (Codolo et al., 2013). Interestingly, NLRP3 deficient mice were found to be resistant to the development of PD symptoms after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injections and dopamine has been described to negatively regulate NLRP3 priming in microglia and astrocytes (Yan et al., 2015).

Activation of the NLRP3 inflammasome has also been described in the context of psychiatric disorders. Individuals suffering from major depressive disorder (MDD) have been shown to display increased levels of pro-inflammatory cytokines IL-1 β , IL-6, TNF α , as well as CRP (Nettis and Pariante, 2020). In mouse models, LPS treatment was shown to induce NLRP3 inflammasome activation and increase in pro-inflammatory cytokine expression, which was accompanied by the induction of depressive-like behavior (Zhang et al., 2014). Furthermore, clinical studies have reported a correlation between IL-1 β and IL-18 levels and Beck Depression Inventory (BDI) scores and postmortem studies have confirmed increased protein and mRNA levels of NLRP3 inflammasome components in the prefrontal cortex of MDD individuals (Alcocer-Gomez et al., 2014; Pandey et al., 2021). Similarly, increased expression of NLRP3 and ASC has been found in postmortem brain tissue from patients diagnosed with

BP, as well as higher protein and mRNA levels of IL-1 β and IL-1R (Kim et al., 2016; Rao et al., 2010). IL-1 β levels were also shown to be increased in the CSF of BP patients compared to healthy controls and these levels were even more elevated in patients with recent manic or hypomanic episodes (Soderlund et al., 2011).

Immune-inflammatory changes have also been implicated in the pathophysiology of SCZ and the NLRP3 inflammasome has been suggested as a potential new biomarker. Early studies have described elevations in plasma levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines in patients suffering from SCZ (Garcia-Alvarez et al., 2016; Maes et al., 1996). Already at early stages of disease onset, patients with FEP display increased levels of NF κ B and decreased levels of the NF κ B inhibitory subunit I κ B α in peripheral blood mononuclear cells (PBMCs) and plasma (Garcia-Bueno et al., 2014a). Interestingly, the deregulation of pro-and anti-inflammatory markers became even more severe after a 1-year follow up (Garcia-Bueno et al., 2014b). Furthermore, CRP levels, a commonly used biomarker of systemic inflammation, as well as NLRP3, IL-1 β and IL-18 expression levels have been shown to be significantly higher in PBMCs of SCZ patients compared to healthy controls (Joseph et al., 2015; Lin et al., 2013; Miller et al., 2014; Ozdamar Unal et al., 2022). Analysis of postmortem tissue has further confirmed increased expression of NLRP3-related proteins in the frontal cortex of SCZ patients (Kim et al., 2016). It has also been described that maternal infection during pregnancy is associated with an increased risk for the development of SCZ (Brown and Derkits, 2010). Based on this finding, a maternal immune activation (MIA) animal model is used to mimic SCZ-like neurological and behavioral changes (Wolff et al., 2011; Zuckerman and Weiner, 2005). Interestingly, it has been shown that MIA is associated with increased NLRP3 inflammasome expression and increased levels of IL-1 β and that perinatal exposure to pro-inflammatory cytokines can lead to activated microglia and neural changes that resemble those observed in SCZ (Boksa, 2010; Talukdar et al., 2021; Ventura et al., 2020; Weir et al., 2015).

In summary, NLRP3 inflammasome activation can be induced by a wide range of different stimuli and has been implicated in many different pathological processes. Therefore, it is important to better understand the innate immune surveillance system in the context of neuroinflammation to be able to target the relevant pathways therapeutically.

3.4.4 NLRP3 Inflammasome inhibitors

Inhibition of the NLRP3 inflammasome pathway reduces microglia-mediated inflammation and is considered as a promising therapeutic approach for neuroinflammatory diseases. NLRP3 activation is a multi-step process and inhibition can be achieved through several different mechanisms including: suppression of inflammasome activating molecules, silencing of upstream signals or direct inhibition of inflammasome complex formation (Hanslik and Ulland,

2020). Examples of direct inhibitors, which specifically target assembly of the NLRP3 inflammasome, are ginsenoside Rg3, oridonin and tranilast. Ginsenoside Rg3 specifically blocks IL-1 β secretion and caspase-1 activation by inhibiting NLRP3 inflammasome assembly and tranilast, initially recognized as an anti-allergic drug, has been shown to directly suppress NLRP3 inflammasome assembly by binding to the NACHT domain of NLRP3 (Huang et al., 2018; Shi et al., 2020). Oridonin is a specific inhibitor that blocks inflammasome assembly and activation by inhibiting the interaction of NEK7 with NLRP3, which is important for NLRP3 oligomerization and ASC recruitment (He et al., 2018). Different indirect inhibitors of the NLRP3 inflammasome have also been described, such as β -hydroxybutyrate (BHB), MCC950 or glyburide. BHB acts by preventing K⁺ efflux, which can cause mitochondrial damage and thereby activate the inflammasome pathway, and by reducing ASC oligomerization (Youm et al., 2015). MCC950 acts by interfering with the ATP hydrolysis motif within the NLRP3 NACHT domain and glyburide suppresses ATP-sensitive K⁺ channels and caspase-1 activation (Coll et al., 2019; Lamkanfi et al., 2009).

Oridonin (Ori), the major active ingredient of the Chinese medicinal herb *Rabdosia rubescens*, is specifically interesting, since it has been shown to reduce microglial activation and AD-associated pathology. Ori is a specific inflammasome inhibitor, which forms a covalent bond with the cysteine 279 in the NACHT domain of NLRP3, thereby blocking the interaction of NLRP3 with NEK7 and inhibiting inflammasome assembly (He et al., 2018). In rat primary microglia, Ori was found to inhibit the release of pro-inflammatory cytokines, as well as DNA binding activity of NF κ B when co-treating the cells with LPS and Ori (Xu et al., 2009). Similar effects were shown in bone marrow-derived macrophages (BMDMs) where Ori exhibited a dose-dependent inhibitory effect on caspase-1 cleavage and IL-1 β secretion after priming and stimulation of the cells with LPS and nigericin (He et al., 2018). Furthermore, Ori was shown to attenuate neuroinflammation and memory deficits in A β ₁₋₄₂-induced AD mice. Treatment with Ori significantly reduced the expression of pro-inflammatory cytokines, as well as nuclear translocation of the NF κ B subunit p65 and attenuated learning and memory impairment in the Morris water maze test (Wang et al., 2014). Interestingly, Ori has also been shown to rescue synaptic loss induced by A β ₁₋₄₂. Thereby, Ori treatment was found to increase expression levels of PSD-95 and synaptophysin in the hippocampus of AD mice, as well as rescue dendritic morphological changes such as spine density, number of branches and total dendritic length (Wang et al., 2016).

3.5 Human stem cell technology

3.5.1 Human induced pluripotent stem cells (iPSCs)

The ability to generate human iPSCs through reprogramming of somatic cells has provided the scientific community with a valuable model system for *in vitro* research and has opened the possibility to generate and investigate every cell type of the human body. The iPSC technology was pioneered by Takahashi and Yamanaka who were able to show that the overexpression of four specific transcription factors, the so-called Yamanaka factors Oct4, KLF4, Sox2, and c-Myc, is sufficient to reprogram somatic cells into a pluripotent state (Takahashi and Yamanaka, 2006). The first human iPSCs were generated by transducing adult dermal fibroblasts with viral vectors carrying the mentioned transcription factors using a retroviral or lentiviral system (Takahashi et al., 2007; Yu et al., 2007). New approaches use transient expression by applying nonintegrative delivery systems such as sendai virus vectors, thereby avoiding insertional mutagenesis and genetic alterations associated with stable integration of transgenes (Chen et al., 2013).

iPSCs have shown great application potential in the fields of regenerative and transplant medicine, human developmental biology, disease modeling and drug discovery screening (Figure 7) (Avior et al., 2016; Kimbrel and Lanza, 2015; Trounson and DeWitt, 2016; Zhu and Huangfu, 2013). Disease modeling using primary patient-derived cells is limited due to the lack of accessibility of specific cell types such as brain cells and heart cells, as well as their limited expandability. In this context, human iPSCs represent an attractive alternative as they can be obtained from easily accessible cell types such as fibroblasts, peripheral blood cells, renal epithelial cells from urine or dental pulp (Staerk et al., 2010; Takahashi et al., 2007; Xue et al., 2013; Yan et al., 2010). PSCs are defined by their ability of unlimited self-renewal and the potential to differentiate into all cell types of the three germ layers (Wobus and Boheler, 2005). Both human embryonic stem cells (ESC) and iPSCs have been used for disease modeling, however, iPSCs have become the preferred option due to the lack of ethical concerns and their easier availability. To date, a number of differentiation protocols have been developed to generate complex human cell types such as neurons, cardiomyocytes, hepatocytes or pancreatic β cells from human iPSCs.

Animal models have provided valuable *in vivo* insights into pathological mechanisms of human diseases. However, substantial species differences have led to drawbacks in the identification of novel therapeutic strategies. The use of human iPSC technology for disease modeling and drug screening is likely to better recapitulate full human disease phenotypes and has reduced the need for animal research (Shi et al., 2017). Another great advantage of human iPSCs is their applicability for patient-specific research, which is especially important when studying diseases with a strong genetic component and high rates of variability across the population

(Wen et al., 2016). Furthermore, recent advances in gene editing technologies, such as CRISPR-Cas9 technology, have enabled the generation of genetically defined human iPSC-based disease models. Site-specific disease-causing mutations can be introduced into iPSCs derived from non-disease affected control individuals or disease-causing mutations can be corrected in patient-derived cells (Hockemeyer and Jaenisch, 2016). These approaches enable the use of isogenic iPSC lines derived from the same genetic background only differing in the introduced mutation or transgene of interest (Shi et al., 2017).

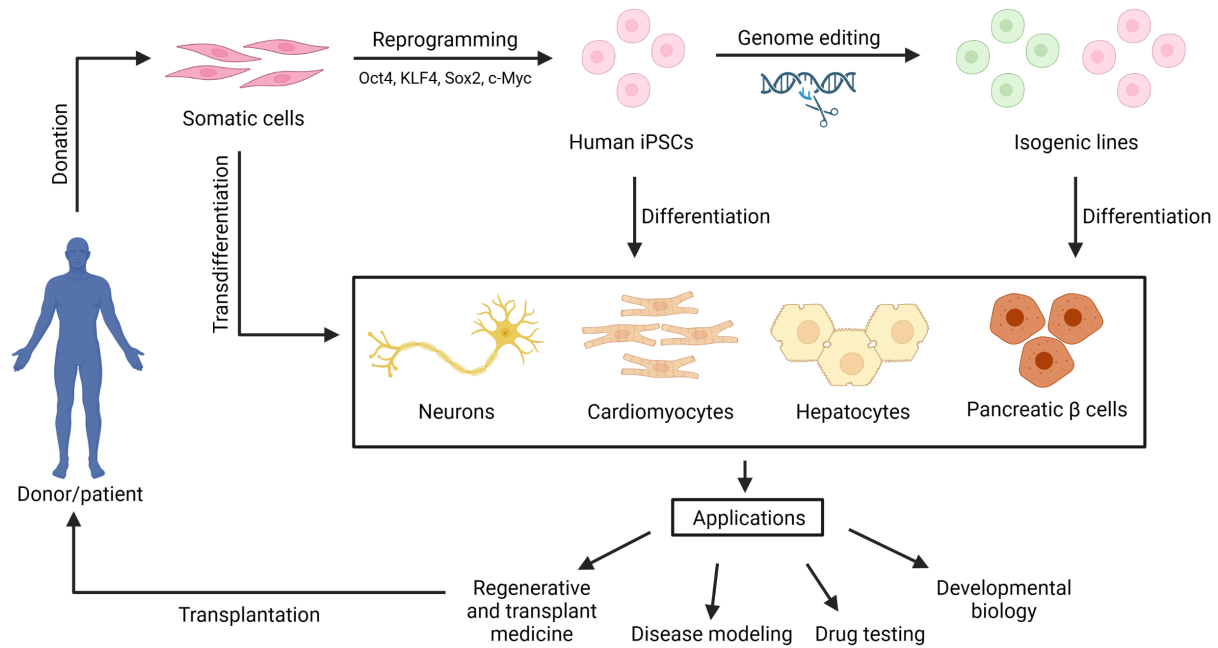


Figure 7: IPSC and direct conversion technology.

Somatic cells such as fibroblasts and peripheral blood mononuclear cells (PBMCs) can be isolated from skin biopsies or blood donation. These cells can then be directly converted into the desired cell types by transdifferentiation approaches or by transition through an iPSC state. Somatic cells can be reprogrammed to iPSCs by forced expression of the transcription factors Oct4, KLF4, Sox2, and c-Myc. Genome editing techniques can be used to generate isogenic lines from the same genetic background. Protocols have been developed to generate complex cell types such as neurons, cardiomyocytes, hepatocytes or pancreatic β cells. These human-specific cells are applied in the fields of regenerative and transplant medicine, disease modeling and drug testing or human developmental biology.

In addition to iPSC technology, direct cell reprogramming, also termed transdifferentiation, has been further developed in the recent years. This approach allows the direct conversion of one somatic cell type into another without the need to transition through an induced pluripotent state (Grath and Dai, 2019). Studies have shown that fibroblasts can be directly converted to a range of cell types such as cardiomyocytes, blood cell progenitors, hepatocytes or neurons by the forced expression of a combination of transcription factors or by applying a specific set of small molecule compounds (Huang et al., 2011; Ieda et al., 2010; Ladewig et al., 2012; Szabo et al., 2010; Vierbuchen et al., 2010). Direct cell reprogramming holds the potential of

preserving epigenetic traits, which can be especially important for the study of age-related degenerative diseases (Huh et al., 2016; Mertens et al., 2015).

So far, many disease models have relied on the use of single disease-relevant cell types. However, in the recent years considerable effort has gone into the development of co-culture systems to enable the investigation of non-cell-autonomous disease characteristics. For example, co-cultures of neurons, astrocytes and microglia have been used to model the pathology of AD, amyotrophic lateral sclerosis (ALS) or SCZ (Guttikonda et al., 2021; Haidet-Phillips et al., 2011; Marchetto et al., 2008; Sellgren et al., 2019). iPSCs are also a key component for the generation of standardized 3D organoid systems, which represent more physiological cellular platforms incorporating multiple cell types of whole tissue niches. Organoids have been generated for multiple organs, including the brain, intestine, liver or lung (Lancaster and Knoblich, 2014). Cells generated in organoids can be functionally more mature due to the interaction of different cell types in the 3D environment, however, they sometimes lack efficiency and reproducibility compared with 2D cultures (Centeno et al., 2018).

3.5.2 Generation of different brain cell types from human iPSCs

iPSCs can be differentiated into all major cell types of the brain including neurons, astrocytes, microglia, oligodendrocytes, pericytes and endothelial cells (Abud et al., 2017; Appelt-Menzel et al., 2017; Begum et al., 2015; Ehrlich et al., 2017; Qian et al., 2017; Tcw et al., 2017).

To date, substantial progress has been made in guiding human iPSCs to regionally and functionally specialized neural subtypes, including glutamatergic, GABAergic, dopaminergic and cholinergic neurons using passive and directed differentiation techniques (Chambers et al., 2009; Diao et al., 2021; Ladewig et al., 2012; Maroof et al., 2013; Munoz et al., 2020; Soldner et al., 2009; Sun et al., 2016). The first step in neural differentiation is the neural induction phase which can be mediated by inhibiting mesodermal and endodermal fates with the use of two small-molecule inhibitors of SMAD-dependent BMP and TGF- β signaling (LDN-193189/noggin and SB-431542/A83-01). The “dual-SMAD inhibition” method is particularly useful for monolayer culture and avoids the formation of embryoid bodies (EBs) in order to reduce culture variability (Chambers et al., 2009). Neuronal subtype specification in the neural tube is determined in a region-specific manner and regional identity of iPSC-derived neural progenitors and neurons can be modulated using specific patterning molecules involved in the formation of the anteroposterior (A-P) and dorsoventral (D-V) axes (Briscoe and Ericson, 2001; Kiecker and Lumsden, 2012; Marshall et al., 1992; Nordstrom et al., 2002). A-P identity can be controlled by Wnt and retinoic acid (RA) signaling and D-V identity can be modulated by regulating sonic hedgehog (Shh) signaling. Thereby, increased activation of Wnt signaling via the Wnt agonist CHIR99021 results in an increased caudalization, which can be used to

generate neural progenitor cells (NPCs) with midbrain or hindbrain identity. Activation of RA signaling has been shown to further enhance a hindbrain fate (Imaizumi et al., 2015; Kirkeby et al., 2012; Li et al., 2009; Lu et al., 2016; Moya et al., 2014). Other commonly used modulators include the Wnt antagonists IWP-2 and XAV-939, which are applied to generate forebrain-specific NPCs (Qi et al., 2017). D-V patterning is governed by Shh signaling, which can be activated by Shh protein or the Shh signaling agonist purmorphamine. Thereby, increased activation of Shh signaling leads to a more ventral cell fate (Kiecker and Lumsden, 2012; Le Dreau and Marti, 2012; Ribes and Briscoe, 2009).

In the past years, several methods for the differentiation of human iPSCs to microglia-like cells have been published, which has opened up new opportunities to study human microglial pathology *in vitro* (Abud et al., 2017; Banerjee et al., 2020; Douvaras et al., 2017; Haenseler et al., 2017; Muffat et al., 2016; Pandya et al., 2017). So far, the study on microglia has been limited to non-human models such as rodents, since the availability of primary human microglia is very limited. Further, microglia-like cell lines are highly proliferative and therefore do not represent a good model system for the mostly non-proliferating terminally differentiated cell type (Timmerman et al., 2018). Microglia are unique in the mononuclear phagocyte system, as they originate from hematopoietic stem cells in the yolk sac and are not derived from the bone marrow. Human microglia migrate to the brain early during development, before the formation of the blood brain barrier, and complete their maturation in the brain environment. It has been shown that microglia and tissue resident macrophages, but not peripheral blood monocyte-derived macrophages, are derived from precursors whose development depends on the transcription factors PU.1 and RUNX1 but does not require the transcriptional activator MYB, indicating different embryonic sources of these cells (Ginhoux et al., 2010; Kierdorf et al., 2013). To generate human iPSC-derived microglia, iPSCs are supplemented with growth factors associated with mesodermal specification, which leads to the development of haemangioblasts and primitive haematopoietic precursors. By adding further growth factors, these precursors are then matured along the myeloid lineage. These protocols are able to generate cells which display similar gene expression patterns of those of human primary microglia. In addition to the expression of known microglial markers, iPSC-derived microglia perform microglial functions, including phagocytosis, secretion of cytokines in response to stimulants and response to ADP or ATP (Pocock and Piers, 2018). The use of microglia-neuron co-cultures has been shown to further enhance the microglial signature (Haenseler et al., 2017). The production of large numbers of microglia-like cells from iPSCs is an important step towards understanding the functional consequences of an increasing number of disease-associated risk factors linked to these cells.

3.5.3 Modeling complement-dependent synaptic pruning using human iPSCs

Patient-derived *in vitro* models have previously been used to better understand dysregulated neuronal-microglial interactions associated to neurodegenerative or psychiatric disorders (Grubman et al., 2020; Guttikonda et al., 2021; Sellgren et al., 2019). Protocols which allow the generation of each cell type separately have opened the possibility to investigate their individual contributions to disease phenotypes by deriving them from patients versus healthy controls or manipulating them separately through gene editing techniques (Sheridan et al., 2022).

In the context of SCZ pathogenesis, impaired synaptic pruning has been demonstrated using iPSC-derived neurons and monocyte-derived microglia-like cells. Monocytes are a fraction of PBMCs which can adopt a microglial-like phenotype when supplemented with specific small molecule compounds, following a direct conversion approach (Etemad et al., 2012; Leone et al., 2006; Ohgidani et al., 2014). This *in vitro* co-culture approach confirmed a SCZ risk-associated increase in neuronal complement deposition, as well as an increased synapse engulfment by monocyte-derived microglia-like cells depending on the *C4* genotype (Sellgren et al., 2019). Microglia were added transiently to neuronal cultures and dendritic spine density was quantified. Furthermore, a method for the isolation of synaptic vesicles (synaptosomes) from iPSC-derived neuronal cultures has previously been described, facilitating large-scale *in vitro* assays to model synaptic pruning (Sellgren et al., 2017). Synaptosomes were first isolated in the 1950s and have since then been used to study synaptic function (Whittaker et al., 1964). Synaptosomes are enriched in pre-and post-synaptic components and have been shown to preserve their *in vivo* functions, including neurotransmitter uptake and release, as well as mitochondrial respiration (Daniel et al., 2012; Johansen et al., 1987; Khattar et al., 2016; Nicholls, 2010). Synaptosomes have also been shown to retain their activity after cryopreservation, making them very useful for assay applications (Gleitz et al., 1993). To model synaptic engulfment by microglia, protocols have been previously established to label synaptosomes with a pH-sensitive dye which becomes fluorescent in the acidic environment of lysosomes (Sellgren et al., 2017). By combining synaptosomes and microglia from patient and control cells, it was shown that the increased engulfment in SCZ patient-derived models can be attributed to neural and microglial factors. Interestingly, it was further demonstrated that the antibiotic minocycline is an efficient inhibitor of microglia phagocytosis and can partially rescue the observed pruning phenotype (Sellgren et al., 2019). These initial studies support the use of *in vitro* models and robust cellular assays for disease modeling and screening of novel therapeutic modulators.

4 Aims of this study

Various lines of evidence, including GWAS studies identifying *C4A* as a risk locus, as well as animal and cellular models, have suggested that SCZ is at least in part a synaptic pruning disorder. These findings have set large efforts in motion to better understand neuron-microglia crosstalk, not only in the context of SCZ, but also with regard to many other neurodevelopmental and neurodegenerative disorders. Microglia-mediated inflammatory responses can have detrimental effects on the brain and pharmacological targeting of microglial activation has become an important focus in drug discovery. Recent advances in the generation of iPSC-derived microglia and neurons have paved the way for patient-derived models and pathway-focused screens in human cells, which might allow the identification of novel biomarkers and more targeted early interventions.

This doctoral thesis focused on the establishment of a fully iPSC-derived *in vitro* model to study synaptic pruning in human cells in the context of SCZ pathogenesis.

The first aim of this thesis was the establishment and optimization of protocols for the generation of human iPSC-derived microglia and cortical neurons and the thorough morphological and functional characterization of both cell types using standard biochemistry techniques, as well as bulk RNA sequencing (RNAseq).

The second part of the study focused on the investigation of the effect of complement component *C4A* expression on microglia-mediated synapse engulfment. Therefore, a *C4A* overexpression cell line was generated and an *in vitro* synaptic pruning assay, as well as specific co-culture conditions were established. In order to reveal potential pathways involved in microglial activation by increased *C4A* expression in neurons, single-nucleus RNAseq (snRNAseq) was applied.

The final goal of this thesis was the application of the established *in vitro* model to validate the efficacy of potential new anti-inflammatory drugs as therapeutic options for SCZ.

5 Materials

5.1 Cell culture

5.1.1 Cell lines

Table 1: Cell lines

Cell line	Source	Donor	Cell type	Reprogramming method
Ctrl#1	Mannheim, Germany	healthy, female, age 44	dermal fibroblasts	Sendai virus
Ctrl#2	Mannheim, Germany	healthy, female, age 25	dermal fibroblasts	Sendai virus
Ctrl#1 C4A	Mannheim, Germany	Ctrl#1	N/A	N/A
Ctrl#1 mCherry	Mannheim, Germany	Ctrl#1	N/A	N/A

5.1.2 Cell culture base media, solutions and small molecules

Table 2: Cell culture base media

Medium	Cat. number	Distributor (headquarter)
Advanced DMEM/F12	12634010	Thermo Fisher Scientific (Waltham, USA)
Advanced MEM	12492013	Thermo Fisher Scientific (Waltham, USA)
DMEM with GlutaMAX	10566016	Thermo Fisher Scientific (Waltham, USA)
DMEM/F12 with glutamine	11320033	Thermo Fisher Scientific (Waltham, USA)
DMEM/F12 with glutamine and HEPES	11330057	Thermo Fisher Scientific (Waltham, USA)
Neurobasal™	21103049	Thermo Fisher Scientific (Waltham, USA)
Opti-MEM™ Reduced Serum Medium	31985062	Thermo Fisher Scientific (Waltham, USA)
X-VIVO™ 15	881024	Lonza (Basel, Switzerland)

Table 3: Commercial ready-to-use cell culture solutions

Compound	Concentration	Cat. number	Distributor (headquarter)
2-Mercaptoethanol	50 mM	31350010	Thermo Fisher Scientific (Waltham, USA)
B-27 supplement	50x	17504044	Thermo Fisher Scientific (Waltham, USA)
BSA solution	7.5%	15260037	Thermo Fisher Scientific (Waltham, USA)
DPBS	1x	14190144	Thermo Fisher Scientific (Waltham, USA)
DPBS + MgCl ₂ and CaCl ₂	1x	D8662	Sigma-Aldrich (St. Louis, USA)
EDTA pH 8.0 UltraPure™	0.5 M	15575020	Thermo Fisher Scientific (Waltham, USA)
FBS	100%	10270106	Thermo Fisher Scientific (Waltham, USA)
Geltrex™	15 mg/ml	A1413302	Thermo Fisher Scientific (Waltham, USA)

GlutaMAX supplement	100x	35050038	Thermo Fisher Scientific (Waltham, USA)
KnockOut™ Serum Replacement	100%	10828028	Thermo Fisher Scientific (Waltham, USA)
MEM-NEAA	100x	11140035	Thermo Fisher Scientific (Waltham, USA)
Penicillin/Streptomycin	100x	15140122	Thermo Fisher Scientific (Waltham, USA)
Sodium pyruvate	100x	11360039	Thermo Fisher Scientific (Waltham, USA)
TrypLE™ Express	1x	12605028	Thermo Fisher Scientific (Waltham, USA)

Table 4: Small molecules and chemicals used for cell culture

Compound	Solvent	Cat. number	Distributor (headquarter)
BMP4	0.1% BSA in H ₂ O	130-111-165	Miltenyi Biotec (Bergisch Gladbach, Germany)
Boric acid	H ₂ O	15583	Thermo Fisher Scientific (Waltham, USA)
Bryostatin 1	Ethanol	203811	EMD Millipore (Burlington, USA)
CaCl ₂	H ₂ O	HN04.1	Carl Roth (Karlsruhe, Germany)
CHIR99021	DMSO	SM13-50	Cell Guidance Systems (Cambridge, UK)
Chloroquine diphosphate	H ₂ O	4106	Tocris Bioscience (Minneapolis, USA)
D(+)-Glucose	H ₂ O	HN06.2	Carl Roth (Karlsruhe, Germany)
DAPT	DMSO	SM15-50	Cell Guidance Systems (Cambridge, UK)
DMSO	N/A	D5879	Sigma-Aldrich (St. Louis, USA)
Ethanol	N/A	32205	Sigma-Aldrich (St. Louis, USA)
FGF-2 (147)	H ₂ O	GFH28	Cell Guidance Systems (Cambridge, UK)
FGF-2 (154)	0.1% BSA in H ₂ O	GFH146	Cell Guidance Systems (Cambridge, UK)
Forskolin	DMSO	SM18-100	Cell Guidance Systems (Cambridge, UK)
GABA	H ₂ O	A5835-337	Sigma-Aldrich (St. Louis, USA)
GM-CSF	H ₂ O	GFH8	Cell Guidance Systems (Cambridge, UK)
HEPES	H ₂ O	9105.4	Carl Roth (Karlsruhe, Germany)
IL-3	H ₂ O	GFH80	Cell Guidance Systems (Cambridge, UK)
IL-34	0.1% BSA in H ₂ O	130-108-977	Miltenyi Biotec (Bergisch Gladbach, Germany)
Insulin	10 mM NaOH	91077C	Sigma-Aldrich (St. Louis, USA)
KCl	H ₂ O	A3582	AppliChem (Darmstadt, Germany)

Materials

Laminin, murine	TBS	L2020	Sigma-Aldrich (St. Louis, USA)
L-Ascorbic acid	H ₂ O	A4544	Sigma-Aldrich (St. Louis, USA)
L-Ascorbic acid 2-phosphate (LAAP)	H ₂ O	A8960	Sigma-Aldrich (St. Louis, USA)
LDN193189	DMSO	72148	StemCell Technologies (Vancouver, Canada)
LM22A	H ₂ O	SML0848	Sigma-Aldrich (St. Louis, USA)
LM22B	DMSO	6037	Tocris Bioscience (Minneapolis, USA)
LPS, <i>E. Coli</i> (O111:B4)	DPBS	LPS25	Sigma-Aldrich (St. Louis, USA)
M-CSF	H ₂ O	GFH3	Cell Guidance Systems (Cambridge, UK)
MgCl ₂	H ₂ O	105833	Merck Millipore (Darmstadt, Germany)
Minocycline hydrochloride	DMSO	M9511	Sigma-Aldrich (St. Louis, USA)
Myo-Inositol	H ₂ O	I5125	Sigma-Aldrich (St. Louis, USA)
NaCl	H ₂ O	31434	Sigma-Aldrich (St. Louis, USA)
Oridonin	DMSO	6924	Tocris Bioscience (Minneapolis, USA)
PD-0332991	DMSO	1116	Selleck Chemicals (Houston, USA)
Pluronic F-127	DPBS	P2443	Sigma-Aldrich (St. Louis, USA)
Polyethyleneimine (PEI)	H ₂ O	P3143	Sigma-Aldrich (St. Louis, USA)
Poly-L-Lysine Hydrobromide (PLL)	H ₂ O	P2636	Sigma-Aldrich (St. Louis, USA)
Polyvinylalcohol	DPBS	P8136	Sigma-Aldrich (St. Louis, USA)
Progesterone	Ethanol	P8783	Sigma-Aldrich (St. Louis, USA)
Puromycin	H ₂ O	540222	EMD Millipore (Burlington, USA)
Putrescine	H ₂ O	51799	Sigma-Aldrich (St. Louis, USA)
SB431542	H ₂ O	SM33	Cell Guidance Systems (Cambridge, UK)
SCF	H ₂ O	GFH4	Cell Guidance Systems (Cambridge, UK)
Sodium selenite	H ₂ O	A8960	Sigma-Aldrich (St. Louis, USA)
TGF- β 1	H ₂ O	GFH39	Cell Guidance Systems (Cambridge, UK)
Transferrin	H ₂ O	T3705	Sigma-Aldrich (St. Louis, USA)
VEGF-165	H ₂ O	GFH44	Cell Guidance Systems (Cambridge, UK)
XAV939	DMSO	SM38	Cell Guidance Systems (Cambridge, UK)
Y-27632	H ₂ O	SM02	Cell Guidance Systems (Cambridge, UK)

5.1.3 Composition of self-made cell culture media and solutions

Table 5: Final composition of cell culture media

Medium (base)	Added components	Final concentration
iPSC medium (DMEM/F12 with glutamine and HEPES)	Pen/Strep LAAP Sodium selenite Insulin Transferrin FGF-2 (154) TGF- β 1	1% (v/v) 64 μ g/ml 14 ng/ml 20 μ g/ml 11 μ g/ml 100 ng/ml 2 ng/ml
NPC medium, phase 1 (Advanced DMEM/F12)	Pen/Strep GlutaMAX supplement B-27 supplement SB431542 LDN193189 XAV939	1% (v/v) 1% (v/v) 1% (v/v) 10 μ M 1 μ M 2 μ M
NPC medium, phase 2 (Advanced DMEM/F12)	Pen/Strep GlutaMAX supplement B-27 supplement LDN193189 XAV939	1% (v/v) 1% (v/v) 1% (v/v) 200 nM 2 μ M
NPC medium, phase 3 (Advanced DMEM/F12)	Pen/Strep GlutaMAX supplement B-27 supplement FGF (147)	1% (v/v) 1% (v/v) 1% (v/v) 20 ng/ml
Neuronal differentiation medium, phase 1 (DMEM/F12 with glutamine)	Pen/Strep GlutaMAX supplement N2-supplement B-27 supplement NEAA Glucose CaCl ₂ Ascorbic acid LM22A LM22B PD-0332991 DAPT	1% (v/v) 1% (v/v) 0.5% (v/v) 1% (v/v) 1% (v/v) 1.6 mg/ml 1.8 mM 200 μ M 1 μ M 1 μ M 2 μ M 5 μ M
Neuronal differentiation medium, phase 2 (Neurobasal™)	Pen/Strep GlutaMAX supplement B-27 supplement Glucose Ascorbic acid LM22A LM22B PD-0332991 DAPT CHIR99021 Forskolin GABA	1% (v/v) 1% (v/v) 2% (v/v) 5 mg/ml 200 μ M 1 μ M 1 μ M 2 μ M 5 μ M 3 μ M 10 μ M 300 μ M

Materials

Neuronal differentiation medium, phase 3 (Neurobasal™)	Pen/Strep GlutaMAX supplement B-27 supplement Glucose Ascorbic acid LM22A LM22B PD-0332991 CHIR99021	1% (v/v) 1% (v/v) 2% (v/v) 5 mg/ml 200 µM 1 µM 1 µM 2 µM 3 µM
Neuronal differentiation medium, phase 4 (Neurobasal™)	Pen/Strep GlutaMAX supplement B-27 supplement Glucose Ascorbic acid LM22A LM22B PD-0332991	1% (v/v) 1% (v/v) 2% (v/v) 5 mg/ml 200 µM 1 µM 1 µM 2 µM
Neuronal differentiation medium, phase 5 (Advanced MEM)	Pen/Strep GlutaMAX supplement B-27 supplement Glucose Ascorbic acid LM22A LM22B PD-0332991 Bryostatin 1	1% (v/v) 1% (v/v) 2% (v/v) 5 mg/ml 200 µM 1 µM 1 µM 2 µM 0.27 nM
EB medium (iPSC medium)	BMP4 VEGF-165 SCF Y-27632	50 ng/ml 50 ng/ml 20 ng/ml 20 µM
Macrophage progenitor medium (X-VIVO™15)	Pen/Strep GlutaMAX supplement 2-Mercaptoethanol M-CSF IL-3	1% (v/v) 1% (v/v) 50 µM 100 ng/ml 25 ng/ml
Co-culture medium (Advanced DMEM/F12)	Pen/Strep GlutaMAX supplement 2-Mercaptoethanol N2-supplement IL-34 GM-CSF	1% (v/v) 1% (v/v) 50 µM 0.5% (v/v) 100 ng/ml 10 ng/ml
HEK-293T medium (DMEM with GlutaMAX)	Pen/Strep GlutaMAX supplement MEM-NEAA Sodium pyruvate FBS	1% (v/v) 1% (v/v) 1% (v/v) 1% (v/v) 10%
Wash medium (DMEM with GlutaMAX)	Pen/Strep	1% (v/v)
iPSC freezing medium	KnockOut™ Serum Replacement iPSC base medium supplemented with Pen/Strep, LAAP and Sodium selenite DMSO Y-27632	50% (v/v) 40% (v/v) 10% (v/v) 20 µM

NPC freezing medium	KnockOut™ Serum Replacement Cytobuffer DMSO Y-27632	70% (v/v) 30% (v/v) 10% (v/v) 20 µM
---------------------	--	--

Table 6: Composition of self-made buffers, supplements and solutions

Name	Added components	Final concentration
Borate buffer	Boric acid H ₂ O adjust pH to 8.4 with NaOH	25 mM
Cytobuffer	Myo-Inositol Polyvinylalcohol DPBS	240 mM 5 mg/ml (w/v) 0.2x
Glucose stock solution	Glucose H ₂ O 0.2 µm sterile-filtered	400 mg/ml
Live cell imaging solution	HEPES pH 7.4 NaCl KCl CaCl ₂ MgCl ₂ Glucose	20 mM 140 mM 2.5 mM 1.8 mM 1 mM 10 mM
N2-supplement	DMEM/F12 with glutamine Pen/Strep Insulin Progesterone Putrescine Sodium selenite Transferrin	70% (v/v) 1% (v/v) 500 µg/ml 630 ng/ml 1.611 mg/ml 520 ng/ml 10 mg/ml
PEI coating solution	Polyethyleneimine (PEI) H ₂ O	1% (v/v)
Pluronic coating solution	Pluronic F-127 DPBS	50 mg/ml

5.2 Molecular biology and biochemistry

5.2.1 Bacterial cultures

Chemically competent *Escherichia coli* (*E.coli*) DH5α were obtained from New England Biolabs (#C2987H).

Table 7: Media used for cultivation of bacteria

Name	Added components	Final concentration
LB medium	LB medium powder Ampicillin	20% (w/v) 100 µg/ml
LB agar	LB medium powder Agar (Bacto™) Ampicillin	20% (w/v) 15% (w/v) 100 µg/ml

Materials

SOC medium	Bacto-Tryptone Yeast Extract NaCl KCl MgCl ₂ MgSO ₄ Glucose	2% (w/v) 0.5% (w/v) 10 mM 2.5 mM 10 mM 10 mM 200 mM
------------	---	---

5.2.2 Plasmids

Table 8: Plasmids used for cloning, nucleofection and lentivirus production

Name	Backbone	Insert	Application	Cat. number	Source
Human C4A cDNA expression vector	pCMV-SPORT6	Complement Fragment 4a (C4A)	cloning	ABIN4214113	genomics-online.com
pCDH-CuO-MCS-T2A-GFP SparQ™ Cloning and Expression Lentivector backbone			cloning	QM521A-1	System Biosciences
psPAX2			Lentivirus production	12260	Addgene (Watertown, USA)
pMD2.G			Lentivirus production	12259	Addgene (Watertown, USA)
pMK243	pBluescript	AAVS1-TetOn-OsTIR1 PGK-TetR PGK-Puromycin-AAVS1	cloning	72835	Addgene (Watertown, USA)
A201 AAV-EF-1α-mCherry			cloning		Group of Prof. Dr. Valery Grinevich (ZI, Mannheim)
AAVS1-T2-pX330			nucleofection	72833	Addgene (Watertown, USA)

5.2.3 Enzymes

Table 9: Enzymes used for cloning and molecular biology

Enzyme	Cat. number	Distributor (headquarter)
Antarctic Phosphatase	M0289	New England Biolabs (Ipswich, USA)
DNase I, amplification grade	AMPD1-1KT	Sigma-Aldrich (Darmstadt, Germany)
DNase I, cell culture	10104159001	Sigma-Aldrich (Darmstadt, Germany)
LongAmp Taq DNA Polymerase	M0323S	New England Biolabs (Ipswich, USA)
Proteinase K	405-001	GeneON Bioscience (Ludwigshafen, Germany)
T4 DNA Ligase	M0202	New England Biolabs (Ipswich, USA)
T4 Polynucleotide Kinase	M0201	New England Biolabs (Ipswich, USA)
<i>HpaI</i>	R0105	New England Biolabs (Ipswich, USA)
<i>XbaI</i>	R0145	New England Biolabs (Ipswich, USA)
<i>BamHI</i>	R0136	New England Biolabs (Ipswich, USA)
<i>NotI</i>	R0189	New England Biolabs (Ipswich, USA)
<i>XmaI</i>	R0180	New England Biolabs (Ipswich, USA)
<i>Sall</i>	R0138	New England Biolabs (Ipswich, USA)

5.2.4 Primers and PCR components

All primers and were obtained from Integrated DNA Technologies, Inc. (Coralville, USA) and were reconstituted at 100 μ M in nuclease-free H₂O for 30 min at 37°C and 400 rpm.

Table 10: Primers for RT-PCR and qPCR

Primer name	Sequence (5'→3')
18S rRNA-for	AAACGGCTACCATCCAAG
18S rRNA-rev	CCTCCAATGGATCCTCGTTA
ARC-for	TCAGCTCCAGTGATTCACGC
ARC-rev	GGGAACCTTGAGACCTGTTGT
BCL11B-for	CAACCCGCAGCACTTGTC
BCL11B-rev	CCTCGTCTTCTTCGAGGATGG
C4 total-for	CCTGAGAAACTGCAGGAGACAT
C4 total-rev	GTGAGTGCCACAGTCTCATCAT
C4A-for	CCTGAGAAACTGCAGGAGACAT
C4A-rev	GTCTAACACTGGACAGGGGTC
C4B-for	CCTGAGAAACTGCAGGAGACAT
C4B-rev	CTATGTATCACTGGAGAGAGGTC
DLG4-for	AGCCCCAGGATATGAGTTGC
DLG4-rev	CCCAGACCTGAGTTACCCCT
FOS-for	CAAGCGGAGACAGACCAACT
FOS-rev	AGTCAGATCAAGGGAAGCCA
GAD1-for	ATCCTGGTTGACTGCAGAGAC
GAD1-rev	CCAGTGGAGAGCTGGTTGAA
GRIA2-for	GGATCCTCATTAAGAACCCCACT
GRIA2-rev	TGAGGGCACTGGTCTTTTCC
GRIA4-for	AGGTGAATGTGGACCCAAGG
GRIA4-rev	AAGGTCAGCTTCATTCTCTTCG
GRIN1-for	GGCAACACCAACATCTGGAA
GRIN1-rev	CCATCCGCATACTTGGAAGAC

GRIN2B-for	CTCACCCCCTTTCCGCTTT
GRIN2B-rev	AAGGCATCCAGTTTCCCTGTT
Mycoplasma-for	GGGAGCAAACAGGATTAGATACCCT
Mycoplasma-rev	TGCACCATCTGTCACTCTGTTAACCTC
NPAS4-for	AACACTACCGCCTGTTGGC
NPAS4-rev	GCAGTAATGGGTCCCTCTGG
RBFOX3-for	GCGCTGAGCCCGTTGAAAT
RBFOX3-rev	CTCCTTCTGGACCGTCCTTG
SLC17A6-for	TCAGATTCCGGGAGGCTACA
SLC17A6-rev	TGGGTAGGTCACACCCTCAA
SLC17A7-for	AGCTGGGATCCAGAGACTGT
SLC17A7-rev	CCGAAAACCTCTGTTGGCTGC
SYN1-for	CAGCTCAACAAATCCCAGTCTC
SYN1-rev	GGTCTCAGCTTTACCTCGT
TBR1-for	ACGAACAACAAAGGAGCTTCA
TBR1-rev	TGGTACTTGTGCAAGGACTGTA

Table 11: Primers and oligonucleotides used for molecular cloning

Primer name	Sequence (5'->3')
EF-1 α -mCherry-HpaI-for	GCTGCAATAAACAAGTTAACACGCGTAAGCTTTGCAAAG
EF-1 α -mCherry-HpaI-rev	GCTGCAATAAACAAGTTAACTTACTTGTACAGCTCGTCC
C4A-XbaI-for	CTCCCAGGTCCAAGTTTGGTATGAGGCTGCTCTGGGGG
C4A-Tag-BamHI-rev	CTTTGTAGTCAGGCGCGCCGCCACCTGGCACCCTGAGTG
Flag-HA-BamHI-for	ATATCGGATCCGGCGCGCCTGACTACAAAG
Flag-HA-NotI-rev	ATAGGCGGCCGCTGCGTAGTCTGGCACGT
T2A-NotI-XmaI-Sall-sense	GGCCGCTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGAC GTGGAGGAGAATCCCGGCCCTCCCGGGATCGG
T2A-NotI-XmaI-Sall-antisense	TCGACCGATCCCGGGAGGGCCGGGATTCTCCTCCACGTCA CCGCATGTTAGAAGACTTCCTCTGCCCTCAGC
Puro-XmaI-for	GACCCCGGGATGACCGAGTACAAGCCC
Puro-Sall-rev	TGTAATCCAGAGGTTGATTGGACGCGCCCGTCAGGCAC

Table 12: RT-PCR reaction mixture

Substance	Concentration	Cat. number	Distributor (headquarter)
Reaction buffer	1x	331610	Biozym (Hessisch Oldendorf, Germany)
dNTPs	each 200 μ M	SL-Set-S-dNTPs	Steinbrenner Laborsysteme (Wiesenbach, Germany)
Primers	each 400 nM	N/A	Integrated DNA Technologies (Coralville, USA)
Taq DNA Polymerase	0.625 U	331610	Biozym (Hessisch Oldendorf, Germany)
cDNA / genomic DNA	10 ng / 200 ng	N/A	N/A
DMSO (for genomic DNA only)	1% (v/v)	D5879	Sigma-Aldrich (St. Louis, USA)

Table 13: qPCR reaction mixture

Substance	Concentration	Cat. number	Distributor (headquarter)
Reaction buffer	1x	M890A	Promega (Madison, USA)
MgCl ₂	2.5 mM	A351B	Promega (Madison, USA)
dNTPs	each 200 µM	SL-Set-S-dNTPs	Steinbrenner Laborsysteme (Wiesenbach, Germany)
Primers	each 200 nM	N/A	Integrated DNA Technologies (Coralville, USA)
DMSO	4% (v/v)	D5879	Sigma-Aldrich (St. Louis, USA)
Syber [®] Green I	1x	S9430	Sigma-Aldrich (St. Louis, USA)
ROX	25 nM	A351513	GENAXXON (Ulm, Germany)
GoTaq [®] DNA Polymerase	0.75 U	M780B	Promega (Madison, USA)
cDNA	10 ng	N/A	N/A

5.2.5 Kits

Table 14: Commercial kits

Kit	Cat. number	Distributor (headquarter)
Cell Line Nucleofector™ Kit V	VCA-1003	Lonza (Basel, Switzerland)
Chromium Next GEM Chip G Single Cell Kit	PN-1000120	10x Genomics (Pleasanton, USA)
Chromium Single Cell 3' Library & Gel Bead Kit v3.1	PN-1000121	10x Genomics (Pleasanton, USA)
ExtractMe [®] Genomic DNA Kit	EM13-050	BLIRT S.A. (Gdańsk, Poland)
iScript™ cDNA synthesis kit	17088991BUN	Bio-Rad Laboratories (Hercules, USA)
Lipofectamine™ 3000 Transfection Reagent	L3000001	Thermo Fisher Scientific (Waltham, USA)
peqGOLD Gel extraction kit	12-2501-01	VWR (Radnor, USA)
peqGOLD Plasmid miniprep kit	732-2780	VWR (Radnor, USA)
Pierce™ BCA Protein-Assay	23225	Thermo Fisher Scientific (Waltham, USA)
PureLink™ HiPure Plasmid Filter Midiprep Kit	K210015	Thermo Fisher Scientific (Waltham, USA)
Qubit RNA BR Assay-Kit	Q10210	Thermo Fisher Scientific (Waltham, USA)
RNA 6000 Nano kit	5067-1511	Agilent Technologies (Santa Clara, USA)
Single Index Kit T Set A	PN-1000213	10x Genomics (Pleasanton, USA)

5.2.6 Antibodies and fluorescent probes

Table 15: Primary antibodies

Antigen	Host	Dilutions	Cat. number	Distributor (headquarter)
Actin	mouse	1:10000	3700	Cell Signaling Technology (Danvers, USA)
Actin	rabbit	1:10000	4970	Cell Signaling Technology (Danvers, USA)
C4	rabbit	1:200 (IF) 1:1000 (WB)	ab173577	Abcam (Cambridge, US)
CD68	mouse	1:200	333802	Biolegend (San Diego, USA)
CTIP2	rat	1:500	ab18465	Abcam (Cambridge, US)
GAPDH	rabbit	1:7000	2118	Cell Signaling Technology (Danvers, USA)
GFAP	mouse	1:500	173011	Synaptic Systems (Göttingen, Germany)
HA-Tag	mouse	1:200 (IF) 1:2000 (WB)	2367	Cell Signaling Technology (Danvers, USA)
Iba1	rabbit	1:500	GTX100042	GeneTex, Irvine, CA, USA
MAP2	chicken	1:7000	822501	Biolegend (San Diego, USA)
Nestin	mouse	1:600	MAB1259	R&D Systems (Minneapolis, USA)
NeuN	rabbit	1:400	MAB377	EMD Millipore (Burlington, USA)
NFκB (p65)	rabbit	1:1000	8242	Cell Signaling Technology (Danvers, USA)
OCT3/4	mouse	1:500	sc-5279	Santa Cruz Biotech (Dallas, USA)
PAX6	rabbit	1:500	901301	Biolegend (San Diego, USA)
pH3	rabbit	1:500	9706	Cell Signaling Technology (Danvers, USA)
PSD-95	rabbit	1:200	810401	Biolegend (San Diego, USA)
S100β	mouse	1:200	S2532	Sigma-Aldrich (St. Louis, USA)
SOX2	rabbit	1:400	3579	Cell Signaling Technology (Danvers, USA)
SSEA4	mouse	1:100	MC-813-70	Hybridoma Bank (Iowa City, USA)
SV2 A	guinea pig	1:3000	119004	Synaptic Systems (Göttingen, Germany)
Synapsin I/II/III	mouse	1:500 (IF) 1:4000 (WB)	853701	Biolegend (San Diego, USA)
Tau	guinea pig	1:750	314004	Synaptic Systems (Göttingen, Germany)
TBR1	rabbit	1:500	20932-1-ap	Proteintech (Rosemont, USA)
TMEM119	mouse	1:200	853301	Biolegend (San Diego, USA)
TREM2	rabbit	1:200	E-AB-16947	Elabscience (Houston, USA)

Table 16: Secondary antibodies

Antigen	Host	Dilution	Cat. number	Distributor (headquarter)
anti-chicken IgY Alexa Fluor-488	goat	1:1000	A11039	Thermo Fisher Scientific (Waltham, USA)
anti-guinea pig IgG Alexa Fluor-647	goat	1:1000	A21450	Thermo Fisher Scientific (Waltham, USA)
anti-mouse IgG Alexa Fluor-488	goat	1:1000	A11001	Thermo Fisher Scientific (Waltham, USA)
anti-mouse IgG Alexa Fluor-568	goat	1:1000	A11004	Thermo Fisher Scientific (Waltham, USA)
anti-mouse IgG Alexa Fluor-647	goat	1:1000	A21236	Thermo Fisher Scientific (Waltham, USA)
anti-rabbit IgG Alexa Fluor-488	goat	1:1000	A11008	Thermo Fisher Scientific (Waltham, USA)
anti-rabbit IgG Alexa Fluor-555	goat	1:1000	A21428	Thermo Fisher Scientific (Waltham, USA)
anti-rat IgG Alexa Fluor-555	goat	1:1000	A21434	Thermo Fisher Scientific (Waltham, USA)
Anti-mouse DyLight™ 680	goat	1:15000	5470S	Cell Signaling Technologies (Danvers, USA)
Anti-mouse DyLight™ 800	goat	1:15000	5257S	Cell Signaling Technologies (Danvers, USA)
Anti-rabbit DyLight™ 680	goat	1:15000	5366S	Cell Signaling Technologies (Danvers, USA)
Anti-rabbit DyLight™ 800	goat	1:15000	5151S	Cell Signaling Technologies (Danvers, USA)
Anti-guinea pig IgG IRDye® 800CW	donkey	1:15000	926- 32411	LI-COR Biosciences GmbH (Lincoln, USA)

Table 17: Fluorescent probes

Name	Dilution / concentration	Cat. number	Distributor (headquarter)
DAPI	300 nM	422801	Biolegend (San Diego, USA)
pHrodo™ Green STP ester	8.9 mM	P35369	Thermo Fisher Scientific (Waltham, USA)
pHrodo™ Green Zymosan Bioparticles	0.5 mg/ml	P35365	Thermo Fisher Scientific (Waltham, USA)

5.2.7 Buffers and solutions

Table 18: Self-made buffers and solutions

Buffer / Solution	Components	Concentration
APS stock solution	APS	10% (w/v) in H ₂ O
DAPI staining solution	DAPI	300 nM in PBS
DEPC-H ₂ O	DEPC	0.1% (v/v) in H ₂ O
DNA isolation lysis buffer	Tris-HCl, pH 8.5 EDTA SDS NaCl	100 mM 5 mM 0.2% (w/v) 200 mM
DNA sample buffer (10X)	Tris-HCl, pH 7.6 Bromophenol blue Glycerol	50 mM 0.25% (w/v) 60%
Mounting solution	Tris-HCl, pH 8.5 Glycerol Mowiol DABCO®	100 mM 25% 10% 0.6%
PBS	NaCl KCl Na ₂ HPO ₄ KH ₂ HPO ₄	137 mM 2.7 mM 10.0 mM 1.8 mM
PFA solution	PFA adjust pH to 7.2	4% (w/v) in PBS
Protein lysis buffer	Tris-HCl, pH 7.4 NaCl SDS EDTA Phosphatase inhibitor Protease inhibitor	50 mM 150 mM 0.2% (w/v) 25 mM 1 tablet / 10 ml 1 tablet / 10 ml
SDS stock solution	SDS	10 % (w/v) in H ₂ O
SDS-PAGE Anode buffer (1X)	Tris-HCl, pH 8.8	200 mM
SDS-PAGE polyacrylamide gel buffer	Tris-HCl pH 8.45 SDS	3 M 0.3% (w/v)
SDS-PAGE sample buffer (6X)	Tris-HCl, pH 6.8 SDS Glycerol 2-Mercaptoethanol Bromophenol blue	93.75 mM 6% 6% 9% 0.25%
SDS-Polyacrylamide separating gel	SDS-PAGE polyacrylamide gel buffer Bis/Acrylamide Glycerol Ammonium persulfate TEMED	33.3% (v/v) 10% (v/v) 10% (v/v) 0.028% (w/v) 0.09% (v/v)
SDS-Polyacrylamide stacking gel	SDS-PAGE polyacrylamide gel buffer Bis/Acrylamide Ammonium persulfate TEMED	24.8% (v/v) 3.84% (v/v) 0.0672% (w/v) 0.224% (v/v)

TAE (1X)	Tris EDTA Acetic acid	40 mM 1 mM 20 mM
TBS (10X)	Tris-HCl, pH 7.4 NaCl KCl	248 mM 1.37 M 26.8 mM
TBS-T (1X)	TBS (10X) Tween® 20	10% (v/v) 0.1% (v/v)
Tris-glycine buffer (10x)	Tris-HCl, pH 8.8 Glycine	250 mM 1.92 M
Triton X-100 stock solution	Triton X-100	10% (v/v) in PBS
Western Blot transfer buffer	Tris-glycine buffer (10X) Methanol SDS	10% (v/v) 20% (v/v) 0.08% (v/v)

5.2.8 Reagents and chemicals

Table 19: Molecular biology reagents and commercial buffers

Reagent	Cat. number	Distributor (headquarter)
1 kb marker	N0468S	New England Biolabs (Ipswich, USA)
100 bp marker, Quick-Load®	N0467S	New England Biolabs (Ipswich, USA)
50% Polyethylene glycol (PEG) - 6000 solution	101443-484	VWR (Radnor, USA)
Adult brain total RNA, human	R1234035-50-BC	BioCat (Heidelberg, Germany)
CutSmart buffer (10X)	B7204S	New England Biolabs (Ipswich, USA)
Fetal brain total RNA, human	1F01-50	Tebu Bio (Le-Perray-en-Yvelines, France)
HEPES buffered saline (2x HBSS)	S0874	Takara Bio Inc. (Tokyo, Japan)
NEBuilder® HiFi DNA Assembly Master Mix (2x)	E2621	New England Biolabs (Ipswich, USA)
peqGREEN DNA/RNA binding dye	peq137-5010	VWR (Radnor, USA)
Polybrene Infection Reagent	TR-1003-G	Merck Millipore (Darmstadt, Germany)
Protein marker PS 10 plus	310003	GeneOn (Ludwigshafen am Rhein, Germany)
RNase AWAY	10666421	Thermo Fisher Scientific (Waltham, USA)
RNA-Solv reagent	R6830-02	VWR (Radnor, USA)
Syn-PER™ Synaptic Protein Extraction Reagent	87793	Thermo Fisher Scientific (Waltham, USA)
Tris-Tricine-SDS buffer (10X)	T1165	Sigma-Aldrich (St. Louis, USA)

Table 20: Chemicals

Chemical	Cat. number	Distributor (headquarter)
2-Mercaptoethanol	805740	Merck Millipore (Darmstadt, Germany)
2-Propanol	1157	Th. Geyer (Renningen, Germany)
30% Bis/acrylamide	3029.1	Carl Roth (Karlsruhe, Germany)
37% HCl	836.1000	Th. Geyer (Renningen, Germany)
Acetic acid	33209	Sigma-Aldrich (St. Louis, USA)
Agar (Bacto™)	214010	BD Biosciences (Franklin Lakes, USA)
Agarose	A9539	Sigma-Aldrich (St. Louis, USA)
Ammonium persulfate	A3678	Sigma-Aldrich (St. Louis, USA)
Ampicillin	A9518	Sigma-Aldrich (St. Louis, USA)
Bacto-Tryptone	211705	BD Biosciences (Franklin Lakes, USA)
Boric acid	15583	Thermo Fisher Scientific (Waltham, USA)
Bovine serum albumin (BSA)	A3294	Sigma-Aldrich (St. Louis, USA)
Bromophenol blue	B8026	Sigma-Aldrich (St. Louis, USA)
Chloroform	32211	Sigma-Aldrich (St. Louis, USA)
DABCO	0718.2	Carl Roth (Karlsruhe, Germany)
DEPC	K028.1	Carl Roth (Karlsruhe, Germany)
EDTA	147850010	Thermo Fisher Scientific (Waltham, USA)
Ethanol	2246.1000	Th. Geyer (Renningen, Germany)
Fetal bovine serum (FBS)	10270-106	Thermo Fisher Scientific (Waltham, USA)
Glycerol	15523	Sigma-Aldrich (St. Louis, USA)
Glycine	10070150	Thermo Fisher Scientific (Waltham, USA)
KCl	1632	Th. Geyer (Renningen, Germany)
KH ₂ PO ₄	1648	Th. Geyer (Renningen, Germany)
LB medium powder	X968.4	Carl Roth (Karlsruhe, Germany)
Methanol	0082.1	Carl Roth (Karlsruhe, Germany)
MgCl ₂	A351B	Promega (Madison, USA)
MgSO ₄	105886	Merck Millipore (Darmstadt, Germany)
Milk powder	T145.3	Carl Roth (Karlsruhe, Germany)
Mowiol® 4-88	0713.1	Carl Roth (Karlsruhe, Germany)
Na ₂ HPO ₄	8622	Th. Geyer (Renningen, Germany)
NaCl	1367	Th. Geyer (Renningen, Germany)
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich (St. Louis, USA)
Phosphatase inhibitor mini tablets	A32957	Thermo Fisher Scientific (Waltham, USA)
Polyethyleneimine (PEI)	P3143	Sigma-Aldrich (St. Louis, USA)
Poly-L-lysine hydrobromide (MW 30,000-70,000)	P2636	Sigma-Aldrich (St. Louis, USA)
Protease inhibitor mini tablets	A32955	Thermo Fisher Scientific (Waltham, USA)
Puromycin dihydrochloride	540222	Merck Millipore (Darmstadt, Germany)
ROX	A351513	GENAXXON (Ulm, Germany)
SDS, pellets	CN30.1	Carl Roth (Karlsruhe, Germany)
Sodium hydroxide, pellets	6771.2	Carl Roth (Karlsruhe, Germany)
TEMED	T9281	Sigma-Aldrich (St. Louis, USA)
TRIS-HCl pH 8.5	4855.5	Carl Roth (Karlsruhe, Germany)
Triton X-100	1.08603.1000	Merck Millipore (Darmstadt, Germany)
Trypan Blue	17-942E	Lonza (Basel, Switzerland)
Tween® 20	P2287	Sigma-Aldrich (Darmstadt, Germany)
Yeast Extract	212750	BD Biosciences (Franklin Lakes, USA)

5.3 Cell culture, molecular biology and biochemistry consumables

Table 21: Consumables

Consumable	Format	Cat. number	Distributor (headquarter)
Blotting membranes	0.45 µm Nitrocellulose	88018	Thermo Fisher Scientific (Waltham, USA)
Bottle top filters	500 ml 1000 ml	15983307 15993307	Thermo Fisher Scientific (Waltham, USA)
Cell scrapers		sc-395251	Santa Cruz Biotechnology (Dallas, USA)
Cell strainers	30 µm	C-8234	neoLab Migge GmbH (Heidelberg, Germany)
Coverslips	12 mm	631-1577	VWR (Radnor, USA)
Cryotubes	1 ml	123280	Greiner (Kremsmünster, Austria)
Luna™ reusable slides		L12011	Logos Biosystems (Anyang, South Korea)
Microscopy slides		H868.1	Carl Roth (Karlsruhe, Germany)
Pasteur pipettes		7691061	Th. Geyer (Renningen, Germany)
PCR strip tubes	8 x 200 µl	710971	Biozym (Hessisch Oldendorf, Germany)
Petri dishes	Ø 10 cm	633180	Greiner (Kremsmünster, Austria)
Pipette tips	10 µl 200 µl 1250 µl	70.1130 70.760.002 70.1186	Sarstedt (Nümbrecht, Germany)
Pipetting reservoirs	25 ml	B3125-50	Parmer GmbH (Cole- Wertheim, Germany)
qPCR plate seals		4ti-0500	Steinbrenner Laborsysteme (Wiesenbach, Germany)
qPCR plates	96-well	4ti-0910/C	Steinbrenner Laborsysteme (Wiesenbach, Germany)
Reaction tubes	0.2 ml 0.5 ml 1.5 ml 2.0 ml	72.737.002 72.699 72.690.001 72.695.200	Sarstedt (Nümbrecht, Germany)
Scalpel Cutfix®		9409814	Th. Geyer (Renningen, Germany)
Screw cap tubes	15 ml 50 ml	62.554.502 62.547.254	Sarstedt (Nümbrecht, Germany)
Serological pipettes	5 ml 10 ml 25 ml 50 ml	86.1253.001 86.1254.001 86.1685.001 7695555	Sarstedt (Nümbrecht, Germany) Th. Geyer (Renningen, Germany)
Syringe filters	0.2 µm	83.1826.001	Sarstedt (Nümbrecht, Germany)
Syringes	50 ml	94.6077.137	Sarstedt (Nümbrecht, Germany)
TC dishes	Ø 10 cm, Cell+	83.3902.300	Sarstedt (Nümbrecht, Germany)
TC plates	6-well 24-well, Cell+	83.3920.005 83.3922.300	Sarstedt (Nümbrecht, Germany)
Western blot filter tissues		115-2166	VWR (Radnor, USA)
U bottom plates	96-well	83.3925.500	Sarstedt (Nümbrecht, Germany)

Virus filter	0.45 µm PVDF	SLHV033R	Merck Millipore (Darmstadt, Germany)
--------------	--------------	----------	--------------------------------------

5.4 Technical equipment

Table 22: Notable laboratory equipment

Name	Appliance	Distributor (headquarter)
5415 D	Centrifuge	Eppendorf (Hamburg, Germany)
5810	Centrifuge, plates	Eppendorf (Hamburg, Germany)
Bioanalyzer 2100	Electrophoresis RNA quality control	Agilent Technologies (Santa Clara, USA)
BP121S	Analytical balance	Sartorius (Göttingen, Germany)
Branson Sonifier 250	Sonication device	Thermo Fisher Scientific (Waltham, USA)
CellDiscoverer 7	Microscope	Carl Zeiss (Oberkochen, Germany)
DFC9000 GT	Camera (microscopy)	(Wetzlar, Germany)
DM6 B	Microscope	Leica (Wetzlar, Germany)
DMIL LED	Microscope, brightfield	Leica (Wetzlar, Germany)
EasyPhor Midi	Agarose gel electrophoresis chamber	Biozym (Hessisch Oldendorf, Germany)
GelStick Touch	Agarose gel imaging system	INTAS Science Imaging Instruments GmbH (Göttingen, Germany)
HERAcell 150i	Incubator	Thermo Fisher Scientific (Waltham, USA)
C170 E3		Binder (Tuttlingen, Germany)
II/2b Device	Nucleofector	Lonza (Basel, Switzerland)
Jupiter Class 1 - Vertical flow clean air cabinet	Class I clean air cabinet	Labogene (Allerød, Denmark)
Luna™	Automated cell counter	Logos Biosystems (Anyang, South Korea)
Milli-Q Q-POD	Water conditioner	EMD Millipore (Burlington, USA)
Milli-Q® Integral 5	Water purifier	Merck KGaA (Darmstadt, Germany)
Mini-PROTEAN® Tetra	PAGE equipment	System Bio-Rad Laboratories (Hercules, USA)
Mr. Frosty™	Freezing container	Thermo Fisher Scientific (Waltham, USA)
NanoDrop™ 1000	Micro-Spectrophotometer	Thermo Fisher Scientific (Waltham, USA)
NU-540 Class II Type A2 Biosafety Cabinet	Biosafety Cabinet	Nuaire (Plymouth, USA)
Odyssey IR imaging system	Western blot imager	LI-COR Biosciences (Lincoln, USA)
Pipet Filler S1	Pipetting aid	Thermo Fisher Scientific Scientific (Waltham, USA)
PowerWave™ XS	Microplate reader	BioTek (Bad Friedrichshall, Germany)
ProfiLab pH597	pH-meter	Xylem Inc. (Rye Brook, USA)

PTC-200	PCR cycler	Bio-Rad Laboratories (Hercules, USA)
QuantStudio 7 Flex	qPCR cycler	Thermo Fisher Scientific (Waltham, USA)
Qubit 4 Fluorometer	DNA, RNA and protein quantification device	Thermo Fisher Scientific (Waltham, USA)
Research [®] plus	Micropipettes	Eppendorf (Hamburg, Germany)
TCS SP5 II	Confocal Microscope	Leica (Wetzlar, Germany)
Thermomixer comfort	Block heater	Eppendorf (Hamburg, Germany)
Thermomixer comfort 5355	Thermomixer	Eppendorf (Hamburg, Germany)
Trans-Blot [®] Turbo [™]	Western blot transfer system	Bio-Rad Laboratories (Hercules, USA)
UF V 700	Freezer -80°C	BINDER GmbH (Tuttlingen, Germany)
VIP plus	Freezer -150°C	Panasonic (Kadoma, Japan)
Z 216 MK	Centrifuge, refrigerated	Hermle (Wehingen, Germany)
Z 366	Centrifuge, cell culture	Hermle (Wehingen, Germany)

5.5 Software

Table 23: Computer software

Computer program	Supplier
ApE – A plasmid editor	M. Wayne Davis
BioRender.com	BioRender (Toronto, Canada)
Chemotaxis and Migration Tool Version 2.0	Ibidi GmbH (Gräfelfing, Germany)
EndNote X8	Clarivate Analytics (London, UK)
Excel 2016	Microsoft (Redmond, USA)
Image Studio v 2.0	Li-Cor (Lincoln, USA)
ImageJ (FIJI)	National Institutes of Health (Rockville, USA)
Leica Application Suite AF	Leica (Wetzlar, Germany)
Leica Application Suite X	Leica (Wetzlar, Germany)
PowerPoint 2016	Microsoft (Redmond, USA)
Prism 5	GraphPad (San Diego, USA)
QuantStudio qPCR software	Thermo Fisher Scientific (Waltham, USA)
R Studio	RStudio PBC, open source
Word 2016	Microsoft (Redmond, USA)
ZEN	Carl Zeiss (Oberkochen, Germany)

6 Methods

6.1 Cell culture

All cells were maintained within humidified incubators at 37°C and 5% CO₂. Recipes of complete cell culture media are listed in Table 5. Composition of self-made buffers supplements and solutions for cell culture are listed in Table 6. Media were stored at 4°C and were pre-warmed to room temperature (RT) before use.

6.1.1 Cultivation of human iPSCs

Ctrl#1 and Ctrl#2 iPSC lines were provided by Dr. Sandra Horschitz. Human iPSCs were cultured as colonies on Geltrex™-coated 6-well plates. Geltrex™ was thawed at 4°C, diluted 1:100 in wash medium and was subsequently added to tissue culture plates overnight at 4°C for coating. iPSCs were cultured in iPSC medium with daily medium changes. Cells were passaged using 0.5 mM EDTA. Thereby, the cells were first washed once with DPBS and were then incubated at 37°C for 4 min with EDTA until they started to detach. EDTA was aspirated and iPSCs were resuspended in iPSC medium which was supplemented with Y-27632 for 24 hours after splitting to promote cell survival. Cells were passaged every 4-7 days at a 1:6-1:10 ratio.

6.1.2 Generation of human iPSC-derived cortical neurons

Ctrl#1 and Ctrl#1 C4A iPSC lines were used for neuronal differentiation. For neural induction the dual-SMAD inhibition approach was used. Thereby, mesodermal and endodermal fates are blocked by inhibition of BMP and TGF- β signaling and cells are pushed into an ectodermal fate (Chambers et al., 2009). Posterization to a hindbrain fate is avoided by additional inhibition of Wnt signaling.

For the generation of NPCs, iPSC cultures were first grown to 70-80% confluency on Geltrex™-coated plates. Media was then changed to NPC medium phase 1 for 8 days with a 1:2 split after 4 days. On day 8, cells were split again and were then cultivated for another 8 days in NPC medium phase 2. On day 16, cells were either frozen in NPC freezing medium or further passaged in NPC medium phase 3 for up to two passages. Cells were passaged using TrypLE™ Express. Thereby, cells were washed once with DPBS and were then incubated with TrypLE™ for 5 min at 37°C. Cells were then flushed from the cell culture plates using wash medium, dissociated to single cells and were then centrifuged at 1200 x g for 3.5 min at RT. Supernatant was removed and cells were resuspended in the corresponding media supplemented with Y-27632. After 24 hours Y-27632 was removed from the media. Media changes were performed daily.

The differentiation protocol for the generation of cortical neurons was based on previously published work by others (Kemp et al., 2016; Telezhkin et al., 2016). Thereby, neuronal

differentiation is initiated by inhibition of Notch signaling and forced cell-cycle exit in the presence of elevated Ca^{2+} levels. Activation of TrkB and Wnt signaling at later stages of the protocol enhances synaptogenesis and network formation. Cortical neuronal differentiation of NPCs was initiated on day 0 by switching to neuronal differentiation medium phase 1. On day 3, cultures were dissociated to single cells using TrypLE™ for 5 min at 37°C. Detached cells were resuspended in wash buffer and centrifuged at 1000 x g for 3.5 min. After removing the supernatant, cells were resuspended in neuronal differentiation medium phase 2 and re-plated on coverslips or tissue culture plates coated with polyethyleneimine (PEI) and laminin. For coating, plates and coverslips were first incubated with PEI coating solution for 10 min at RT. After washing 3x with H_2O , laminin (diluted 1:400 in DPBS + MgCl_2 and CaCl_2) was added overnight at 4°C. Prior to coating, coverslips were pre-treated with 37% hydrochloric acid for 1-2 hours at RT with gentle shaking. After multiple washing steps with H_2O and ethanol coverslips were air-dried and autoclaved. Cells were seeded at a density of 200.000 cells / well on coverslips in 24-well plates or 4 million cells / well in 6-well plates. Thereby, cell numbers were determined using an automated cell counter (Luna™). Cells were supplemented with Y-27632 for 24 hours after the split. On day 10, medium was changed to neuronal differentiation medium phase 3 and from day 17 cells were cultured in neuronal differentiation medium phase 4. Subsequently, neurons were further matured until day 50 in neuronal differentiation medium phase 4 with medium changes twice per week.

6.1.3 Generation of human iPSC-derived microglia

The protocol for the generation of iPSC-derived microglia was based on published work by others with minor adaptations (Haenseler et al., 2017). Ctrl#1 mCherry and Ctrl#2 iPSC lines were used for microglial differentiation. The Ctrl#1 mCherry line was used for live imaging of co-cultures. Ctrl#2 was used for all other experiments. Human iPSCs were dissociated to single cells with TrypLE™, resuspended in wash media and centrifuged at 1200 x g for 3.5 min. Cells were resuspended in EB medium and plated into U-bottom 96-well plates at a density of 20.000 cells / well to form EBs. Cell numbers were determined using an automated cell counter (Luna™). Prior to cell seeding, U-bottom 96-well plates were coated with Pluronic coating solution at RT for 15 min. A 50% media change was performed on day 2 and on day 4 EBs were replated into non-coated 6-well plates with 15 EBs / well. EBs were transferred using 1250 µl pipette tips. Pipette tips were cut to increase the diameter size and reduce the force on the EBs. All remaining EB medium was removed and EBs were supplied with 3 ml / well macrophage progenitor medium. Medium was changed every 3 to 5 days depending on the consumption. Macrophage progenitor cells appeared in the supernatant after 2-4 weeks and could be harvested for many weeks, up to a few months. For maturation of microglia in mono-culture, progenitor cells were harvested, filtered through a 30 µm cell strainer and plated at a

density of 200.000 cells / well on coverslips in 24-well plates or 4 million cells / well in 6-well plates coated with PEI / laminin (for coating procedure see 6.1.2). Macrophage progenitor cells were differentiated to mature microglia over a course of 1 week in co-culture medium.

6.1.4 Co-cultures of human iPSC-derived cortical neurons and microglia

Macrophage progenitor cells were harvested from the supernatant, filtered through a 30 µm cell strainer and added to day 50 iPSC-derived cortical neurons. 100.000 macrophage progenitors were added to 200.000 neurons / well on coverslips in 24-well plates and 2 million macrophage progenitors were added to 4 million neurons / well in 6-well plates. Co-cultures were maintained for a duration of 1-2 weeks in co-culture medium.

6.1.5 Treatments of microglia, cortical neurons and co-cultures

Mono-cultures of mature microglia were stimulated with 100 ng/ml *E.Coli* (O111:B4) LPS for 24 hours to induce an inflammatory response and thereby test the functional response of the cells. LPS was added to co-culture medium after 1 week of final microglia maturation. Mono-cultures of mature microglia were further treated with different concentrations of the NLRP3 inflammasome inhibitor oridonin (1, 5 and 10 µM) in combination with 100 ng/ml LPS in co-culture medium for 24 hours to assess the anti-inflammatory effects of oridonin. For *in vitro* synaptic pruning assays, mature microglia mono-cultures were treated with synaptosomes (see sections 6.1.6 and 6.5.4) and 5 or 10 µM oridonin for 5 hours. For phagocytosis assays, mature microglia were treated with pHrodo™ Green Zymosan Bioparticles (see section 6.1.6) and different concentrations of the tetracycline antibiotic minocycline (60, 80 and 100 µM) for 2-2.5 hours.

Microglia-neuron co-cultures and pure neuronal cultures were treated with 1 µM oridonin for 1-2 weeks. Microglia-neuron co-cultures were further treated with 60 µM minocycline for 1 week. All co-culture treatments were performed in co-culture medium. DMSO was used as a vehicle (VEH) control for all oridonin and minocycline treatments. Oridonin and minocycline concentrations used in this study were taken from the literature (He et al., 2018; Sellgren et al., 2019; Wang et al., 2005; Wang et al., 2016).

6.1.6 Phagocytosis assay and *in vitro* synaptic pruning assay

For the phagocytosis assay, pHrodo™ Green Zymosan Bioparticles were added to mature microglia mono-cultures at a final concentration of 20 µg/ml in live cell imaging solution. The pHrodo™ Green Zymosan Bioparticle stock solution was prepared by reconstituting 1 mg of bioparticles in 2 ml of live cell imaging solution for a stock concentration of 0.5 mg/ml. Microglia were imaged for 2-2.5 hours with the Celldiscoverer 7 (Zeiss) microscope.

For the *in vitro* synaptic pruning assay, 5 µl of labeled synaptosome solution (see section 6.5.4) was added to mature microglia mono-cultures in 500 µl live cell imaging solution in one well of

a 24-well plate. Microglia were imaged for 5 hours with the Celldiscoverer 7 (Zeiss) microscope.

6.1.7 Cultivation of HEK-293T cells

HEK-293T cells were cultured on PLL (0.1 mg/ml poly-L-lysine in 25 mM borate buffer (pH 8.4)) -coated 10 cm dishes in HEK-293T medium. When growing confluent, cells were split 1:20 using TrypLE™. Media was changed every second day. HEK-293T cells were used for lentivirus production.

6.1.8 Cryopreservation of cells

Human iPSCs were frozen in iPSC freezing medium. Thereby, cells were detached with EDTA as described in section 6.1.1. NPCs were detached from cell culture plates using TrypLE™ as described in section 6.1.2 and were frozen in NPC freezing medium. HEK-293T cells were detached in the same way as NPCs and were frozen in HEK-293T medium with 10% DMSO. 500 µl of cell suspension was transferred to one cryotube. All cells were frozen in Mr. Frosty™ freezing containers containing 2-propanol and were transferred to a -150°C freezer for long-term storage.

Cryopreserved cells were thawed at 37°C and were then quickly transferred to 10 ml wash medium. After centrifugation at 500 x g for iPSCs and 1200 x g for NPCs and HEK-293T cells, cells were re-suspended in their corresponding medium supplemented with 10 µM Y-27632. Medium was changed after 24 hours to remove cell debris and Y-27632.

6.2 Molecular biology

All recipes of self-made buffers and solutions are listed in Table 18.

6.2.1 Genomic DNA (gDNA) isolation

For gDNA isolation, cells were washed 1x with ice-cold DPBS and were subsequently scraped from the cell culture dish using a cell scraper. After centrifugation at 5000 x g for 5 min, the cell pellet was re-suspended in 150 µl DNA isolation lysis buffer supplemented with 1 µl proteinase K. Cells were incubated with the lysis buffer at 37°C for 1 hour shaking at 500 rpm in a thermomixer. Proteinase K was inactivated by increasing the temperature to 95°C for 10 min. 105 µl 2-propanol was added for 30 min at RT for DNA precipitation. Samples were centrifuged at 12.000 x g for 10 min and the resulting pellet was subsequently washed twice with 75% ethanol (v/v in H₂O). After air-drying the pellet at RT, it was finally re-suspended in 100 µl H₂O at 37°C and 400 rpm shaking for 30 min. All DNA samples were stored at -20°C.

6.2.2 RNA Isolation and cDNA synthesis

RNA isolation was performed under a chemical hood and the workspace was cleaned with RNase AWAY prior to starting. For RNA isolation, cells were washed 1x with ice-cold DPBS and were subsequently scraped from the cell culture dish using a cell scraper. After centrifugation at 5000 x g for 5 min, the cell pellet was re-suspended in 1 ml RNA-Solv reagent and either frozen at -80°C for later processing or lysed for 5 min at RT. 100 µl chloroform was added and samples were vortexed for 15 s. After a 10 min incubation at RT, samples were centrifuged at 12.000 x g for 5 min at 4°C to allow phase separation. The aqueous phase, containing RNA and DNA was transferred to a new test tube. 500 µl 2-propanol was added and samples were carefully mixed by inverting before incubating them overnight at -20°C for nucleic acid precipitation. Subsequently, samples were centrifuged at 12.000 x g for 15 min at 4°C. After removing the supernatant, pellets were washed twice with 1 ml 75% ethanol (v/v in DEPC-H₂O) with centrifugation steps at 12.000 x g for 10 min and 4°C in between. After air-drying the pellets, they were resuspended in 20 µl DEPC-H₂O at 37°C for 15 min and 400 rpm in a thermomixer. Genomic DNA was digested using the DNase I Amplification Grade kit. 2.5 µl DNase I and 2.5 µl 10x reaction buffer were added to each sample and incubated for 15 min at RT. The reaction was stopped by adding 2.5 µl stop solution at 70°C for 10 min. RNA concentration was measured using a NanoDrop™ 1000 spectrophotometer. RNA samples were stored at -80°C.

cDNA was synthesized from 1 µg of RNA using the iScript™ cDNA synthesis kit. The reaction mixture specified in Table 24 was incubated in a thermal cycler according to the protocol described in Table 25. cDNA was diluted 1:5 in nuclease-free H₂O.

Table 24: cDNA synthesis reaction mixture

Component	Volume (total 20 µl)
5x iScript™ reaction mix	4 µl
iScript™ reverse transcriptase (RT)	1 µl
RNA template	variable (1 µg total)
Nuclease-free H ₂ O	variable

Table 25: Cycling program for cDNA synthesis

Step	Temperature	Duration
Priming	25°C	5 min
Reverse transcription	46°C	20 min
Enzyme inactivation	95°C	1 min
	4°C	∞

6.2.3 Reverse transcription PCR (RT-PCR)

All substances used for RT-PCR, as well as their final concentrations in the reaction mixture, are listed in Table 12. Amplification of target amplicons from cDNA or gDNA was performed using the RT-PCR cycling program specified in Table 26. Annealing temperature and elongation time was adjusted based on primers and expected amplicon size. All primers are listed in Table 10. PCR products were analyzed by agarose gel electrophoresis as described in 6.2.6.

Table 26: RT-PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	
Denaturation	95°C	20 sec	35
Annealing	60°C	20 sec	
Elongation	72°C	1 min/500 bp	
Final elongation	72°C	10 min	
	4°C	∞	

6.2.4 Quantitative PCR (qPCR)

SYBR Green-based qPCR analysis was performed using a QuantStudio 7 Flex system. A GoTaq® DNA Polymerase was used for amplification. All substances used for qPCR, as well as their final concentrations in the reaction mixture, are listed in Table 12. All primers are listed in Table 10. The threshold cycle (Ct) values and melting curves were calculated using the QuantStudio software. The $\Delta\Delta C_t$ method was applied to determine fold changes (Livak and Schmittgen, 2001) and gene expression was normalized to 18S rRNA.

Table 27: qPCR program

Steps	Temperature	Time	Cycles
Hold stage	50°C	2 min	
	95°C	10 min	
PCR stage	95°C	15 sec	40
	60°C	15 sec	
	72°C	30 sec	
Melt curve stage	95°C	15 sec	
	60°C	1 min	
	95°C	15 sec	

6.2.5 Mycoplasma PCR

Human iPSCs were regularly checked for mycoplasma contamination by RT-PCR (Ossewaarde et al., 1996; van Kuppeveld et al., 1992). Therefore, gDNA was isolated as described in section 6.2.1 and was used as a template for a PCR reaction. A mycoplasma contamination was detectable by a 270 bp band which represents the mycoplasma-specific

16S rRNA. Primer sequences are listed in Table 10 and details of the mycoplasma PCR cycling program are listed in Table 28. PCR products were analyzed by agarose gel electrophoresis as described in 6.2.6.

Table 28: Mycoplasma PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min	
Denaturation	95°C	15 sec	35
Annealing	50°C	15 sec	
Elongation	72°C	10 sec	
Final elongation	72°C	3 min	
	4°C	∞	

6.2.6 Agarose gel electrophoresis and gel extraction of DNA

RT-PCR products were analyzed by agarose gel electrophoresis using 1-2% agarose in 1xTAE buffer supplemented with pEqGREEN at a dilution of 1:15.000. After a completed PCR reaction, 10x DNA sample buffer was added to the PCR reaction mix to a final concentration of 1x. Depending on the expected amplicon size a 100 bp or 1 kbp marker was loaded next to the samples as a reference. DNA fragments were separated in the agarose gel at 100 V for 50 min and gels were imaged using a GelStick Touch imaging system (INTAS Science Imaging Instruments GmbH).

If necessary, DNA fragments were isolated from agarose gels using the pEqGOLD Gel extraction kit according to the manufacturer's instructions.

6.2.7 Quality control for bulk RNAseq

For RNA bulk sequencing experiments, RNA concentration was determined using a Qubit 4 Fluorometer with the Qubit RNA BR Assay-Kit according to the manufacturer's instructions. To assess the RNA integrity, samples were prepared using the RNA 6000 Nano kit and were subsequently analyzed using a Bioanalyzer 2100 according to the manufacturer's instructions.

6.2.8 Bulk RNAseq

Two biological replicates of microglia treated or not treated with LPS were analyzed. RNA quality was assessed as described in 6.2.7. Samples with RIN values ≥ 7.3 were sent to the High Throughput Sequencing Unit of the Genomics & Proteomics Core Facility, German Cancer Research Center (DKFZ) to be processed. Libraries were prepared with the TruSeq Stranded protocol (Illumina) and sequenced to 50 bp single reads on one lane on the Illumina HiSeq 4K platform. Sequencing data was run through an RNAseq processing workflow by the Omics IT and Data Management Core Facility, German Cancer Research Center (DKFZ). Total counts per feature were imported to R (R Core Team, 2020) and analyzed using DESeq2 (Love et al., 2014). All features without any counts were removed, for differential testing with

DESeq2 the formula “~Batch+Condition” was used. Heatmaps for bulk RNAseq expression data show z-scaled TPM normalized counts.

Bulk RNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

6.2.9 Nuclei isolation

Co-cultures of iPSC-derived microglia and neurons with and without C4A overexpression were harvested with TrypLE™ Express. Cells were pelleted, washed 1x in PBS and transferred to 1.5 ml safe-lock tubes. All remaining liquid was removed and cell pellets were snap frozen in liquid nitrogen and transferred to -80°C. Nuclei were isolated according to a protocol kindly provided by the single-cell Open Lab at the German Cancer Research Center (DKFZ; Heidelberg, Germany). Buffers used for nuclei isolation are listed in Table 29 and Table 30. On ice, cell pellets were shortly homogenized in 350 µl TST buffer and filtered through a 40 µm cell strainer into a 2 ml tube. The cell strainer was subsequently rinsed with another 350 µl TST buffer to wash out remaining cells. 1 ml of 1x ST buffer was added and the cells were spun down at 500xg for 5 min and 4°C. The supernatant was removed and cells were washed again with 1ml 1x ST buffer. The pellet was then resuspended in 1 ml EZ buffer, incubated 3 min on ice and washed again with 1 ml 1x ST buffer as described above. Finally, the pellet was resuspended in 200 µl 1x ST buffer and filtered through a 20 µm cell strainer. Number and quality of nuclei was assessed using an automated cell counter (Luna™). A total number of 20.000 nuclei at a final concentration of 500 nuclei per µl were used for loading on a 10x Chromium Next GEM Chip.

Table 29: 2x ST buffer

Volume	Reagent	Final concentration
584 µl	5M NaCl	292 mM
200 µl	1M Tris HCl, pH 7.5	20 mM
20 µl	1M CaCl ₂	2 mM CaCl ₂
420 µl	1M MgCl ₂	42 mM MgCl ₂
8.776 µl	H ₂ O	
10 ml	Total volume	

Table 30: TST buffer

Volume	Reagent	Final concentration
1 ml	2x ST buffer	1x
60 µl	1% Tween-20	0.03%
10 µl	2% BSA	0.01%
930 µl	H ₂ O	
2 ml	Total volume	

6.2.10 Single-nucleus RNAseq (snRNAseq)

cDNA library preparation was done at the single-cell Open Lab at the German Cancer Research Center (DKFZ; Heidelberg, Germany) according to the 10x Genomics Chromium Next GEM single cell 3' reagent kits v3.1 user guide (10x Genomics, PN-1000121). The prepared cDNA libraries were processed by the High Throughput Sequencing Unit of the Genomics & Proteomics Core Facility of the German Cancer Research Center (DKFZ). The libraries were sequenced on the Illumina NovaSeq 6K platform on a S1 flow cell (paired-end 100) to a depth of 420M-500M reads per library. Fastq files were parsed to cellranger (10x Genomics) count in order to generate a count matrix. FastQC was used for general sequencing quality control (Andrews et al., 2015). If not stated otherwise, data analysis was performed using the Seurat v4.1.0 package (Hao et al., 2021) in R v4.0.3 (R Core Team, 2020). Count matrices were filtered with following parameters: Any feature that was expressed in less than three cells was removed from the analysis. Any cell with less than 500 expressed features, more than 10% mitochondrial genes expressed, or less than 600 total UMI counts was removed from further analysis. The data was normalized using `sctransform`.

Dimensional reduction was performed using UMAP with `dims=1:30`. Shared nearest neighbor graph was constructed with `dims=1:30`. Clusters were generated with `resolution=0.1`.

Cell type identification was performed by inspection of bona fide marker gene expression for microglia (MERTK, GPR34, TREM2, PROS1, C1QA, GAS6, TMEM119, ITGAM, CD14, TLR4, SPI1, CD163, CD86, CD74, CD40, CD68, AIF1, PTPRC, CSF1R) and neurons (RBFOX3, SYN1, DLG4, SLC17A7, SLC17A6, GAD1, BCL11B, TBR1, GRIN1, GRIN2B, GRIA2, GRIA4, MAPT, MAP2, NPAS4, ARC, FOS).

GO analysis was performed using the R packages AnnotationDbi (Pagès et al., 2020), org.Hs.eg.db (Carlson, 2020), clusterProfiler (Yu et al., 2012). Plots were generated with ggplot2 (Wickham, 2016).

Zscore for GO terms was calculated as the difference between the number of up-/down-regulated genes in a term divided by the square root of the number of genes associated with the GO term.

SnRNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

6.2.11 Transformation of chemically competent *E. Coli*

For amplification of plasmid DNA *E.coli* bacteria were used. Bacteria were thawed on ice for 5 min before adding 5 µl of purified plasmid DNA for 15 min on ice. Bacteria were then exposed to 42°C for 42 sec to allow DNA uptake. Bacteria were put on ice for 2 min before adding 300 µl SOC medium. Subsequently, bacteria were incubated at 37°C for 1 hour at 700 rpm in a thermomixer. Bacteria were then plated onto pre-warmed LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Single bacteria colonies were picked and further

grown in 5 ml LB medium for mini preparation or 200 µl LB medium for midi preparation. 100 µg/ml ampicillin was added and bacterial cultures were incubated at 37°C and 400 rpm shaking overnight. For plasmid DNA isolation from bacteria see section 6.2.12.

6.2.12 Plasmid DNA isolation

For small-scale isolation of plasmid DNA from bacteria a peqGOLD Plasmid miniprep kit was used. Plasmid DNA was isolated according to the manufacturer's instructions from 2 ml liquid bacterial culture. The extracted DNA was eluted in 30 µl H₂O was used to analyze bacterial clones in the process of molecular cloning. For large-scale isolation of plasmid DNA, the PureLink™ HiPure Plasmid Filter Midiprep Kit was used. DNA was isolated from 200 ml liquid bacterial culture following the manufacturer's instructions. Plasmid DNA was eluted in 100 µl H₂O. DNA concentration was measured using a NanoDrop™ 1000 spectrophotometer and DNA was stored at -20°C.

6.2.13 Lipofectamine transfection

For lipofectamine transfection the Lipofectamine™ 3000 Transfection Reagent Kit was used according to the manufacturer's protocol. For one well of a 6-well plate, 62.5 µl Opti-MEM™ medium was mixed with 2.5 µl Lipofectamine transfection reagent and in a separate test tube 62.5 µl Opti-MEM™ medium was mixed with 2.5 µg DNA and 2.5 µl of the P3000 reagent. The DNA mixture was then added to the Lipofectamine mixture and they were incubated for 15 min at RT. 125 µl transfection mix was then added to one 6-well. Medium was changed after 24 hours. Puromycin (0.33 µg/ml) selection was started after 48 hours.

6.3 Generation of a mCherry fluorescent reporter cell line

6.3.1 Molecular cloning

For the generation of a fluorescent reporter cell line a vector driving the expression of mCherry under the control of a constitutively active EF-1α promoter was used. Therefore, the EF-1α-mCherry sequence was amplified from the A201 AAV-EF-1α-mCherry plasmid kindly provided by the group of Prof. Dr. Valery Grinevich (ZI, Mannheim, Germany) and was subsequently cloned into the pBluescript backbone pMK243. For plasmid details see Table 8 and Figure 8. For primer details see Table 11. First, the pMK243 backbone, which has two HpaI restriction sites, was digested at 37°C for 1 hour with the HpaI restriction enzyme to achieve a linearization and for removal of the *TetOn-OsTIR PGK-TetR* sequence from the backbone. For details about the reaction mixture see Table 31. The reaction products were separated by a 1% agarose gel electrophoresis and the backbone was isolated by gel extraction as described in section 6.2.6. The backbone was then dephosphorylated by adding 2 µl Antarctic Phosphatase Reaction Buffer (10X) and 1 µl Antarctic Phosphatase for 30 min at 37°C. The

reaction was stopped by heat-inactivation at 80°C for 2 min. The EF-1 α -mCherry sequence was amplified using the LongAmp Taq DNA Polymerase and specific primers for Gibson assembly cloning. For details see Table 32 and Table 33. After amplification, the PCR product was separated by a 1% agarose gel electrophoresis and was isolated by gel extraction. The EF-1 α -mCherry insert and the pMK243 backbone were ligated for 30 min at 50°C using the Gibson assembly reaction mixture listed in Table 34.

Table 31: Restriction digest of pMK243

Component	Volume
CutSmart buffer (10x)	2 μ l
HpaI	1 μ l
pMK243 plasmid	1 μ l (1 μ g)
H ₂ O	16 μ l
Total	20 μ l

Table 32: LongAmp reaction mixture

Component	Concentration
LongAmp Reaction Buffer (5x)	1x
dNTPs	300 μ M
Primers	400 nM each
LongAmp Taq DNA Polymerase	2.5 U
Plasmid DNA	10 ng
H ₂ O	To 25 μ l

Table 33: LongAmp PCR cycling program

Step	Temperature	Time	Cycles
Initial denaturation	94°C	30 sec	
Denaturation	94°C	30 sec	30
Annealing	60°C	30 sec	
Elongation	65°C	50 sec / kb	
Final extension	65°C	10 min	
	4°C	∞	

Table 34: Gibson assembly reaction mixture for pMK243- EF-1 α -mCherry

Component	Volume
NEBuilder® HiFi DNA Assembly Master Mix (2x)	10 μ l
Backbone vector DNA	0.02 pmol
Insert DNA	0.06 pmol
H ₂ O	variable
Total	to 20 μ l

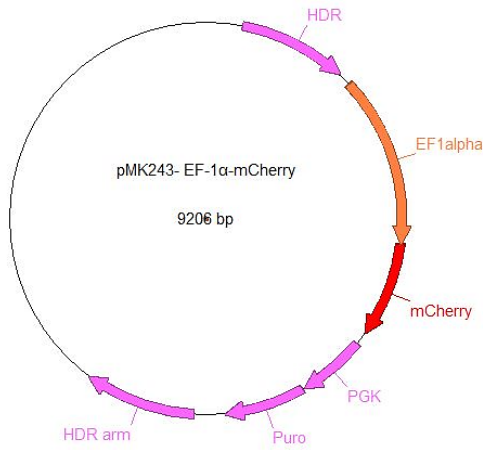


Figure 8: Plasmid map of pMK243- EF-1α-mCherry.

6.3.2 Sanger sequencing

After cloning, final plasmid DNA constructs were sent for sequencing to the Microsynth Seqlab GmbH (Göttingen, Germany). 12 µl of plasmid DNA at a concentration of 40-100 ng/µl was mixed with 3 µl of the relevant sequencing primers at a concentration of 10 µM. FASTA sequencing files were analyzed using the ApE plasmid editor software.

6.3.3 Nucleofection and clonal selection

Nucleofection of the Ctrl#1 iPSC line was performed using a Lonza Cell Line Nucleofector 2b and the Cell Line Nucleofector Kit V (Lonza, VCA-1003) according to the manufacturer's protocol. Cells were dissociated to single cells with TrypLE™ for 5 min at 37°C and were counted with automated cell counter (Luna™). 1×10^6 cells were centrifuged at 500 x g for 5 min. The pellet was resuspended in a mixture of 80 µl Nucleofector solution V, 20 µl of supplement 1 and 1 µg of each of the following plasmids: pMK243-EF-1α-mCherry and AAVS1-T2-pX330. The cell suspension was transferred to a nucleofection cuvette avoiding air bubbles and the program B-023 was applied. Cells were then plated in iPSC medium without Pen/Strep and with Y-27632 (5 µM) at four different densities. 400.000, 300.000, 200.000 and 100.000 cells were plated onto four different wells of a 6-well plate. Non-nucleofected cells were plated on the two remaining wells of the 6-well plate. One well was used as a selection control and the other one was grown confluent and harvested as a control for SNP analysis. Two days after nucleofection media was changed to iPSC medium with 1% Pen/Strep and Y-27632 (5 µM). Puromycin (0.33 µg/ml) was added to the media after 3 days and Y-27632 was removed. Puromycin selection was continued for 1 week and mCherry positive clones were picked manually and expanded.

6.3.4 SNP analysis

To identify potential karyotypic abnormalities, a single nucleotide polymorphism (SNP) analysis was performed. Therefore, gDNA was extracted using the ExtractMe® Genomic DNA Kit according to the manufacturer's instructions. 900 ng of DNA (60 ng/μl) was sent to the Life & Brain GmbH (Bonn, Germany) and was analyzed using an Illumina Infinium® Global Screening Array–24v1.0 BeadChip. Data was kindly processed and analyzed by Josef Frank (Department of Genetic Epidemiology, ZI Mannheim, Germany).

6.4 Generation of a C4A overexpression cell line

6.4.1 Molecular cloning

For the generation of a C4A overexpression cell line a lentiviral vector was used. For details about plasmids see Table 8 and Figure 9. For primers and oligonucleotides see Table 11. First, the pCDH-CuO-MCS-T2A-GFP SparQ™ cloning and expression lentivector backbone was digested at 37°C for 1 hour with the NotI and Sall restriction enzymes, as described in Table 35, to achieve a linearization of the plasmid and for removal of the T2A-GFP sequence from the backbone. The reaction products were separated by a 1% agarose gel electrophoresis and the backbone was isolated by gel extraction as described in section 6.2.6. The T2A sequence was then re-introduced into the vector with additional restriction sites for subsequent cloning steps. For this, the T2A-NotI-XmaI-Sall sense and antisense oligonucleotides were phosphorylated and annealed by incubation with the T4 polynucleotide kinase using the reaction mixture listed in Table 36 and incubated for 30 min at 37°C and then heated to 95°C for 5 min, followed by slow cooling to 25°C at 5°C/sec. The annealed oligonucleotides and the backbone were then ligated as described in Table 37. In the next step, a puromycin resistance gene was added after the T2A sequence using classical cloning techniques to enable selection of the cells. The puromycin sequence was amplified from a plasmid available in the lab using the LongAmp Taq DNA Polymerase as described in section 6.3.1. It was cloned into the backbone using the XmaI and Sall restriction enzymes. After all digestion and amplification steps, reaction products were separated by 1% agarose gel electrophoresis and isolated by gel extraction as described above. For ligation, the T4 DNA Ligase was used according to the manufacturer's protocol. The ligation mixture listed in Table 38 was incubated for 1 hour at RT. Subsequently, an HA-and Flag-Tag sequence was inserted using the same classical cloning techniques with the BamHI and NotI restriction enzymes. The C4A cDNA sequence was cloned into the backbone using the Gibson assembly approach. Therefore, the C4A cDNA insert was amplified from a human C4A cDNA expression vector using the LongAmp Taq DNA Polymerase and specific primers for Gibson assembly cloning as described in section 6.3.1. The pCDH-CuO-Flag-HA-T2A-Puro backbone was digested using the XbaI and BamHI restriction enzymes. Backbone and insert were separated by a 1% agarose gel electrophoresis

and isolated by gel extraction. The C4A insert and the backbone were ligated for 30 min at 50°C using the Gibson assembly reaction mixture listed in Table 39. The final pCDH-CuO-C4A-Flag-HA-T2A-Puro plasmid was sent for sanger sequencing as described in section 6.3.2. For antibody testing and validation of the construct, HEK-293T cells were transfected with the C4A expression vector using the Lipofectamine™ 3000 Transfection Reagent as described in section 6.2.13.

Table 35: Restriction digest of pCDH-CuO-MCS-T2A-GFP SparQ™

Component	Volume
CutSmart Buffer (10x)	2 µl
NotI	1 µl
Sall	1 µl
pCDH-CuO-MCS-T2A-GFP SparQ™ plasmid	1 µl (1 µg)
H ₂ O	15 µl
Total	20 µl

Table 36: Reaction mixture for oligonucleotide phosphorylation

Component	Volume
T2A-NotI-XmaI-Sall- sense (100 µM)	1 µl
T2A-NotI-XmaI-Sall- antisense (100 µM)	1 µl
T4 Polynucleotide Kinase Reaction Buffer (10x)	1 µl
H ₂ O	6.5 µl
T4 Polynucleotide Kinase	0.5 µl
Total	10 µl

Table 37: Ligation reaction T2A-NotI-XmaI-Sall oligos with backbone

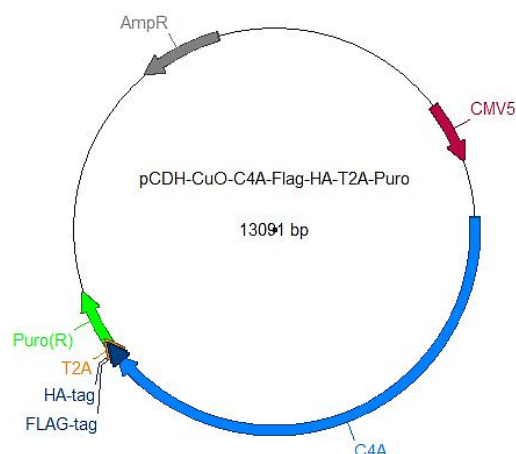
Component	Volume
Linearized pCDH-CuO-MCS-T2A-GFP SparQ™ backbone	variable (50 ng)
Phosphorylated and annealed T2A-NotI-XmaI-Sall oligos (1:200)	1 µl
T4 DNA Ligase Buffer (10x)	1.1 µl
H ₂ O	variable
T4 DNA Ligase	1 µl
Total	11 µl

Table 38: Ligation mixture for T4 DNA Ligase

Component	20 µl reaction
T4 DNA Ligase Buffer (10x)	2 µl
Backbone vector DNA	0.02 pmol
Insert DNA	0.06 pmol
H ₂ O	to 20 µl
T4 DNA Ligase	1 µl

Table 39: Gibson assembly reaction mixture

Component	Volume
NEBuilder® HiFi DNA Assembly Master Mix (2x)	10 μ l
Backbone vector DNA	0.03 pmol
Insert DNA	0.06 pmol
H ₂ O	variable
Total	to 20 μ l

**Figure 9: Plasmid map of pCDH-CuO-C4A-Flag-HA-T2A-Puro.**

6.4.2 Lentivirus production

All work involving lentiviruses was performed according to Laboratory Biosafety Level (BSL) - 2. For lentivirus production HEK-293T cells were grown to 70-80% confluency on 10 cm cell culture dishes coated with PLL (see section 6.1.7). 30 min prior to transfection fresh medium supplemented with 25 μ M chloroquine diphosphate was added to the cells. A mixture of three transfection plasmids was prepared. 7.9 μ g of a psPAX2 lentiviral packaging plasmid, 3.5 μ g of a pMD2.G VSV-G envelope expressing plasmid and 10 μ g of the C4A expression lentivector were used. For plasmid details see Table 8. 130 μ l of a 2 M CaCl₂ solution was added and the DNA mixture was filled up to a total volume of 1 ml with H₂O. Plasmids were transfected into HEK-293T cells via calcium phosphate transfection. Therefore, 1 ml of 2x HEPES buffered saline solution (HBSS) was filled into a 15 ml screw cap tube. A 5 ml serological pipette was used to blow air into the solution and the DNA mixture was added dropwise. The mixture was incubated for 30 min at RT and was then added dropwise to the HEK-293T cells. A medium change was performed after 8 hours and conditioned medium was subsequently collected on days 2 and 3 after transfection. Conditioned medium was pooled and centrifuged for 10 min at 1600 x g at 4°C. The supernatant was subsequently filtered through a 0.45 μ m PVDF filter. Lentivirus purification was performed using polyethylene glycol (PEG)-6000 precipitation. 24 ml supernatant was mixed with 6 ml 50% PEG-6000 solution, 2.5 ml 4 M NaCl and 2.7 ml DPBS. The solution was incubated for 1.5 hours at 4°C and was mixed by inverting every 30

min. After centrifugation at 3200 x g for 30 min, the pellet was re-suspended in 100 µl DPBS + MgCl₂ and CaCl₂. 20 µl Aliquots were stored at -80°C.

6.4.3 Infection and selection of human iPSCs

The Ctrl#1 human iPSC line was split 1:6 one day before lentivirus infection as described in 6.1.1. Medium was changed to 1 ml iPSC medium supplemented with 10 µg/ml Polybrene Infection Reagent shortly before infection. One aliquot (20 µl) of lentivirus (see section 6.4.2) was added to one well of a 6-well plate. Medium was changed to fresh iPSC medium after 24 hours and after 48 hours puromycin (0.33 µg/ml) was added for selection. Non-infected control cells were used as a selection control.

6.5 Biochemistry

All recipes of self-made buffers and solutions are listed in Table 18.

6.5.1 Immunocytochemistry

All immunocytochemistry stainings were performed on 12 mm coverslips which were prepared as described in 6.1.2. After washing once with PBS, cells were fixed with 4% PFA for 10 min at RT. After washing three times with PBS, cells were subsequently blocked and permeabilized using a blocking solution (10% FBS in PBS with 0.1% Triton-X) for 1 hour at RT. Primary antibodies were diluted in blocking solution and were incubated overnight at 4°C in a wet chamber. After washing three times with PBS, coverslips were incubated with Alexa Fluor-labeled secondary antibodies. All secondary antibodies were diluted 1:1000 in blocking solution and were incubated 45 min at RT. Secondary antibodies were removed by washing twice with PBS. Cell nuclei were stained with DAPI for 10 min at RT and coverslips were washed twice with PBS and once with H₂O. Coverslips were mounted on glass slides using Mowiol®/DABCO and were left to solidify overnight at RT. All primary and secondary antibodies and their dilutions are listed in Table 15 and Table 16.

6.5.2 Cell lysis and protein quantification

Cells were washed once with ice-cold PBS and were scraped off cell culture plates using a cell scraper. After centrifugation at 5000 x g for 5 min and 4°C, cell pellets were re-suspended in 100 µl protein lysis buffer and were incubated for 1 hour on ice. Lysates were then sonicated with 5 pulses (duty cycle 20%, output control 5.5) using a Branson Ultrasonics™ sonifier. Subsequently, samples were centrifuged at 16.000 x g and 4°C and supernatant was transferred to a new test tube. Protein concentration was determined with a Pierce™ BCA Protein-Assay according to the manufacturer's protocol. In short, samples were diluted 1:5 in H₂O and were incubated with assay reagents for 30 min at 37°C in a 96-well plate. The absorption was measured at 562 nm using a PowerWave™ XS microplate reader. Protein

concentrations were calculated by using a BSA standard dilution series as a reference. Protein samples were stored at 4°C.

6.5.3 SDS-PAGE and Western blotting

For protein analysis, lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, samples were denatured in SDS-PAGE sample buffer at 95°C for 5 min. For C4 blots 40 µg of protein was loaded in each well and for synaptosome blots 15 µg of protein was loaded in each well. The SDS-PAGE was first run at 30 V for 20 min to allow stacking of the proteins and was then increased to 120 V for 1.5-2 h depending on the molecular weight of the proteins analyzed. Proteins were then transferred onto 0.45 µm nitrocellulose blotting membranes by semi-dry blotting using a Trans-Blot® Turbo™ Western blot transfer system for 45 min at 20 V and 1 A. Membranes were blocked for 1 h at RT in 5% (w/v) milk powder in TBS-T (blocking buffer) and were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Membranes were washed three times for 10 min with TBS-T and DyLight™ infrared (IR)-conjugated secondary antibodies, diluted in TBS-T, were added at RT for 1 h. After three final washing steps, proteins were visualized using a LI-COR Odyssey IR imaging system. All primary and secondary antibodies and their dilutions are listed in Table 15 and Table 16.

6.5.4 Extraction of synaptosomes and labeling with pHrodo

Synaptosomes were isolated from neuronal cultures at day 50 using the Syn-PER™ Synaptic Protein Extraction Reagent according to the manufacturer's protocol. Cells were washed twice with ice-cold PBS and 200 µl of Syn-PER™ reagent was added to one well of a 6-well plate. Cells from 2 wells were pooled for each synaptosome extraction. Cells were scraped from cell culture plates using a cell scraper and were centrifuged at 1200 x g for 10 min at 4°C. The supernatant was transferred into a new test tube and was centrifuged at 15.000 x g for 20 min at 4°C. After removing the supernatant, the synaptosome pellet was re-suspended in 17 µl Syn-PER™ reagent supplemented with 5% (v/v) DMSO and 2 µl of a 10x phosphatase and protease inhibitor solution. Synaptosomes were stored at -80°C.

Synaptosomes were subsequently labeled with the amine-reactive dye pHrodo (pHrodo™ Green STP ester) according to the data sheet instructions and as described previously by others (Sellgren et al., 2019; Sellgren et al., 2017). pHrodo is a fluorogenic dye which is non-fluorescent in an extracellular environment with a pH of 7.4, but becomes increasingly fluorescent in the acidic environment of phagosomes or lysosomes. 2 µl of a 1 M sodium bicarbonate solution was added to 20 µl of extracted synaptosome solution. 500 µg of lyophilized pHrodo™ Green STP ester was reconstituted in 75 µl DMSO (8.9 mM Stock) and 1 µl of the pHrodo solution was added to each synaptosome sample. After 2 hours incubation at RT, 300 rpm gentle agitation and protection from light, labeled synaptosomes were centrifuged

at 15.000 x g for 20 min at 4°C. After removing the supernatant, synaptosomes were washed three times with a 0.1 M sodium bicarbonate solution with subsequent centrifugation steps at 15.000 x g for 20 min at 4°C. Labeled synaptosomes were re-suspended in 19 µl Syn-PER™ Synaptic Protein Extraction Reagent with 5% (v/v) DMSO and were stored at -80°C.

6.6 Microscopy and image analysis

6.6.1 Fluorescence microscopy

All immunofluorescence stainings were imaged using a DM6 B fluorescence microscope (Leica) and the corresponding Las X imaging software. Images were acquired at a 10x, 20x or 40x magnification. The number of synapsin and PSD-95 particles per unit length, as well as their co-localization were analyzed using the ImageJ plugin ComDet v.0.4.1. For quantification of microglial morphology, images were first thresholded to create a binary mask and were then analyzed using the ImageJ shape descriptor function determining perimeter, circularity and Feret's diameter. All other immunofluorescence analyses were done manually using ImageJ.

6.6.2 Live cell imaging

For all live cell imaging experiments, a Celldiscoverer 7 microscope (Carl Zeiss) was used. Before starting an imaging session, the microscope chamber was equilibrated to 37°C and 5% CO₂ and medium was replaced by pre-warmed live cell imaging solution. For microglia phagocytosis assays, *in vitro* synaptic pruning assays and imaging of co-cultures, images were automatically acquired every 10 min for 2-10 hours with 3 images taken per well using a 10x magnification. For quantification of phagocytosis and *in vitro* synaptic pruning assays, fluorescence intensity was measured for each image using ImageJ (Fiji). The fluorescence intensity was normalized to the total cell number per image based on brightfield images. Microglial movement was tracked by first determining cell positions using the ImageJ manual tracking plugin. Manual tracks were then analyzed using the Chemotaxis and Migration Tool Version 2.0 (Ibidi).

6.7 Statistical analysis and visualization

Bar graphs and scatter plots were generated with GraphPad Prism 5 and depict mean ± standard error of the mean (SEM). Quantifications were performed on at least three biological replicates. Kolmogorov-Smirnov test for normality was performed on all data sets. If criteria for normal distribution were met, significance testing between two groups was performed by applying an unpaired two-tailed t test. A one-way ANOVA with Tukey's multiple comparison test was performed for multiple groups. If criteria for normal distribution were not met, non-parametric testing was performed. A two-tailed Mann-Whitney U test was performed if two groups were compared. A Kruskal-Wallis test followed by a Dunn's post hoc test was

performed if multiple groups influenced by a single variable were compared. For comparisons of data sets with more than one variable a two-way ANOVA with Bonferroni post-hoc testing was applied. The exact number of replicates and the applied statistical tests are specified in the individual figure legends. All statistical analyses were performed with the help of GraphPad Prism 5. Significance levels against the respective controls are * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns: not significant ($P > 0.05$). Schematic representations were generated with the help of BioRender.com.

7 Results

7.1 Generation and characterization of human iPSC-derived microglia

For the generation of microglia-like cells from human iPSCs a previously published protocol was used with minor adaptations (Haenseler et al., 2017). Human iPSCs of healthy control individuals were grown into EBs and cultured with bone morphogenic protein 4 (BMP4), vascular endothelial growth factor (VEGF) and stem cell factor (SCF), to induce mesoderm, as well as endothelial and hematopoietic precursors. EBs were plated onto cell culture dishes and high concentrations of macrophage colony-stimulating factor (M-CSF) and IL-3 were added to promote myeloid differentiation. After approximately 2-3 weeks macrophage precursors emerged into the culture supernatants. These cells could then be harvested for multiple weeks up to a few months and were further matured for 7 days in the presence of colony stimulating factor 1 receptor (CSF1R) ligand IL-34 and granulocyte macrophage colony stimulating factor (GM-CSF), both of which activate survival signaling pathways and are expressed by neurons and astrocytes, respectively. After final maturation, iPSC-derived microglia displayed a microglia-like ramified morphology (Figure 10).

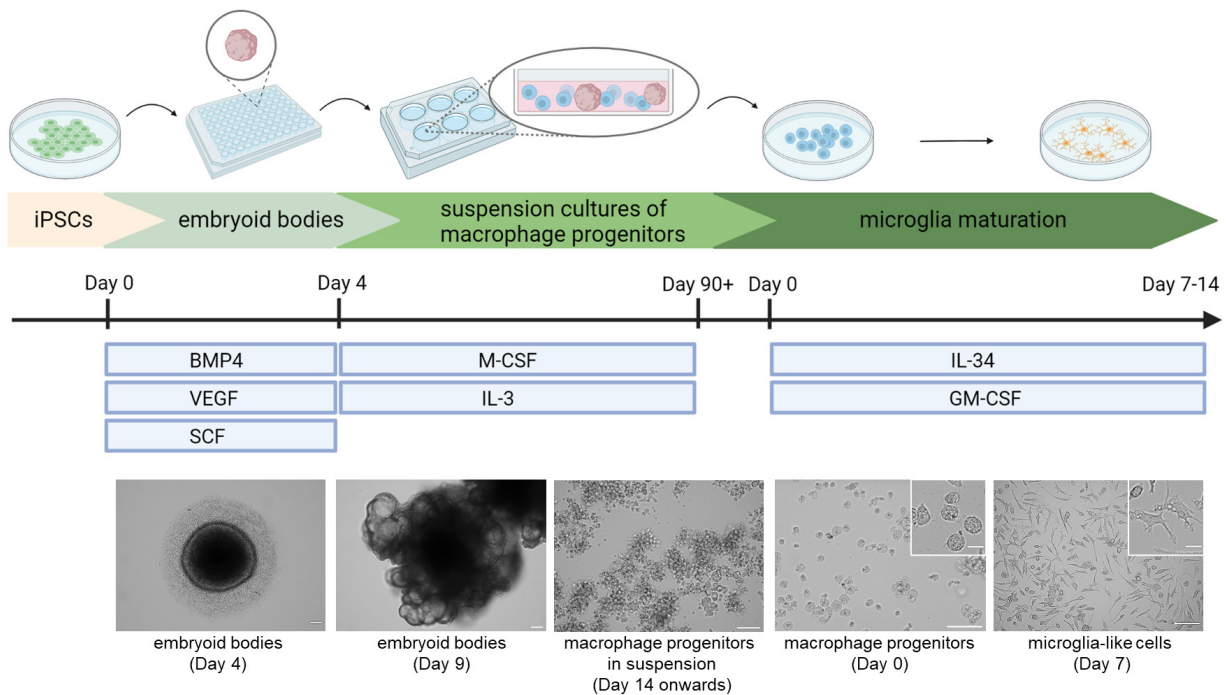


Figure 10: Differentiation protocol for the generation of human iPSC-derived microglia.

Schematic representation of the differentiation protocol for the generation of human iPSC-derived microglia showing the most important steps and the general timeline. Macrophage progenitor cells can be collected from suspension cultures for at least 90 days. Macrophage progenitors are further matured to microglia-like cells for 7-14 days in microglia maturation medium. Exemplary brightfield images illustrate the different stages of the microglia differentiation protocol. Scale bars, 100 μ m and 20 μ m [zoomed in].

Results

Expression of typical microglia markers was assessed by immunocytochemistry. $94 \pm 1\%$ of the cells were positive for Iba1, $96 \pm 0.8\%$ positive for CD68, $99 \pm 0.5\%$ positive for TREM2 and $95 \pm 1\%$ positive for TMEM119 (means $\pm$ SEM) (Figure 11A). To characterize the gene expression pattern of human iPSC-derived microglia, whole transcriptome bulk RNAseq experiments were performed after 7 days of final maturation in the presence of IL-34 and GM-CSF. Control microglia (Ctrl1, Ctrl2) were compared to LPS-treated samples (LPS1, LPS2). Canonical microglia markers were analyzed and compared to bulk RNAseq data from day 45 human iPSC-derived cortical neurons (d45_1, d45_2, d45_3). Expression of microglia marker genes including *TREM2*, *TMEM119*, *CD68* and *AIF1* (Iba1) was highly enriched in microglia samples compared to neuronal samples. Homeostasis-associated genes such as *GPR34*, *TMEM119* and *CSF1R* were downregulated in microglia after treatment with LPS (Figure 11B).

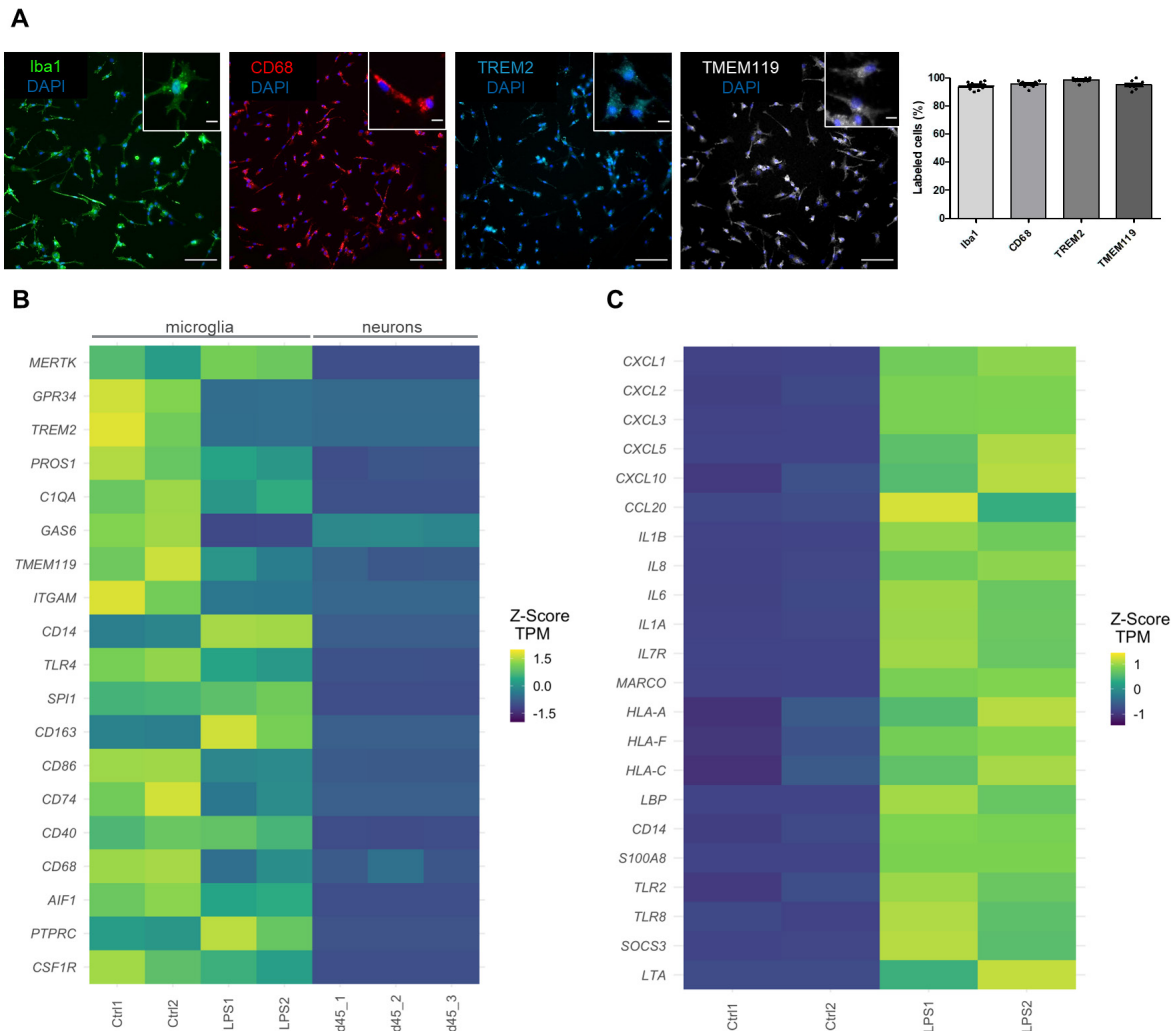


Figure 11: iPSC-derived microglia express canonical microglia markers and respond to pro-inflammatory stimuli.

(A) Microglia-like cells express common microglia markers such as Iba1 (94%), CD68 (96%), TREM2 (99%) and TMEM119 (95%) ($n = 9$ images with 1267-1524 cells per marker from three biological replicates, dots for % of labeled cells per analyzed image, means $\pm$ SEM). (B) Z-scores of TPM counts from RNA sequencing (RNAseq) experiments show the expression of canonical microglia markers.

Results

Control microglia samples (Ctrl1, Ctrl2) are compared to LPS-treated microglia (LPS1, LPS2) and microglia samples are compared to samples from day 45 human iPSC-derived cortical neurons (d45_1, d45_2, d45_3). **(C)** Z-scores of markers related to inflammation and immune response show differences between controls (Ctrl1, Ctrl2) and LPS-treated samples (LPS1, LPS2) indicating a response of human iPSC-derived microglia to pro-inflammatory stimuli in RNAseq experiments. Scale bars, 100 μm (A) and 10 μm [zoomed in A]. Bulk RNAseq data of human iPSC-derived cortical neurons was generated by Dr. Ruven Wilkens. Bulk RNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

Z-scores of inflammation-related genes were significantly differentially expressed between controls (Ctrl1, Ctrl2) and LPS-treated samples (LPS1, LPS2) (Figure 11C). For example, the expression of genes encoding C-X-C and C-C subfamilies of chemokines (e.g., *CXCL1*, *CCL20*), interleukins (e.g., *IL1B*, *IL8*, *IL6*, *IL1A*), PRRs (e.g., *MARCO*), MHC class I molecules (e.g., *HLA-A*, *HLA-F*) and LPS receptors (*LBP* and *CD14*) were significantly upregulated after LPS treatment. To link the identified differentially expressed genes to different biological pathways, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed. GO enrichment analysis for biological processes (BP), molecular functions (MF) and cellular components (CC) confirmed the observed LPS-induced inflammatory response. Thereby, pathways related to the response to bacterial molecules, cytokine production, NF κ B-signaling or immune receptor activity, as well as the endosomal-lysosomal system, were associated to LPS treatment (Figure 12A-C). KEGG analysis identified pathways associated to NF κ B-signaling, TNF- and chemokine signaling, Toll-like receptor signaling, pathways of neurodegeneration and pathogenic *E.Coli* infection to be enriched after LPS treatment (Figure 12D).

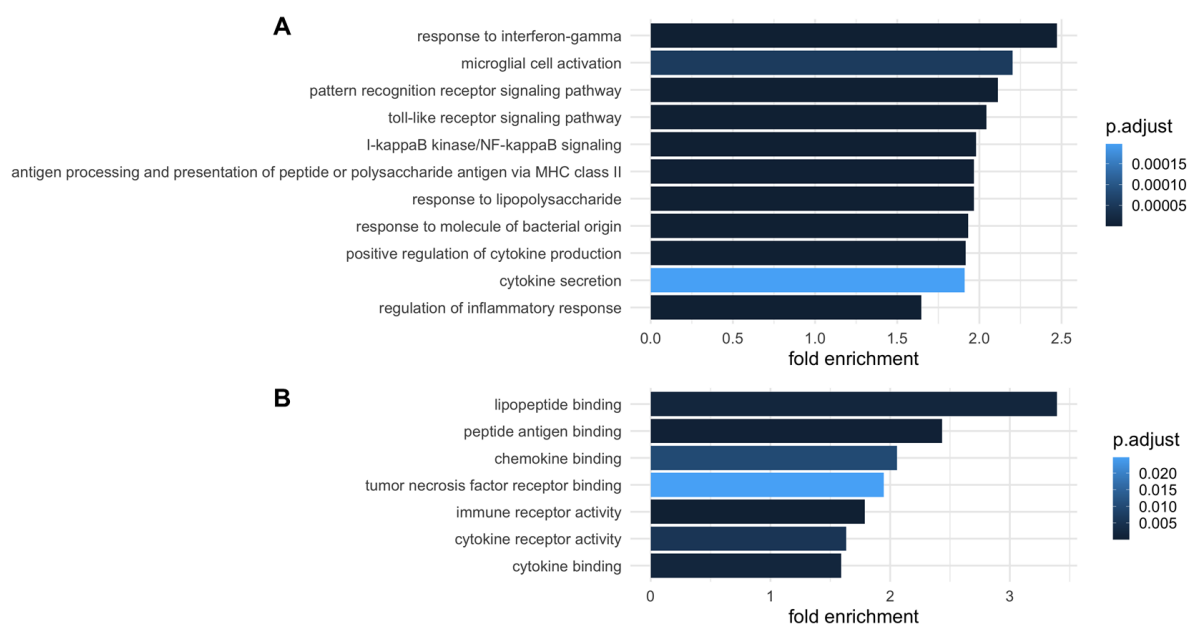


Figure continued on next page

Results

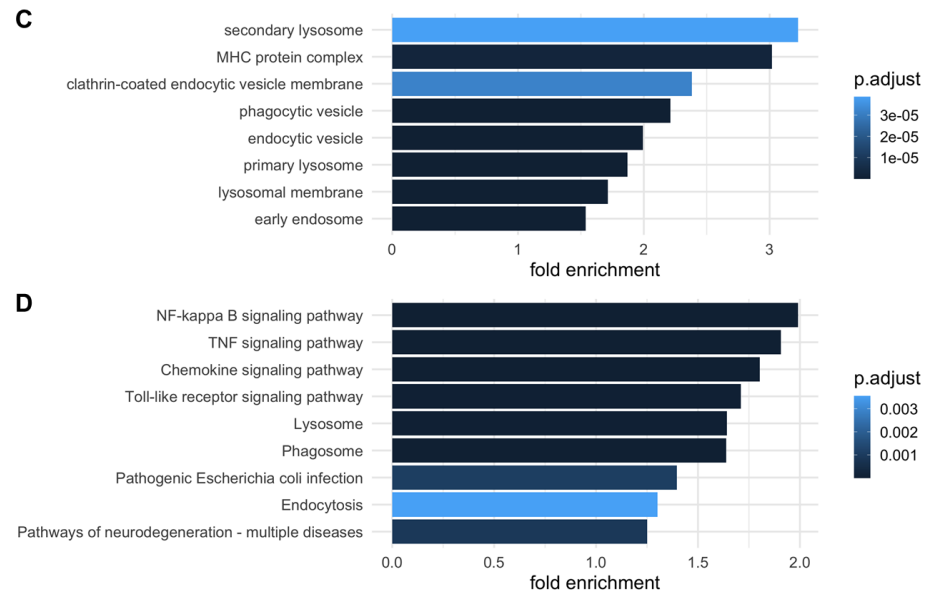


Figure 12: Differentially expressed genes in LPS-treated microglia.

(A-C) GO enrichment analysis for biological processes (BP), molecular functions (MF) and cellular components (CC) comparing control human iPSC-derived microglia with LPS-treated microglia in whole transcriptome bulk RNA sequencing (RNAseq) experiments. (D) KEGG pathway enrichment analysis comparing control and LPS-treated microglia. Bulk RNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

Functional activity of iPSC-derived microglia was further demonstrated by showing their phagocytic activity. The cells were treated with pHrodo-labeled zymosan bioparticles, a cell wall component of *Saccharomyces cerevisiae*, and were subsequently imaged for 5 hours in live cell imaging experiments. pH-sensitive particles became visible within 30 min with increasing fluorescence intensity per cell over time indicating a phagocytic activity of the cells (Figure 13).

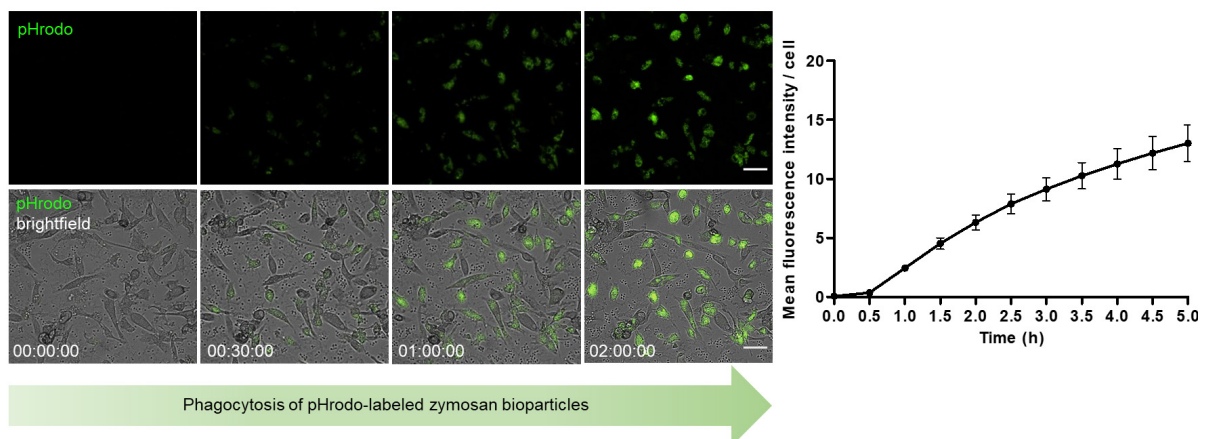


Figure 13: IPSC-derived microglia are phagocytically competent.

The progressive acidification of the phagosomes was detected using zymosan bioparticles coupled to pH-sensitive fluorophores. pH-sensitive particles became visible inside the cells within 30 min indicating the development of mature phagosomes (n = 4 biological replicates with 122-176 cells per replicate, means ± SEM). Scale bars, 50 µm. Time scale, hh:minmin:secsec.

Furthermore, treatment with 100 ng/ml *E.Coli* LPS for 24 hours caused a significant change in microglia morphology, indicative of a response to pro-inflammatory stimuli. The percentage of cells displaying a multipolar, hyper-ramified shape increased from $18 \pm 2\%$ in control cells to $69 \pm 3\%$ in LPS-treated cells. The percentage of elongated, bipolar microglia decreased from $41 \pm 2\%$ in control cells to $14 \pm 1\%$ in LPS-treated cells and the percentage of microglia displaying a round morphology decreased from $40 \pm 2\%$ to $17 \pm 1\%$ after LPS treatment (means $\pm$ SEM) (Figure 14A). Shape descriptor parameters further confirmed the morphological change of Iba1-positive cells after LPS treatment. LPS-treated microglia displayed a significantly decreased circularity with no significant change in whole cell perimeter or Feret's diameter, which represents the longest distance between any two points along the region of interest (ROI) boundary (Figure 14B).

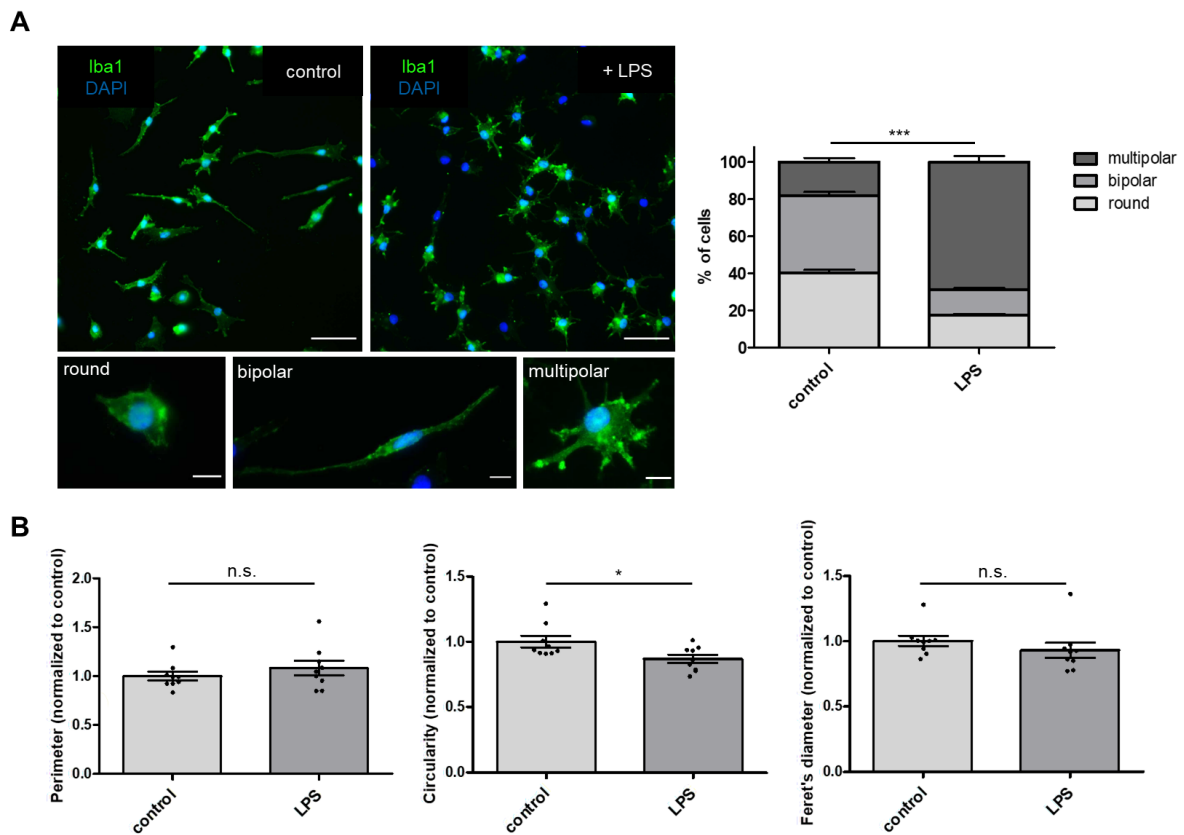


Figure 14: IPSC-derived microglia undergo morphological change after LPS-treatment.

(A) A 24-hour treatment with 100 ng/ml LPS causes microglia to adopt a multipolar, hyper-ramified morphology ($n = 9$ images with 571-695 cells per condition from three biological replicates, Mann-Whitney U-test, $P < 0.001$ for “multipolar”, “bipolar” and “round”, means $\pm$ SEM). (B) Quantifications of shape descriptor parameters (perimeter, circularity and Feret's diameter) show differences between controls and LPS-treated cells ($n = 9$ images with 900-1214 cells per condition from three biological replicates, two-tailed t test, dots represent individual analyzed images, means $\pm$ SEM). Scale bars, 50 μ m (A) and 10 μ m [zoomed in A]. * $P < 0.05$ and *** $P < 0.001$. n.s., not significant.

7.2 Generation of human cortical neuronal cultures from iPSCs

Human iPSCs from healthy control individuals were also used to generate forebrain-type neural progenitors by inhibiting mesodermal and endodermal fates with the use of two small-molecule inhibitors of SMAD signaling (LDN-193189 and SB-431542) (Chambers et al., 2009). Addition of Wnt pathway inhibitor XAV-939 blocked the formation of neural crest cells and enhanced the induction of forebrain precursors (Qi et al., 2017). This highly efficient approach allowed the use of adherent culture conditions as opposed to EB formation. Neural progenitors were then further differentiated into mature cortical neurons by small molecule-driven cell-cycle arrest using a combination of the Notch pathway inhibitor DAPT, cyclin-dependent kinase (CDK)4/6 inhibitor PD-0332991, as well as CREB and Wnt pathway activators Forskolin and CHIR99021, respectively. Fetal conditions were further simulated by exposure to GABA and increased concentrations of CaCl_2 . Small-molecule agonists of TrkB (LM22A and LM22B) and the PKC activator Bryostatin1 were added to promote neuronal survival, differentiation and synaptic functions (Figure 15). The applied protocol was based on previously published work by Kemp and Teleshkin (Kemp et al., 2016; Telezhkin et al., 2016).

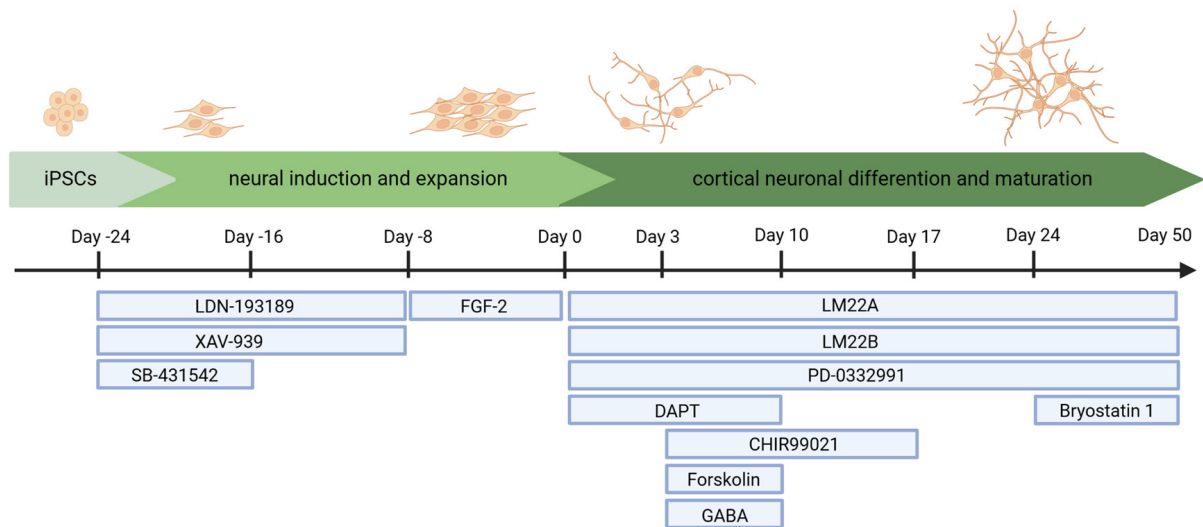


Figure 15: Differentiation protocol for the generation of human iPSC-derived cortical neurons.

Schematic representation of the differentiation protocol for the generation of mature human iPSC-derived cortical neurons showing the general timeline, as well as the most important steps and small molecules. After 24 days of neural induction and expansion, neuronal differentiation and maturation was initiated by exchanging neural progenitor medium with neural differentiation medium (Day 0). Neurons were matured for 50 days before performing experiments.

Neural progenitor cells were characterized by immunofluorescence stainings for common progenitor markers such as Pax6, Sox2 and Nestin, as well as the proliferation marker pH3 (Figure 16A). After cortical neuronal differentiation, highly pure cultures were obtained with only minimal astrocytic content, as shown by GFAP and S100 β immunofluorescence stainings (Figure 16B). Human iPSC-derived cortical neurons expressed pan-neuronal markers MAP2,

Tau and NeuN, as well as synaptic markers such as synapsin and PSD-95 after 50 days of final differentiation, as shown by immunofluorescence stainings. $44 \pm 2\%$ of the neurons expressed the layer VI cortical layer marker TBR1 and $28 \pm 2\%$ of the cells expressed the layer V cortical layer marker CTIP2 (means $\pm$ SEM) (Figure 16C).

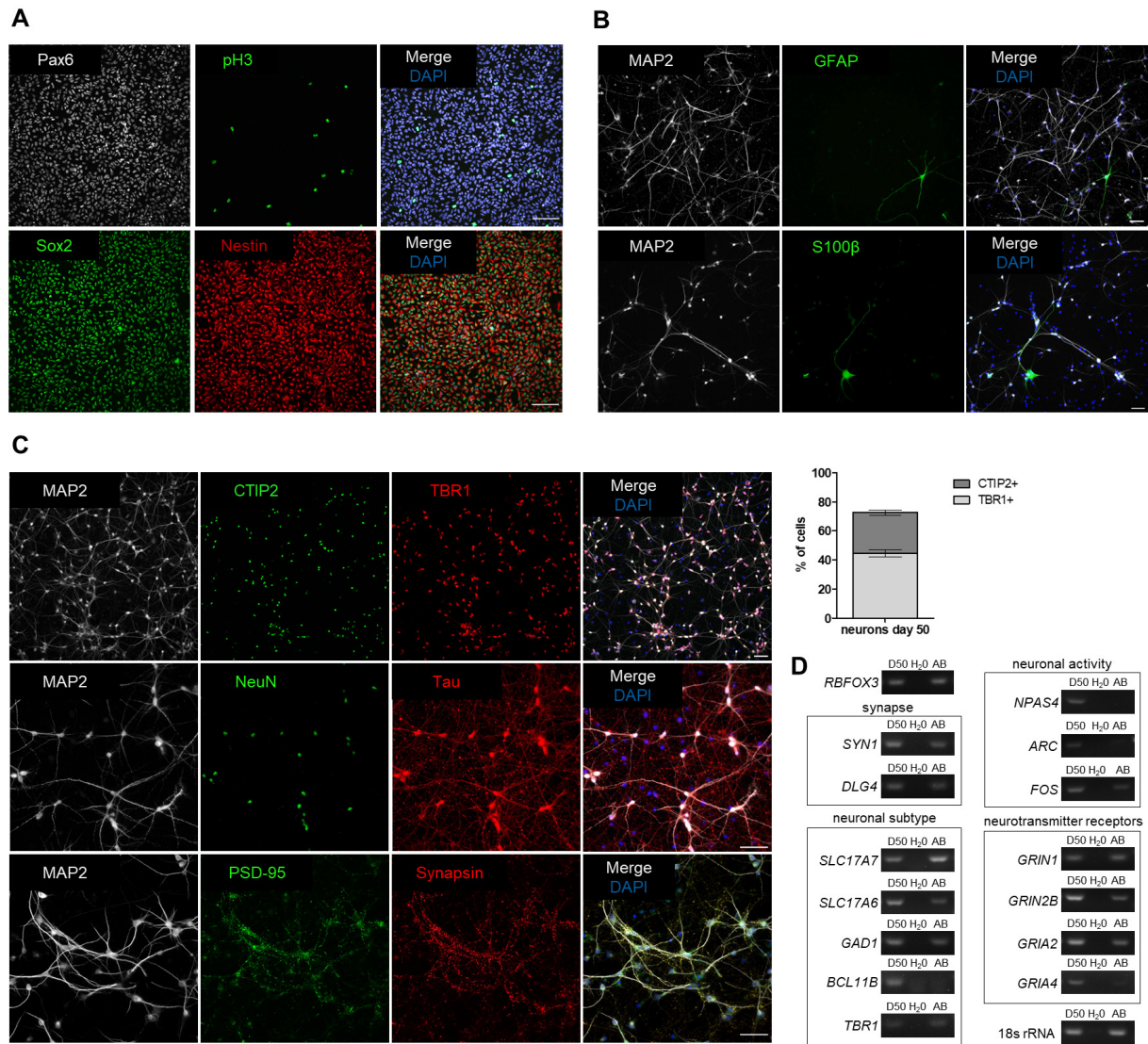


Figure 16: Differentiation and characterization of human iPSC-derived cortical neurons.

(A) Representative immunostainings for neural progenitor markers Pax6, Sox2, Nestin and proliferation marker pH3. (B) Representative immunostainings for astrocyte markers GFAP and S100β in cortical neuronal cultures at day 50. (C) Representative immunostainings of day 50 neuronal cultures. Neurons express pan-neuronal markers and cortical layer markers. Quantification of the percentage of TBR1+ and CTIP2+ nuclei (n = 9 images with 5291 cells from three biological replicates, means $\pm$ SEM). (D) RT-PCR analysis of canonical neuronal marker genes in neurons at day 50 of differentiation. AB: adult brain. Scale bars, 100 μm (A) and 50 μm (B and C).

RT-PCR analysis was performed at day 50 of neuronal differentiation for further characterization of the cells. The neuronal marker gene *RBFOX3* (NeuN), as well as genes encoding synaptic proteins (*SYN1* (synapsin), *DLG4* (PSD-95)) were expressed, as well as

neuronal activity-related immediate early genes (*NPAS4*, *ARC*, *FOS*). The neuronal cultures contained different neuronal subtypes as shown by the expression of the glutamatergic markers *SLC17A6* (vGlut2) and *SLC17A7* (vGlut1) and the GABAergic marker *GAD1*. NMDA (*GRIN1*, *GRIN2B*) and AMPA-receptor genes (*GRIA2*, *GRIA4*) were expressed at day 50 and the regional identity of the generated neuronal cultures was confirmed by *BCL11B* (CTIP2) and *TBR1* expression (Figure 16D).

7.3 Overexpression of complement component C4A in human iPSC-derived cortical neurons

The effects of neuronal complement component C4A expression on microglia in the context of synaptic pruning and SCZ pathogenesis were investigated by lentiviral overexpression of C4A in neurons. Thereby, neurons overexpressing C4A were compared to a non-transduced control. To generate an overexpression cell line, the *C4A* sequence was cloned into a lentivector backbone adding a hemagglutinin (HA) and Flag-Tag, as well as a puromycin resistance gene for easier detection and selection of infected cells (Figure 17A). The final construct was first tested by lipofectamine transfection and subsequent immunofluorescence stainings in HEK293T cells (Figure 17B). Human iPSCs of healthy control individuals were used for the generation of a C4A overexpression cell line. Cells were investigated for genomic and karyotypic integrity and analyzed for single nucleotide polymorphisms before using them for experiments (Figure 17C). Furthermore, immunostainings were performed for pluripotency markers Oct3/4, SSEA4 and Sox2 to validate their developmental potential (Figure 17D). After lentiviral infection and selection with puromycin, C4A overexpression in human iPSCs was confirmed by immunofluorescence stainings for C4 and the HA-Tag (Figure 17E). RT-PCR analysis was performed using specific primers for the detection of the *C4A* and *C4B* isoforms, as well as total *C4* gene expression. Samples from infected iPSCs and transfected HEK293T cells were compared to control iPSCs confirming the overexpression of *C4A* (Figure 17F).

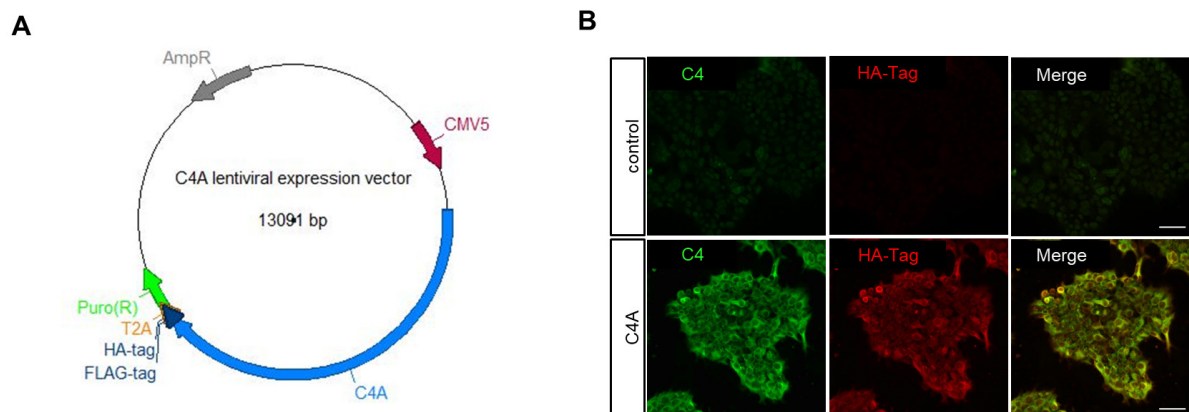


Figure continued on next page

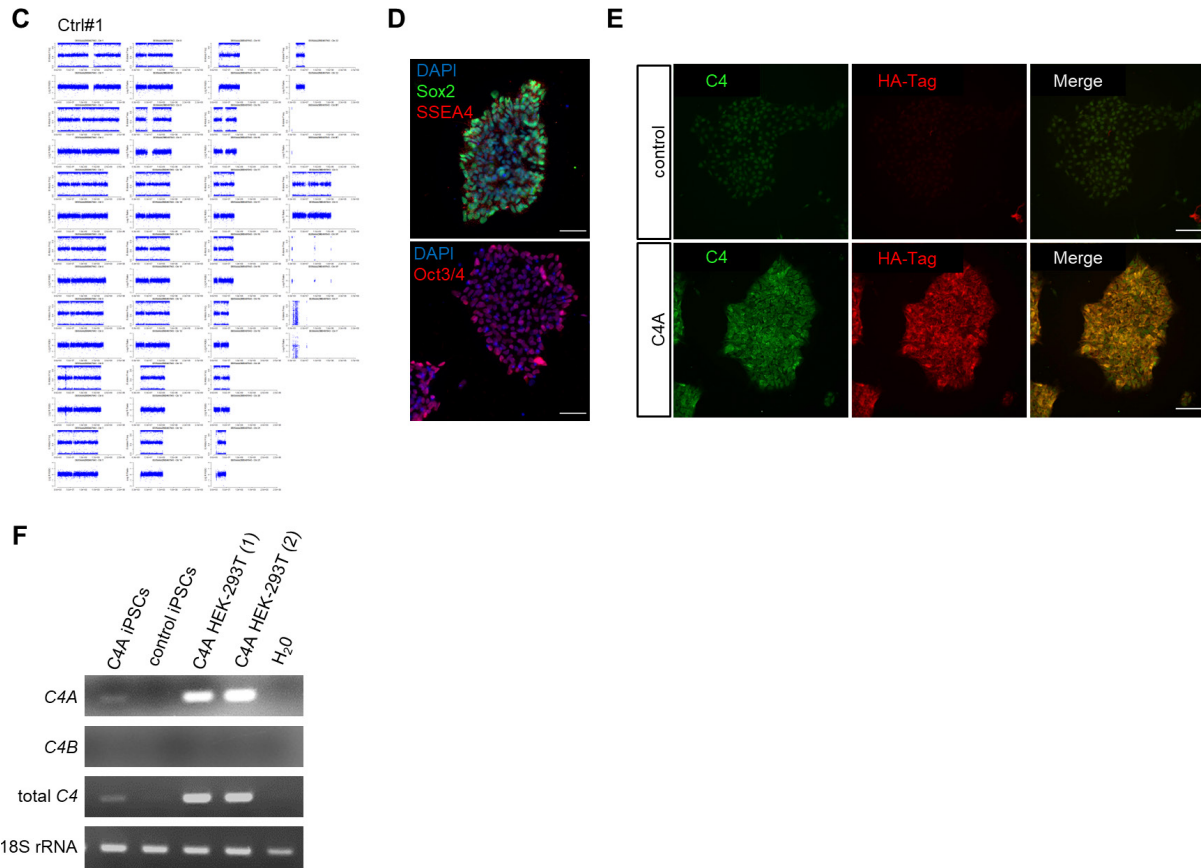


Figure 17: Generation of a C4A overexpression cell line.

(A) Plasmid map of the lentiviral expression vector used for the generation of a C4A overexpression cell line. C4A was tagged using a hemagglutinin (HA)-tag and a Flag-Tag for easier detection. (B) Validation of the lentiviral vector and the C4 and HA-Tag antibodies by transfection of the construct into HEK293T cells. Representative immunostainings for C4 and the HA-tag in transfected (C4A) and non-transfected cells (control). (C) High resolution single-nucleotide polymorphism (SNP) analysis of the Ctrl#1 iPSC line used for the generation of a C4A overexpression cell line. (D) Ctrl#1 human iPSCs stained for pluripotency markers Oct3/4, SSEA4 and Sox2. (E) Representative immunostainings for C4 and the HA-tag in human iPSCs overexpressing C4A and in control iPSCs. (F) RT-PCR analysis of the two isoforms of the *C4* gene (*C4A* and *C4B*), as well as total *C4* in iPSCs and HEK293T cells. iPSCs and HEK293T cells overexpressing C4A are compared to control iPSCs. Scale bars, 50 μ m (B and D) and 100 μ m (E).

Overexpression of C4A at different time points of neuronal differentiation was further validated by Western blot experiments. C4 expression was detected in iPSCs, neural progenitor cells and neurons at different timepoints of differentiation in the overexpression cell line. There was no expression of C4 in control iPSCs, neural progenitors and neurons. Specificity of the C4 antibody was confirmed by co-localization of the signal with the HA-Tag. C4 expression could be detected up to day 60 of neuronal differentiation (Figure 18A). Immunofluorescence stainings were performed at day 50 for C4 overexpressing neurons and non-transduced control neurons. The stainings revealed a localization of C4 at neuronal cell bodies and processes in C4 overexpressing cells (Figure 18B). Interestingly, C4 also co-localized with the pre-synaptic marker synapsin in mature iPSC-derived neurons (Figure 18C). qPCR analysis of *C4A*, *C4B*

and total *C4* gene expression in control and *C4A* overexpressing neurons at day 50 detected a 6-fold increase in *C4A* expression and a 3.5-fold increase in total *C4* expression in the overexpression cell line compared to the non-transduced control. *C4B* expression was not significantly changed in *C4A* overexpressing cells compared to control cells (Figure 18D).

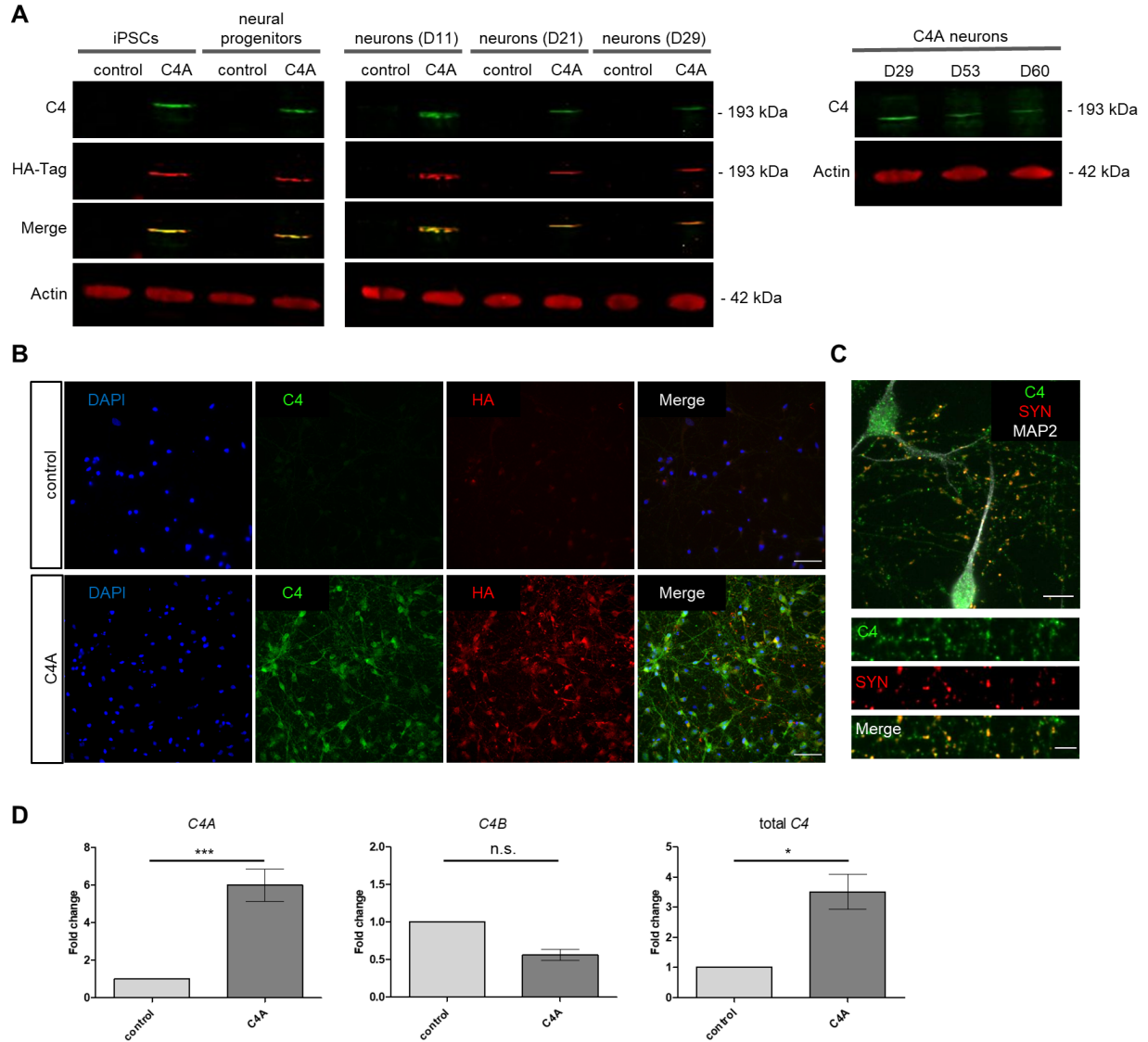


Figure 18: Overexpression of *C4A* in human iPSC-derived cortical neurons.

(A) Representative Western blots for C4 and the HA-tag in iPSCs, neural progenitors and cortical neurons at different time points. Control cells are compared to *C4A* overexpressing cells. (B) Representative immunostainings detecting C4 or the HA-tag in *C4A*-overexpressing neurons and control neurons at day 50. (C) Representative immunostaining showing the co-localization of C4 with synapsin (SYN) in iPSC-derived cortical neurons. (D) qPCR analysis of *C4A*, *C4B* and total *C4* gene expression in control and *C4A* overexpressing neurons at day 50. Fold change was calculated using the $\Delta\Delta C_t$ method, with 18S rRNA as an endogenous control and normalization to control neurons ($n = 3$ biological replicates with technical PCR triplicates, two-tailed t test, means $\pm$ SEM). Scale bars, 50 μ m (B), 10 μ m (C) and 5 μ m [zoomed in (C)]. * $P < 0.05$ and *** $P < 0.001$. n.s., not significant.

7.4 Increased engulfment of synaptosomes from C4A overexpressing neurons in an *in vitro* synaptic pruning assay

In order to investigate differences in synaptic pruning activity in response to high levels of C4A expression, an *in vitro* synaptic pruning assay was established. Given the known role of complement proteins in marking synapses for their removal by microglia, higher C4A expression in neurons was expected to lead to an increased phagocytic activity of microglia. Synaptosomes were isolated from mature iPSC-derived cortical neurons at day 50 of differentiation by homogenization and repeated centrifugation using a Syn-PER synaptic protein extraction reagent (Thermo Fisher Scientific) as described by others (Sellgren et al., 2019; Sellgren et al., 2017).

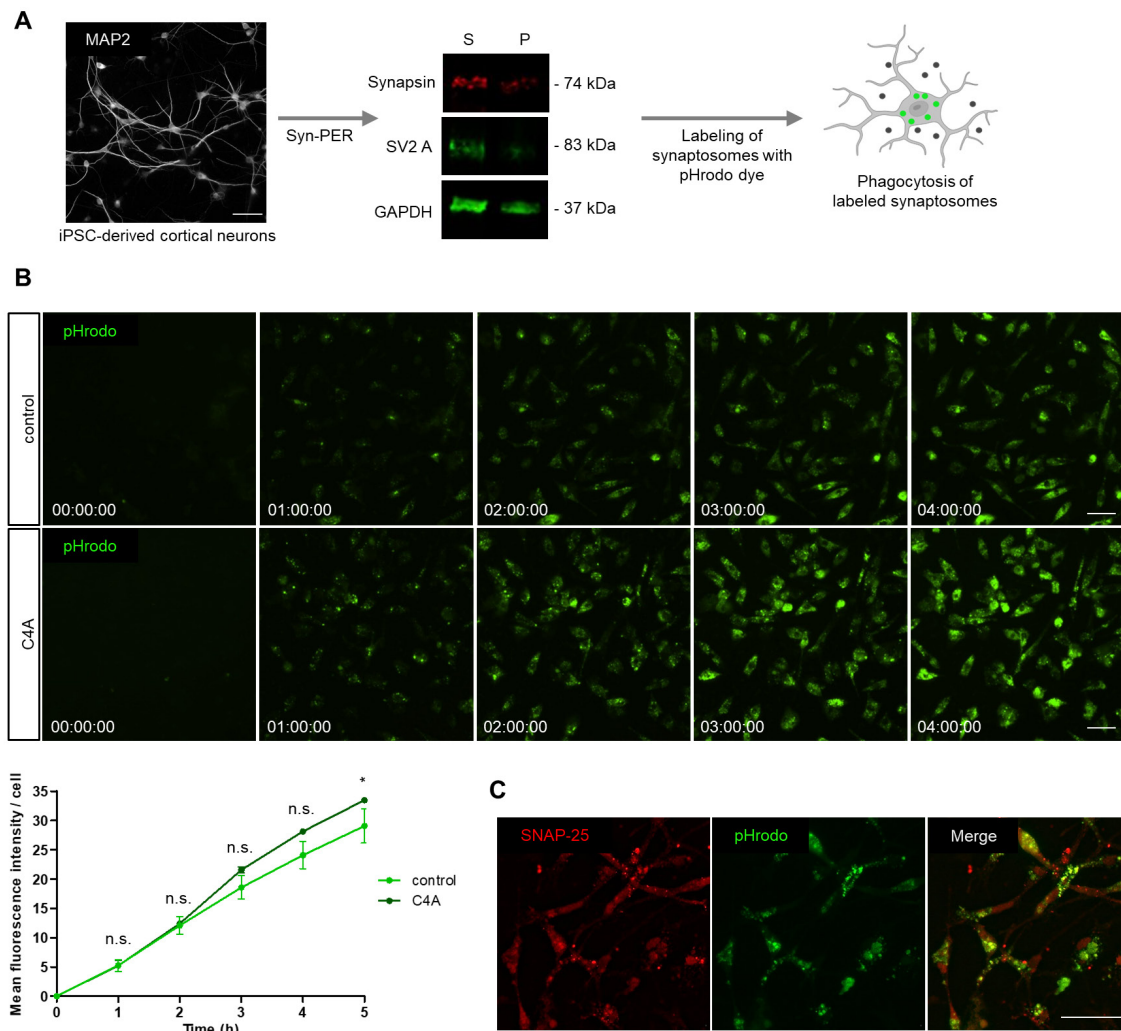


Figure 19: Overexpression of C4A promotes phagocytosis of synaptosomes by microglia in an *in vitro* synaptic pruning assay.

(A) Schematic overview of an *in vitro* synaptic pruning assay. Synaptosomes were prepared using a Syn-PER synaptic protein extraction reagent and subsequently labeled with the pH-sensitive dye pHrodo. Western blot confirmed enrichment of the synaptic markers synapsin and SV2 A in synaptosomes (S) compared to total protein (P) from neuronal cultures. Microglia were exposed to

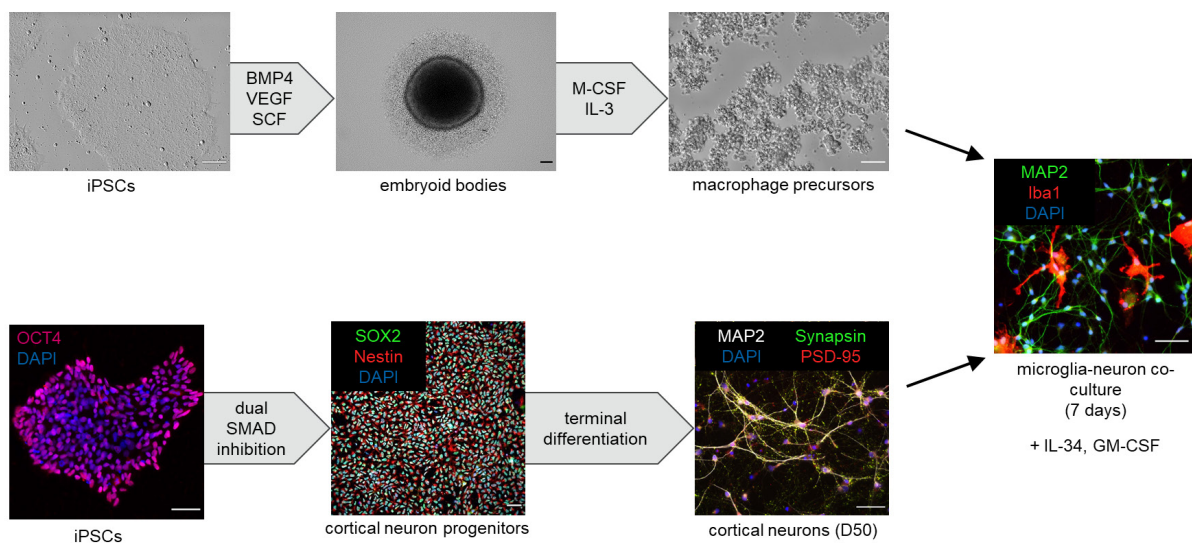
synaptosomes and phagocytic activity was quantified by measuring the fluorescence intensity per cell of the pHrodo dye. **(B)** Representative images and quantification of pHrodo-labeled synaptosome uptake in human iPSC-derived microglia during live imaging experiments. Uptake of synaptosomes from control neurons was compared to synaptosomes from C4A overexpressing neurons. Images were automatically acquired every 10 min for 5 hours ($n = 3$ biological replicates with 596-645 cells per condition, two-way ANOVA with Bonferroni's multiple comparison test, means $\pm$ SEM). **(C)** Representative immunostaining showing co-localization of pHrodo-labeled synaptosomes with the synaptic protein SNAP-25 in iPSC-derived microglia. Scale bars, 50 μ m (A, B and C). Time scale, hh:minmin:secsec. * $P < 0.05$. n.s., not significant.

Western blot analysis was performed to confirm the enrichment of synaptic proteins such as synapsin and synaptic vesicle protein 2 A (SV2 A) in the synaptosome fraction (S) compared to the remaining pellet fraction (P). Extracted synaptosomes were labeled with the ammine-reactive pH-sensitive dye pHrodo and subsequently added to iPSC-derived microglia cultures in live imaging experiments (Figure 19A). Synaptosomes were isolated and labeled from both control and C4A overexpressing neurons. The labeled synaptosomes became visible within 1 hour with increasing fluorescence intensity per cell over time indicating phagocytosis by microglia. Interestingly, after 3 hours of live imaging the fluorescence intensity was higher in microglia exposed to synaptosomes from C4A overexpressing neurons compared to synaptosomes isolated from control neurons. This difference in phagocytic activity became significant after 5 hours of live imaging (Figure 19B). Immunofluorescence stainings showed the co-localization of pHrodo fluorescence with the synaptic marker protein SNAP-25 in human iPSC-derived microglia after 5 hours of live imaging confirming the specific phagocytosis of pHrodo-labeled synaptic structures (Figure 19C).

7.5 Co-cultures of human iPSC-derived microglia with cortical neurons

In order to generate a more physiological *in vitro* model for synaptic pruning in the context of SCZ pathogenesis, specific co-culture conditions were established for iPSC-derived microglia with mature cortical neurons. Therefore, macrophage precursor cells were added to mature day 50 iPSC-derived cortical neurons at a ratio of 1:2. The two cell types were cultured together for 7 days in a medium which was optimized for co-cultures containing IL-34 and GM-CSF for microglia maturation (Figure 20A). Co-culture experiments were performed with control neurons and neurons overexpressing complement component C4A (Figure 20B).

A



B

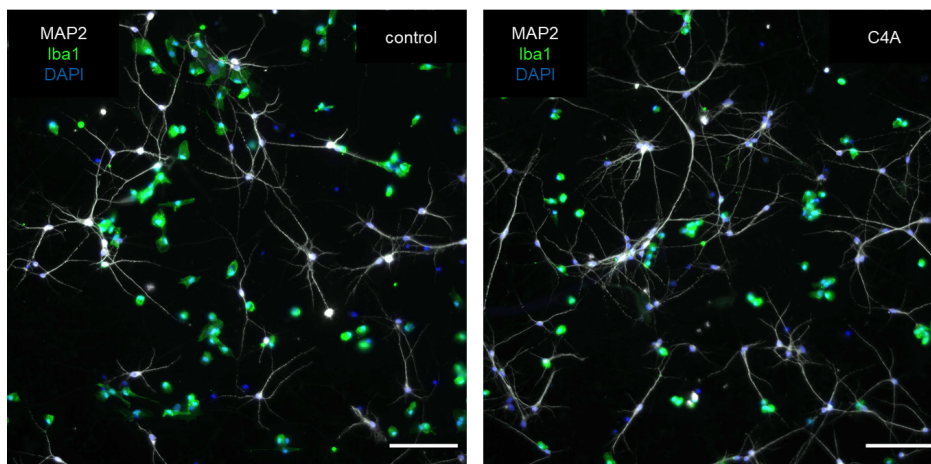


Figure 20: Establishment of a microglia-neuron co-culture system.

(A) Schematic representation of a co-culture protocol for human iPSC-derived microglia and cortical neurons. Macrophage precursor cells are added to day 50 mature cortical neurons for 7 days. **(B)** Representative immunostainings of co-cultures of microglia with control neurons and neurons overexpressing C4A. Scale bars, 50 μ m (white), 100 μ m (black) (A) and 100 μ m (B).

Results

Live imaging of co-cultures was performed using microglia differentiated from a human iPSC line expressing mCherry under the control of a constitutively active EF-1 α promoter (Figure 21A). In co-culture with iPSC-derived cortical neurons, microglia displayed a typical dynamic behavior, continuously making contact with neuronal processes and sensing their environment (Figure 21B). When comparing mono-culture microglia with microglia cultured in co-cultures, it became evident that co-culture microglia displayed a significantly higher motility with an accumulated distance of $1063 \pm 69 \mu\text{m}$ per 10 hours compared to $289 \pm 27 \mu\text{m}$ per 10 hours in mono-cultures (means $\pm$ SEM). The Euclidean distance, which describes the distance from start to endpoint in a straight line, was also increased in co-cultures with $137 \pm 30 \mu\text{m}$ per 10 hours compared to $42 \pm 5 \mu\text{m}$ per 10 hours in mono-cultures (means $\pm$ SEM) (Figure 21C).

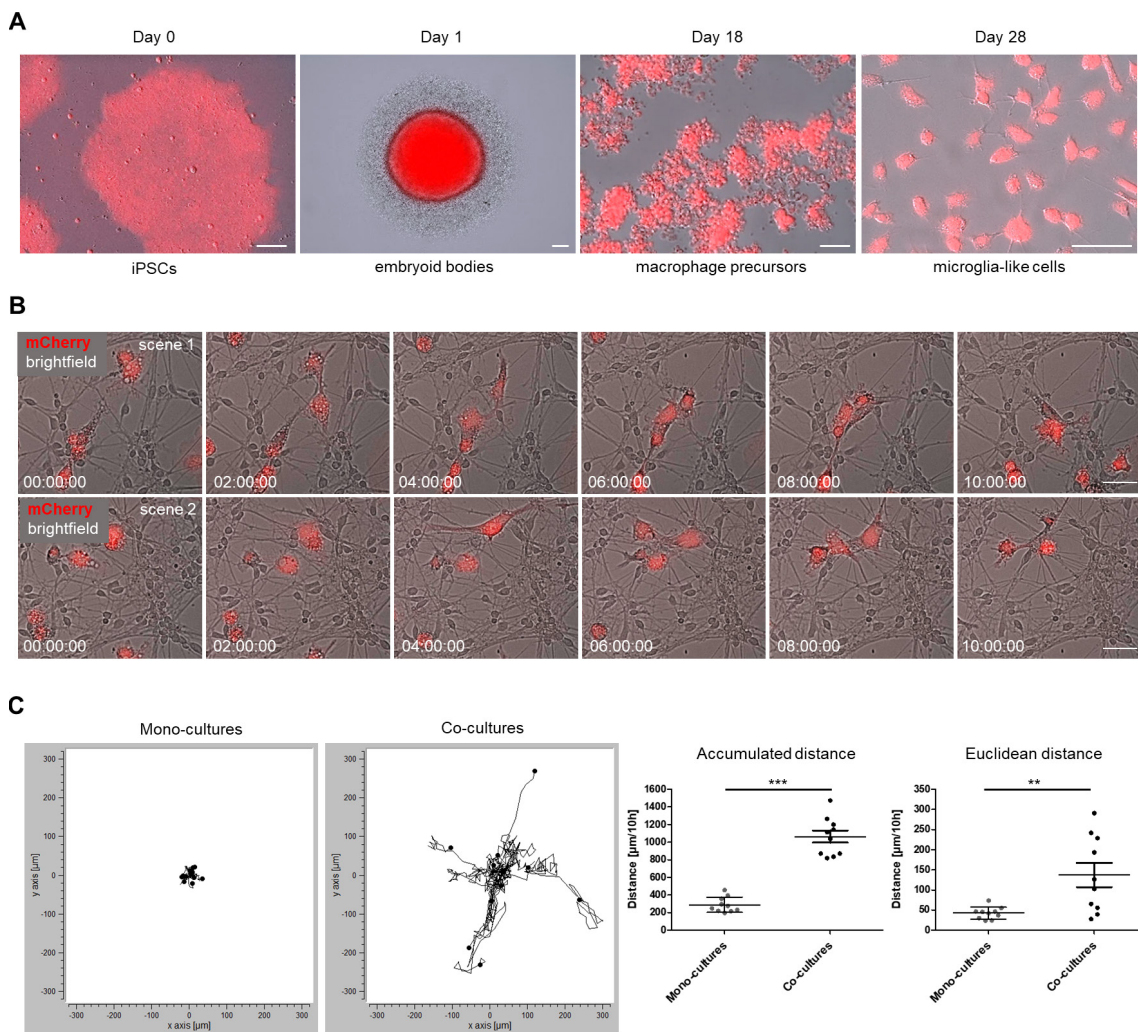


Figure 21: Live imaging of microglia-neuron co-cultures.

(A) Generation of iPSC-derived microglia using an iPSC line expressing mCherry under the control of a constitutively active EF-1 α promoter. (B) Representative images from live cell imaging experiments of microglia-neuron co-cultures using mCherry-labeled microglia. Images were automatically acquired every 10 min for 10 hours. (C) Tracks of mono-culture microglia compared to co-culture microglia. Quantifications of accumulated and Euclidean distance (distance in a straight line from start to end point) ($n = 10$ tracks, Mann-Whitney U-test, dots for individual cells, means $\pm$ SEM). Scale bars, 100 μm (A) and 50 μm (B). Time scale, hh:minmin:secsec. ** $P < 0.01$ and *** $P < 0.001$.

7.6 Overexpression of C4A promotes synaptic loss in co-cultures of iPSC-derived cortical neurons and microglia by increased microglial activation

In order to further examine the effects of increased C4A expression on synaptic pruning activity by microglia and to validate the findings from the *in vitro* synaptic pruning assay, microglia-neuron co-culture experiments were performed. Thereby, synapse densities were assessed in co-cultures with control neurons and in co-cultures with neurons overexpressing C4A.

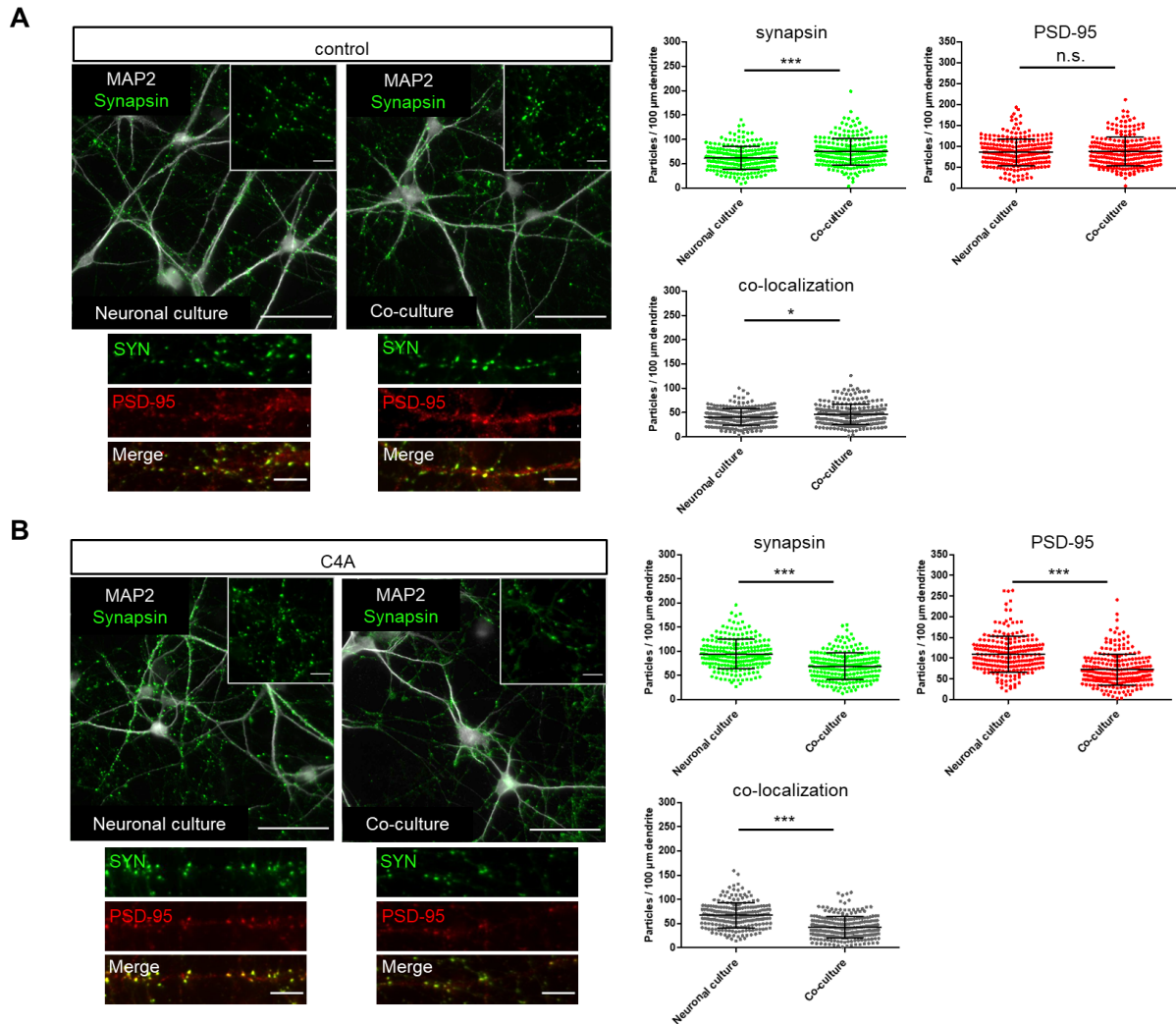


Figure 22: Overexpression of C4A promotes synaptic pruning in neuron-microglia co-cultures.

(A) Representative immunostainings and respective quantification of pre-synaptic (synapsin; SYN) and post-synaptic (PSD-95) particles per 100 μm dendrite, as well as their co-localization, in control human iPSC-derived cortical neurons with and without co-culture with human iPSC-derived microglia ($n = 220\text{--}240$ randomly selected dendrites examined per group from three biological replicates, Mann-Whitney U-test, dots for individual dendrites, means $\pm$ SEM). (B) Immunostainings and quantification of synaptic markers and their co-localization in C4A overexpressing neurons with and without co-culture with microglia ($n = 220\text{--}240$ randomly selected dendrites examined per group from three biological replicates, Mann-Whitney U-test, dots for individual dendrites, means $\pm$ SEM). Scale bars, 50 μm (A and B) and 10 μm [zoomed in (A and B)]. * $P < 0.05$ and *** $P < 0.001$. n.s., not significant.

The number of pre-synaptic synapsin particles, post-synaptic PSD-95 particles, as well as their co-localization were quantified as particles per unit length. Thereby, co-localization of pre-and postsynaptic particles was used as a common readout for mature synapses. After 7 days of co-culture there was a significant increase in the number of synapsin particles, as well as their co-localization with PSD-95 in co-cultures with control neurons compared to pure neuronal cultures (Figure 22A). In contrast, neurons overexpressing C4A displayed a significantly reduced number of synapsin and PSD-95 particles per 100 μm dendrite, as well as a reduced co-localization of pre-and post-synaptic puncta in co-cultures compared to neuronal mono-cultures (Figure 22B).

In order to gain insight into potential pathways involved in microglia activation after exposure to high expression levels of C4A in neurons, immunostainings for the p65 subunit of the NF κ B protein complex were performed. The NF κ B transcription factor is an important regulator of immune response and a known driver of NLRP3 inflammasome expression. Several recent reports provide evidence that microglia-specific activation of the inflammasome pathway plays a role in various neurodegenerative and neuroinflammatory conditions (Fusco et al., 2020). Using the established *in vitro* co-culture system, the involvement of the NLRP3 inflammasome pathway in SCZ pathogenesis was evaluated by quantification of the percentage of nuclear and cytosolic NF κ B (p65) in microglia. Thereby, nuclear p65 is an indication for the activation of the NF κ B pathway.

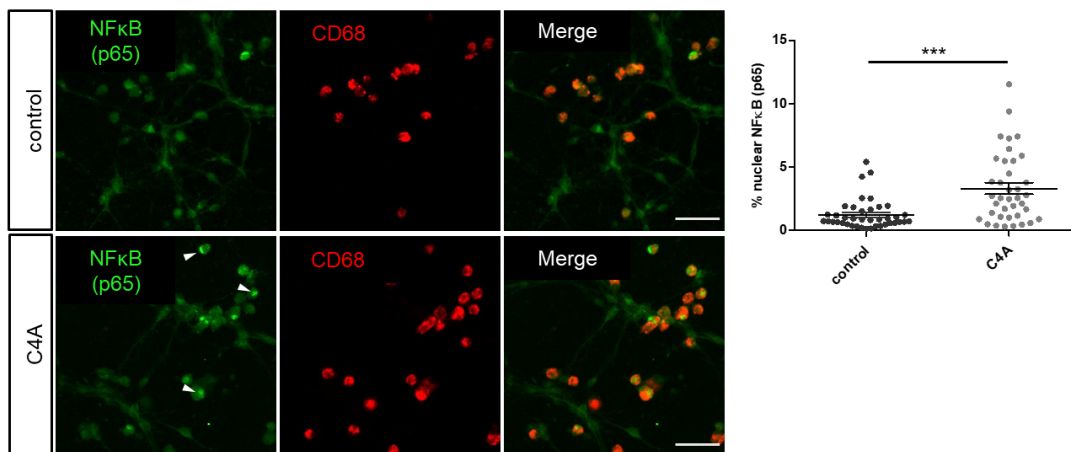


Figure 23: Overexpression of C4A in neurons leads to increased NF κ B pathway activation in microglia.

Representative immunostainings of NF κ B (p65) and CD68 in co-cultures of human iPSC-derived microglia with C4A overexpressing human iPSC-derived neurons or control neurons. Quantification of the percentage of nuclear NF κ B (p65) in microglia after co-culture with both cell lines ($n = 38-40$ images with 15901-18480 cells per condition from four biological replicates, dots for % of nuclear NF κ B (p65) per analyzed image, Mann-Whitney U-test, means $\pm$ SEM). Scale bars, 50 μm . *** $P < 0.001$.

Interestingly, CD68-positive microglia in co-cultures with C4A overexpressing neurons showed a significant increase in nuclear NF κ B (p65) compared to microglia in co-cultures with control

neurons, indicating an activation of the NFkB pathway in microglia after exposure to C4A. The percentage of nuclear NFkB increased from $1.2 \pm 0.2\%$ in co-cultures with control neurons to $3.3 \pm 0.4\%$ in co-cultures with C4A overexpressing neurons (means $\pm$ SEM) (Figure 23). In order to further characterize iPSC-derived microglia in co-cultures with control and C4A overexpressing neurons, snRNAseq was performed. Seurat clustering resulted in two distinct subpopulations of nuclei. Cellular identity was determined based on their expression pattern revealing a large microglia cluster and a smaller neuronal cluster for both co-culture conditions (Figure 24A). Cell type identification was performed by analyzing canonical neuronal and microglial gene expression such as *RBFOX3*, *MAPT* and *MAP2* for neurons and *TREM2*, *ITGAM* and *AIF1* for microglia (Figure 24B).

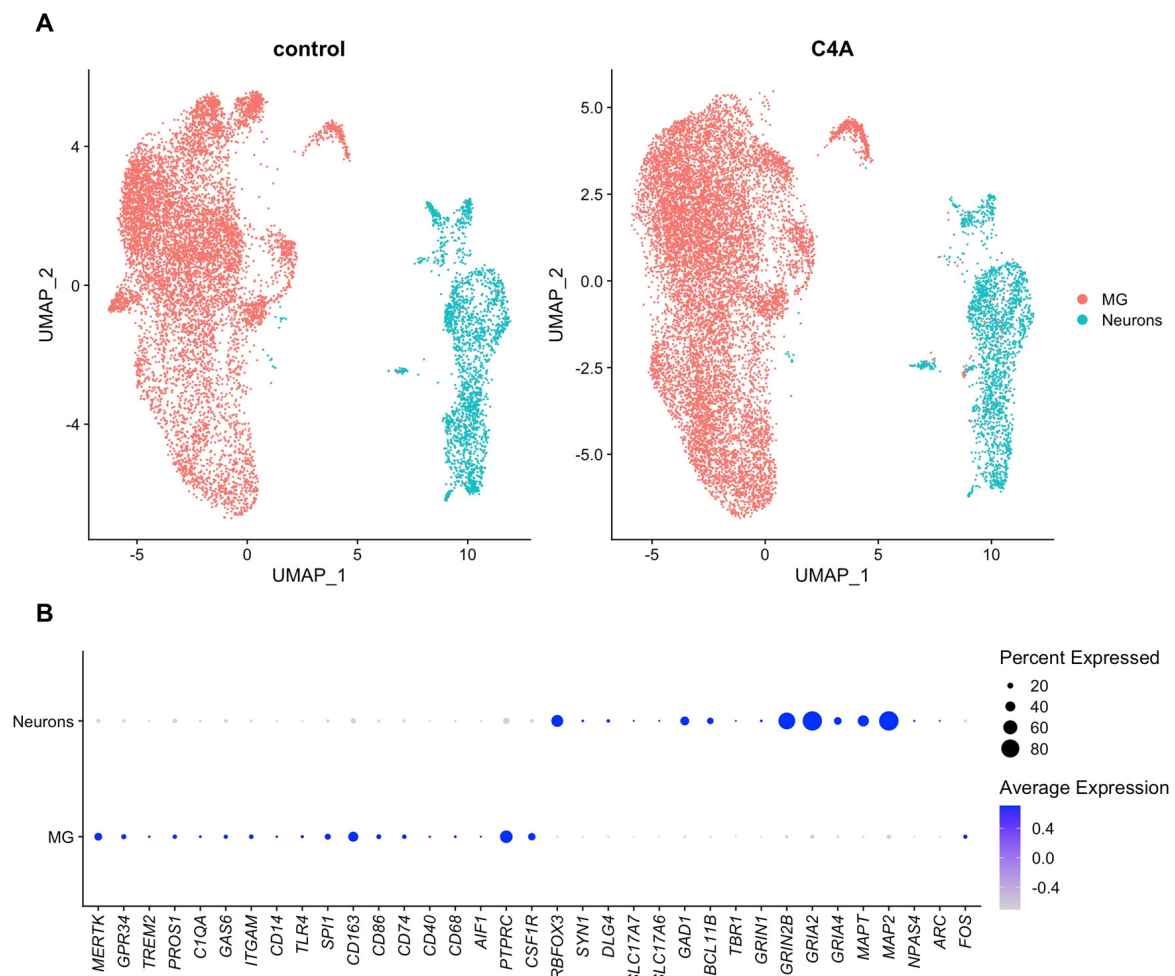


Figure 24: Single-nucleus RNAseq (snRNAseq) cell type identification analysis.

(A) Dimensional reduction plot in UMAP dimensions. Seurat clustering of snRNAseq data from co-cultures with control and C4A overexpressing neurons revealed two cell populations. Cell type identification was performed by inspection of bona fide marker gene expression for microglia and neurons. The identified microglial (MG) cluster is shown in red and the neuronal cluster is shown in blue. **(B)** Dotplot of canonical neuronal and microglial (MG) marker gene expression. Color intensity of each dot represents the average log-scaled expression of each gene across all cells of the two clusters. Size of the dots represent the fraction of cells in each cluster in which transcripts for the genes were detected. SnRNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

Results

Microglial activation by C4A was further confirmed by snRNAseq. GO enrichment analysis comparing microglia from co-cultures with control and C4A overexpressing neurons revealed significant differences in biological processes and molecular functions such as microglial cell activation, phagocytosis, immune response-activating signal transduction and regulation of NIK/NFκB signaling (Figure 25A). Interestingly, the expression of genes involved in complement binding, including the C3-receptor subunits *ITGAM* and *ITGB2*, was higher in microglia from C4A co-cultures, as well as the expression of genes associated with NFκB and NLRP3 inflammasome activation, such as *TLR2*, the purinergic receptor P2X7 (*P2RX7*) and dead-box helicase 3 X-linked (*DDX3X*), a gene encoding a stress granule protein which can interact with NLRP3 to drive inflammasome activation (Figure 25B).

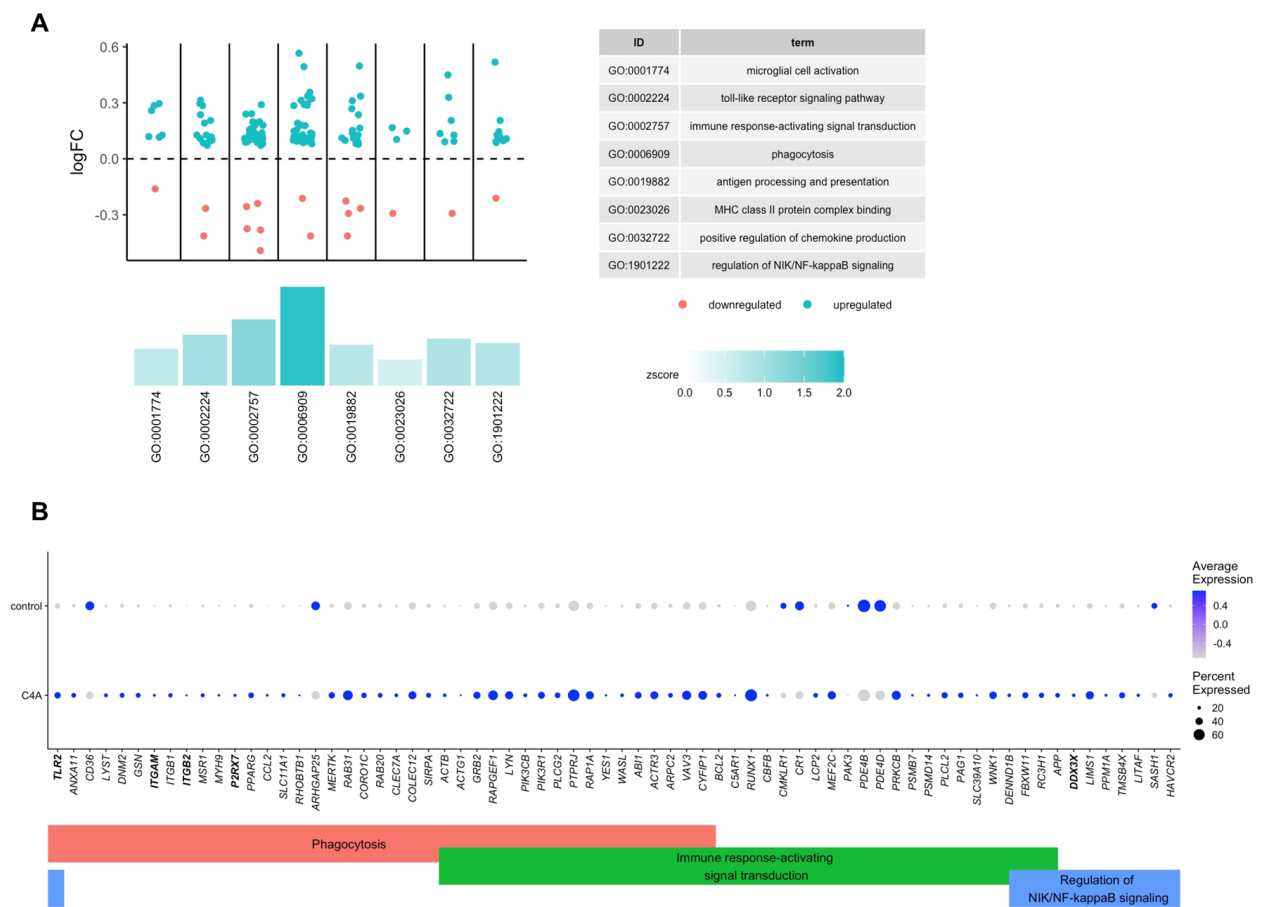


Figure 25: Comparative analysis of single-nucleus microglial transcriptomics from co-cultures with control and C4A overexpressing neurons confirms microglial activation by C4A.

(A) Log-fold changes of differentially expressed genes and z-scores for GO terms regarding microglial activation, phagocytosis and immune signaling. The height and color of the bar indicate the z-score. The scatterplots display the relative expression levels (logFC) for significant genes in each term (blue, upregulated genes; red, downregulated genes). (B) Dot plot showing a selection of significantly differentially expressed genes between microglia from co-cultures with control and C4A overexpressing neurons. Genes are classified in their corresponding GO terms 'Phagocytosis', 'Immune response-activating signal transduction' and 'Regulation of NIK/NF-kappaB signaling'. The color intensity of each dot represents the average log-scaled expression of each gene across all cells of the microglia cluster, and the size of the dot represents the fraction of cells in the cluster in which transcripts for that gene were detected. SnRNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

7.7 NLRP3 inflammasome inhibitor oridonin attenuates inflammatory response in iPSC-derived microglia and inhibits synapse engulfment

The NLRP3 inflammasome is a multiprotein complex, which is composed of the protein subunits NEK7, NLRP3, ASC and caspase-1. Activation and assembly of the complex leads to the self-cleavage and activation of pro-caspase 1. Caspase-1 then facilitates IL-1 β and IL-18 maturation by cleavage of their inactive pro-isomers pro-IL1 β and pro-IL-18 into their active forms (Figure 26A). Multiple signals can activate the NLRP3 inflammasome, however the most commonly investigated are TLR and NF κ B signaling (O'Brien et al., 2020). Whole transcriptome bulk RNAseq data confirmed the expression of the main components of the NLRP3 inflammasome, as well as the NF κ B subunit p65 (*RELA*) and downstream target genes *IL1B* and *IL18* in iPSC-derived microglia. The expression of *NEK7*, *NLRP3*, *CASP1*, *IL1B*, *IL18* and *RELA* was significantly increased after treatment of microglia with 100 ng/ml LPS for 24 hours, as shown by z-scores of TPM counts (Figure 26B). ASC (*PYCARD*) expression was significantly downregulated after LPS treatment.

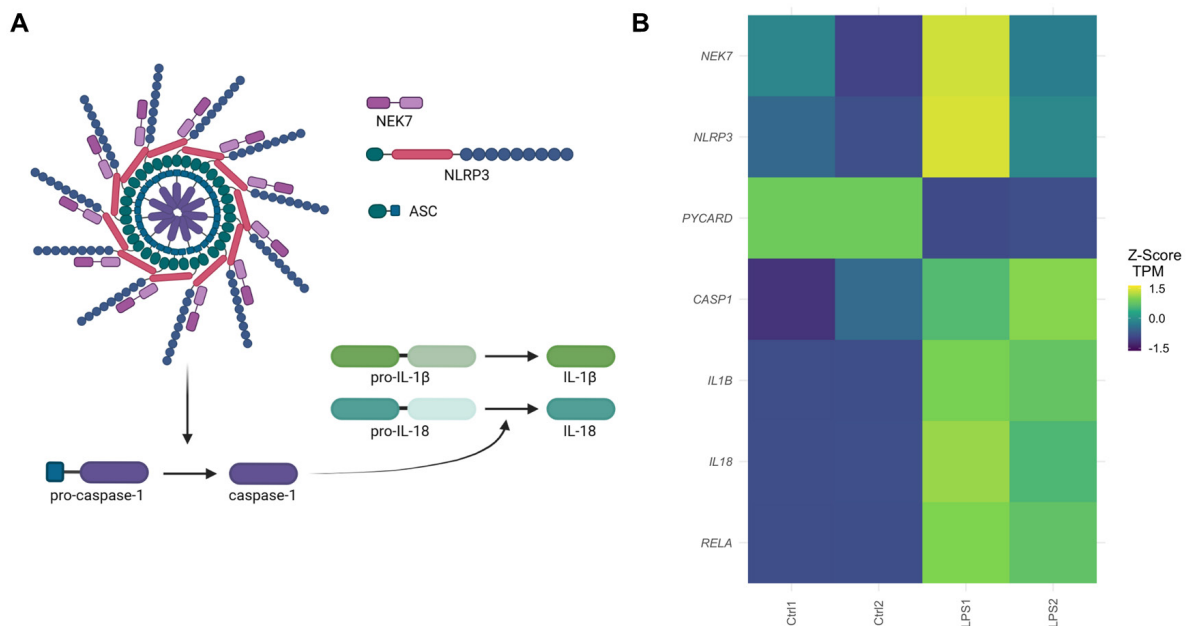


Figure 26: Expression of the NLRP3 inflammasome in iPSC-derived microglia.

(A) Illustration of the NLRP3 inflammasome, which is formed after activation by binding of NEK7, NLRP3, ASC (*PYCARD*) and caspase-1 into a multi-protein-complex. The active NLRP3 inflammasome allows cleavage of pro-caspase 1 into its active isomer caspase 1, which in turn cleaves pro-IL-1 β and pro-IL-18 into their active forms IL-1 β and IL-18. (B) Z-scores of TPM counts from bulk RNA sequencing experiments show the expression of components of the NLRP3 inflammasome, as well as the NF κ B subunit p65 (*RELA*) and downstream target genes *IL1B* and *IL18* in iPSC-derived microglia. Control microglia samples (Ctrl1, Ctrl2) are compared to LPS-treated microglia (LPS1, LPS2). Bulk RNAseq data analysis was performed in collaboration with Dr. Anne Hoffrichter.

Oridonin (Ori), the major active ingredient of the traditional Chinese medicinal herb *Rabdosia rubescens*, has been shown to covalently bind to NLRP3, thereby inhibiting inflammasome assembly (He et al., 2018). To investigate the anti-inflammatory effects of Ori on human iPSC-derived microglia mono-cultures, cells were treated either with LPS alone or in combination with different concentrations of the NLRP3 inhibitor. In line with the results in section 7.1, treatment of human iPSC-derived microglia with 100 ng/ml LPS for 24 hours caused a significant increase in the percentage of cells displaying a multipolar, hyper-ramified shape from $9 \pm 1\%$ in control cells to $55 \pm 4\%$ in LPS-treated cells. The percentage of bipolar microglia decreased from $43 \pm 3\%$ in control cells to $7 \pm 1\%$ in LPS-treated cells and the percentage of microglia displaying a round morphology decreased from $47 \pm 3\%$ to $37 \pm 4\%$ after LPS treatment (means $\pm$ SEM). Interestingly, co-treatment of microglia with LPS and Ori (10 μ M) for 24 hours was sufficient to suppress the LPS-induced change in microglia morphology. The percentage of cells displaying a multipolar shape significantly decreased to $11 \pm 3\%$ after co-treatment with Ori. Furthermore, the percentage of bipolar microglia increased to $27 \pm 2\%$ and the percentage of microglia displaying a round morphology increased to $62 \pm 5\%$ in LPS + Ori treated cells (means $\pm$ SEM) (Figure 27A). Analysis of shape descriptor parameters revealed a significantly reduced whole cell perimeter and Feret's diameter, as well as a significantly increased circularity in LPS + Ori treated microglia compared to control cells and LPS-treated cells (Figure 27B).

In order to validate the nuclear translocation of NF κ B subunit p65 as a readout for NLRP3 inflammasome activation, human iPSC-derived microglia mono-cultures were treated either with LPS alone or with LPS and different concentrations of Ori (1, 5 or 10 μ M). For each condition the percentage of nuclear NF κ B (p65) was quantified. After LPS treatment the percentage of nuclear NF κ B (p65) significantly increased from $2 \pm 0.3\%$ in control microglia to $82 \pm 3\%$ in LPS-treated cells (means $\pm$ SEM). When co-treating microglia with LPS and Ori, the percentage of nuclear NF κ B (p65) decreased in a concentration-dependent manner compared to LPS treatment alone. For co-treatment with LPS and 1 μ M Ori the percentage decreased to $80 \pm 3\%$, for co-treatment with 5 μ M Ori to $62 \pm 2\%$ and for 10 μ M Ori to $45 \pm 4\%$ (means $\pm$ SEM) (Figure 27C).

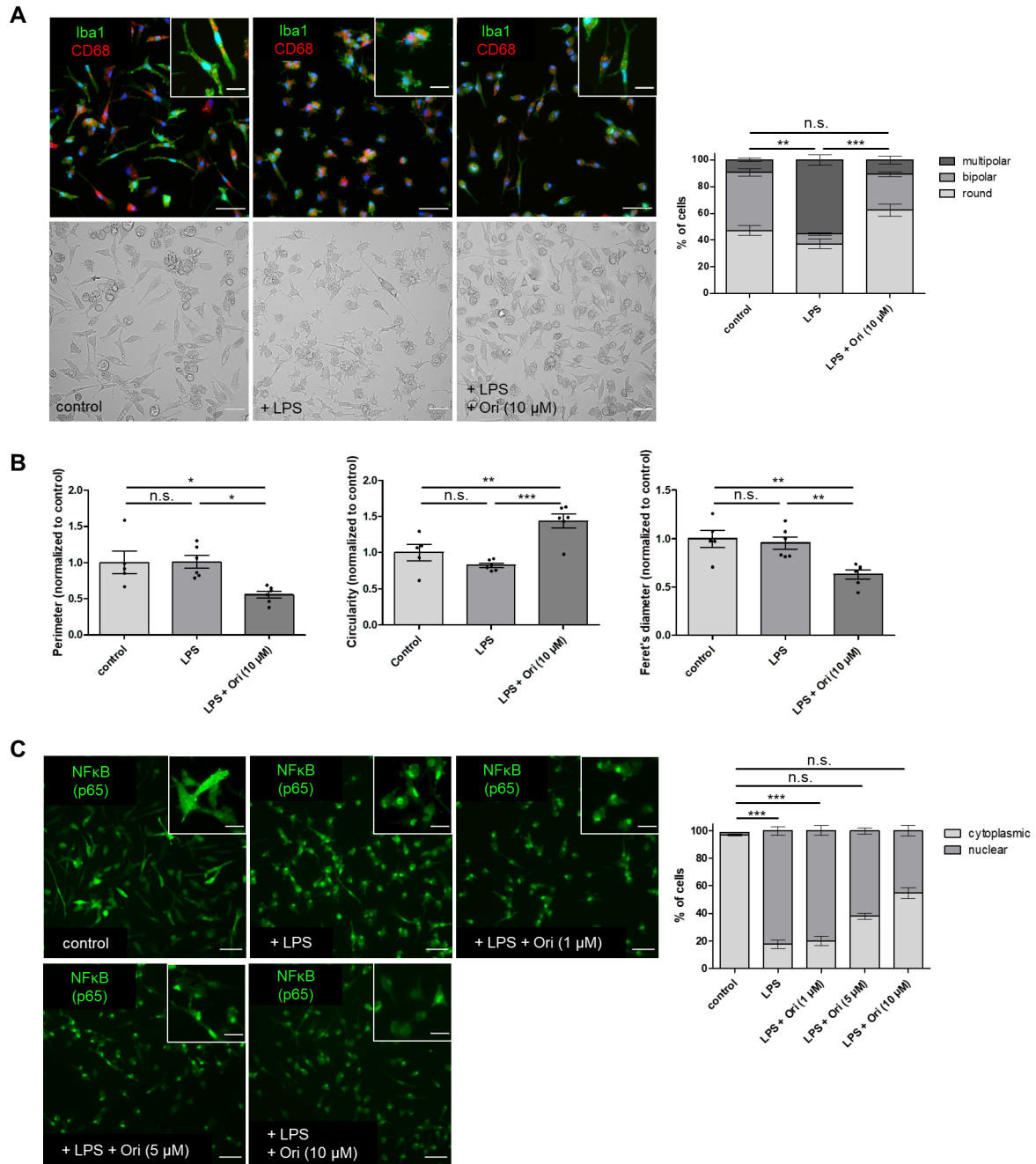


Figure 27: Oridonin attenuates LPS-induced changes in iPSC-derived microglia.

(A) Representative immunofluorescence stainings and respective quantifications of human iPSC-derived microglia morphology after treatment with LPS (100 ng/ml) alone or co-treatment with LPS and 10 μ M oridonin (Ori) for 24 hours ($n = 9$ images with 1223-1440 cells per condition from three biological replicates, Kruskal-Wallis test with Dunn's multiple comparison test, $P < 0.01$ and $P < 0.001$ for "multipolar", means $\pm$ SEM). (B) Quantifications of shape descriptor parameters (perimeter, circularity and Feret's diameter) show differences between control and LPS-treated cells and cells treated with LPS + Ori (10 μ M) ($n = 5-6$ images with 603-805 cells per condition from three biological replicates, one-way ANOVA with Tukey's multiple comparison test, dots represent individual analyzed images, means $\pm$ SEM). (C) Representative immunostainings and quantification of the percentage of nuclear and cytoplasmic NF κ B (p65) in human iPSC-derived microglia after treatment with LPS alone or co-treatment with LPS and different concentrations of Ori (1, 5 and 10 μ M) for 24 hours ($n = 9$ images with 1150-1382 cells per condition from three biological replicates, Kruskal-Wallis test with Dunn's multiple comparison test, means $\pm$ SEM). Scale bars, 50 μ m (A and C) and 20 μ m [zoomed in (A and C)]. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. n.s., not significant.

Furthermore, the effects of Ori on the engulfment of synaptic structures by human iPSC-derived microglia was evaluated using the *in vitro* synaptic pruning assay described in section 7.4. Microglia mono-cultures were treated with pHrodo-labeled synaptosomes from control neurons and C4A overexpressing neurons. For live cell imaging experiments, images were acquired every 10 min for a total duration of 5 hours. Treatment with 5 or 10 μ M Ori significantly decreased the engulfment of labeled synaptosomes in a concentration-dependent manner for both control and C4A conditions. After 1 hour of treatment with 5 μ M Ori the mean fluorescence intensity per cell was significantly reduced by 85% and after 5 hours by 73% for control synaptosomes compared to the VEH control. After 1 hour of treatment with 10 μ M Ori the mean fluorescence intensity per cell was significantly reduced by 82% and after 5 hours by 86% for control synaptosomes (Figure 28A). For treatment with 5 μ M Ori and C4A synaptosomes the mean fluorescence intensity per cell was significantly reduced by 73% compared to the VEH control after 1 hour. After 5 hours the fluorescence intensity was reduced by 78%. After treatment with 10 μ M Ori fluorescence intensity was significantly decreased by 89% after 1 hour and 91% after 5 hours compared to the VEH control for the C4A condition (Figure 28B).

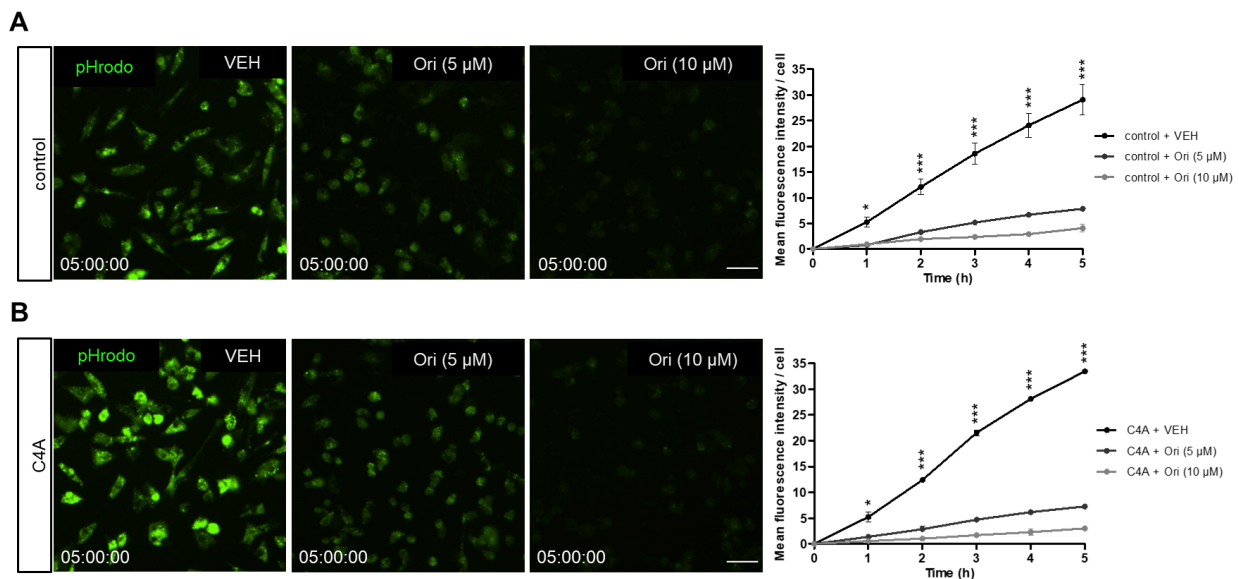


Figure 28: Oridonin inhibits synapse engulfment in an *in vitro* synaptic pruning assay.

(A) Representative images and quantification of the uptake of pHrodo-labeled synaptosomes from control neurons by human iPSC-derived microglia during live imaging experiments. Cells were treated with VEH or 5 and 10 μ M oridonin (Ori). Images were automatically acquired every 10 min for 5 hours ($n = 3$ biological replicates with 593-729 cells per condition, two-way ANOVA with Bonferroni's multiple comparison test, means $\pm$ SEM). **(B)** Representative images and quantification of the uptake of pHrodo-labeled synaptosomes from C4A overexpressing neurons by microglia. Cells were treated with VEH or 5 and 10 μ M Ori. Images were automatically acquired every 10 min for 5 hours ($n = 3$ biological replicates with 596-703 cells per condition, two-way ANOVA with Bonferroni's multiple comparison test, means $\pm$ SEM). Scale bars, 50 μ m (A and B). Time scale, hh:minmin:secsec. * $P < 0.05$ and *** $P < 0.001$.

7.8 Anti-inflammatory effects of oridonin and minocycline reduce microglial activation after exposure to C4A in co-cultures with cortical neurons

The effects of NLRP3 inflammasome inhibitor Ori on synaptic pruning in the context of SCZ pathogenesis was further evaluated using the established microglia-neuron co-culture conditions described in section 7.5. Thereby, co-cultures with neurons overexpressing C4A were treated with 1 μ M Ori for 7 days.

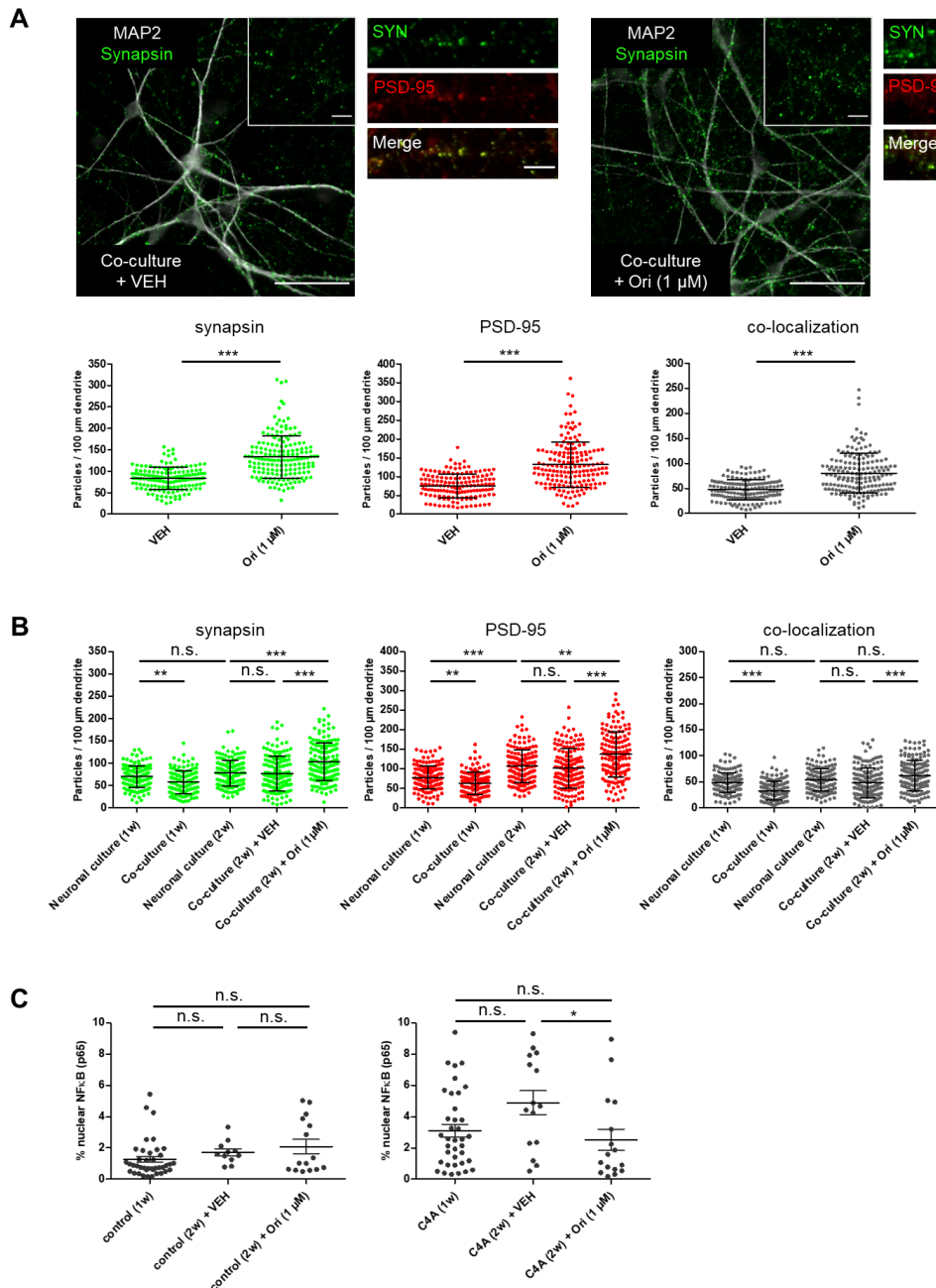


Figure 29: Effects of oridonin on synapse density and NFkB nuclear translocation in neuron-microglia co-cultures.

(A) Representative immunostainings and respective quantification of pre-synaptic (synapsin; SYN) and post-synaptic (PSD-95) particles per 100 μ m dendrite, as well as their co-localization, in C4A overexpressing human iPSC-derived cortical neurons after co-culture with human iPSC-derived

microglia. Co-cultures were treated with VEH or 1 μ M oridonin (Ori) ($n = 150$ -170 randomly selected dendrites examined per group from three biological replicates, Mann-Whitney U-test, dots for individual dendrites, means $\pm$ SEM). **(B)** Quantification of synaptic markers and their co-localization in C4A overexpressing iPSC-derived neurons with and without 1 week (1w) or 2 weeks (2w) of co-culture with microglia. Co-cultures were treated with VEH or Ori (1 μ M) starting from week 2 ($n = 140$ -150 randomly selected dendrites examined per group from three biological replicates, Kruskal-Wallis test with Dunn's multiple comparison test, dots for individual dendrites, means $\pm$ SEM). **(C)** Quantification of the percentage of nuclear NF κ B (p65) in CD68+ microglia after 1 or 2 weeks of co-culture with either control neurons or C4A overexpressing neurons. Co-cultures were treated with VEH or Ori (1 μ M) starting from week 2 ($n = 11$ -40 images from 3-4 biological replicates with 2558-18480 cells per condition, dots for % of nuclear NF κ B (p65) per analyzed image, Kruskal-Wallis test with Dunn's multiple comparison test, means $\pm$ SEM). Scale bars, 50 μ m (A) and 10 μ m [zoomed in (A)]. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. n.s., not significant.

Interestingly, the number of synapsin and PSD-95 particles, as well as their co-localization, was significantly increased in neurons from Ori-treated co-cultures compared to VEH-treated co-cultures (Figure 29A). In order to investigate if Ori can also restore synaptic density after an initial excessive synaptic pruning phase, neurons overexpressing C4A were co-cultured with microglia for a total duration of 2 weeks, starting Ori or VEH treatment only from week 2. Thereby, the number of synapsin and PSD-95 particles and their co-localization was significantly reduced after 1 week of co-culture compared to pure neuronal cultures, as seen in previous experiments. However, after 2 weeks of co-culture the difference in synapse densities between pure neuronal cultures and VEH-treated co-cultures was no longer significant indicating a decreased activation of microglial cells over time. This effect could also in part be due to the general increase in synaptic particles in more mature neuronal cultures over time, which was significant for PSD-95. Interestingly, the number of pre- and post-synaptic particles was significantly higher in Ori-treated co-cultures compared to pure neuronal cultures and VEH-treated co-cultures at the same time point. There was also a trend for an increased co-localization which became significant when comparing VEH-and Ori-treated co-cultures (Figure 29B). Furthermore, Ori treatment was able to significantly reduce the percentage of nuclear NF κ B (p65) in CD68-positive microglia in co-cultures with C4A overexpressing neurons when starting treatment from week 2. There was no change in the percentage of nuclear NF κ B (p65) in co-cultures with control neurons (Figure 29C). Ori-treated co-cultures were further compared to co-cultures treated with 60 μ M minocycline (Mino), a tetracycline antibiotic which has been shown to have beneficial anti-inflammatory effects in various neurodegenerative and neurodevelopmental diseases. The number of synapsin particles in C4A overexpressing neurons from Mino-treated co-cultures was also significantly increased compared to VEH-treated cells. However, there was no significant change in the number of PSD-95 particles or the co-localization of pre-and post-synaptic structures (Figure 30A). These findings indicate that Ori is more efficient in improving synaptic densities in neuron-microglia co-cultures compared to Mino. General effects of Mino on microglia phagocytosis were

assessed by treating human iPSC-derived microglia mono-cultures with pHrodo-labeled zymosan particles in combination with different concentrations of Mino in live cell imaging experiments. Thereby, images were acquired every 10 min for a total duration of 2.5 hours. After 1 hour the mean fluorescence intensity per cell was significantly decreased by all three Mino concentrations (60, 80 and 100 μ M) compared to the VEH control. The effect remained significant in a concentration-dependent manner after 2.5 hours of live imaging. These results imply that the observed effect of Mino on pre-synaptic density could be due to a general inhibition of microglial phagocytic activity (Figure 30B).

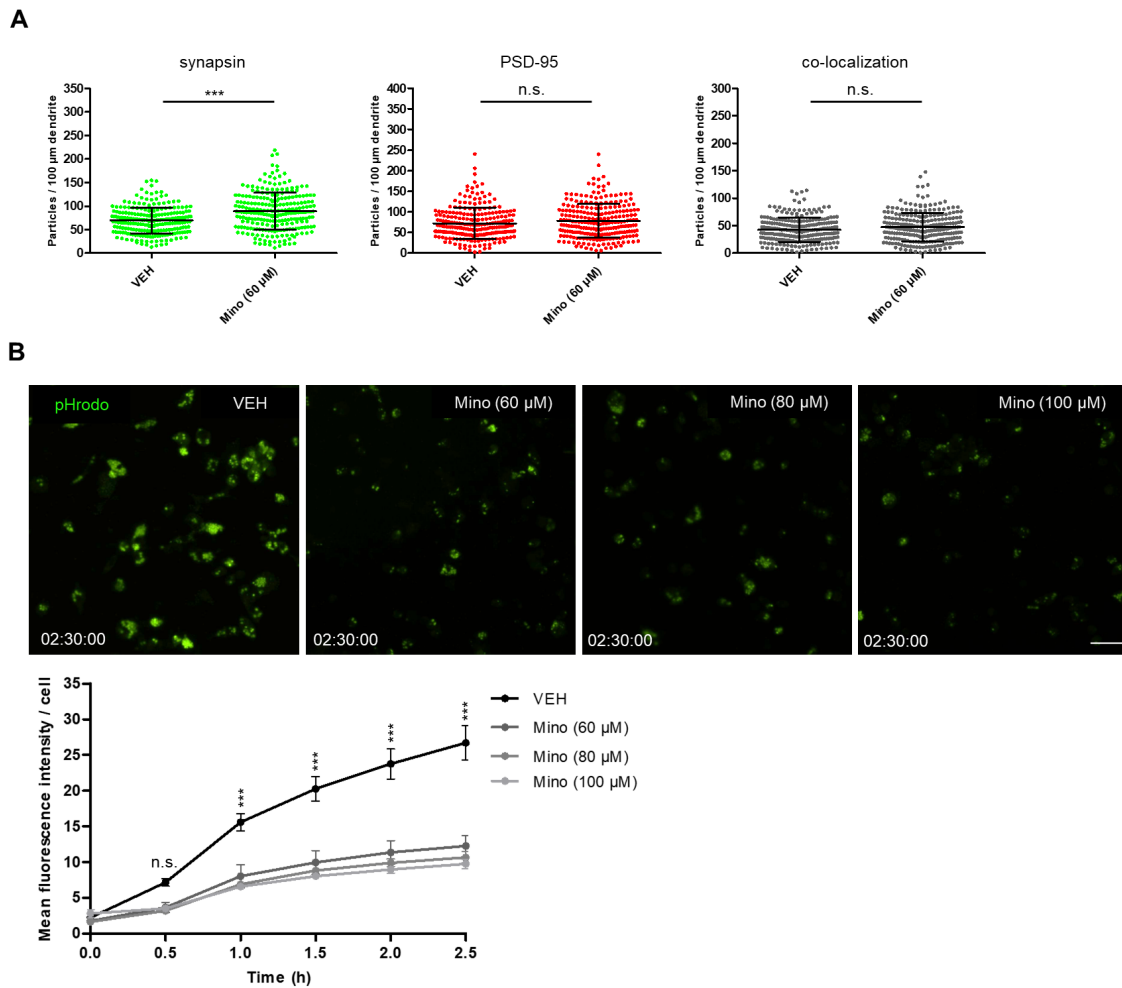


Figure 30: Effects of minocycline on microglial synaptic pruning and phagocytic activity.

(A) Quantification of synapsin and PSD-95 particles, as well as their co-localization, in C4A overexpressing human iPSC-derived cortical neurons after co-culture with human iPSC-derived microglia. Co-cultures were treated with VEH or 60 μ M minocycline (Mino) ($n = 220$ -240 randomly selected dendrites examined per group from three biological replicates, Mann-Whitney U-test, dots for individual dendrites, means $\pm$ SEM). (B) Representative images and quantification of pHrodo-labeled zymosan uptake in iPSC-derived microglia during live imaging experiments. Cells were treated with VEH or different concentrations of Mino (60, 80 and 100 μ M). Images were automatically acquired every 10 min for 2.5 hours ($n = 3$ biological replicates with 543-743 cells per condition, two-way ANOVA with Bonferroni's multiple comparison test, means $\pm$ SEM). Scale bar, 50 μ m (B). Time scale, hh:minmin:secsec. *** $P < 0.001$. n.s., not significant.

Results

Co-culture experiments were also performed using control iPSC-derived cortical neurons. Control neurons displayed a significantly higher number of synapsin particles in co-cultures with microglia compared to pure neuronal cultures, as seen in previous experiments. Interestingly, pre- and post-synaptic particles and their co-localization were even more increased in co-cultures with Ori treatment compared to VEH-treated co-cultures indicating an additional positive effect of Ori on control neurons (Figure 31A).

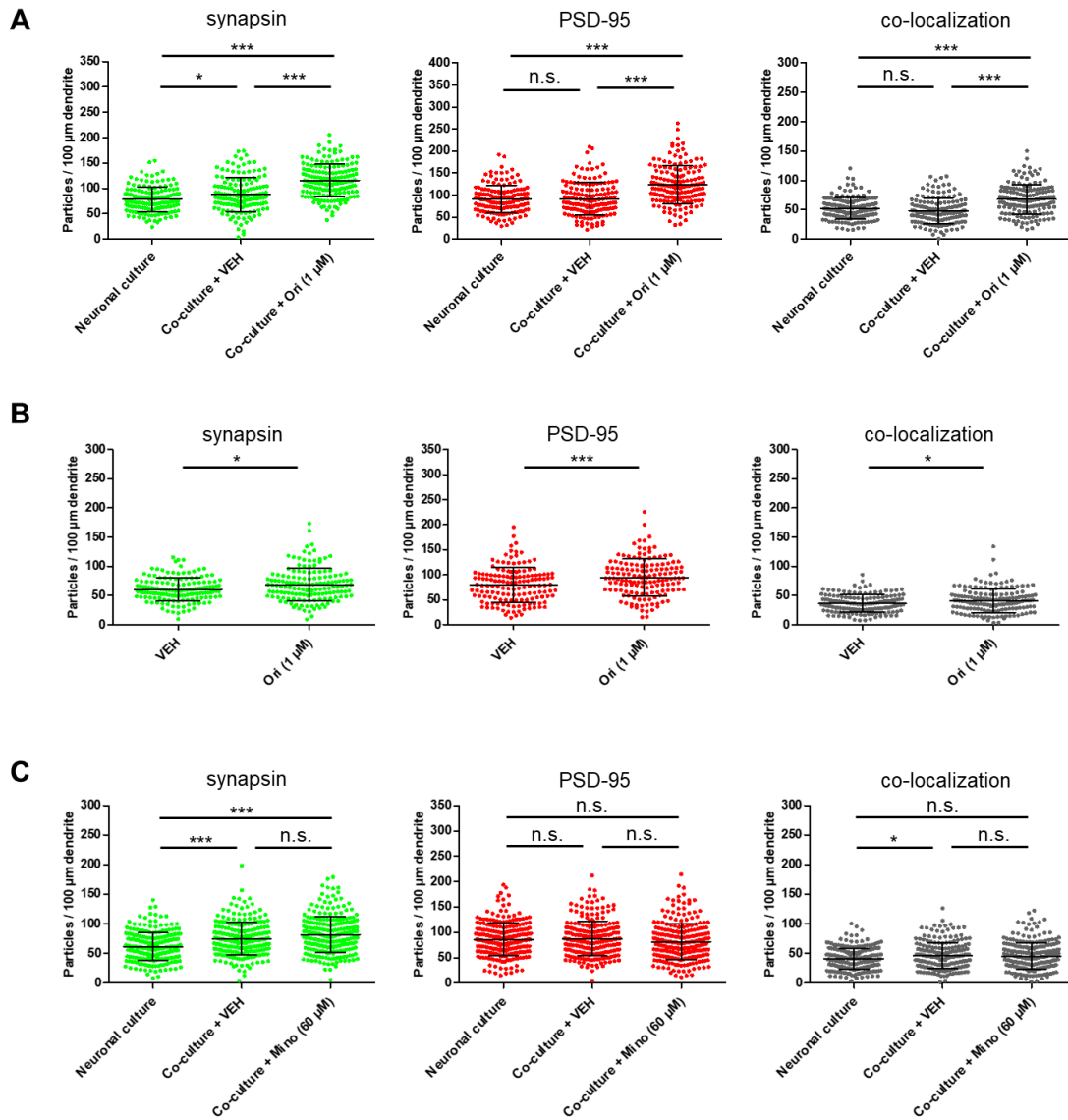


Figure 31: Effects of oridonin and minocycline on synapse density in control human iPSC-derived cortical neurons.

(A) Quantification of pre-synaptic (synapsin) and post-synaptic (PSD-95) particles, as well as their co-localization, in control iPSC-derived cortical neurons with and without co-culture with iPSC-derived microglia. Co-cultures were treated with VEH or 1 μ M oridonin (Ori) (n = 150-170 randomly selected dendrites examined per group from three biological replicates, Kruskal-Wallis test with Dunn's multiple comparison test, dots for individual dendrites, means $\pm$ SEM). (B) Quantification of synaptic markers and their co-localization in control human iPSC-derived cortical neurons. Pure neuronal cultures were treated with VEH or Ori (1 μ M) (n = 150 randomly selected dendrites examined per group from three biological replicates, Mann-Whitney U-test, dots for individual dendrites, means $\pm$ SEM).

(C) Quantification of synaptic markers and their co-localization in control neurons with and without co-culture with microglia and treatment with VEH or 60 μ M minocycline (Mino) (n = 220-240 randomly selected dendrites examined per group from three biological replicates, Kruskal-Wallis test with Dunn's multiple comparison test, dots for individual dendrites, means $\pm$ SEM). * P < 0.05 and *** P < 0.001. n.s., not significant.

This effect was further validated by treatment of pure neuronal control cultures with Ori. Neurons treated with Ori (1 μ M) for 1 week also showed a significant increase in synaptic markers and their co-localization when compared to VEH-treated neurons (Figure 31B). However, this effect was less striking compared to the aforementioned treatment of co-cultures with Ori. On the contrary, when treating the control co-cultures with Mino instead of Ori, there was no change in the number of pre-and post-synaptic particles compared to the VEH control (Figure 31C). These results indicate that Ori can exert superior beneficial effects on synaptogenesis compared to Mino and could therefore represent a promising new approach to protect cortical neurons against excessive synaptic pruning in the context of SCZ pathogenesis.

8 Discussion

Microglia have been shown to be of great importance during synaptic remodeling (Schafer et al., 2012) and have been discussed to be key players in the development of SCZ and other psychiatric disorders (Rahimian et al., 2021). SCZ has a strong genetic component and especially the MHC locus and specific *C4* alleles with an effect on *C4A* expression have been associated with an increased risk for the development of the disease (Sekar et al., 2016).

The here presented work introduces a powerful *in vitro* model to study synaptic pruning in the context of SCZ pathogenesis in human cells. It was shown, that increased *C4A* expression in iPSC-derived cortical neurons leads to an activation of the NF κ B pathway in microglia resulting in an excessive synaptic loss by increased synaptic pruning. Furthermore, the NLRP3 inflammasome was identified as a potential new therapeutic target for SCZ. Thereby, the NLRP3 inflammasome inhibitor oridonin was shown to inhibit *C4A*-induced microglial activation and rescue synaptic densities in co-cultures of human iPSC-derived cortical neurons and microglia.

8.1 Generation of human iPSC-derived cortical neurons and microglia

The first aim of this thesis was the establishment and validation of differentiation protocols for the generation of human iPSC-derived microglia and mature cortical neurons.

Recent advances in the generation of iPSC-derived microglia have led to a growing toolset to study synaptic pruning-associated disorders in human cells. This development has been especially important since different groups have demonstrated significant differences between murine and human microglia (Gosselin et al., 2017; Smith and Dragunow, 2014). Following these observations, several protocols have been published in the recent years for the differentiation of human iPSCs to microglia-like cells (Abud et al., 2017; Banerjee et al., 2020; Douvaras et al., 2017; Haenseler et al., 2017; Muffat et al., 2016; Pandya et al., 2017). It is known that microglia originate from hematopoietic stem cells in the yolk sac instead of the bone marrow and therefore new methods to generate microglia-like cells transition from iPSCs over hematopoietic and erythromyeloid progenitors (Kierdorf et al., 2013; Schulz et al., 2012).

The protocol for the generation of iPSC-derived microglia established in this thesis resulted in highly pure cultures of microglia-like cells expressing important canonical microglia markers, as shown by immunofluorescence stainings and bulk RNAseq experiments. iPSC-derived microglia displayed a high phagocytic activity as demonstrated by the uptake of fluorescently labeled zymosan bioparticles and responded to LPS stimulation by change of morphology and upregulation of pro-inflammatory cytokine expression.

Although iPSC-derived microglia perform key cell-specific functions and express important microglia markers, there are still a few limitations which should be kept in mind when utilizing these cells for *in vitro* disease modeling and drug discovery. First, there are still concerns about the similarity between iPSC-derived microglia and human primary microglia. Although the transcriptomic signatures are similar, microglia are highly dynamic cells which immediately respond to changes in their environment and display a higher base level of activation due to *in vitro* culture conditions compared to *in vivo* microglia. Microglia *in vitro* cultures have been shown to exhibit a down-regulation of core signature and homeostasis-associated genes and an up-regulation of disease-and injury-related genes (Bohlen et al., 2017). *In vitro* artefacts have been reduced by the development of differentiation protocols that spare the use of fetal bovine serum which has previously been added to media to promote proliferation and survival of the cells (Bohlen et al., 2017; Morini et al., 2021). In parallel, additional important factors have been recently identified to improve microglial identity *in vitro* by systematic assessment of media components in single-cell RNA sequencing experiments. Especially astrocyte-derived TGF- β 1 was shown to significantly improve the expression of key microglial signature genes and should therefore be implemented in future experiments (Washer et al., 2022).

Substantial progress has also been made in the development of efficient differentiation protocols to obtain neuronal cultures of specific regional identity from human iPSCs. In this study, mature iPSC-derived cortical neuronal cultures were generated by applying a combination of small molecules to pattern neural progenitor cells towards a forebrain identity and subsequently driving cell-cycle arrest to promote neuronal differentiation. The established protocol resulted in highly pure neuronal cultures with only minimal astrocytic content, as shown by GFAP and S100 β immunofluorescence stainings. After 50 days of differentiation, neurons expressed pan-neuronal markers, as well as layer V and VI cortical layer markers. Furthermore, iPSC-derived cortical neurons displayed a robust synaptogenesis as shown by the co-localization of pre-and postsynaptic marker proteins synapsin and PSD-95.

Although the successful establishment of functional synapses has been demonstrated for various protocols generating iPSC-derived neuronal cultures, there are still concerns regarding their maturity. Dendritic spines are rarely observed in iPSC-derived neurons and PSD-95 labeling often appears more diffuse in neurites implying an immaturity of the cells. The lack of astrocytes may represent a restriction in this regard, as they are known to play an important role in synaptogenesis (Baldwin and Eroglu, 2017). Prolonged *in vitro* maturation represents a major challenge for disease modeling using iPSC-derived cells and increased efforts have gone into the development of protocols with accelerated neuronal maturation. A recent study identified specific factors targeting chromatin remodeling and calcium-dependent transcription that efficiently promote maturation of iPSC-derived neurons, but also non-neuronal lineages.

A central hypothesis of this study is the presence of an epigenetic program in immature neurons preventing their fast maturation in humans (Hergenreder et al., 2022). This finding could facilitate the use of iPSC-technology to model human disease by reducing long culture periods to generate functional cells.

The approach of using pure iPSC-derived neuronal cultures in combination with human iPSC-derived microglia, as presented in this thesis, enables the specific investigation of microglia activation by neuronal complement expression without interference of other cell types. However, in future studies it will be important to develop more complex and physiological disease models such as tri-cultures with astrocytes or microglia-containing human brain organoids to further dissect the involvement of different cell types in SCZ pathogenesis. Astrocytes have been shown to have complex interactions with microglia and neurons and contribute to synaptogenesis, complement expression and neuroinflammation in the brain (Guttikonda et al., 2021). Therefore, further investigation of their role in complement-dependent synaptic pruning is of high relevance when attempting to better understand cellular processes occurring during the development of the disease.

Taken together, the differentiation protocols established in this thesis gave rise to synaptically connected cortical neurons and functionally active microglia-like cells expressing the most important cell-type specific canonical markers. Thereby, despite the lack of other important cell types such as astrocytes, these human cell cultures fulfilled the requirements to serve as an *in vitro* model to study synaptic pruning in the context of SCZ pathogenesis.

8.2 Generation of a human iPSC-derived *in vitro* model to study synaptic pruning-associated changes in SCZ

The second part of this work focused on the establishment of an *in vitro* model to investigate the effect of complement component C4A on microglia-mediated synapse engulfment in the context of SCZ pathogenesis.

Structural and functional brain changes such as a reduction of cortical gray matter volume and decreased neuronal connectivity, as well as increased microglial activity are characteristics of SCZ (Birur et al., 2017; Monji et al., 2009). Considering the typical time of onset during adolescence and early adulthood, researchers have hypothesized that excess synaptic pruning could trigger the disease (Cannon et al., 2015; Keshavan et al., 2020a). The role for the classical complement cascade to ‘tag’ synapses for removal by microglia cells has been identified and thoroughly studied in the mouse retinogeniculate system. C1q, C3 and C4 KO mice were found to have defects in synaptic elimination and complement component C3 was shown to localize to subsets of synapses in the developing brain (Sekar et al., 2016; Stevens et al., 2007). Furthermore, KO of CR3, a complement receptor only expressed by microglia in

the brain, resulted in similar deficits in synapse engulfment, indicating the process to be largely microglia-mediated (Schafer et al., 2012). Thus, the majority of animal studies investigating the role of the complement system in synaptic pruning have relied on loss-of function approaches, though SCZ is associated to increased C4 expression. More recently, the relevance of overexpression models has been emphasized in mouse studies showing reduced cortical synapse densities in mice overexpressing human C4A due to increased synaptic pruning by microglia resulting in SCZ-like changes in mouse behavior (Comer et al., 2020; Yilmaz et al., 2021).

In this thesis, the effects of neuronal complement component C4A expression on microglia in the context of synaptic pruning and SCZ pathogenesis were investigated by lentiviral overexpression of C4A in iPSC-derived cortical neurons. The comparison of a lentiviral overexpression cell line with the corresponding non-transduced control enabled the reduction of genetic variability and facilitated a genotype-phenotype correlation. Using this experimental setting, more detailed insights into specific pathways leading to microglial activation could be gained, enabling the investigation of potential new pharmacological interventions. As SCZ is a complex and heterogeneous disorder, the use of SCZ patient-derived cells can result in a high variability between cell lines making a clear interpretation of results very difficult. Therefore, disease models employing an isogenic setting present a major advantage in this regard. One advantage of viral-based vectors is the stable integration of a transgene into the host genome, thereby achieving a robust and sustained expression even after cell division (Fus-Kujawa et al., 2021). However, lentiviral transduction bears the risk of insertional mutagenesis at the integration site which should be considered when interpreting the results (Poletti and Mavilio, 2018).

A correlation of increased neuronal C4A expression with increased C3 deposition and enhanced synaptic engulfment by microglia has been previously demonstrated by others using human *in vitro* cell culture models (Sellgren et al., 2019). Thereby, monocyte-derived microglia-like cells were used, which are generated by following a direct conversion approach. In this thesis, main findings of this study were confirmed using the first fully iPSC-derived human synaptic pruning model incorporating microglia that originate from yolk sac-derived erythromyeloid progenitors.

First, synaptosomes were isolated from mature iPSC-derived neuronal cultures and labeled with a pH-sensitive fluorescent dye to visualize engulfment by microglia. This *in vitro* synaptic pruning assay was developed by Sellgren et al. showing an increased engulfment of SCZ patient-derived synaptosomes by monocyte-derived microglia-like cells depending on the C4 genotype (Sellgren et al., 2019; Sellgren et al., 2017). In the here presented work,

synaptosomes derived from neurons overexpressing C4A were more efficiently phagocytosed compared to those derived from controls, which is in line with the aforementioned results.

Synaptosomes have been widely used by the neuroscientific community to study synaptic structure and function and they offer the advantage that they can be maintained frozen which is especially beneficial for high throughput drug screening approaches (Daniel et al., 2012; Gleitz et al., 1993). However, there are some drawbacks which should be discussed. First, there is no way to control for the exact amount of labeled synaptosomes added to microglia cells, except for standardizing cell numbers before synaptosome extraction and performing exemplary Western blots to show a comparable enrichment of synaptic structures in the synaptosome fractions of different cell lines. Furthermore, it was shown that synaptosomes expose phosphatidylserine and show caspase activation rapidly after preparation which is likely to alter results (Gyls et al., 2004; Morini et al., 2021). Moreover, it has to be taken into account that microglia are highly phagocytic cells and general phagocytosis of potentially labeled cellular debris has to be distinguished from specific synapse engulfment via the microglial C3 receptor. Therefore, it is important to further confirm the relevance of the synaptic pruning assay by pre-treating the cells with an anti-CD11b antibody to specifically inhibit C3-CR3 interaction which should decrease microglial phagocytosis of synaptosomes.

In order to generate a more physiological *in vitro* model to study synaptic pruning in human cells, a co-culture protocol for iPSC-derived microglia and cortical neurons was established. Thereby, media composition and cell numbers were optimized to enable the combination of both cell types over a duration of 1-2 weeks. The data presented in this thesis confirms that C4A overexpression in neurons is a driver of increased synaptic engulfment by microglia which is in line with observations by Sellgren and colleagues that show an increased reduction in spine density in co-cultures with neurons derived from SCZ patients (Sellgren et al., 2019).

Interestingly, in the here presented work, microglia had beneficial effects on neuronal connectivity in co-cultures with control neurons which stands in contrast to previous work by others, where spine densities were also reduced in the control condition (Sellgren et al., 2019). These differences could be explained by variances in the experimental outlines. In this work, synapse densities were assessed after a co-culture duration of 7 days, whereas Sellgren et al. performed co-culture experiments for 48 hours. Though microglia are best known as the immune cells of the brain, they also play an important role in maintaining homeostasis and promoting neurogenesis by secreting neurotrophic factors such as brain-derived neurotrophic factor (BDNF), insulin like growth factor 1 (IGF-1), arginase 1 (Arg-1) and TGF- β (Li and Barres, 2018; Pons and Rivest, 2020; Thion et al., 2018). BDNF is known to support neuronal growth, differentiation and synaptogenesis, IGF-1 is an inhibitor of apoptosis and Arg-1 has been shown to decrease NO levels and oxidative stress (Pons and Rivest, 2020; Thored et al.,

2009; Ueno et al., 2013). Furthermore, microglia act as important phagocytes by clearing the brain from cellular debris and apoptotic cells (Neumann et al., 2009). The differences seen when comparing this work with previous work by others, could reflect short- and long-term effects of microglia in co-cultures with neurons. While microglia play an important role in the formation of mature neural circuits by eliminating excess numbers of synapses during normal brain development, the secretion of aforementioned neurotrophic factors could counteract the decrease in synapse density leading to an increased neural connectivity in long-term co-cultures of control iPSC-derived cortical neurons and microglia.

In summary, a fully iPSC-derived *in vitro* model for synaptic pruning in the context of SCZ pathogenesis was established using lentiviral overexpression of C4A to analyze the specific contribution of C4A abundance in synaptosome and synapse engulfment by microglia. Using a more physiological differentiation approach to generate iPSC-derived microglia, important findings in the literature were reproduced by showing a positive correlation of increased C4A expression with the synaptic pruning activity of microglia. In long-term direct co-culture experiments of control iPSC-derived cortical neurons with microglia, microglial cells had a beneficial effect on neuronal connectivity which might be due to the secretion of neurotrophic factors.

8.3 The microglial NLRP3 inflammasome as a potential drug target in SCZ

In this work, the application of a newly developed human iPSC-based *in vitro* synaptic pruning model led to the identification of the NLRP3 inflammasome as a potential new therapeutic option for SCZ. It was shown that enhanced C4A levels in iPSC-derived cortical neurons activate the NF κ B pathway in microglia, an important mediator of NLRP3 inflammasome activation. Single-nucleus RNAseq data further confirmed increased microglial activation in co-cultures with C4A overexpressing neurons. NF κ B pathway activation was demonstrated by an increase in nuclear translocation of the NF κ B subunit p65, as well as by significant differences in NF κ B signaling in GO enrichment analysis. Interestingly, the expression of the NLRP3-associated genes *DDX3X* and *P2RX7* was significantly increased in microglia in co-cultures with C4A overexpressing neurons compared to controls. *DDX3X* has been identified as a stress granule protein which can interact with NLRP3 to drive inflammasome activation. It has been shown that, depending on the availability of *DDX3X* in macrophages, cell stress signals can be interpreted to decide between the formation of pro-survival stress granules or inflammasome assembly (Samir et al., 2019). *P2RX7* is a microglial ATP-gated ion channel which has been implicated in neuropsychiatric mood disorders such as depression (Bhattacharya and Jones, 2018). Microglial *P2RX7* activation has been shown to mediate NLRP3 inflammasome activation and IL-1 β release and brain penetrant *P2RX7* antagonists

have entered clinical development to target neuroinflammation in CNS pathophysiology (Bhattacharya et al., 2013; Pelegrin, 2021).

Though evidence from this study suggests a novel role for the NLRP3 inflammasome in SCZ pathogenesis, future studies should include more specific cellular and biochemical assays. Detection of ASC specks, which are large structures formed by the inflammasome adaptor protein ASC, can be visualized by immunofluorescence stainings and are commonly used to identify inflammasome activation. Furthermore, ELISA assays for IL-1 β and IL-18 represent a useful approach to obtain quantitative information on cytokine concentrations present in the supernatant of cells, which can be a readout for caspase 1 activation (Zito et al., 2020). It would also be of great value to further dissect the involvement of the NLRP3 inflammasome in activating microglia to more excessively phagocytose synapses by performing experiments with specific NLRP3 activators. In this regard, co-cultures could be treated with ATP or LPS possibly causing a similar synaptic pruning phenotype as seen with increased levels of C4A.

Potential crosstalk between complement signaling pathways and the NLRP3 inflammasome have been previously implicated in AD. Thereby, it was suggested that aggregated A β can activate complement, leading to microglial phagocytosis and synapse pruning (Yang et al., 2020). However, the exact mechanism of complement-mediated inflammasome activation and its role in the context of synaptic pruning-associated disorders has not been investigated. The human *in vitro* model established in this thesis can serve as a valuable tool to further analyze the molecular mechanisms underlying these processes and can support the investigation of novel therapeutic strategies to inhibit microglial activation in the context of neuropsychiatric and neurodegenerative diseases.

8.4 Inhibition of microglia activation in a human iPSC-derived model of synaptic pruning

In seeking ways to inhibit microglia activation in the context of SCZ pathogenesis, the field has focused on treatment with tetracycline antibiotic minocycline for many years (Inta et al., 2017; Tikka et al., 2001). Minocycline has previously been shown to inhibit microglia activation and decrease the engulfment of synaptic structures in cell culture models using patient-derived human iPSCs (Sellgren et al., 2019). Although clinical studies have reported beneficial effects of minocycline in alleviating negative symptoms of SCZ (Chaudhry et al., 2012; Kelly et al., 2015; Liu et al., 2014), a number of experimental studies on rodents have shown detrimental effects, especially at early postnatal stages (Arnoux et al., 2014; Inta et al., 2016; Tsuji et al., 2004), emphasizing the need of more investigations and the development of novel drugs. In the work presented in this thesis, oridonin increased synapse densities more efficiently than

minocycline, offering the exciting prospect of inhibiting microglia activation in the context of SCZ pathogenesis via a novel mechanism.

Beneficial effects of inhibiting microglia activation with the specific NLRP3 inhibitor oridonin have been previously described in the context of AD (Wang et al., 2014; Wang et al., 2016; Zhang et al., 2013). Using the iPSC-derived *in vitro* model established in this thesis, anti-inflammatory and neuroprotective effects of oridonin were confirmed. First, it was shown that main components of the NLRP3 inflammasome are expressed by iPSC-derived microglia *in vitro* and that there is a significant change in expression after LPS treatment, indicating an activation of the pathway. In proof of principle experiments, it was further shown that oridonin attenuates LPS-induced changes in microglia morphology and NFκB (p65) nuclear translocation in human iPSC-derived microglia. These findings support previous reports showing similar anti-inflammatory effects of oridonin on rat primary microglia (Xu et al., 2009) and other cell types such as human renal proximal tubular epithelial cells (HK-2 cells) (Huang et al., 2020).

In this work, the effect of oridonin on the engulfment of synaptic structures was first investigated using the established *in vitro* synaptic pruning assay. Interestingly, oridonin significantly decreased the phagocytosis of labeled synaptosomes by iPSC-derived microglia in a dose-dependent manner. An inhibitory effect on microglial phagocytosis was also seen by treatment with minocycline, as described before by others (Sellgren et al., 2019). Moreover, the here presented work demonstrates a beneficial effect of oridonin on synapse densities in C4A overexpressing cortical neurons after co-culture with microglia, possibly by inhibiting NLRP3 inflammasome activation in microglia, which was concluded from a decrease in nuclear translocation of NFκB (p65).

Although the inhibitory effect of oridonin on microglial phagocytosis in the *in vitro* synaptic pruning assay is in line with the decrease in synapse engulfment in co-culture experiments, molecular mechanisms underlying this process will need further investigation. Effects of oridonin on phagocytosis have previously been investigated in the context of anti-cancer properties where it was shown to have an opposing effect. Thereby, oridonin enhanced the phagocytosis of apoptotic tumor cells by macrophages. This effect was, however, shown to be specific for apoptotic cells and not for other phagocytic targets (Liu et al., 2005). Since oridonin can directly and indirectly interact with different proteins and receptors, it is not unlikely that its effects can vary depending on the cellular process and cell types targeted.

In SCZ pathogenesis, a reduction of synapse density often already occurs before onset of disease symptoms and before patients can be diagnosed. In this work, it was shown that oridonin can also restore the number of functional synapses at a time point where synaptic engulfment by microglia has already largely happened. Interestingly, the number of synaptic

particles was even higher in oridonin-treated co-cultures compared to untreated pure neuronal cultures of the same age for both C4A and control conditions. This additional beneficial effect on synaptogenesis was further demonstrated by treatment of pure neuronal control cultures with oridonin, which also increased synaptic density. Oridonin has previously been shown to activate the BDNF/TrkB/CREB pathway, thereby exerting neuroprotective effects, which could explain these findings (Wang et al., 2016). In contrary, there was no additional increase in synapse densities in control co-cultures after treatment with the tetracycline antibiotic minocycline. Direct effects of minocycline on pure neuronal cultures were not investigated in this work and will need further evaluation. Minocycline has been described to exert beneficial anti-inflammatory, anti-apoptotic and antioxidant properties in the pathogenesis of several neurological diseases. However, the exact neuroprotective mechanism remains to be clarified and some studies even indicate contradictory (beneficial or detrimental) effects depending on the model used (Cheng et al., 2015; Diguët et al., 2004; Yong et al., 2004).

When analyzing co-cultures of microglia with C4A overexpressing neurons, it became evident that differences in synapse densities between pure neuronal cultures and VEH-treated co-cultures were no longer significant after 2 weeks. Synapse densities of pure neuronal cultures are known to increase with age according to the maturation status of the cells, which might counteract the synaptic pruning phenotype seen at earlier timepoints. Furthermore, this finding can result from a decreased activation of microglial cells in the co-cultures over time. In this context, it is interesting to note that neurons can actively control microglia function by producing 'off' signals, thereby protecting the brain from overactivated immune cells under inflammatory conditions. Examples for neurotrophins and signaling molecules secreted by neurons to influence microglia activity are CX3CL1, TGF- β , CD22 and BDNF (Biber et al., 2007).

In this thesis, oridonin has demonstrated a promising potential to inhibit microglia activation in the context of synaptic pruning and SCZ pathogenesis. However, it has also become evident that oridonin exhibits multifunctional effects, many of which are still poorly understood. Future research on this topic should include the investigation of long-term effects of oridonin, in order to validate if microglia can restore their regular function after discontinuation of treatment. Furthermore, a detailed investigation of the effects of oridonin on other cell types such as astrocytes should be performed by using more complex disease models. Despite promising effects of oridonin, its clinical development has been restricted due to limited aqueous solubility which reduces its bioavailability and may limit the use as a neuroprotective agent *in vivo* (Xu et al., 2018). Thus, more research should be directed towards enhancing pharmacokinetic properties to improve the delivery through oral and CNS barriers.

In summary, this study shows the value of a human-specific iPSC-derived model to study the involvement of microglia in the complex pathogenesis of SCZ. The findings presented in this thesis indicate that the inhibition of microglial NF- κ B signaling via the NLRP3 inflammasome inhibitor oridonin can protect cortical neurons against excessive synaptic pruning and give new insights into possible molecular pathways involved in the disease. Furthermore, the established human *in vitro* model offers a platform to perform high throughput screening for the identification of novel pharmacological entities, as well as for personalized medicine approaches using patient-derived cells.

9 Summary and outlook

In this thesis, a fully iPSC-derived human synaptic pruning *in vitro* model for SCZ-risk-associated synaptic loss was developed incorporating cortical neurons and microglia. Lentiviral overexpression of C4A in iPSC-derived neurons was used to analyze the specific contribution of C4A abundance in synaptosome and synapse engulfment by microglia.

As summarized in Figure 32, the here presented work demonstrates that:

1. human iPSC-derived cell culture models are powerful tools to study synaptic pruning in the context of SCZ pathogenesis.
2. microglial engulfment of synaptosomes derived from cortical neurons overexpressing C4A is increased compared to synaptosomes from control neurons.
3. microglial cells have beneficial or detrimental effects on neuronal connectivity in direct co-cultures with human iPSC-derived cortical neurons depending on neuronal C4A expression levels.
4. enhanced C4A expression levels in iPSC-derived cortical neurons activate inflammatory pathways in microglia, including the NF κ B pathway, an important mediator of NLRP3 inflammasome activation.
5. the inhibition of NLRP3 inflammasome activation prevents C4A-mediated synaptic engulfment by microglia.

Combining the use of a human iPSC-derived *in vitro* model with the overexpression of SCZ risk factor C4A provided novel insights into specific pathways leading to microglia activation and enabled the evaluation of potential new pharmacological interventions. The presented findings will, however, need further validation using more complex cell culture systems such as tri-cultures or human brain organoids, as well as *in vivo* disease models. A more in depth understanding of the mechanisms underlying NLRP3 inflammasome activation and regulation will be critical to develop effective treatments.

NF- κ B signaling and NLRP3 inflammasome activation are basic inflammatory response pathways regulating many different cellular processes and further work will have to be invested to identify ways of specifically inhibiting microglia activated from complement without affecting normal immune cell functions. Using general NLRP3 inflammasome inhibitors to prevent SCZ may cause severe side effects and raises questions regarding the balance between efficacy and safety of the treatment. When targeting hyperactive synaptic pruning by inhibiting microglia activation, it also has to be considered that potential therapeutics would only be effective in

very early phases of SCZ pathogenesis. In this regard, it will be important to develop reliable biomarkers and strategies to identify patients early before the onset of symptoms.

Despite the limitations and open questions, this thesis sets the base for the development of new therapeutic strategies targeting neuroinflammation and establishes a robust human cell culture model to study synaptic pruning in the context of SCZ pathogenesis. Future research in this field will also be of great value regarding treatment options for other psychiatric disorders such as ASD, BP and MDD, which have also been associated with complement dysregulation, neuroinflammation and altered synaptic pruning (Sierra et al., 2022).

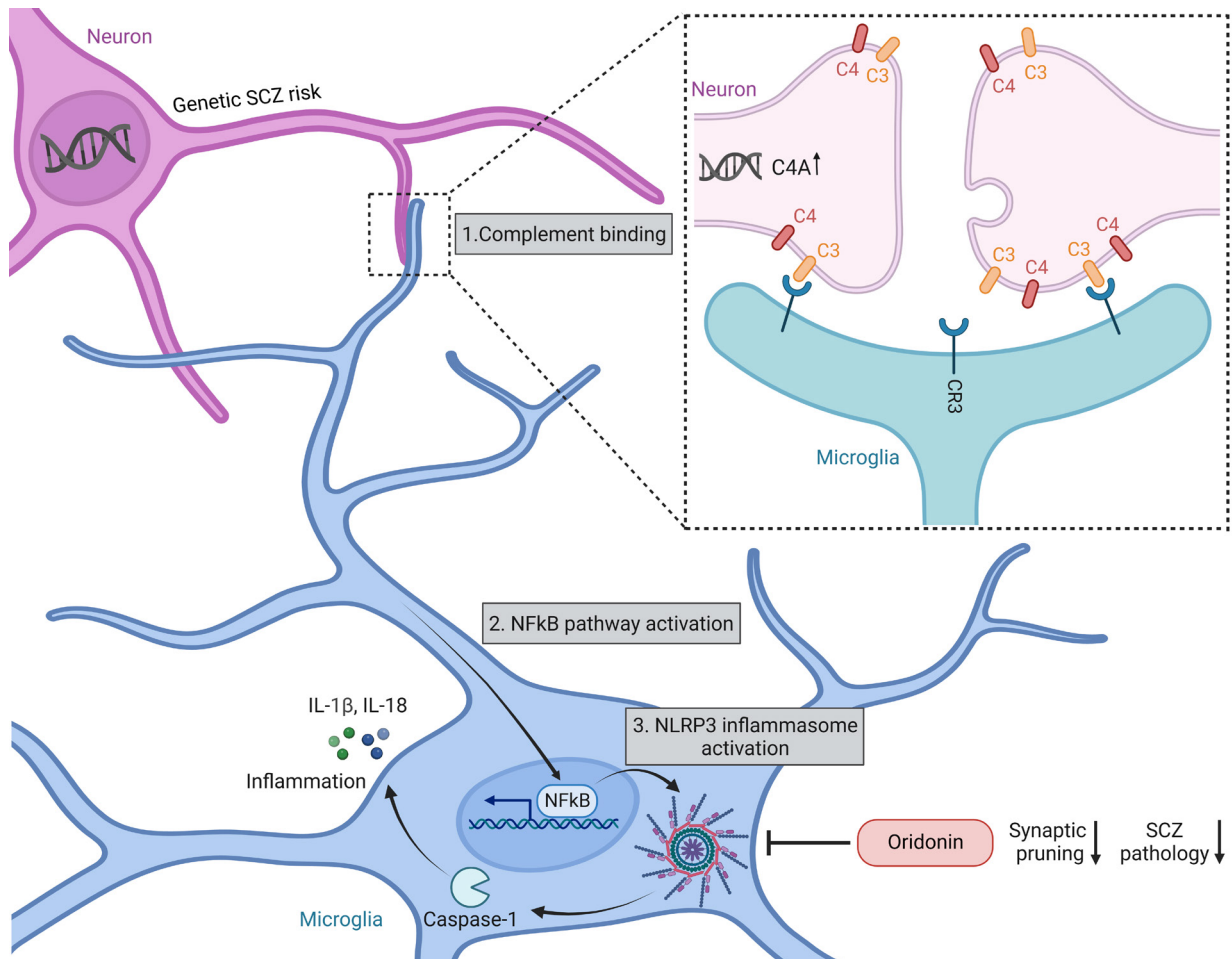


Figure 32: Proposed model for microglial NLRP3 inflammasome activation by increased neuronal complement expression implicating a potential new therapeutic approach.

10 References

- Abud, E. M., Ramirez, R. N., Martinez, E. S., Healy, L. M., Nguyen, C. H. H., Newman, S. A., Yeromin, A. V., Scarfone, V. M., Marsh, S. E., Fimbres, C., *et al.* (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* **94**, 278-293 e279.
- Alcocer-Gomez, E., de Miguel, M., Casas-Barquero, N., Nunez-Vasco, J., Sanchez-Alcazar, J. A., Fernandez-Rodriguez, A., and Cordero, M. D. (2014). NLRP3 inflammasome is activated in mononuclear blood cells from patients with major depressive disorder. *Brain Behav Immun* **36**, 111-117.
- Andreeva, L., David, L., Rawson, S., Shen, C., Pasricha, T., Pelegrin, P., and Wu, H. (2021). NLRP3 cages revealed by full-length mouse NLRP3 structure control pathway activation. *Cell* **184**, 6299-6312 e6222.
- Andrews, S., Krueger, F., Segonds-Pichon, A., Biggins, L., Krueger, C., and Wingett, S. (2015). FastQC: a quality control tool for high throughput sequence data. . <http://www.bioinformatics.babraham.ac.uk/projects/fastqc> (Accessed December 10, 2020).
- Appelt-Menzel, A., Cubukova, A., Gunther, K., Edenhofer, F., Piontek, J., Krause, G., Stuber, T., Walles, H., Neuhaus, W., and Metzger, M. (2017). Establishment of a Human Blood-Brain Barrier Co-culture Model Mimicking the Neurovascular Unit Using Induced Pluri- and Multipotent Stem Cells. *Stem Cell Reports* **8**, 894-906.
- Arnoux, I., Hoshiko, M., Sanz Diez, A., and Audinat, E. (2014). Paradoxical effects of minocycline in the developing mouse somatosensory cortex. *Glia* **62**, 399-410.
- Avior, Y., Sagi, I., and Benvenisty, N. (2016). Pluripotent stem cells in disease modelling and drug discovery. *Nat Rev Mol Cell Biol* **17**, 170-182.
- Baker, P. J., Boucher, D., Bierschenk, D., Tebartz, C., Whitney, P. G., D'Silva, D. B., Tanzer, M. C., Monteleone, M., Robertson, A. A., Cooper, M. A., *et al.* (2015). NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. *Eur J Immunol* **45**, 2918-2926.
- Baldwin, K. T., and Eroglu, C. (2017). Molecular mechanisms of astrocyte-induced synaptogenesis. *Curr Opin Neurobiol* **45**, 113-120.
- Banerjee, P., Paza, E., Perkins, E. M., James, O. G., Kenkhuis, B., Lloyd, A. F., Burr, K., Story, D., Yusuf, D., He, X., *et al.* (2020). Generation of pure monocultures of human microglia-like cells from induced pluripotent stem cells. *Stem Cell Res* **49**, 102046.
- Begum, A. N., Guoynes, C., Cho, J., Hao, J., Lutfy, K., and Hong, Y. (2015). Rapid generation of sub-type, region-specific neurons and neural networks from human pluripotent stem cell-derived neurospheres. *Stem Cell Res* **15**, 731-741.
- Belt, K. T., Carroll, M. C., and Porter, R. R. (1984). The structural basis of the multiple forms of human complement component C4. *Cell* **36**, 907-914.
- Belt, K. T., Yu, C. Y., Carroll, M. C., and Porter, R. R. (1985). Polymorphism of human complement component C4. *Immunogenetics* **21**, 173-180.
- Benros, M. E., Nielsen, P. R., Nordentoft, M., Eaton, W. W., Dalton, S. O., and Mortensen, P. B. (2011). Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study. *Am J Psychiatry* **168**, 1303-1310.

- Beraud, D., and Maguire-Zeiss, K. A. (2012). Misfolded alpha-synuclein and Toll-like receptors: therapeutic targets for Parkinson's disease. *Parkinsonism Relat Disord* 18 Suppl 1, S17-20.
- Bhattacharya, A., and Jones, D. N. C. (2018). Emerging role of the P2X7-NLRP3-IL1beta pathway in mood disorders. *Psychoneuroendocrinology* 98, 95-100.
- Bhattacharya, A., Wang, Q., Ao, H., Shoblock, J. R., Lord, B., Aluisio, L., Fraser, I., Nepomuceno, D., Neff, R. A., Welty, N., *et al.* (2013). Pharmacological characterization of a novel centrally permeable P2X7 receptor antagonist: JNJ-47965567. *Br J Pharmacol* 170, 624-640.
- Bialas, A. R., and Stevens, B. (2013). TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. *Nat Neurosci* 16, 1773-1782.
- Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007). Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci* 30, 596-602.
- Birur, B., Kraguljac, N. V., Shelton, R. C., and Lahti, A. C. (2017). Brain structure, function, and neurochemistry in schizophrenia and bipolar disorder-a systematic review of the magnetic resonance neuroimaging literature. *NPJ Schizophr* 3, 15.
- Bjartmar, L., Huberman, A. D., Ullian, E. M., Renteria, R. C., Liu, X., Xu, W., Prezioso, J., Susman, M. W., Stellwagen, D., Stokes, C. C., *et al.* (2006). Neuronal pentraxins mediate synaptic refinement in the developing visual system. *J Neurosci* 26, 6269-6281.
- Blevins, H. M., Xu, Y., Biby, S., and Zhang, S. (2022). The NLRP3 Inflammasome Pathway: A Review of Mechanisms and Inhibitors for the Treatment of Inflammatory Diseases. *Front Aging Neurosci* 14, 879021.
- Bohlen, C. J., Bennett, F. C., Tucker, A. F., Collins, H. Y., Mulinyawe, S. B., and Barres, B. A. (2017). Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. *Neuron* 94, 759-773 e758.
- Boksa, P. (2010). Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain Behav Immun* 24, 881-897.
- Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* 11, 43-49.
- Brown, A. S., and Derkits, E. J. (2010). Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. *Am J Psychiatry* 167, 261-280.
- Cannon, T. D., Chung, Y., He, G., Sun, D., Jacobson, A., van Erp, T. G., McEwen, S., Addington, J., Bearden, C. E., Cadenhead, K., *et al.* (2015). Progressive reduction in cortical thickness as psychosis develops: a multisite longitudinal neuroimaging study of youth at elevated clinical risk. *Biol Psychiatry* 77, 147-157.
- Cannon, T. D., van Erp, T. G., Bearden, C. E., Loewy, R., Thompson, P., Toga, A. W., Huttunen, M. O., Keshavan, M. S., Seidman, L. J., and Tsuang, M. T. (2003). Early and late neurodevelopmental influences in the prodrome to schizophrenia: contributions of genes, environment, and their interactions. *Schizophr Bull* 29, 653-669.
- Carlson, M. (2020). org.Hs.eg.db: Genome wide annotation for Human. R package version 3.12.0.

- Carroll, M. C., Belt, T., Palsdottir, A., and Porter, R. R. (1984). Structure and organization of the C4 genes. *Philos Trans R Soc Lond B Biol Sci* 306, 379-388.
- Carroll, M. C., Fathallah, D. M., Bergamaschini, L., Alicot, E. M., and Isenman, D. E. (1990). Substitution of a single amino acid (aspartic acid for histidine) converts the functional activity of human complement C4B to C4A. *Proc Natl Acad Sci U S A* 87, 6868-6872.
- Centeno, E. G. Z., Cimarosti, H., and Bithell, A. (2018). 2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling. *Mol Neurodegener* 13, 27.
- Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27, 275-280.
- Chaudhry, I. B., Hallak, J., Husain, N., Minhas, F., Stirling, J., Richardson, P., Dursun, S., Dunn, G., and Deakin, B. (2012). Minocycline benefits negative symptoms in early schizophrenia: a randomised double-blind placebo-controlled clinical trial in patients on standard treatment. *J Psychopharmacol* 26, 1185-1193.
- Chen, I. P., Fukuda, K., Fusaki, N., Iida, A., Hasegawa, M., Lichtler, A., and Reichenberger, E. J. (2013). Induced pluripotent stem cell reprogramming by integration-free Sendai virus vectors from peripheral blood of patients with craniometaphyseal dysplasia. *Cell Reprogram* 15, 503-513.
- Chen, J., and Chen, Z. J. (2018). PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. *Nature* 564, 71-76.
- Chen, L. F., and Greene, W. C. (2004). Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5, 392-401.
- Chen, Y., Zhao, Z., Lin, F., Wang, L., Lin, Z., and Yue, W. (2021). Associations Between Genotype and Peripheral Complement Proteins in First-Episode Psychosis: Evidences From C3 and C4. *Front Genet* 12, 647246.
- Cheng, S., Hou, J., Zhang, C., Xu, C., Wang, L., Zou, X., Yu, H., Shi, Y., Yin, Z., and Chen, G. (2015). Minocycline reduces neuroinflammation but does not ameliorate neuron loss in a mouse model of neurodegeneration. *Sci Rep* 5, 10535.
- Chung, W. S., Clarke, L. E., Wang, G. X., Stafford, B. K., Sher, A., Chakraborty, C., Joung, J., Foo, L. C., Thompson, A., Chen, C., *et al.* (2013). Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* 504, 394-400.
- Codolo, G., Plotegher, N., Pozzobon, T., Brucale, M., Tessari, I., Bubacco, L., and de Bernard, M. (2013). Triggering of inflammasome by aggregated alpha-synuclein, an inflammatory response in synucleinopathies. *PLoS One* 8, e55375.
- Coll, R. C., Hill, J. R., Day, C. J., Zamoshnikova, A., Boucher, D., Massey, N. L., Chitty, J. L., Fraser, J. A., Jennings, M. P., Robertson, A. A. B., and Schroder, K. (2019). MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition. *Nat Chem Biol* 15, 556-559.
- Comer, A. L., Jinadasa, T., Sriram, B., Phadke, R. A., Kretsge, L. N., Nguyen, T. P. H., Antognetti, G., Gilbert, J. P., Lee, J., Newmark, E. R., *et al.* (2020). Increased expression of schizophrenia-associated gene C4 leads to hypoconnectivity of prefrontal cortex and reduced social interaction. *PLoS Biol* 18, e3000604.

- Cong, Q., Soteros, B. M., Wollet, M., Kim, J. H., and Sia, G. M. (2020). The endogenous neuronal complement inhibitor SRPX2 protects against complement-mediated synapse elimination during development. *Nat Neurosci* 23, 1067-1078.
- Coulthard, L. G., Hawksorth, O. A., and Woodruff, T. M. (2018). Complement: The Emerging Architect of the Developing Brain. *Trends Neurosci* 41, 373-384.
- Cruz, C. M., Rinna, A., Forman, H. J., Ventura, A. L., Persechini, P. M., and Ojcius, D. M. (2007). ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282, 2871-2879.
- Dangel, A. W., Mendoza, A. R., Baker, B. J., Daniel, C. M., Carroll, M. C., Wu, L. C., and Yu, C. Y. (1994). The dichotomous size variation of human complement C4 genes is mediated by a novel family of endogenous retroviruses, which also establishes species-specific genomic patterns among Old World primates. *Immunogenetics* 40, 425-436.
- Daniel, J. A., Malladi, C. S., Kettle, E., McCluskey, A., and Robinson, P. J. (2012). Analysis of synaptic vesicle endocytosis in synaptosomes by high-content screening. *Nat Protoc* 7, 1439-1455.
- Dean, K., and Murray, R. M. (2005). Environmental risk factors for psychosis. *Dialogues Clin Neurosci* 7, 69-80.
- Diao, X., Wang, F., Becerra-Calixto, A., Soto, C., and Mukherjee, A. (2021). Induced Pluripotent Stem Cell-Derived Dopaminergic Neurons from Familial Parkinson's Disease Patients Display alpha-Synuclein Pathology and Abnormal Mitochondrial Morphology. *Cells* 10.
- Diguet, E., Gross, C. E., Tison, F., and Bezard, E. (2004). Rise and fall of minocycline in neuroprotection: need to promote publication of negative results. *Exp Neurol* 189, 1-4.
- Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., Sun, H., Wang, D. C., and Shao, F. (2016). Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* 535, 111-116.
- Doorduyn, J., de Vries, E. F., Willemsen, A. T., de Groot, J. C., Dierckx, R. A., and Klein, H. C. (2009). Neuroinflammation in schizophrenia-related psychosis: a PET study. *J Nucl Med* 50, 1801-1807.
- Douvaras, P., Sun, B., Wang, M., Kruglikov, I., Lallous, G., Zimmer, M., Terrenoire, C., Zhang, B., Gandy, S., Schadt, E., *et al.* (2017). Directed Differentiation of Human Pluripotent Stem Cells to Microglia. *Stem Cell Reports* 8, 1516-1524.
- Ehrlich, M., Mozafari, S., Glatza, M., Starost, L., Velychko, S., Hallmann, A. L., Cui, Q. L., Schambach, A., Kim, K. P., Bachelin, C., *et al.* (2017). Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors. *Proc Natl Acad Sci U S A* 114, E2243-E2252.
- Etemad, S., Zamin, R. M., Ruitenberg, M. J., and Filgueira, L. (2012). A novel in vitro human microglia model: characterization of human monocyte-derived microglia. *J Neurosci Methods* 209, 79-89.
- Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2018). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48, 35-44 e36.

- Faust, T. E., Gunner, G., and Schafer, D. P. (2021). Mechanisms governing activity-dependent synaptic pruning in the developing mammalian CNS. *Nat Rev Neurosci* 22, 657-673.
- Fillman, S. G., Weickert, T. W., Lenroot, R. K., Catts, S. V., Bruggemann, J. M., Catts, V. S., and Weickert, C. S. (2016). Elevated peripheral cytokines characterize a subgroup of people with schizophrenia displaying poor verbal fluency and reduced Broca's area volume. *Mol Psychiatry* 21, 1090-1098.
- Fus-Kujawa, A., Prus, P., Bajdak-Rusinek, K., Teper, P., Gawron, K., Kowalczyk, A., and Sieron, A. L. (2021). An Overview of Methods and Tools for Transfection of Eukaryotic Cells in vitro. *Front Bioeng Biotechnol* 9, 701031.
- Fusco, R., Siracusa, R., Genovese, T., Cuzzocrea, S., and Di Paola, R. (2020). Focus on the Role of NLRP3 Inflammasome in Diseases. *Int J Mol Sci* 21.
- Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., Robertson, A. A., Cooper, M. A., Graf, T., and Hornung, V. (2016). Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity* 44, 833-846.
- Gallego, J. A., Blanco, E. A., Morell, C., Lencz, T., and Malhotra, A. K. (2021). Complement component C4 levels in the cerebrospinal fluid and plasma of patients with schizophrenia. *Neuropsychopharmacology* 46, 1140-1144.
- Garcia-Alvarez, L., Garcia-Portilla, M. P., Gonzalez-Blanco, L., Saiz Martinez, P. A., de la Fuente-Tomas, L., Menendez-Miranda, I., Iglesias, C., and Bobes, J. (2016). Differential blood-based biomarkers of psychopathological dimensions of schizophrenia. *Rev Psiquiatr Salud Ment* 9, 219-227.
- Garcia-Bueno, B., Bioque, M., Mac-Dowell, K. S., Barcones, M. F., Martinez-Cengotitabengoa, M., Pina-Camacho, L., Rodriguez-Jimenez, R., Saiz, P. A., Castro, C., Lafuente, A., *et al.* (2014a). Pro-/anti-inflammatory dysregulation in patients with first episode of psychosis: toward an integrative inflammatory hypothesis of schizophrenia. *Schizophr Bull* 40, 376-387.
- Garcia-Bueno, B., Bioque, M., MacDowell, K. S., Santabarbara, J., Martinez-Cengotitabengoa, M., Moreno, C., Saiz, P. A., Berrocoso, E., Gasso, P., Fe Barcones, M., *et al.* (2014b). Pro-/antiinflammatory dysregulation in early psychosis: results from a 1-year follow-up study. *Int J Neuropsychopharmacol* 18.
- Gejman, P. V., Sanders, A. R., and Duan, J. (2010). The role of genetics in the etiology of schizophrenia. *Psychiatr Clin North Am* 33, 35-66.
- Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., *et al.* (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330, 841-845.
- Gleitz, J., Beile, A., Wilffert, B., and Tegtmeier, F. (1993). Cryopreservation of freshly isolated synaptosomes prepared from the cerebral cortex of rats. *J Neurosci Methods* 47, 191-197.
- Glenner, G. G., and Wong, C. W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 120, 885-890.
- Gosselin, D., Skola, D., Coufal, N. G., Holtman, I. R., Schlachetzki, J. C. M., Sajti, E., Jaeger, B. N., O'Connor, C., Fitzpatrick, C., Pasillas, M. P., *et al.* (2017). An environment-dependent transcriptional network specifies human microglia identity. *Science* 356.

- Gracias, J., Orhan, F., Horbeck, E., Holmen-Larsson, J., Khanlarkani, N., Malwade, S., Goparaju, S. K., Schwieler, L., Demirel, I. S., Fu, T., *et al.* (2022). Cerebrospinal fluid concentration of complement component 4A is increased in first episode schizophrenia. *Nat Commun* 13, 6427.
- Grath, A., and Dai, G. (2019). Direct cell reprogramming for tissue engineering and regenerative medicine. *J Biol Eng* 13, 14.
- Grubman, A., Vandekolk, T. H., Schroder, J., Sun, G., Hatwell-Humble, J., Chan, J., Oksanen, M., Lehtonen, S., Hunt, C., Koistinaho, J. E., *et al.* (2020). A CX3CR1 Reporter hESC Line Facilitates Integrative Analysis of In-Vitro-Derived Microglia and Improved Microglia Identity upon Neuron-Glia Co-culture. *Stem Cell Reports* 14, 1018-1032.
- Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., and Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A* 83, 4913-4917.
- Guttikonda, S. R., Sikkema, L., Tchieu, J., Saurat, N., Walsh, R. M., Harschnitz, O., Ciceri, G., Sneeboer, M., Mazutis, L., Setty, M., *et al.* (2021). Fully defined human pluripotent stem cell-derived microglia and tri-culture system model C3 production in Alzheimer's disease. *Nat Neurosci* 24, 343-354.
- Gyllys, K. H., Fein, J. A., Wiley, D. J., and Cole, G. M. (2004). Rapid annexin-V labeling in synaptosomes. *Neurochem Int* 44, 125-131.
- Haenseler, W., Sansom, S. N., Buchrieser, J., Newey, S. E., Moore, C. S., Nicholls, F. J., Chintawar, S., Schnell, C., Antel, J. P., Allen, N. D., *et al.* (2017). A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. *Stem Cell Reports* 8, 1727-1742.
- Haidet-Phillips, A. M., Hester, M. E., Miranda, C. J., Meyer, K., Braun, L., Frakes, A., Song, S., Likhite, S., Murtha, M. J., Foust, K. D., *et al.* (2011). Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nat Biotechnol* 29, 824-828.
- Halassa, M. M., Fellin, T., and Haydon, P. G. (2007). The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol Med* 13, 54-63.
- Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., Fitzgerald, K. A., Latz, E., Moore, K. J., and Golenbock, D. T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9, 857-865.
- Hanslik, K. L., and Ulland, T. K. (2020). The Role of Microglia and the Nlrp3 Inflammasome in Alzheimer's Disease. *Front Neurol* 11, 570711.
- Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W. M., 3rd, Zheng, S., Butler, A., Lee, M. J., Wilk, A. J., Darby, C., Zager, M., *et al.* (2021). Integrated analysis of multimodal single-cell data. *Cell* 184, 3573-3587 e3529.
- Hatzimanolis, A., Foteli, S., Stefanatou, P., Ntigrintaki, A. A., Ralli, I., Kollias, K., Nikolaou, C., Gazouli, M., and Stefanis, N. C. (2022). Deregulation of complement components C4A and CSMD1 peripheral expression in first-episode psychosis and links to cognitive ability. *Eur Arch Psychiatry Clin Neurosci* 272, 1219-1228.
- He, H., Jiang, H., Chen, Y., Ye, J., Wang, A., Wang, C., Liu, Q., Liang, G., Deng, X., Jiang, W., and Zhou, R. (2018). Ordonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. *Nat Commun* 9, 2550.

- Heilbronner, U., Samara, M., Leucht, S., Falkai, P., and Schulze, T. G. (2016). The Longitudinal Course of Schizophrenia Across the Lifespan: Clinical, Cognitive, and Neurobiological Aspects. *Harv Rev Psychiatry* 24, 118-128.
- Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T. C., *et al.* (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493, 674-678.
- Hergenreder, E., Zorina, Y., Zhao, Z., Munguba, H., Calder, E. L., Baggiolini, A., Minotti, A. P., Walsh, R. M., Liston, C., Levitz, J., *et al.* (2022). Combined small molecule treatment accelerates timing of maturation in human pluripotent stem cell-derived neurons. *bioRxiv* preprint doi: <https://doi.org/10.1101/20220602494616>.
- Hockemeyer, D., and Jaenisch, R. (2016). Induced Pluripotent Stem Cells Meet Genome Editing. *Cell Stem Cell* 18, 573-586.
- Hoffmann, A., Leung, T. H., and Baltimore, D. (2003). Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *EMBO J* 22, 5530-5539.
- Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. (2002). The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298, 1241-1245.
- Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., Fitzgerald, K. A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9, 847-856.
- Huang, J. H., Lan, C. C., Hsu, Y. T., Tsai, C. L., Tzeng, I. S., Wang, P., Kuo, C. Y., and Hsieh, P. C. (2020). Oridonin Attenuates Lipopolysaccharide-Induced ROS Accumulation and Inflammation in HK-2 Cells. *Evid Based Complement Alternat Med* 2020, 9724520.
- Huang, P., He, Z., Ji, S., Sun, H., Xiang, D., Liu, C., Hu, Y., Wang, X., and Hui, L. (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature* 475, 386-389.
- Huang, Y., Jiang, H., Chen, Y., Wang, X., Yang, Y., Tao, J., Deng, X., Liang, G., Zhang, H., Jiang, W., and Zhou, R. (2018). Tranilast directly targets NLRP3 to treat inflammasome-driven diseases. *EMBO Mol Med* 10.
- Huh, C. J., Zhang, B., Victor, M. B., Dahiya, S., Batista, L. F., Horvath, S., and Yoo, A. S. (2016). Maintenance of age in human neurons generated by microRNA-based neuronal conversion of fibroblasts. *Elife* 5.
- Huh, G. S., Boulanger, L. M., Du, H., Riquelme, P. A., Brotz, T. M., and Shatz, C. J. (2000). Functional requirement for class I MHC in CNS development and plasticity. *Science* 290, 2155-2159.
- Husemann, J., Loike, J. D., Anankov, R., Febbraio, M., and Silverstein, S. C. (2002). Scavenger receptors in neurobiology and neuropathology: their role on microglia and other cells of the nervous system. *Glia* 40, 195-205.
- Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142, 375-386.

- Imaizumi, K., Sone, T., Ibata, K., Fujimori, K., Yuzaki, M., Akamatsu, W., and Okano, H. (2015). Controlling the Regional Identity of hPSC-Derived Neurons to Uncover Neuronal Subtype Specificity of Neurological Disease Phenotypes. *Stem Cell Reports* 5, 1010-1022.
- Inta, D., Lang, U. E., Borgwardt, S., Meyer-Lindenberg, A., and Gass, P. (2017). Microglia Activation and Schizophrenia: Lessons From the Effects of Minocycline on Postnatal Neurogenesis, Neuronal Survival and Synaptic Pruning. *Schizophr Bull* 43, 493-496.
- Inta, I., Vogt, M. A., Vogel, A. S., Bettendorf, M., Gass, P., and Inta, D. (2016). Minocycline exacerbates apoptotic neurodegeneration induced by the NMDA receptor antagonist MK-801 in the early postnatal mouse brain. *Eur Arch Psychiatry Clin Neurosci* 266, 673-677.
- Isenman, D. E., and Young, J. R. (1984). The molecular basis for the difference in immune hemolysis activity of the Chido and Rodgers isotypes of human complement component C4. *J Immunol* 132, 3019-3027.
- Ishii, T., and Haga, S. (1984). Immuno-electron-microscopic localization of complements in amyloid fibrils of senile plaques. *Acta Neuropathol* 63, 296-300.
- Jacomb, I., Stanton, C., Vasudevan, R., Powell, H., O'Donnell, M., Lenroot, R., Bruggemann, J., Balzan, R., Galletly, C., Liu, D., *et al.* (2018). C-Reactive Protein: Higher During Acute Psychotic Episodes and Related to Cortical Thickness in Schizophrenia and Healthy Controls. *Front Immunol* 9, 2230.
- Jo, E. K., Kim, J. K., Shin, D. M., and Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol* 13, 148-159.
- Johansen, L., Roberg, B., and Kvamme, E. (1987). Uptake and release for glutamine and glutamate in a crude synaptosomal fraction from rat brain. *Neurochem Res* 12, 135-140.
- Joseph, J., Depp, C., Martin, A. S., Daly, R. E., Glorioso, D. K., Palmer, B. W., and Jeste, D. V. (2015). Associations of high sensitivity C-reactive protein levels in schizophrenia and comparison groups. *Schizophr Res* 168, 456-460.
- Kalinowski, A., Liliental, J., Anker, L. A., Linkovski, O., Culbertson, C., Hall, J. N., Pattni, R., Sabatti, C., Noordsy, D., Hallmayer, J. F., *et al.* (2021). Increased activation product of complement 4 protein in plasma of individuals with schizophrenia. *Transl Psychiatry* 11, 486.
- Karin, M., and Delhase, M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin Immunol* 12, 85-98.
- Karlsgodt, K. H., Sanz, J., van Erp, T. G., Bearden, C. E., Nuechterlein, K. H., and Cannon, T. D. (2009). Re-evaluating dorsolateral prefrontal cortex activation during working memory in schizophrenia. *Schizophr Res* 108, 143-150.
- Karlsgodt, K. H., Sun, D., and Cannon, T. D. (2010). Structural and Functional Brain Abnormalities in Schizophrenia. *Curr Dir Psychol Sci* 19, 226-231.
- Katsnelson, M. A., Rucker, L. G., Russo, H. M., and Dubyak, G. R. (2015). K⁺ efflux agonists induce NLRP3 inflammasome activation independently of Ca²⁺ signaling. *J Immunol* 194, 3937-3952.
- Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q. T., *et al.* (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526, 666-671.

- Kelley, N., Jeltema, D., Duan, Y., and He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *Int J Mol Sci* 20.
- Kelly, D. L., Sullivan, K. M., McEvoy, J. P., McMahon, R. P., Wehring, H. J., Gold, J. M., Liu, F., Warfel, D., Vyas, G., Richardson, C. M., *et al.* (2015). Adjunctive Minocycline in Clozapine-Treated Schizophrenia Patients With Persistent Symptoms. *J Clin Psychopharmacol* 35, 374-381.
- Kemp, P. J., Rushton, D. J., Yarova, P. L., Schnell, C., Geater, C., Hancock, J. M., Wieland, A., Hughes, A., Badder, L., Cope, E., *et al.* (2016). Improving and accelerating the differentiation and functional maturation of human stem cell-derived neurons: role of extracellular calcium and GABA. *J Physiol* 594, 6583-6594.
- Kesavardhana, S., and Kanneganti, T. D. (2017). Mechanisms governing inflammasome activation, assembly and pyroptosis induction. *Int Immunol* 29, 201-210.
- Keshavan, M., Lizano, P., and Prasad, K. (2020a). The synaptic pruning hypothesis of schizophrenia: promises and challenges. *World Psychiatry* 19, 110-111.
- Keshavan, M. S., Collin, G., Guimond, S., Kelly, S., Prasad, K. M., and Lizano, P. (2020b). Neuroimaging in Schizophrenia. *Neuroimaging Clin N Am* 30, 73-83.
- Khattar, N. K., Yablonska, S., Baranov, S. V., Baranova, O. V., Kretz, E. S., Larkin, T. M., Carlisle, D. L., Richardson, R. M., and Friedlander, R. M. (2016). Isolation of functionally active and highly purified neuronal mitochondria from human cortex. *J Neurosci Methods* 263, 1-6.
- Kiecker, C., and Lumsden, A. (2012). The role of organizers in patterning the nervous system. *Annu Rev Neurosci* 35, 347-367.
- Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., Wieghofer, P., Heinrich, A., Riemke, P., Holscher, C., *et al.* (2013). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat Neurosci* 16, 273-280.
- Kim, H. K., Andreazza, A. C., Elmi, N., Chen, W., and Young, L. T. (2016). Nod-like receptor pyrin containing 3 (NLRP3) in the post-mortem frontal cortex from patients with bipolar disorder: A potential mediator between mitochondria and immune-activation. *J Psychiatr Res* 72, 43-50.
- Kimbrel, E. A., and Lanza, R. (2015). Current status of pluripotent stem cells: moving the first therapies to the clinic. *Nat Rev Drug Discov* 14, 681-692.
- Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O., and Parmar, M. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. *Cell Rep* 1, 703-714.
- Kovacs, R. A., Vadaszi, H., Bulyaki, E., Torok, G., Toth, V., Matyas, D., Kun, J., Hunyadi-Gulyas, E., Fedor, F. Z., Csincsi, A., *et al.* (2020). Identification of Neuronal Pentraxins as Synaptic Binding Partners of C1q and the Involvement of NP1 in Synaptic Pruning in Adult Mice. *Front Immunol* 11, 599771.
- Ladewig, J., Mertens, J., Kesavan, J., Doerr, J., Poppe, D., Glaue, F., Herms, S., Wernet, P., Kogler, G., Muller, F. J., *et al.* (2012). Small molecules enable highly efficient neuronal conversion of human fibroblasts. *Nat Methods* 9, 575-578.

- Lamkanfi, M., Mueller, J. L., Vitari, A. C., Misaghi, S., Fedorova, A., Deshayes, K., Lee, W. P., Hoffman, H. M., and Dixit, V. M. (2009). Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J Cell Biol* 187, 61-70.
- Lancaster, M. A., and Knoblich, J. A. (2014). Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* 345, 1247125.
- Laskaris, L., Zalesky, A., Weickert, C. S., Di Biase, M. A., Chana, G., Baune, B. T., Bousman, C., Nelson, B., McGorry, P., Everall, I., *et al.* (2019). Investigation of peripheral complement factors across stages of psychosis. *Schizophr Res* 204, 30-37.
- Latz, E., Xiao, T. S., and Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nat Rev Immunol* 13, 397-411.
- Law, S. K., Dodds, A. W., and Porter, R. R. (1984). A comparison of the properties of two classes, C4A and C4B, of the human complement component C4. *EMBO J* 3, 1819-1823.
- Le Dreau, G., and Marti, E. (2012). Dorsal-ventral patterning of the neural tube: a tale of three signals. *Dev Neurobiol* 72, 1471-1481.
- Lee, H., Brott, B. K., Kirkby, L. A., Adelson, J. D., Cheng, S., Feller, M. B., Datwani, A., and Shatz, C. J. (2014). Synapse elimination and learning rules co-regulated by MHC class I H2-Db. *Nature* 509, 195-200.
- Lee, S. J. (2008). Origins and effects of extracellular alpha-synuclein: implications in Parkinson's disease. *J Mol Neurosci* 34, 17-22.
- Lehrman, E. K., Wilton, D. K., Litvina, E. Y., Welsh, C. A., Chang, S. T., Frouin, A., Walker, A. J., Heller, M. D., Umemori, H., Chen, C., and Stevens, B. (2018). CD47 Protects Synapses from Excess Microglia-Mediated Pruning during Development. *Neuron* 100, 120-134 e126.
- Leone, C., Le Pavec, G., Meme, W., Porcheray, F., Samah, B., Dormont, D., and Gras, G. (2006). Characterization of human monocyte-derived microglia-like cells. *Glia* 54, 183-192.
- Leung, T. H., Hoffmann, A., and Baltimore, D. (2004). One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. *Cell* 118, 453-464.
- Levkovitz, Y., Levi, U., Braw, Y., and Cohen, H. (2007). Minocycline, a second-generation tetracycline, as a neuroprotective agent in an animal model of schizophrenia. *Brain Res* 1154, 154-162.
- Li, Q., and Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nat Rev Immunol* 18, 225-242.
- Li, X. J., Zhang, X., Johnson, M. A., Wang, Z. B., Lavaute, T., and Zhang, S. C. (2009). Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. *Development* 136, 4055-4063.
- Lin, C. C., Chang, C. M., Liu, C. Y., and Huang, T. L. (2013). Increased high-sensitivity C-reactive protein levels in Taiwanese schizophrenic patients. *Asia Pac Psychiatry* 5, E58-63.
- Liu, F., Guo, X., Wu, R., Ou, J., Zheng, Y., Zhang, B., Xie, L., Zhang, L., Yang, L., Yang, S., *et al.* (2014). Minocycline supplementation for treatment of negative symptoms in early-phase schizophrenia: a double blind, randomized, controlled trial. *Schizophr Res* 153, 169-176.
- Liu, T., Zhang, L., Joo, D., and Sun, S. C. (2017). NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* 2, 17023-.

- Liu, Y. Q., You, S., Zhang, C. L., Tashiro, S., Onodera, S., and Ikejima, T. (2005). Oridonin enhances phagocytosis of UV-irradiated apoptotic U937 cells. *Biol Pharm Bull* 28, 461-467.
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550.
- Lu, A., and Wu, H. (2015). Structural mechanisms of inflammasome assembly. *FEBS J* 282, 435-444.
- Lu, J., Zhong, X., Liu, H., Hao, L., Huang, C. T., Sherafat, M. A., Jones, J., Ayala, M., Li, L., and Zhang, S. C. (2016). Generation of serotonin neurons from human pluripotent stem cells. *Nat Biotechnol* 34, 89-94.
- Maes, M., Bosmans, E., Ranjan, R., Vandoolaeghe, E., Meltzer, H. Y., De Ley, M., Berghmans, R., Stans, G., and Desnyder, R. (1996). Lower plasma CC16, a natural anti-inflammatory protein, and increased plasma interleukin-1 receptor antagonist in schizophrenia: effects of antipsychotic drugs. *Schizophr Res* 21, 39-50.
- Malik, A., and Kanneganti, T. D. (2017). Inflammasome activation and assembly at a glance. *J Cell Sci* 130, 3955-3963.
- Marchetto, M. C., Muotri, A. R., Mu, Y., Smith, A. M., Cezar, G. G., and Gage, F. H. (2008). Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell* 3, 649-657.
- Maroof, A. M., Keros, S., Tyson, J. A., Ying, S. W., Ganat, Y. M., Merkle, F. T., Liu, B., Goulburn, A., Stanley, E. G., Elefanty, A. G., *et al.* (2013). Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* 12, 559-572.
- Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A., and Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* 360, 737-741.
- McCutcheon, R. A., Krystal, J. H., and Howes, O. D. (2020). Dopamine and glutamate in schizophrenia: biology, symptoms and treatment. *World Psychiatry* 19, 15-33.
- Melbourne, J. K., Rosen, C., Feiner, B., and Sharma, R. P. (2018). C4A mRNA expression in PBMCs predicts the presence and severity of delusions in schizophrenia and bipolar disorder with psychosis. *Schizophr Res* 197, 321-327.
- Mertens, J., Paquola, A. C. M., Ku, M., Hatch, E., Bohnke, L., Ladjevardi, S., McGrath, S., Campbell, B., Lee, H., Herdy, J. R., *et al.* (2015). Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. *Cell Stem Cell* 17, 705-718.
- Messias, E. L., Chen, C. Y., and Eaton, W. W. (2007). Epidemiology of schizophrenia: review of findings and myths. *Psychiatr Clin North Am* 30, 323-338.
- Millan, M. J., Andrieux, A., Bartzokis, G., Cadenhead, K., Dazzan, P., Fusar-Poli, P., Gallinat, J., Giedd, J., Grayson, D. R., Heinrichs, M., *et al.* (2016). Altering the course of schizophrenia: progress and perspectives. *Nat Rev Drug Discov* 15, 485-515.

- Miller, B. J., Buckley, P., Seabolt, W., Mellor, A., and Kirkpatrick, B. (2011). Meta-analysis of cytokine alterations in schizophrenia: clinical status and antipsychotic effects. *Biol Psychiatry* 70, 663-671.
- Miller, B. J., Culpepper, N., and Rapaport, M. H. (2014). C-reactive protein levels in schizophrenia: a review and meta-analysis. *Clin Schizophr Relat Psychoses* 7, 223-230.
- Mitchell, S., Vargas, J., and Hoffmann, A. (2016). Signaling via the NFkappaB system. *Wiley Interdiscip Rev Syst Biol Med* 8, 227-241.
- Monji, A., Kato, T., and Kanba, S. (2009). Cytokines and schizophrenia: Microglia hypothesis of schizophrenia. *Psychiatry Clin Neurosci* 63, 257-265.
- Morini, R., Bizzotto, M., Perrucci, F., Filipello, F., and Matteoli, M. (2021). Strategies and Tools for Studying Microglial-Mediated Synapse Elimination and Refinement. *Front Immunol* 12, 640937.
- Moya, N., Cutts, J., Gaasterland, T., Willert, K., and Brafman, D. A. (2014). Endogenous WNT signaling regulates hPSC-derived neural progenitor cell heterogeneity and specifies their regional identity. *Stem Cell Reports* 3, 1015-1028.
- Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., Bakiasi, G., Tsai, L. H., Aubourg, P., Ransohoff, R. M., and Jaenisch, R. (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. *Nat Med* 22, 1358-1367.
- Munoz-Planillo, R., Kuffa, P., Martinez-Colon, G., Smith, B. L., Rajendiran, T. M., and Nunez, G. (2013). K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38, 1142-1153.
- Munoz, S. S., Engel, M., Balez, R., Do-Ha, D., Cabral-da-Silva, M. C., Hernandez, D., Berg, T., Fifita, J. A., Grima, N., Yang, S., *et al.* (2020). A Simple Differentiation Protocol for Generation of Induced Pluripotent Stem Cell-Derived Basal Forebrain-Like Cholinergic Neurons for Alzheimer's Disease and Frontotemporal Dementia Disease Modeling. *Cells* 9.
- Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A., Opitz, B., van der Meer, J. H., van de Veerdonk, F. L., Ferwerda, G., Heinhuis, B., Devesa, I., *et al.* (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113, 2324-2335.
- Nettis, M. A., and Pariante, C. M. (2020). Is there neuroinflammation in depression? Understanding the link between the brain and the peripheral immune system in depression. *Int Rev Neurobiol* 152, 23-40.
- Neumann, H., Kotter, M. R., and Franklin, R. J. (2009). Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain* 132, 288-295.
- Nicholls, D. G. (2010). Stochastic aspects of transmitter release and bioenergetic dysfunction in isolated nerve terminals. *Biochem Soc Trans* 38, 457-459.
- Nilsson, J., Gobom, J., Sjodin, S., Brinkmalm, G., Ashton, N. J., Svensson, J., Johansson, P., Portelius, E., Zetterberg, H., Blennow, K., and Brinkmalm, A. (2021). Cerebrospinal fluid biomarker panel for synaptic dysfunction in Alzheimer's disease. *Alzheimers Dement (Amst)* 13, e12179.

- Nimgaonkar, V. L., Prasad, K. M., Chowdari, K. V., Severance, E. G., and Yolken, R. H. (2017). The complement system: a gateway to gene-environment interactions in schizophrenia pathogenesis. *Mol Psychiatry* 22, 1554-1561.
- Nonaka, M., Nakayama, K., Yeul, Y. D., and Takahashi, M. (1985). Complete nucleotide and derived amino acid sequences of the fourth component of mouse complement (C4). Evolutionary aspects. *J Biol Chem* 260, 10936-10943.
- Nordstrom, U., Jessell, T. M., and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat Neurosci* 5, 525-532.
- North, H. F., Bruggemann, J., Cropley, V., Swaminathan, V., Sundram, S., Lenroot, R., Pereira, A. M., Zalesky, A., Bousman, C., Pantelis, C., *et al.* (2021). Increased peripheral inflammation in schizophrenia is associated with worse cognitive performance and related cortical thickness reductions. *Eur Arch Psychiatry Clin Neurosci* 271, 595-607.
- O'Barr, S., and Cooper, N. R. (2000). The C5a complement activation peptide increases IL-1beta and IL-6 release from amyloid-beta primed human monocytes: implications for Alzheimer's disease. *J Neuroimmunol* 109, 87-94.
- O'Brien, W. T., Pham, L., Symons, G. F., Monif, M., Shultz, S. R., and McDonald, S. J. (2020). The NLRP3 inflammasome in traumatic brain injury: potential as a biomarker and therapeutic target. *J Neuroinflammation* 17, 104.
- Ohgidani, M., Kato, T. A., Setoyama, D., Sagata, N., Hashimoto, R., Shigenobu, K., Yoshida, T., Hayakawa, K., Shimokawa, N., Miura, D., *et al.* (2014). Direct induction of ramified microglia-like cells from human monocytes: dynamic microglial dysfunction in Nasu-Hakola disease. *Sci Rep* 4, 4957.
- Ossewaarde, J. M., de Vries, A., Bestebroer, T., and Angulo, A. F. (1996). Application of a Mycoplasma group-specific PCR for monitoring decontamination of Mycoplasma-infected Chlamydia sp. strains. *Appl Environ Microbiol* 62, 328-331.
- Ozdamar Unal, G., Hekimler Ozturk, K., and Inci, H. E. (2022). Increased NLRP3 inflammasome expression in peripheral blood mononuclear cells of patients with schizophrenia: a case-control study. *Int J Psychiatry Clin Pract*, 1-7.
- Pagès, H., Carlson, M., Falcon, S., and Li, N. (2020). AnnotationDbi: Manipulation of SQLite-based annotations in Bioconductor. R package version 1.52.0. <https://bioconductor.org/packages/AnnotationDbi>.
- Pandey, G. N., Zhang, H., Sharma, A., and Ren, X. (2021). Innate immunity receptors in depression and suicide: upregulated NOD-like receptors containing pyrin (NLRPs) and hyperactive inflammasomes in the postmortem brains of people who were depressed and died by suicide. *J Psychiatry Neurosci* 46, E538-E547.
- Pandya, H., Shen, M. J., Ichikawa, D. M., Sedlock, A. B., Choi, Y., Johnson, K. R., Kim, G., Brown, M. A., Elkahouloun, A. G., Maric, D., *et al.* (2017). Differentiation of human and murine induced pluripotent stem cells to microglia-like cells. *Nat Neurosci* 20, 753-759.
- Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., *et al.* (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456-1458.
- Pelegriin, P. (2021). P2X7 receptor and the NLRP3 inflammasome: Partners in crime. *Biochem Pharmacol* 187, 114385.

- Penn, A. A., Riquelme, P. A., Feller, M. B., and Shatz, C. J. (1998). Competition in retinogeniculate patterning driven by spontaneous activity. *Science* 279, 2108-2112.
- Perea, G., Navarrete, M., and Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci* 32, 421-431.
- Perry, V. H., and O'Connor, V. (2008). C1q: the perfect complement for a synaptic feast? *Nat Rev Neurosci* 9, 807-811.
- Picchioni, M. M., and Murray, R. M. (2007). Schizophrenia. *BMJ* 335, 91-95.
- Pocock, J. M., and Piers, T. M. (2018). Modelling microglial function with induced pluripotent stem cells: an update. *Nat Rev Neurosci* 19, 445-452.
- Poletti, V., and Mavilio, F. (2018). Interactions between Retroviruses and the Host Cell Genome. *Mol Ther Methods Clin Dev* 8, 31-41.
- Pons, V., and Rivest, S. (2020). Beneficial Roles of Microglia and Growth Factors in MS, a Brief Review. *Front Cell Neurosci* 14, 284.
- Presumey, J., Bialas, A. R., and Carroll, M. C. (2017). Complement System in Neural Synapse Elimination in Development and Disease. *Adv Immunol* 135, 53-79.
- Qi, Y., Zhang, X. J., Renier, N., Wu, Z., Atkin, T., Sun, Z., Ozair, M. Z., Tchieu, J., Zimmer, B., Fattahi, F., *et al.* (2017). Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. *Nat Biotechnol* 35, 154-163.
- Qian, T., Maguire, S. E., Canfield, S. G., Bao, X., Olson, W. R., Shusta, E. V., and Palecek, S. P. (2017). Directed differentiation of human pluripotent stem cells to blood-brain barrier endothelial cells. *Sci Adv* 3, e1701679.
- R Core Team (2020). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria URL <https://www.R-project.org/>.
- Rahimian, R., Wakid, M., O'Leary, L. A., and Mechawar, N. (2021). The emerging tale of microglia in psychiatric disorders. *Neurosci Biobehav Rev* 131, 1-29.
- Ransohoff, R. M., and Perry, V. H. (2009). Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* 27, 119-145.
- Rao, J. S., Harry, G. J., Rapoport, S. I., and Kim, H. W. (2010). Increased excitotoxicity and neuroinflammatory markers in postmortem frontal cortex from bipolar disorder patients. *Mol Psychiatry* 15, 384-392.
- Ribes, V., and Briscoe, J. (2009). Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback. *Cold Spring Harb Perspect Biol* 1, a002014.
- Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., Civin, W. H., Brachova, L., Bradt, B., Ward, P., and *et al.* (1992). Complement activation by beta-amyloid in Alzheimer disease. *Proc Natl Acad Sci U S A* 89, 10016-10020.
- Samir, P., Kesavardhana, S., Patmore, D. M., Gingras, S., Malireddi, R. K. S., Karki, R., Guy, C. S., Briard, B., Place, D. E., Bhattacharya, A., *et al.* (2019). DDX3X acts as a live-or-die checkpoint in stressed cells by regulating NLRP3 inflammasome. *Nature* 573, 590-594.

- Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci U S A* 97, 12705-12710.
- Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., and Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* 74, 691-705.
- Schizophrenia Working Group of the Psychiatric Genomics, C. (2014). Biological insights from 108 schizophrenia-associated genetic loci. *Nature* 511, 421-427.
- Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E., Pollard, J. W., *et al.* (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336, 86-90.
- Sekar, A., Bialas, A. R., de Rivera, H., Davis, A., Hammond, T. R., Kamitaki, N., Tooley, K., Presumey, J., Baum, M., Van Doren, V., *et al.* (2016). Schizophrenia risk from complex variation of complement component 4. *Nature* 530, 177-183.
- Sellgren, C. M., Gracias, J., Watmuff, B., Biag, J. D., Thanos, J. M., Whittredge, P. B., Fu, T., Worringer, K., Brown, H. E., Wang, J., *et al.* (2019). Increased synapse elimination by microglia in schizophrenia patient-derived models of synaptic pruning. *Nat Neurosci* 22, 374-385.
- Sellgren, C. M., Sheridan, S. D., Gracias, J., Xuan, D., Fu, T., and Perlis, R. H. (2017). Patient-specific models of microglia-mediated engulfment of synapses and neural progenitors. *Mol Psychiatry* 22, 170-177.
- Sheridan, S. D., Horng, J. E., and Perlis, R. H. (2022). Patient-Derived In Vitro Models of Microglial Function and Synaptic Engulfment in Schizophrenia. *Biol Psychiatry* 92, 470-479.
- Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., and Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526, 660-665.
- Shi, Y., Inoue, H., Wu, J. C., and Yamanaka, S. (2017). Induced pluripotent stem cell technology: a decade of progress. *Nat Rev Drug Discov* 16, 115-130.
- Shi, Y., Wang, H., Zheng, M., Xu, W., Yang, Y., and Shi, F. (2020). Ginsenoside Rg3 suppresses the NLRP3 inflammasome activation through inhibition of its assembly. *FASEB J* 34, 208-221.
- Shulman, J. M., De Jager, P. L., and Feany, M. B. (2011). Parkinson's disease: genetics and pathogenesis. *Annu Rev Pathol* 6, 193-222.
- Sierra, D. P., Tripathi, A., and Pillai, A. (2022). Dysregulation of complement system in neuropsychiatric disorders: A Mini Review. *Biomarkers in Neuropsychiatry*, 100056.
- Smith, A. M., and Dragunow, M. (2014). The human side of microglia. *Trends Neurosci* 37, 125-135.
- Soderlund, J., Olsson, S. K., Samuelsson, M., Walther-Jallow, L., Johansson, C., Erhardt, S., Landen, M., and Engberg, G. (2011). Elevation of cerebrospinal fluid interleukin-1ss in bipolar disorder. *J Psychiatry Neurosci* 36, 114-118.

- Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G. W., Cook, E. G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., *et al.* (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 136, 964-977.
- Spataro, J., Mullen, P. E., Burgess, P. M., Wells, D. L., and Moss, S. A. (2004). Impact of child sexual abuse on mental health: prospective study in males and females. *Br J Psychiatry* 184, 416-421.
- Spauwen, J., Krabbendam, L., Lieb, R., Wittchen, H. U., and van Os, J. (2006). Impact of psychological trauma on the development of psychotic symptoms: relationship with psychosis proneness. *Br J Psychiatry* 188, 527-533.
- Staerk, J., Dawlaty, M. M., Gao, Q., Maetzel, D., Hanna, J., Sommer, C. A., Mostoslavsky, G., and Jaenisch, R. (2010). Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell* 7, 20-24.
- Stellwagen, D., and Shatz, C. J. (2002). An instructive role for retinal waves in the development of retinogeniculate connectivity. *Neuron* 33, 357-367.
- Stephan, A. H., Barres, B. A., and Stevens, B. (2012). The complement system: an unexpected role in synaptic pruning during development and disease. *Annu Rev Neurosci* 35, 369-389.
- Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., Micheva, K. D., Mehalow, A. K., Huberman, A. D., Stafford, B., *et al.* (2007). The classical complement cascade mediates CNS synapse elimination. *Cell* 131, 1164-1178.
- Stoltzner, S. E., Grenfell, T. J., Mori, C., Wisniewski, K. E., Wisniewski, T. M., Selkoe, D. J., and Lemere, C. A. (2000). Temporal accrual of complement proteins in amyloid plaques in Down's syndrome with Alzheimer's disease. *Am J Pathol* 156, 489-499.
- Sun, A. X., Yuan, Q., Tan, S., Xiao, Y., Wang, D., Khoo, A. T., Sani, L., Tran, H. D., Kim, P., Chiew, Y. S., *et al.* (2016). Direct Induction and Functional Maturation of Forebrain GABAergic Neurons from Human Pluripotent Stem Cells. *Cell Rep* 16, 1942-1953.
- Sun, D., Stuart, G. W., Jenkinson, M., Wood, S. J., McGorry, P. D., Velakoulis, D., van Erp, T. G., Thompson, P. M., Toga, A. W., Smith, D. J., *et al.* (2009). Brain surface contraction mapped in first-episode schizophrenia: a longitudinal magnetic resonance imaging study. *Mol Psychiatry* 14, 976-986.
- Sun, S. C. (2012). The noncanonical NF-kappaB pathway. *Immunol Rev* 246, 125-140.
- Sun, S. C., and Ley, S. C. (2008). New insights into NF-kappaB regulation and function. *Trends Immunol* 29, 469-478.
- Szabo, E., Rampalli, S., Risueno, R. M., Schnerch, A., Mitchell, R., Fiebig-Comyn, A., Levadoux-Martin, M., and Bhatia, M. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* 468, 521-526.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872.
- Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676.

- Talukdar, P. M., Abdul, F., Maes, M., Berk, M., Venkatasubramanian, G., Kutty, B. M., and Debnath, M. (2021). A proof-of-concept study of maternal immune activation mediated induction of Toll-like receptor (TLR) and inflammasome pathways leading to neuroprogressive changes and schizophrenia-like behaviours in offspring. *Eur Neuropsychopharmacol* 52, 48-61.
- Tcw, J., Wang, M., Pimenova, A. A., Bowles, K. R., Hartley, B. J., Lacin, E., Machlovi, S. I., Abdelaal, R., Karch, C. M., Phatnani, H., *et al.* (2017). An Efficient Platform for Astrocyte Differentiation from Human Induced Pluripotent Stem Cells. *Stem Cell Reports* 9, 600-614.
- Telezhkin, V., Schnell, C., Yarova, P., Yung, S., Cope, E., Hughes, A., Thompson, B. A., Sanders, P., Geater, C., Hancock, J. M., *et al.* (2016). Forced cell cycle exit and modulation of GABAA, CREB, and GSK3beta signaling promote functional maturation of induced pluripotent stem cell-derived neurons. *Am J Physiol Cell Physiol* 310, C520-541.
- Thion, M. S., Ginhoux, F., and Garel, S. (2018). Microglia and early brain development: An intimate journey. *Science* 362, 185-189.
- Thored, P., Heldmann, U., Gomes-Leal, W., Gisler, R., Darsalia, V., Taneera, J., Nygren, J. M., Jacobsen, S. E., Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2009). Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. *Glia* 57, 835-849.
- Tikka, T., Fiebich, B. L., Goldsteins, G., Keinänen, R., and Koistinaho, J. (2001). Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci* 21, 2580-2588.
- Timmerman, R., Burm, S. M., and Bajramovic, J. J. (2018). An Overview of in vitro Methods to Study Microglia. *Front Cell Neurosci* 12, 242.
- Tremblay, M. E., Lowery, R. L., and Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol* 8, e1000527.
- Triantafilou, K., Hughes, T. R., Triantafilou, M., and Morgan, B. P. (2013). The complement membrane attack complex triggers intracellular Ca²⁺ fluxes leading to NLRP3 inflammasome activation. *J Cell Sci* 126, 2903-2913.
- Trounson, A., and DeWitt, N. D. (2016). Pluripotent stem cells progressing to the clinic. *Nat Rev Mol Cell Biol* 17, 194-200.
- Tsuji, M., Wilson, M. A., Lange, M. S., and Johnston, M. V. (2004). Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model. *Exp Neurol* 189, 58-65.
- Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., and Yamashita, T. (2013). Layer V cortical neurons require microglial support for survival during postnatal development. *Nat Neurosci* 16, 543-551.
- Uhlhaas, P. J., and Singer, W. (2010). Abnormal neural oscillations and synchrony in schizophrenia. *Nat Rev Neurosci* 11, 100-113.
- Vainchtein, I. D., Chin, G., Cho, F. S., Kelley, K. W., Miller, J. G., Chien, E. C., Liddel, S. A., Nguyen, P. T., Nakao-Inoue, H., Dorman, L. C., *et al.* (2018). Astrocyte-derived interleukin-33 promotes microglial synapse engulfment and neural circuit development. *Science* 359, 1269-1273.

- van Berckel, B. N., Bossong, M. G., Boellaard, R., Kloet, R., Schuitemaker, A., Caspers, E., Luurtsema, G., Windhorst, A. D., Cahn, W., Lammertsma, A. A., and Kahn, R. S. (2008). Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study. *Biol Psychiatry* 64, 820-822.
- van der Ende, E. L., Xiao, M., Xu, D., Poos, J. M., Panman, J. L., Jiskoot, L. C., Meeter, L. H., Dopfer, E. G., Papma, J. M., Heller, C., *et al.* (2020). Neuronal pentraxin 2: a synapse-derived CSF biomarker in genetic frontotemporal dementia. *J Neurol Neurosurg Psychiatry* 91, 612-621.
- van Kuppeveld, F. J., van der Logt, J. T., Angulo, A. F., van Zoest, M. J., Quint, W. G., Niesters, H. G., Galama, J. M., and Melchers, W. J. (1992). Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl Environ Microbiol* 58, 2606-2615.
- Veerhuis, R., Nielsen, H. M., and Tenner, A. J. (2011). Complement in the brain. *Mol Immunol* 48, 1592-1603.
- Ventura, L., Freiburger, V., Thiesen, V. B., Dias, P., Dutra, M. L., Silva, B. B., Schlindwein, A. D., and Comim, C. M. (2020). Involvement of NLRP3 inflammasome in schizophrenia-like behaviour in young animals after maternal immune activation. *Acta Neuropsychiatr* 32, 321-327.
- Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Sudhof, T. C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035-1041.
- Walss-Bass, C., Lokesh, G. L. R., Dyukova, E., Gorenstein, D. G., Roberts, D. L., Velligan, D., and Volk, D. E. (2019). X-Aptamer Technology Identifies C4A and ApoB in Blood as Potential Markers for Schizophrenia. *Mol Neuropsychiatry* 5, 52-59.
- Wang, A. L., Yu, A. C., Lau, L. T., Lee, C., Wu, M., Zhu, X., and Tso, M. O. (2005). Minocycline inhibits LPS-induced retinal microglia activation. *Neurochem Int* 47, 152-158.
- Wang, C., Yue, H., Hu, Z., Shen, Y., Ma, J., Li, J., Wang, X. D., Wang, L., Sun, B., Shi, P., *et al.* (2020). Microglia mediate forgetting via complement-dependent synaptic elimination. *Science* 367, 688-694.
- Wang, S., Yang, H., Yu, L., Jin, J., Qian, L., Zhao, H., Xu, Y., and Zhu, X. (2014). Oridonin attenuates Abeta1-42-induced neuroinflammation and inhibits NF-kappaB pathway. *PLoS One* 9, e104745.
- Wang, S., Yu, L., Yang, H., Li, C., Hui, Z., Xu, Y., and Zhu, X. (2016). Oridonin Attenuates Synaptic Loss and Cognitive Deficits in an Abeta1-42-Induced Mouse Model of Alzheimer's Disease. *PLoS One* 11, e0151397.
- Washer, S. J., Perez-Alcantara, M., Chen, Y., Steer, J., James, W. S., Trynka, G., Bassett, A. R., and Cowley, S. A. (2022). Single-cell transcriptomics defines an improved, validated monoculture protocol for differentiation of human iPSC to microglia. *Sci Rep* 12, 19454.
- Weinhard, L., di Bartolomei, G., Bolasco, G., Machado, P., Schieber, N. L., Neniskyte, U., Exiga, M., Vadisiute, A., Raggioli, A., Schertel, A., *et al.* (2018). Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. *Nat Commun* 9, 1228.
- Weir, R. K., Forghany, R., Smith, S. E., Patterson, P. H., McAllister, A. K., Schumann, C. M., and Bauman, M. D. (2015). Preliminary evidence of neuropathology in nonhuman primates prenatally exposed to maternal immune activation. *Brain Behav Immun* 48, 139-146.

- Wen, Z., Christian, K. M., Song, H., and Ming, G. L. (2016). Modeling psychiatric disorders with patient-derived iPSCs. *Curr Opin Neurobiol* 36, 118-127.
- Whitfield, C. L., Dube, S. R., Felitti, V. J., and Anda, R. F. (2005). Adverse childhood experiences and hallucinations. *Child Abuse Negl* 29, 797-810.
- Whittaker, V. P., Michaelson, I. A., and Kirkland, R. J. (1964). The separation of synaptic vesicles from nerve-ending particles ('synaptosomes'). *Biochem J* 90, 293-303.
- Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, <http://ggplot2.org>.
- Wobus, A. M., and Boheler, K. R. (2005). Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 85, 635-678.
- Wolf, S. A., Boddeke, H. W., and Kettenmann, H. (2017). Microglia in Physiology and Disease. *Annu Rev Physiol* 79, 619-643.
- Wolff, A. R., Cheyne, K. R., and Bilkey, D. K. (2011). Behavioural deficits associated with maternal immune activation in the rat model of schizophrenia. *Behav Brain Res* 225, 382-387.
- Wright, I. C., Rabe-Hesketh, S., Woodruff, P. W., David, A. S., Murray, R. M., and Bullmore, E. T. (2000). Meta-analysis of regional brain volumes in schizophrenia. *Am J Psychiatry* 157, 16-25.
- Xiao, G., Harhaj, E. W., and Sun, S. C. (2001). NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 7, 401-409.
- Xu, J., Wold, E. A., Ding, Y., Shen, Q., and Zhou, J. (2018). Therapeutic Potential of Oridonin and Its Analogs: From Anticancer and Antiinflammation to Neuroprotection. *Molecules* 23.
- Xu, Y., Xue, Y., Wang, Y., Feng, D., Lin, S., and Xu, L. (2009). Multiple-modulation effects of Oridonin on the production of proinflammatory cytokines and neurotrophic factors in LPS-activated microglia. *Int Immunopharmacol* 9, 360-365.
- Xue, Y., Cai, X., Wang, L., Liao, B., Zhang, H., Shan, Y., Chen, Q., Zhou, T., Li, X., Hou, J., *et al.* (2013). Generating a non-integrating human induced pluripotent stem cell bank from urine-derived cells. *PLoS One* 8, e70573.
- Yan, X., Qin, H., Qu, C., Tuan, R. S., Shi, S., and Huang, G. T. (2010). iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev* 19, 469-480.
- Yan, Y., Jiang, W., Liu, L., Wang, X., Ding, C., Tian, Z., and Zhou, R. (2015). Dopamine controls systemic inflammation through inhibition of NLRP3 inflammasome. *Cell* 160, 62-73.
- Yang, A. C., and Tsai, S. J. (2017). New Targets for Schizophrenia Treatment beyond the Dopamine Hypothesis. *Int J Mol Sci* 18.
- Yang, J., Wise, L., and Fukuchi, K. I. (2020). TLR4 Cross-Talk With NLRP3 Inflammasome and Complement Signaling Pathways in Alzheimer's Disease. *Front Immunol* 11, 724.
- Yang, J., Zhao, Y., and Shao, F. (2015). Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Curr Opin Immunol* 32, 78-83.

- Yilmaz, M., Yalcin, E., Presumey, J., Aw, E., Ma, M., Whelan, C. W., Stevens, B., McCarroll, S. A., and Carroll, M. C. (2021). Overexpression of schizophrenia susceptibility factor human complement C4A promotes excessive synaptic loss and behavioral changes in mice. *Nat Neurosci* **24**, 214-224.
- Yong, V. W., Wells, J., Giuliani, F., Casha, S., Power, C., and Metz, L. M. (2004). The promise of minocycline in neurology. *Lancet Neurol* **3**, 744-751.
- Youm, Y. H., Nguyen, K. Y., Grant, R. W., Goldberg, E. L., Bodogai, M., Kim, D., D'Agostino, D., Planavsky, N., Lupfer, C., Kanneganti, T. D., *et al.* (2015). The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med* **21**, 263-269.
- Yu, G., Wang, L. G., Han, Y., and He, Q. Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284-287.
- Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920.
- Zhan, Y., Paolicelli, R. C., Sforzini, F., Weinhard, L., Bolasco, G., Pagani, F., Vyssotski, A. L., Bifone, A., Gozzi, A., Ragozzino, D., and Gross, C. T. (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat Neurosci* **17**, 400-406.
- Zhang, Y., Liu, L., Peng, Y. L., Liu, Y. Z., Wu, T. Y., Shen, X. L., Zhou, J. R., Sun, D. Y., Huang, A. J., Wang, X., *et al.* (2014). Involvement of inflammasome activation in lipopolysaccharide-induced mice depressive-like behaviors. *CNS Neurosci Ther* **20**, 119-124.
- Zhang, Z. Y., Daniels, R., and Schluesener, H. J. (2013). Oridonin ameliorates neuropathological changes and behavioural deficits in a mouse model of cerebral amyloidosis. *J Cell Mol Med* **17**, 1566-1576.
- Zhu, Z., and Huangfu, D. (2013). Human pluripotent stem cells: an emerging model in developmental biology. *Development* **140**, 705-717.
- Zito, G., Buscetta, M., Cimino, M., Dino, P., Bucchieri, F., and Cipollina, C. (2020). Cellular Models and Assays to Study NLRP3 Inflammasome Biology. *Int J Mol Sci* **21**.
- Zuckerman, L., and Weiner, I. (2005). Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. *J Psychiatr Res* **39**, 311-323.

11 Contributions and previous work

Quality control, processing and visualization of all bulk and single-nucleus RNAseq data was performed by Dr. Anne Hoffrichter. This includes data presented in **Figures 11, 12, 24, 25 and 26**.

Bulk RNAseq data of human iPSC-derived cortical neurons included in **Figure 11** was generated by Dr. Ruven Wilkens.

The data presented in this thesis is largely part of a manuscript which is currently under review. This involves data presented in **Figures 11-14, 16-20, 22, 23, 25, 27-31**. Figure legends for these figures were obtained and adapted from Wangemann et al. (under review) and were originally written by myself.

12 Acknowledgements

First, I would like to thank Prof. Dr. Philipp Koch for welcoming me as a doctoral student in his lab, for his scientific guidance and for giving me the freedom to develop my project independently and to pursue my own ideas.

I thank Dr. Georg Köhr, Dr. Ana Oliveira and Prof. Dr. Christoph Schuster for gladly accepting to be examiners of this thesis and members of my defense committee and I thank Prof. Dr. Michael Platten for giving me valuable input during my TAC meetings.

A huge thank you to Anne for analyzing and visualizing all of the sequencing data and for always offering help whenever needed.

A special thanks to Klara, Elena and Malin for going through all of this together, keeping the mood up in stressful times and for keeping me company in our little “exile” on the 2nd floor.

Thank you to Raquel, Annasara, Fabio, Karen, Anne and Klara for being my boulder buddies and keeping me fit during all those years. This journey would have been a lot less fun without you!

Thanks to Andrea for being the biggest microglia fan in the lab and always helping with lots of enthusiastic input.

Thank you to Ruven, Bettina, Marco, Ammar, Isa, Isabel, Sandra and all of the newly joined members of the lab for creating a warm and fun working atmosphere and for always offering help and support when needed.

Thank you to Gina and Helene for the great technical support and organization of the lab.

Finally, I want to greatly thank my family for their unconditional love, for the wonderful times we spend together and for always encouraging and supporting me no matter what. It means a lot!

And last but not least, a million thanks to Joseph who supported me throughout the whole process and who always knew the right moments to pull me away from my laptop to go on climbing trips or other fun adventures. Thank you for your patience during stressful times and for always knowing how to make me laugh and to not take things too seriously.

Thank you!