

THE ROLE OF TLDc PROTEINS IN NEUROINFLAMMATION

by

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2 Declaration

I, Dominic S. A. Simpson, hereby declare that all work presented in this thesis is my own, except where stated. To the best of my knowledge, this thesis contains no material previously published by any other person except where due acknowledgement has been made. Significant contributions are outlined here in brief: Expert analysis of RNA sequencing data and data visualisation were performed by Szymon Stodolak in the Sir William Dunn School of Pathology at the University of Oxford. Animal husbandry (including mating, weaning and cage changes), initial genotyping and a portion of phenotyping tests (<30%) were performed by technicians at the Mary Lyon Centre, MRC Harwell.

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3 COVID-19 Pandemic Impact Statement

The COVID-19 lockdown significantly impacted my research by disrupting access to crucial laboratory equipment, the implementation of large-scale *in vivo* studies, the ability to collaborate with colleagues, and the execution of planned experiments. The temporary closure of the Mammalian Genetics Unit (MGU) necessitated halting all mouse intercross breeding at the MRC Harwell Mary Lyon Centre (MLC) for six months, thereby delaying plans for a large-scale, complex *in vivo* study. Fortunately, during the lockdown, I was able to focus on a literature review, which was published in the summer of 2020 (Simpson & Oliver, 2020).

The pandemic's impact was compounded by the strategic decision by the Medical Research Council (MRC) to close the MGU at MRC Harwell, where my DPhil research was initially based. This necessitated a limitation of my mouse experiment plans and a shift in focus to stem cell studies. This transition, although carefully managed by my supervisors and the Oxford-MRC DTP team, required me to adapt my methodology and approach, leading to delays in my research timeline. Moreover, the closure of the unit compelled me to prematurely terminate extensive *in vivo* studies that had required significant time and effort to plan and execute.

In conclusion, while I experienced considerable disruption to my laboratory research, especially in large-scale *in vivo* studies, I am grateful that I was able to redirect my efforts and publish a literature review. I am also thankful for the excellent training I received in both *in vivo* and human stem cell research at the MGU, MLC, and the Martin Centre for Stem Cell Research.

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4 Abstract

The TLDc domain defines a family of proteins, including OXR1 and NCOA7, which are crucial for neuronal function^{1,2}. Disruption or loss-of-function (LOF) mutations in these proteins are linked to neurological disorders such as intellectual disability, ataxia, and autism spectrum disorder (ASD)³⁻⁵. Additionally, TLDc proteins have been implicated in inflammatory responses, evidenced by pronounced microglial activation in *Oxr1*^{-/-} mice⁶, and the identification of NCOA7-B, a short isoform of NCOA7, as a novel interferon (IFN)-stimulated gene (ISG)⁷. At the molecular level, TLDc proteins interact with the V-ATPase^{2,8-10}, a vital multimeric proton pump which acidifies intracellular compartments. However, the functional consequences of TLDc binding in mammalian disease models are poorly defined. This thesis examines these interactions in three models: the subcellular localisation and binding of NCOA7-B to the V-ATPase; ASD-associated behavioural phenotypes in the maternal immune activation (MIA) model using *Ncoa7*^{DEL} mice; and quantification of lysosomal function and pH in *OXR1*^{-/-} human induced pluripotent stem cell (iPSC)-derived microglia.

1. Identifying NCOA7-B interaction with the V-ATPase in microglia

This study confirmed the IFN-responsive nature of the *Ncoa7-B* transcript across various models, including mouse cortical tissue and microglial cells derived from mice and human iPSC. Endogenous NCOA7-B protein has not yet been detected, due to the lack of specific probes. Thus, its expression was investigated using epitope tagging techniques. Whilst detectable at the genomic and transcript level, FLAG-NCOA7-B protein was not observable in IFN β -treated mouse microglial cells. As an alternative method, NCOA7-B was GFP tagged in 293T cells and GFP expression was increased

upon IFN β stimulation, suggesting upregulation of NCOA7-B. These findings indicate that NCOA7-B may play an unexplored role in microglial IFN response.

2. ASD-associated behavioural phenotypes in MIA using *Ncoa7^{DEL}* mice

The *NCOA7* gene locus is a potential hub in ASD patients and regulates neuronal development^{2,11,12}. This research explored gene-environment interactions in an MIA model, induced with the viral mimetic poly(I:C). Elevated *Ncoa7-B* transcript expression and trends toward an impaired IL6 response were observed in the foetal brain. Behaviourally, MIA-*Ncoa7^{DEL}* mice exhibited increased anxiety-like behaviour and impaired motor learning, underscoring a potential interaction of the *Ncoa7* locus and maternal inflammation in ASD-related endophenotypes.

3. Lysosomal pH and function in *OXRI*^{-/-} human iPSC-derived microglia

To examine the role of OXR1 and its TLDc domain, *OXRI*^{-/-} and *OXRI* TLDc truncation (*OXRI^{TLDc}*^{-/-}) human iPSC were generated and differentiated to microglia. Probing of the endolysosomal function of OXR1-LOF microglia found deficiencies in endocytosis and an accumulation of lysosomes, as well as cell death during the differentiation process from macrophage precursors to microglia. Although lysosomal pH was unaffected by LOF mutations, lentiviral reconstitution of *OXRI*, or overexpression in WT microglia, RNA-seq revealed upregulation of transcripts encoding MHC Class II peptide presentation proteins and chemokine signalling, underlining a potential functional outcome of endolysosomal dysfunction in human microglia.

Collectively, these data elucidate novel roles for OXR1 and NCOA7 in the regulation of V-ATPase activity and inflammatory responses in microglia.

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8 List of Abbreviations

ALS	Amyotrophic lateral sclerosis
ASD	Autism spectrum disorder
BCA	Bicinchoninic acid
CD χ	Cluster of differentiation χ
CDS	Coding sequence
CNS	Central nervous system
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's phosphate buffered saline
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid
GFAP	Glial fibrillary acidic protein
gRNA	Guide ribonucleic acid
HBSS	Hank's balanced salt solution
HDR	Homology-directed repair
NDD	Neurodevelopmental disorder
IFN	Interferon
INAR	Interferon- α/β receptor
IL	Interleukin
iPSC	Induced pluripotent stem cells
ISG	Interferon stimulated gene
LC3	Microtubule-associated protein 1A/1B-light chain 3
LOF	Loss of function
LPS	Lipopolysaccharide
MAPK	Mitogen-associated protein kinase
mEAK7	mTOR Associated Protein, Eak-7 Homolog
MEF	Mouse embryonic fibroblast
MS	Multiple sclerosis
mTORC	Mammalian target of rapamycin complex
NCOA7	Nuclear Receptor Coactivator 7
NCOA7-B	Relates to the short, interferon-inducible isoform of NCOA7
NCOA7-FL	Relates to the longer isoforms of NCOA7

OS	Oxidative stress
OXR1	Oxidation resistance 1
PBMC	Peripheral blood mononuclear cells
Poly(I:C)	Polyinosinic:polycytidylic acid
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
TLDc	Tre2/Bub2/Cdc16, lysin motif, domain catalytic
TLR	Toll-like receptor
VLP	Virion-like particle

9 Introduction

9.1 TLDC proteins are required for neuronal function in mammals

9.1.1 The TLDC domain defines a novel family of proteins

The Tre2/Bub2/Cdc16 (TBC), Lysin Motif (LysM), Domain catalytic (TLDC) domain-containing proteins are identified by a highly conserved domain, expressed throughout eukaryotes^{13–15} (Figure 9.1). The family includes Oxidation Resistance 1 (*OXR1*), Nuclear Receptor Coactivator 7 (*NCOA7*), *TBC1D24*, *KIAA1609* (or *TLDC1*), *C20ORF118* (or *TLDC2*), and mTOR Associated Protein, Eak-7 Homolog (*mEAK7*)^{15,16}.

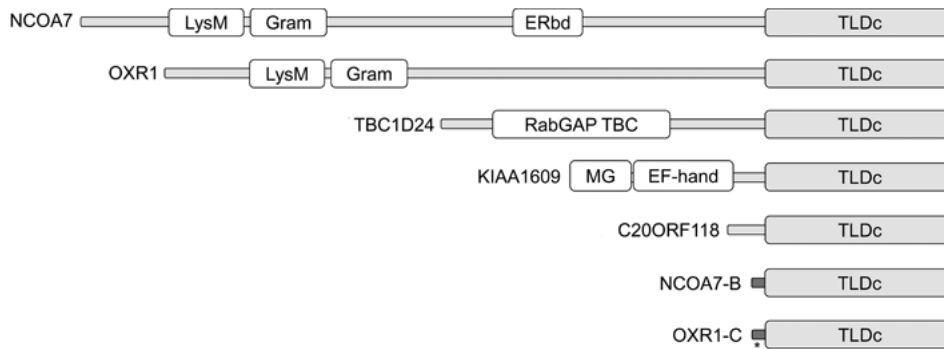


Figure 9.1. Domain structure of mammalian TLDC proteins.

TLDC – Tre2/Bub2/Cdc16 (TBC), Lysin Motif (LysM), Domain Catalytic; LysM: lysin motif; ERbd – oestrogen receptor binding domain; RabGAP – GTPase activating protein; N-M – N-myristoylation domain. The accession numbers corresponding to the human isoforms as shown are: OXR1 (NP_001185461), OXR1-C (NP_001185464), NCOA7 (NP_001186548), NCOA7-B (NP_001186551), TBC1D24 (NP_001186036), KIAA1609 (NP_065998) and C20ORF118 (NP_001291712). The unique first coding exons of NCOA7-B and OXR1-C are shown in dark grey. Not to scale. Reproduced from Finelli & Oliver (2017)¹⁷ and distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).

Initially characterised in *OXR1*, this domain imparts resistance to oxidative stress (OS), including in neurons^{13–15}. The stress-resistance properties of *OXR1* were first demonstrated through ectopic expression in *E. coli*¹³ and have since been attributed to homologues in numerous species, including *D. rerio*^{18,19}, *D. melanogaster*^{3,20}, *C.*

*elegans*²¹, *M. musculus*¹ and in human cell lines^{22,23}. The underlying mechanisms of stress resistance in neurons are complex, potentially involving innate immune signalling²¹, lysosomal dysfunction³, upregulation of antioxidant proteins via the p21 signalling pathway²³, inhibition of apoptosis through suppression of p53 signalling²⁴, maintenance of mitochondrial function^{22,23,25}, cysteine-mediated reactive oxygen species (ROS) sequestration¹, glycolysis regulation²⁶, and post-translational modifications of peroxiredoxin²⁷. These mechanisms enhance resistance to OS to facilitate neuronal survival. OS resistance mechanisms of TLDC proteins are extensively reviewed by Volkert & Crowley²⁸ and are likely to be cell-type and context-specific.

9.1.2 Dysfunction of TLDC proteins NCOA7 and OXR1 cause neurological disease

NCOA7 and *OXR1*, the primary focus of this thesis, are hypothesised to be paralogues originating from a gene duplication event¹⁴. Both are highly expressed in the brain and mutated in neurological diseases resulting from loss of function (LOF)^{1,2}. Furthermore, they exhibit similar functions in conferring OS resistance in neurons. Overexpression of these proteins in murine neuronal cell lines and primary neurons effectively confers resistance to arsenite-induced OS, while their knockdown via RNA interference (RNAi) increases vulnerability to oxidative damage, with *OXR1* levels directly correlated with the extent of neuronal death¹⁵. Disruption of the *Oxr1* locus in mice leads to oxidative DNA damage in cerebellar granule neurons, precipitating neurodegeneration, ataxia, and death¹. Additionally, mutations in human *OXR1*, discussed further in Section 13.2, are associated with reduced cerebellar volume, severe neurodevelopmental delay, hearing loss and ataxia³⁻⁵.

Conversely, while knockdown of *Ncoa7* alone does not increase sensitivity to OS, its overexpression markedly diminishes arsenite-induced neuronal death, with NCOA7-

B (the short splice variant of *NCOA7*, consisting almost entirely of the TLDc domain) providing the most significant protective effect among the studied TLDc proteins¹⁵. Deletion of the *Ncoa7* locus in mice results in a less severe phenotype compared to *Oxr1*^{-/-}, with no apparent neuronal death or motor dysfunction². However, *Ncoa7*^{DEL/DEL} mice display increased anxiety-like behaviours and deficits in social interaction. This phenotype was associated with lysosomal dysfunction in neurons and a reduction in inhibitory synapse density within the somatosensory and visual cortices². In patients, an *NCOA7* LOF mutation is associated with autism spectrum disorder (ASD)^{12,29}, a connection explored in more depth in Section 12.2. Together, these observations underline the functional relationship between these paralogous TLDc proteins in neuronal systems and additional mechanisms, beyond OS resistance, in TLDc function.

Despite their similar roles, these proteins exhibit anticorrelated expression patterns in the mouse brain; *OXR1* is predominantly found in cerebellar granule cells, while *NCOA7* is prevalent in cerebellar Purkinje cells (Figure 9.2)^{1,2}. This pattern of expression may be indicative of functional redundancy between the genes, where the expression of at least one isoform is necessary for neuronal function. Notably, the combined knockout of *Ncoa7* and *Oxr1* is embryonically lethal², underscoring the crucial, overlapping roles of these proteins in neuronal survival.

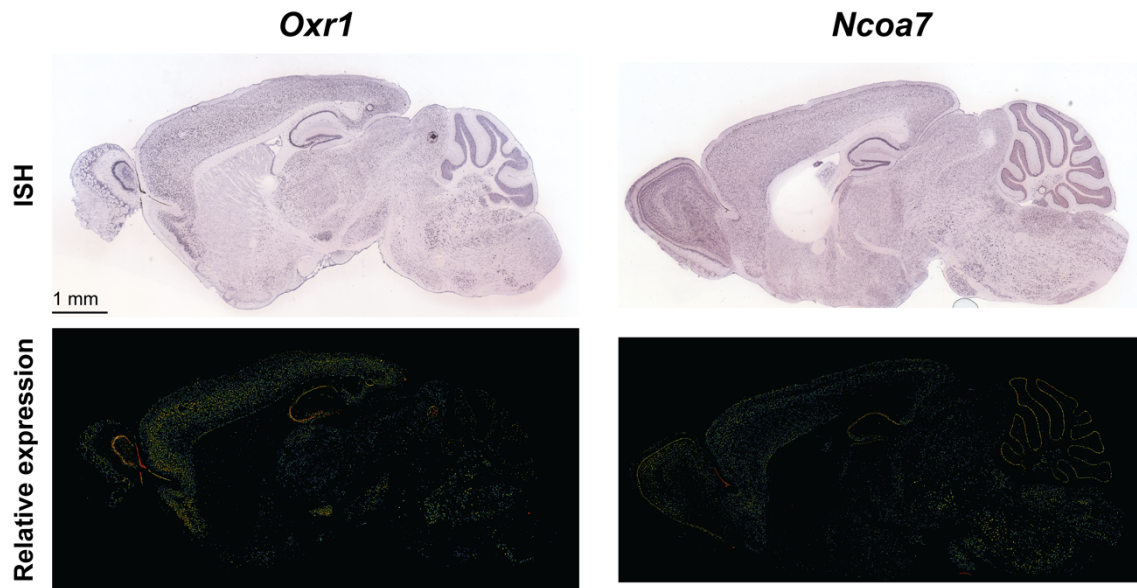


Figure 9.2. Expression patterns of *Oxr1* and *Ncoa7* transcript in the mouse brain.

Figures demonstrating patterns of transcript expression (*in situ* hybridisation, ISH) in sagittal brain sections from a male C57BL/6J mouse at P56. Figure produced using data from the Allen Brain Atlas³⁰. Accessed at: <https://mouse.brain-map.org/gene/show/82023> (OXR1) and <https://mouse.brain-map.org/gene/show/84287> (NCOA7). Reproduced in accordance with Allen Terms of Use and Citation Policy.

Additionally, *NCOA7* and *OXR1* have also been found expressed as short, splice variants which consist almost exclusively of the TLDC domain (NCOA7-B and OXR1-C) and a short, unique first exon^{1,7,31,32} (Figure 9.1). Whilst their expression patterns and interaction with the full-length isoforms remain poorly defined, functional compensation in TLDC deletion by the full-length and short isoforms has been observed. For example, OXR1-C or NCOA7-B overexpression is sufficient to rescue OS in murine neuronal cell lines¹⁵ and OXR1-C reconstitution in *Oxr1*^{-/-} mice confers protection against OS and abrogates the phenotype¹. It remains to be determined whether NCOA7-B reconstitution abrogates neuronal dysfunction in *Ncoa7*^{DEL/DEL} mice. This isoform-specific functional redundancy may be translatable, as the phenotypic severity of *OXR1* patient mutations is associated with the disruption of specific isoforms. Where mutations affecting the TLDC

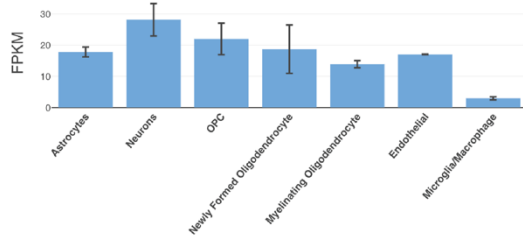
domain result in severe neurodevelopmental delay, ataxia and epilepsy^{3,5}; polymorphisms, where OXR1-C is not predicted to be disrupted, are associated with milder phenotypes, including severe hearing loss but normal intellectual ability and motor coordination⁴. This further suggests a compensatory capacity among *OXR1* isoforms.

In conclusion, *NCOA7* and *OXR1* may not only share a genetic origin but also perform critical, overlapping functions in neuronal health, impacting both cell survival and neurological phenotype. The TLDc domain is likely to be critical to the mechanism of these functions since its disruption results in severe neurological disease and some aspects of function can be restored in knockout models. Their study offers potential insights into the molecular mechanisms underpinning neuroprotection and the consequences of their dysfunction, highlighting the importance of these proteins in maintaining neuronal integrity and function. However, the molecular mechanisms underpinning the role of *NCOA7* and *OXR1* in neuronal development and survival remain poorly defined. This thesis thus underscores the necessity of further investigation into the specific roles and interactions of TLDc domain-containing proteins within the brain.

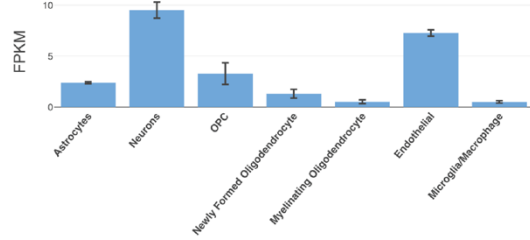
9.1.3 The role of *NCOA7* and *OXR1* in microglia is poorly characterised

Despite significant expression in non-neuronal cells (Figure 9.3), especially in human microglia, the function of *NCOA7* and *OXR1* in glia is poorly understood. Very recent evidence indicates a crucial role of *NCOA7* and *OXR1* in regulating inflammation, with particular consequence for microglia, the brain parenchymal macrophages and orchestrators of neuroinflammation. Whilst this is reviewed in depth in chapter introductions, this section provides an overview of current evidence implicating TLDc proteins in neuroinflammation.

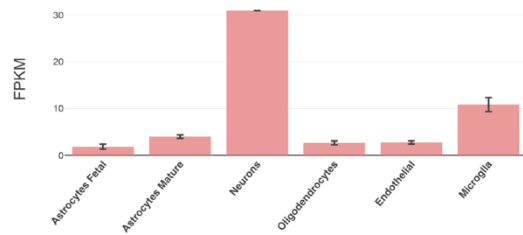
***Oxr1* - Mus musculus**



***Ncoa7* - Mus musculus**



***OXR1* - Homo sapiens**



***NCOA7* - Homo sapiens**

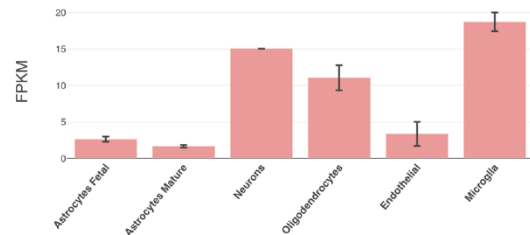


Figure 9.3. Relative transcript expression of NCOA7 and OXR1 in human and mouse brain cell types. Figure reproduced from brainnaseq.org and data from Zhang et al. (2014)³³ (mouse) and Zhang et al. (2016)³⁴ (human) with authors permission. In the studies, cells were isolated from adult mouse brain and RNA-seq performed on isolated cell types. Similarly brain cells from human foetal, juvenile and adult brain samples were sequenced.

9.1.3.1 Deletion of *OXR1* in mice causes neuroinflammation

Recent, unpublished evidence indicates that *OXR1* disruption in the brain is sufficient to cause neuroinflammation. The deletion of *Oxr1* in mice causes marked microgliosis throughout the cerebellum, which is the principal region of neuronal death in *Oxr1*^{-/-} mice^{1,6} (Figure 9.4). These microglia are characterised by an amoeboid and bushy morphology, indicative of a proinflammatory, neurotoxic phenotype. Additionally, bulk RNA-seq of *Oxr1*^{-/-} mouse cerebellum before the onset of neuronal death has demonstrated upregulation of transcripts encoding neuroinflammatory-associated genes⁶. This finding suggests neuroinflammatory processes precede neuronal death, suggesting neurotoxic microglia may cause or accentuate neuronal loss. This evidence indicates a

potential role for *OXRI* in the regulation of microglial inflammatory processes. Furthermore, whilst *Oxr1* disruption is neuroinflammatory, its overexpression in spinal neurons limits neuroinflammation, improves functional outcomes and delays death in ALS model mice^{35–37}, suggesting *OXRI* may regulate microglial activation in a non-cell-autonomous manner. Thus, the level of expression of *Oxr1* in the mouse brain is intimately related to neuroinflammation, with abrogation of expression resulting in inflammation and overexpression limiting inflammatory markers and neuronal death. Crucially, to date, limited evidence from humans indicates a function for *OXRI* in inflammation, thus exploring the translatability of these findings to human disease is crucial.

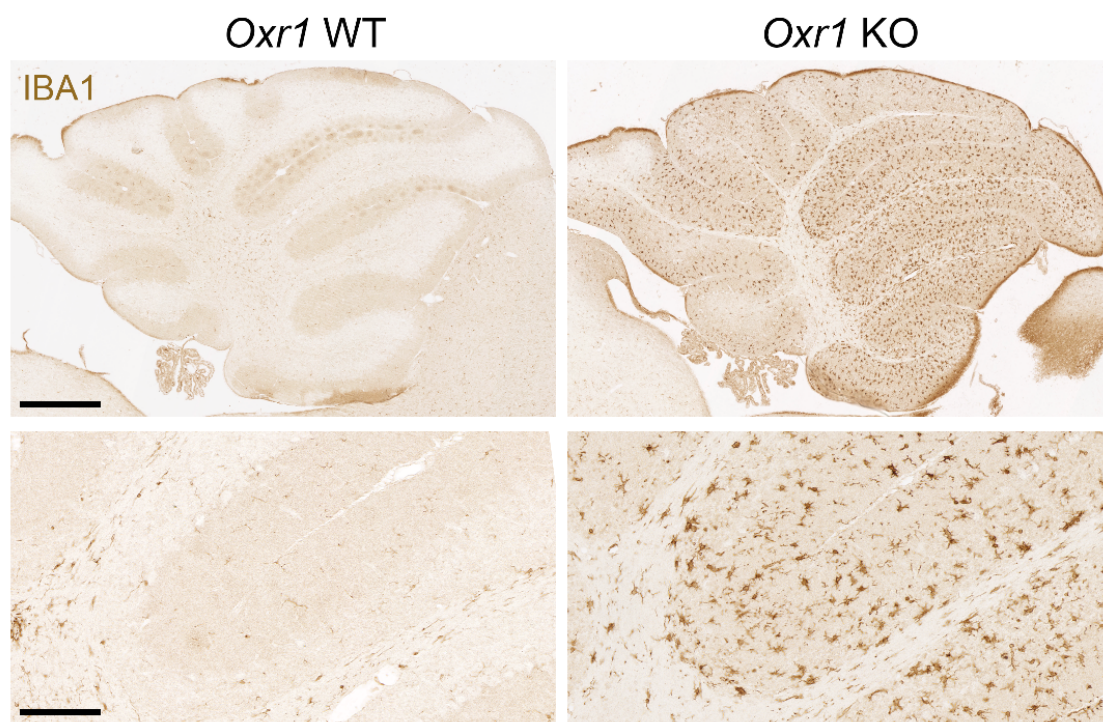


Figure 9.4. Microgliosis in the *Oxr1*^{-/-} mouse brain.

Representative Iba1 immunostaining (DAB) of sagittal cerebellar sections from *OXRI*-WT and -KO mice at P22. Scale bars: 300 μ m (top panels) and 60 μ m (bottom panels). Reproduced from Bucknor & Oliver (2024)³⁸ and in accordance with the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

9.1.3.2 *NCOA7-B is a novel interferon-stimulated gene strongly induced in macrophages*

Additionally, the short isoform of *NCOA7*, NCOA7-B, is significantly associated with inflammatory activation, particularly in response to viral-related stimuli such as interferons (IFNs). Notably, NCOA7-B inhibits endosome-mediated viral entry and membrane fusion and has been demonstrated to limit SARS-CoV-2 infection in lung epithelial cells^{39,40}. Moreover, *NCOA7* is upregulated in epithelial cells following rhinovirus infection and in LPS- and IFN β -stimulated macrophages, the peripheral counterparts of microglia^{41,42}. NCOA7-B is hypothesised to function as a novel interferon-stimulated gene (ISG), with its expression being induced by a range of type I IFNs, including IFN α and IFN β ^{7,43,44}. A significant induction (>60-fold) of NCOA7-B is observed in peripheral blood mononuclear cells and macrophages treated with interferon- β -1b (IFN β 1b) or LPS⁴². The expression of this isoform in ovarian follicles is also regulated by mitogen-associated protein kinases (MAPKs), p38, and ERK, which are key mediators of the pro-inflammatory response in microglia³¹, though it is unknown whether this pathway is conserved in myeloid cells. Together, this evidence implies a conserved proinflammatory activation pathway involving NCOA7-B in myeloid cells, with potential functional consequences for viral infection and IFN-associated diseases, such as multiple sclerosis^{7,45}. It is important to explore whether this upregulation is observable in microglia and the potential function of NCOA7-B in the microglial response to proinflammatory stimuli. This holds particular relevance for neurodegenerative diseases, where the type I interferon response to hallmark aggregated protein plaques is an emerging mechanism of neurodegeneration⁴⁶⁻⁴⁸.

9.1.3.3 *Exploration of NCOA7-B and OXR1 is warranted in neuroinflammation*

Together, these findings underscore the potential role of TLDc proteins in neuroinflammation, meriting further exploration through specific genetic manipulation in microglia and models of inflammation. This would enhance understanding of the molecular pathways governing neurological dysfunction when TLDc protein expression is altered. Investigating the connection between oxidative resistance and inflammatory functions of TLDc proteins could provide comprehensive insights into their biological significance and contribute to a deeper understanding of their roles in cellular and systemic responses.

9.2 The TLDc domain is a novel V-ATPase interacting domain

9.2.1 Recent evidence has demonstrated TLDc binding to the V-ATPase in multiple models

Beyond OS resistance, the TLDc domain has recently been identified as a novel vacuolar-type H⁺-ATPase (V-ATPase) interacting peptide⁸. Whilst the specific effects of binding are unclear and are discussed in each chapter's introduction, this section provides an overview of V-ATPase function and evidence for TLDc protein interaction. Initially identified in a screen for kidney V-ATPase interactors¹⁰, NCOA7 and OXR1 proteins have since been demonstrated to interact with the V-ATPase in various systems and species, including yeast, mouse and human. The potential effects of binding are discussed in the final section of this thesis; however, the table below summarises the body of evidence of TLDc domain binding to the V-ATPase (Table 9.1).

Table 9.1. Interactions of NCOA7 and OXR1 with V-ATPase subunits.

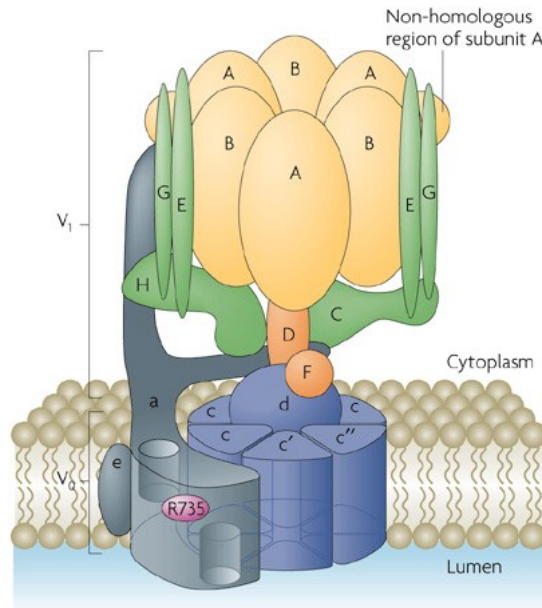
Oxr1p is the yeast homologue of OXR1, consisting almost entirely of the TLDc domain. TLD_{cOXR1/NCOA7} refers to recombinant TLDc domains originating from either protein. *Ex vivo* i.p. denotes immunoprecipitation (i.p.) outside of a biological system. *In vivo* i.p. denotes binding in an animal model. *In vitro* i.p. denotes binding in a cellular model.

TLDc protein	TLDc protein species	V-ATPase protein species	Model system	Interacting subunits	Predicted directionality of effect	References
Oxr1p	Yeast	Human	<i>Ex vivo</i> i.p.	AB, C, EG2 (peripheral stator)	Inhibition by disassembly	Khan et al. (2022) ⁴⁹
TLD _{cOXR1} TLD _{cNCOA7}	Human	Human	<i>Ex vivo</i> binding assay	V1/V0	Inhibition by disassembly	Oot & Wilkens (2024) ⁹
TLD _{cOXR1} TLD _{cNCOA7}	Mouse	Mouse	<i>Ex vivo</i> i.p.	B1 (Kidney-specific)	n/a	Eaton et al. (2021) ⁸
NCOA7-FL	Mouse	Mouse	<i>In vivo</i> i.p.	A, B2, C.	Stabilisation and increased subunit expression	Castroflorio et al. (2020) ²
NCOA7-FL OXR1-FL	Mouse	Mouse	<i>In vivo</i> i.p. ICC	B1 (Kidney-specific)	Stabilisation and increased subunit expression	Merkulova et al. (2015) ¹⁰ and Merkulova et al. (2018) ⁵⁰
NCOA7-B	Human	Human (cell line)	<i>In vitro</i> i.p.	B2, E1, A, G2	Stabilisation and vesicular acidification	Doyle et al. (2018) ³⁹

9.2.2 The V-ATPase is a multimeric proton pump

The V-ATPase is a highly conserved enzyme complex crucial for acidifying a variety of intracellular compartments in eukaryotic cells. This proton pump is heteromultimeric, composed of 14 subunits forming two domains: a soluble, peripheral V_1 domain responsible for ATP hydrolysis; and an integral, membrane-bound V_0 domain that translocates protons across the membrane by rotary action⁵¹ (Figure 9.5). V_1 is formed from eight subunits, denoted in uppercase (A, B, C, D, E, F, G, H), whereas V_0 comprises six different subunits denoted in lowercase (a, d, e, c, c' and c''). Tissue-specific isoforms of these subunits exist, for example, the neuronal-specific G2 and a1 subunits^{52,53}, though their functional significance is poorly defined.

The V-ATPase plays a pivotal role in various physiological processes including endocytosis, neurotransmitter release, and the activation of lysosomal enzymes by regulation of vesicular pH⁵¹. Crucially, subunits d, D and F form the central stalk, serving as a rotor to couple energy from ATP-hydrolysis to proton translocation⁵⁴. Reversible protonation of glutamic acid residues in the c-ring (formed by the c and c'' subunits in mammals) and complex rotation drive translocation upon ATP hydrolysis by subunit A and B interaction⁵¹.



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Figure 9.5. Structure of mammalian V-ATPase.

The vacuolar (V-)ATPase is formed of a peripheral domain (V1, yellow and orange), responsible for ATP hydrolysis, and an integral domain (V0, blue and grey), involved in proton translocation. The core of the V1 domain is composed of a hexameric arrangement of alternating A and B subunits, which bind and hydrolyse ATP. The V0 domain includes a 'c-ring' that rotates to translocate proteins. The V1 and V0 domains are connected by a central stalk, composed of subunits D and F of V1 and subunit d of V0, which acts as a rotor to couple ATP hydrolysis to proton movement, and peripheral stalks, composed of subunits C, E, G, H and the N-terminal domain of subunit a which act as a stator to stabilise the domain during rotation. Reproduced from Forgac (2007)⁵¹ with permission (license number: 5847570592321)

9.2.3 V-ATPase function in macrophages and microglia

In immune cells, the V-ATPase has specific functions that are critical for immune responses. It is integral to phagocytosis, a key aspect of the innate immune response where pathogens are engulfed and destroyed, where phagosome maturation is correlated with V-ATPase recruitment to the phagosome membrane⁵⁵. The resulting phagosomal acidification by V-ATPase is essential for the activation of hydrolytic enzymes that degrade ingested pathogens⁵⁶. Whilst knockdown of specific V-ATPase subunits in RAW 264.7 human immortalised macrophages was demonstrated to have no effect on lysosomal acidification and phagosome-lysosomal fusion⁵⁷, functional compensation by other V-ATPase subunits and residual expression cannot be discounted. Furthermore, the enzyme's function extends to regulating the processing and presentation of antigens in dendritic cells, where V-ATPase A and E subunit expression is increased and lysosomal pH is reduced in mature dendritic cells⁵⁸. This process is suggested to involve PI 3-kinase/mTOR signalling since pharmacological inhibition of PI 3-kinase or mTOR impairs V-ATPase assembly in dendritic cells⁵⁹. This V-ATPase-mediated regulation of antigen presentation has not yet been explored in other professional antigen-presenting cells, such as macrophages and microglia and is an avenue for further research. A further understanding of the detailed mechanisms of V-ATPase regulation in immune cells, including by interacting partners, offers potential therapeutic avenues for controlling immune responses, enhancing pathogen clearance, and moderating inflammatory diseases.

9.2.4 Regulation of V-ATPase activity

The activity of the V-ATPase is finely regulated by reversible disassembly of the V₁ and V₀ domains to form a holoenzyme, a process modulated by cellular demands. For example, lysosomal acidification in the maturation of dendritic cells is dependent on

holoenzyme assembly⁵⁸. Whilst in yeast, this process is controlled by the RAVE complex (regulator of the ATPase of vacuolar and endosomal membranes), the regulation of disassembly in mammals is yet to be fully defined^{60,61}. Though current evidence suggests this role is played, in part, by a rabconnectin complex, which has homologous regions with RAVE, interacts with the V-ATPase and promotes vesicular acidification in cells^{60,62}. Thus, it is of current interest to identify and characterise regulators of V-ATPase in mammalian systems and here, the TLDc domain has provided an avenue for intense research.

9.2.5 Evaluation of TLDc domain function by structural modification

The crystal structure of the human TLDc domain from the short *NCOA7* isoform, NCOA7-B, demonstrated a globular structure consisting of two N-terminal domain α -helices followed by ten β -strands forming two antiparallel β -sheets^{18,63}. Whilst it is difficult to hypothesise the function of the TLDc domain from its structure alone, this model is invaluable for comparing TLDc domain conservation and mutation mapping, with predictions of polymorphism effects at the molecular level. For example, alanine substitution of highly conserved residues G815, G845 and G896 of the TLDc domain of NCOA7-B abolished V-ATPase binding, whilst substitution of S817, L926 and E938 decreased the interaction⁸. Interestingly, these highly conserved residues have also been demonstrated to be crucial to the neuroprotective mechanism of NCOA7-B in OS-induced neuronal death¹⁵.

9.2.6 Thesis aims

The TLDc domain is shared between two proteins, NCOA7 and OXR1, that converge on neurological function, inflammatory processes and V-ATPase regulation. Whilst the OS resistance functions of these proteins are well characterised, their role in

inflammation is incompletely defined, especially in microglia where these genes are highly expressed. Since the V-ATPase plays a crucial role in phagocyte function, the overarching aim of this thesis is to explore the relationship between OXR1 and NCOA7 and the V-ATPase in neuroinflammation. This was achieved through three specific aims, with findings summarised below:

1. To quantify NCOA7-B protein expression and identify NCOA7-B binding to the V-ATPase by epitope tagging in microglia.

Despite successful FLAG-tag detection at the genomic and transcriptomic level, FLAG-NCOA7-B was not observable using a variety of detection methods. However, successful tagging of NCOA7-B with GFP in 293T cells demonstrated an IFN-stimulated GFP signal. This novel finding suggests that NCOA7-B protein expression is increased in IFN-stimulated cells and correlated with transcript levels.

2. To explore the role of ASD-associated LOF in *NCOA7* in an inflammatory model of ASD.

The combination of MIA and *Ncoa7* deletion resulted in anxiety-like phenotypes in the light-dark box and impaired motor learning in the rotarod test, suggesting a gene-environment interaction between gestational inflammation and *Ncoa7* deletion.

3. To examine the role of *OXR1* in microglia, by generating *OXR1*^{-/-} human induced pluripotent stem cells (iPSC), differentiating them to microglia, then characterising lysosomal function, pH and global transcriptional changes by RNA-seq.

This found deficiencies in endocytosis of proteinaceous substrates and an accumulation of lysosomes, as well as death of microglia during the differentiation process *in vitro*. Additionally, V-ATPase density on intracellular membranes was increased, providing a potential mechanism for endolysosomal dysfunction. Finally, transcripts encoding MHC Class II peptide presentation and chemokine signalling were upregulated in *OXR/LOF* microglia, underlining a potential functional outcome of *OXR/LOF* in human microglia.

10 Materials & Methods

10.1 Methods used *in vivo*

10.1.1 Mouse Strains and husbandry

10.1.1.1 *Husbandry*

Mice were generated and maintained under UK Home Office Project License P7133CD66 (license holder: Dr Peter Oliver) with local ethical guidelines issued by the Medical Research Council (MRC) Harwell Institute. Mice had access to food and water *ad libitum* in individually ventilated cages (maximum of five mice per cage) and were housed under a controlled 12-hour light-dark cycle, temperature ($21 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$). All mice were maintained on a C57BL6/J (Jackson Laboratory sub-strain) background and backcrossed over ten generations before intercrossing to limit confounds due to genetic drift. Husbandry, including mating, weaning and initial genotyping was performed by MRC Harwell Institute technicians with oversight from Dr Peter Oliver and myself.

10.1.1.2 *Ncoa7^{DEL} mice*

This mouse was generated by the Wellcome Trust Centre for Human Genetics, University of Oxford. The *Ncoa7* deletion (*Ncoa7^{DEL}*) harbours a 157 kb deletion of the entire *Ncoa7* locus introduced by CRISPR-Cas9. The details of the generation of this mouse are described previously², in brief guide-RNAs were designed immediately upstream and downstream of the *Ncoa7* start and stop codons. Cas9 mRNA (10 ng/ μL) and in vitro transcribed guide RNAs (gRNAs) (5 ng/ μL) were delivered by pronuclear injection into fertilised C57BL/6J oocytes. In the resulting litters, a founder was identified, and the deletion was carried to the next generation.

10.1.1.3 Intraperitoneal injection of lipopolysaccharide

To model inflammation, mice were injected i.p. with 250 µg/mL lipopolysaccharide (LPS in sterile 0.9% saline, Sigma Aldrich, L4391) in the lower right quadrant (volume of dose was 10 µL per gram of body weight) for 4 hours. Mice were then sacrificed by cervical dislocation and tissue harvested immediately post-mortem.

10.1.2 Maternal immune activation (MIA)

Timed *Ncoa7^{+ / DEL}* inter-cross breeding groups (two does with one buck) were established. After timed mating, dams were confirmed pregnant on E12.5 by palpation. Dams were randomly assigned to either 20 mg/kg polyinosinic:polycytidylic acid (poly(I:C)) (Bio-Techne, 4287) or 0.9% NaCl and injected intraperitoneally on E12.5 in the lower left quadrant using 30 G x 8mm needles. The volume of injection was 5 µL/g of body weight. For expression studies, animals were sacrificed by cervical dislocation 3 hours post-dose. Maternal tissue was immediately harvested and frozen in liquid nitrogen and the uterus, containing embryos, was extracted in ice-cold PBS. Embryo heads and placentas were sub-dissected in ice-cold PBS and frozen on dry ice. For behavioural and phenotyping studies, mice were left undisturbed in a low-traffic room until parturition. After parturition, pups were genotyped, identified by ear-clipping and weaners were randomised to same-sex cages at P21.

10.1.2.1 Behavioural phenotyping

I performed most behavioural phenotyping, with <30% performed by phenotyping technicians at the Medical Research Centre Mary Lyon Centre. All fear conditioning was performed by formally trained phenotyping technicians due to the stressful nature of this test. All investigators were blind to genotype during all tests. Since, to our knowledge, behavioural analysis of MIA offspring has not been performed on a homozygous gene knockout background, a standardised phenotyping pipeline was carried

out on three cohorts of age-matched female *Ncoa7^{+/+}* and *Ncoa7^{DELI/DEL}* offspring of pregnant dams treated with poly(I:C) or saline in the order described below. For video tracking, all experiments were analysed using Ethovision XT (Noldus, The Netherlands). For all tests, mice were habituated to the test room for at least 20 minutes, tests were performed individually and the apparatus was cleaned with 70% ethanol in between mice to minimise olfactory cues. All phenotyping was performed in the morning, to avoid circadian confounds. All phenotyping tests were carried out according to MLC standard operating procedures.

10.1.2.2 Open field maze

Mice were placed in square, opaque Plexiglas arenas (44 x 44 cm) in 150-200 lux conditions and were video-tracked for 20 minutes. For analysis, the arena was divided into the inner zone (11 x 11 cm central square) and the remaining outer zone.

10.1.2.3 Elevated-zero maze

Mice were placed in the open arm of an elevated ring-shaped runway with open (30 cm x 5 cm) and closed quadrants (30 cm x 5 cm x 15 cm) at 50 ± 5 lux and video tracked for 5 minutes. For analysis, preference for the open or closed quadrant was scored.

10.1.2.4 Light-dark box

Mice were placed in the open arena of a box with an open (40 cm x 40 cm x 20 cm) at 100 ± 10 lux and closed (40 cm x 20 cm x 40 cm) area covered from light and video tracked for 10 minutes. For analysis, their preference for the light or dark area was scored.

10.1.2.5 Y-maze forced alternation

Mice were placed in the same arm of a Y-shaped, transparent maze (3 identical arms 35 cm x 5 cm x 15 cm) at 15-20 lux with access to one, randomised arm blocked for

10 minutes. Mice were then returned to their home cage for a 2-minute interval, during which the arena was cleaned, and then returned to the maze, with access to all arms, for 5 minutes. For analysis, time spent in the novel arm, versus familiar was compared.

10.1.2.6 Three-chamber social novelty test

Mice were placed in the centre chamber (23 cm x 40 cm x 22 cm per chamber) of a transparent, three-chambered arena with access to the peripheral chambers via openings, for 10 minutes at 10 ± 2 lux throughout the arena. Mice were returned to the home cage for 2 minutes, during which the arena was cleaned and an object (empty single-mouse cage) and a novel mouse (sex- and age-matched C57BL/6 mouse in a single mouse cage) were placed in either the left- or right-hand chamber (randomised). For analysis, the preference for the object or novel mouse was scored by the time spent in each chamber and the number of interactions.

10.1.2.7 Rotarod

Mice were placed on an accelerating rotarod (Ugo Basile), with rotor speed increasing from 4 rpm to 40 rpm over 5 minutes. The trial was repeated once per day for 3 days. For analysis, the time taken for the mouse to lose coordination (i.e. drop off the rod, or cling to and spin with the rod) was recorded.

10.1.2.8 Context- and cue-dependent fear conditioning

Mice were placed in the test chamber of a Fear Conditioning System (Ugo Basile) to habituate for 2 minutes. A single tone (5 kHz, 80 dB, 20 seconds) was played to serve as the conditioning stimulus. During the last second of the tone, an electrical foot shock (0.5 mA) was applied to serve as the unconditioned stimulus. The mouse was left in the chamber for a further 2 minutes. 24 hours after conditioning, mice were placed in the same chamber and freezing behaviour recorded for 5 minutes. Mice were then returned

to their home cage for 4 hours. Mice were placed in a plexiglass cylinder for 6 minutes with vanilla fragrance spread at the top of the cylinder during which the mouse was exposed to the conditioning stimulus. Freezing behaviour and activity were recorded.

10.1.3 Pathology and histology

10.1.3.1 Immunohistochemistry

Mice were placed under terminal anaesthesia by i.p. injection of pentobarbital (100 mg/mL Euthatal, Larkmead Veterinary, Didcot, UK), followed by transcardial perfusion with PBS, followed by 4% paraformaldehyde (Thermo Fisher, 28908) in PBS. Tissue was removed and post-fixed in 10% neutral buffered formalin (Leica, 380077027), cryoprotected in 30% sucrose for 3 days at 4°C and then either vibratome (Leica, VT100S) sectioned at 30 µm or cryo-sectioned (Thermo Fisher, CryoStar NX70) in the parasagittal plane at 12 µm in optimal cutting temperature (OCT) compound (VWR, 361603E) onto SuperFrost Plus slides (Thermo Fisher, J1830AMNZ).

Sections were blocked and permeabilised for 1 hour in 10% normal goat serum (VectorLabs S-1000), 0.3% Triton X-100 in PBS (blocking buffer) at room temperature. Primary antibody was applied in blocking buffer overnight at 4°C. Sections were then washed three times in 0.3% Triton X-100 in PBS, followed by incubation with secondary antibody for 1 hour at room temperature. Sections were washed again three times and mounted in ProLong Glass Antifade Mountant with NucBlue Stain (Invitrogen, P36983). Antibodies used in immunohistochemistry are listed in Table 10.1.

Table 10.1. Antibodies used in immunohistochemistry

Target	Host, clonality	Supplier	Catalogue number	Dilution used
Iba1	Rabbit, polyclonal	Wako (Fujifilm)	019-19741	1:1000
CD68	Rat, polyclonal	Biolegend	137001	1:500
Rabbit	Goat, polyclonal	Invitrogen	A32731	1:1,000

Rat	Goat, polyclonal	Invitrogen	A21247	1:1,000
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10.1.3.2 *Preparation of tissue for BaseScope*

Mice were sacrificed by cervical dislocation and brain dissected. The brain was immediately frozen in OCT compound on dry ice and then cryo-sectioned at 8 or 12 μ m.

10.1.3.3 *BaseScope*

BaseScope is a proprietary *in situ* hybridisation method that uses target RNA-specific oligo probes that bind in tandem, offering greater specificity than standard *in situ* hybridisation methods. The signal is then amplified by a cascade of signal amplification molecules to confer increased sensitivity. The procedure was carried out as per instructions, using the BaseScope Duplex Detection Kit (ACD, 322436) and custom, proprietary probes for NCOA7-FL (BA-Mm-Ncoa7-tv1-E4E5), designed to bind to the full-length, canonical transcript of NCOA7; and NCOA7-B (BA-Mm-Ncoa7-tv2-E1E2-C2), designed to detect the short, interferon-inducible isoform. Briefly, mounted sections and cells cultured on chamber slides were fixed in 4% paraformaldehyde in PBS for 1 hour at 4°C. Proprietary probes were then hybridised for 2 hours at 40°C, and signal was amplified using a sequence of proprietary buffers before detecting red and blue signal via alkaline phosphatase- and horseradish peroxidase-based catalysis of colorimetric substrate. Sections were counterstained in 50% haematoxylin, Gill No. 1 (Santa Cruz, 517-28-2) solution for 1 minute and then mounted in VectaMount (Vector Laboratories, H-5000) under cover glass.

10.1.4 **Microscopy at MRC Harwell**

Imaging of tissue sections was performed using an LSM-700 (Zeiss) inverted laser scanning confocal microscope with 4 laser lines: 5 mW diode violet laser 405 nm; 10mW diode green laser 488 nm; 10mW diode red laser 555 nm and 5 mW diode far red laser

639 nm using the PlanApochromat 20x (dry, 0.8 NA), 40x (oil, 1.4 NA) and 63x (oil, 1.4 NA). Images were collected using multitrack mode to avoid fluorescence crosstalk with pinhole diameter set to 1 Airy unit for optical sectioning and laser power and gain set to limit detector saturation.

10.2 Cell culture and methods used *in vitro*

As standard, all cells were cultured at 37 °C and 5% CO₂. Where available, Cellosaurus.org Research Resource Identifiers (RRID) are listed after cell line names.

10.2.1 HEK293T and HeLa culture

HEK293T (293T, RRID:CVCL_0063, obtained from ATCC at p5) were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Sigma Aldrich, D6429) supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS, Sigma Aldrich, F4135). Cells were passaged every 3-4 days using TrypLE Express (Gibco, 12604013) 15 times before returning to a master stock.

10.2.2 Immortalised murine microglial cell culture and drug treatments

Murine immortalised BV-2 (RRID:CVCL_0182, p2) or SIM-A9 (RRID:CVCL_5I31, p2) microglia (both from Generon) were maintained in DMEM-F12 supplemented with 10% HI-FBS. SIM-A9 cells were maintained in DMEM-F12-10% HI-FBS with the addition of 5% heat-inactivated horse serum (Gibco, 26050088). Cells were maintained at 37°C, 5% CO₂ and passaged no more than 15 times before returning to the parent stock. BV-2 cells are a commonly used, adherent mouse microglial cell line originating from a C57BL/6 mouse that were immortalised by transforming with a v-raf/v-myc carrying retrovirus⁶⁴. SIM-A9 cells are a newer mouse microglial cell line, that is semi-adherent and also derived from a C57BL/6 mouse that was spontaneously immortalised in culture⁶⁵.

To model inflammation, cells were treated with 500 ng/mL lipopolysaccharide (LPS, Sigma Aldrich, L4391) or 10 µg/mL Poly(I:C) both dissolved in Hank's Balanced Salt Solution (HBSS). For interferon (IFN)-β induction, cells were treated with 100

ng/mL recombinant human IFN- β (Bio-Techne, 8499-IF-010). Treatments were for 6 hours (transcript quantification) or 24 hours (protein quantification).

10.2.3 Epitope tagging of NCOA7-B with DYKDDDDK (FLAG) peptide in SIM-A9 cells

10.2.3.1 Knock-in design

See for details of oligonucleotides used in the generation of FLAG-NCOA7-B SIM-A9 cells. A gRNA sequence was designed proximal to the start codon of *Ncoa7-B*, the short, interferon-inducible splice variant of *Ncoa7* with its own alternative transcriptional start site (ENSMUST00000216172.2). An appropriate protospacer sequence was found using CRISPOR⁶⁶ and Cas-OFFinder⁶⁷ on the first exon of *Ncoa7-B* (ENSMUSE00000666505) that was proximal enough to the start site to accommodate the homology-directed repair (HDR) donor template. The ssDNA HDR donor template was designed to append a DYKDDDDK-G(6) sequence immediately after the ATG of exon 1 with ~75 bp homology arms.

10.2.3.2 RNP formation and delivery

Ribonucleoprotein (RNP) complexes were formed as per section 10.2.7.2. Nucleofection with the SF Cell Line 96-well Nucleofector Kit (Lonza, V4SC-2096) was used to deliver RNP complexes to SIM-A9 cells. 1×10^6 SIM-A9 cells (passage 7) were resuspended in 100 μ L SF Cell Line Nucleofector Solution with RNP and HDR donor template, both at a final concentration of 4 μ M. 20 μ L of cell suspension was gently added to the nucleofection strip well and cells were electroporated using program CM-158, incubated at room temperature for 5 minutes and then resuspended with 80 μ L warm, complete medium. 25 μ L of cells were transferred from the nucleofection well to a pre-incubated 96-well plate containing 75 μ L complete medium, supplemented with IDT

HDR Enhancer v2 (IDT, 10007910). Cells were expanded and colonies formed by limited dilution cloning in 96-well plates.

10.2.3.3 Screening and validation of successfully edited clones

Successful HDR was screened for using junction PCR, in which the forward primer (NCOA7B-Scrn-F) was situated in the novel DNA sequence (encoding DYKDDDDK-G(6)) and the reverse primer situated downstream of the homology arms to avoid amplification of non-integrated donor template. Once successful candidate clones were identified, the region was sequenced to confirm the integration and preservation of the host genome. Three heterozygous clones which had successfully undergone HDR with the genome otherwise intact were identified.

Table 10.2. Oligonucleotides used in the generation of FLAG-NCOA7-B SIM-A9 cells

Oligonucleotide name	Description	Sequence
NCOA7-C1	Guide RNA	5' -TTCTCACTGCTCACAATGAG-3'
FLAG-NCOA7B-HDR-Donor	HDR Donor Template	5' - TACCTTCACAAAAGGCTCTTGGTCAGGC CTGGCACAATAGAAAATCTGAATGTCCA GGGGCAGTCCTCTGCCTCTTCCGCCTCC TCCCCCGCCCTTGTCGTCATCGTCTTTG TAGTCCATGGTGCTGGGACAGAAGACTG TCCACAGAGAAGAATCGGTCTTTCTCTG GTTGCCTGCACACCAGCCACTGACT-3'
NCOA7B-Scrn-F	Screening primer for successful HDR	5' -AGACGATGACGACAAGGGCG-3'
NCOA7B-Scrn-R	Screening primer for successful HDR	5' -GGAATTCCAAACGCACGCCA-3'
NCOA7B-Seq-F	HDR site sequencing primer	5' -GTCCGCCCTGAGCAAAGACCCC-3'
NCOA7B-Seq-R	HDR site sequencing primer	5' -GGAATTCCAAACGCACGCCA-3'

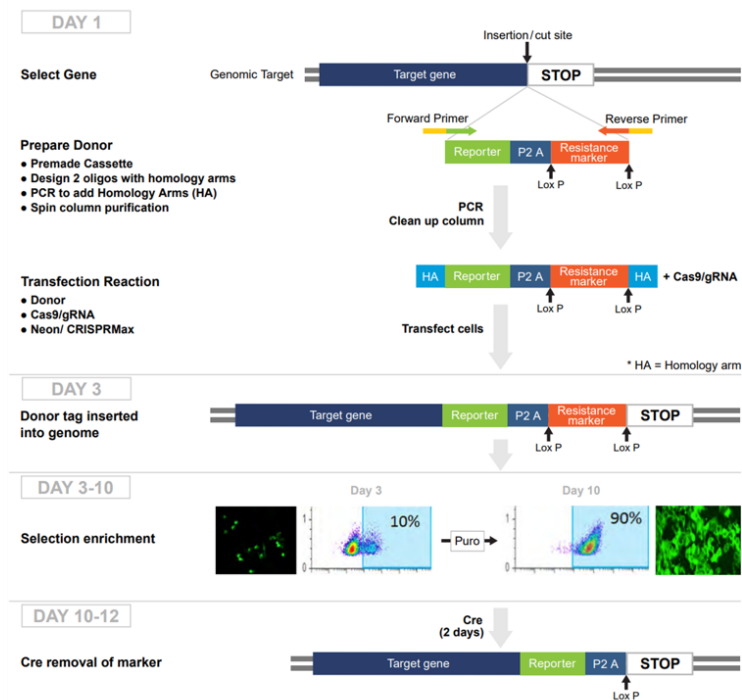
10.2.4 Tagging NCOA7-B with GFP in 293T cells

The TrueTag Donor DNA Kit, GFP (Invitrogen, A42992) was used to insert a GFP-tag at the N-terminus of NCOA7-B. This technology uses locus-specific primers to append homology arms to a Puro-T2A-GFP construct in PCR (Figure 10.1). The PCR product is then column-purified and delivered to cells as a repair template for HDR in

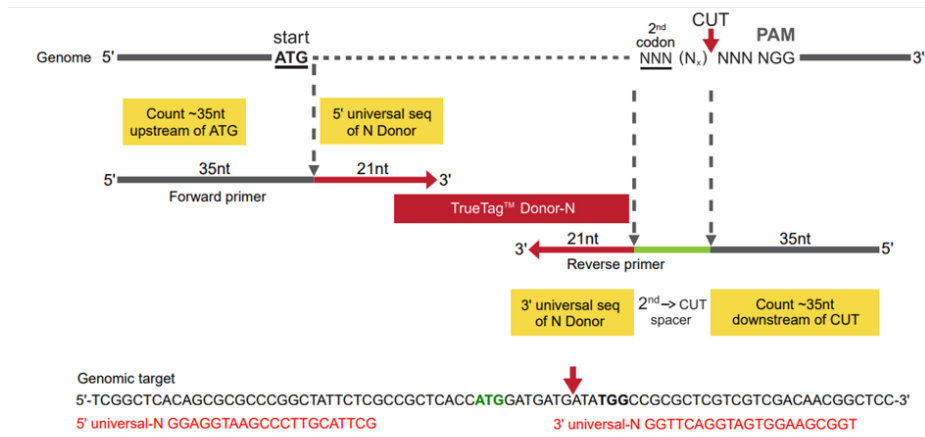
CRISPR-Cas9 editing. Primers and gRNAs (Invitrogen) used in the generation and verification of GFP-NCOA7B-293T cells are listed in Table 10.3. The donor template was generated as per manufacturer's instructions. Briefly, provided donor template was amplified in 30 cycles of PCR using the TrueTag Homology Arm Primers listed in Table 10.3. The PCR product was then column-purified in a GeneJET purification column and eluted in nuclease-free water. RNP was then delivered using Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Invitrogen, CMAX00008). Briefly, RNP was complexed of equimolar (7.5 pmol) Alt-R S.p. Cas9 Nuclease V3 and gRNA in the presence of 500 ng purified donor template. Transfection complexes were formed at room temperature for 10 minutes and then delivered to cells. Successfully edited cells were selected for using 1 µg/mL puromycin (Invivogen, ant-pr-1) for 7 days.

Table 10.3. Oligonucleotides used in TrueTag generation of GFP-NCOA7B-293T cells

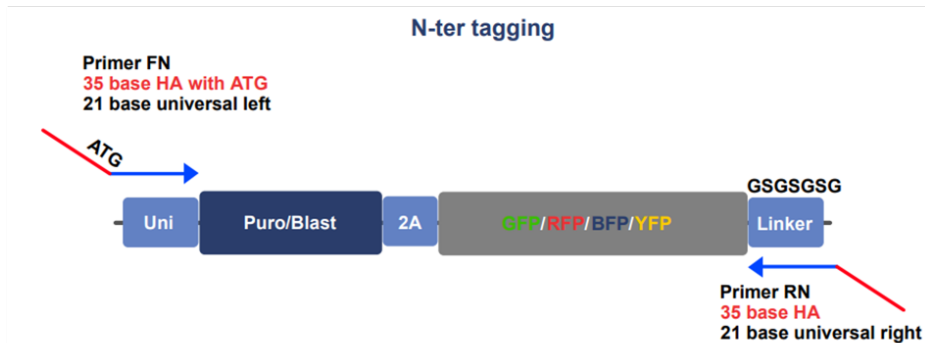
Oligonucleotide name	Description	Sequence
NCOA7-C1	Guide RNA	5' -TTCTCACTGCTCACAATGAG-3'
NCOA7-TrueTag-Fwd	TrueTag Homology Arm Primer	5' - TTAGAGAGAGAGCCACACTTCTCACTGC TCACAATGGGAGGTAAGCCCTTGCATTC G-3'
NCOA7-TrueTag-Rev	TrueTag Homology Arm Primer	5' - AAAATCTGGATGTCCAAGGGTAATCTTT GGCCTCTACCGCTTCCACTACCTGAACC -3'
NCOA7-Seq-Fwd	Cleavage detection/Sequencing Primer	5' -GCAGGAAGTGTGTTGCTCT-3'
NCOA7-Seq-Rev	Cleavage detection/Sequencing Primer	5' -GCAACCACACCATAACCCTC-3'
TrueTag-NPuroGFP-Fwd	TrueTag Sequencing/Verification primer	5' -GTCCGCCCTGAGCAAAGACCCC-3'
TrueTag-NPuroGFP-Rev	TrueTag Sequencing/Verification primer	5' -GGGTAATCGGCGAAGGCAGCGG-3'



A. TrueTag workflow



B. Homology arm design example.



C. Donor template design.

Figure 10.1. Invitrogen TrueTag workflow and design.

10.2.5 Transfection of 293T and HeLa cells

293T or HeLa cells were transfected using Lipofectamine 2000 (Invitrogen, 11668027), as per manufacturer's instructions. Briefly, 3×10^5 cells per well were seeded in a 6-well plate a day before transfection. On the day of transfection, 2.5 μ g construct was delivered per well at a DNA (μ g) to lipid (μ L) ratio of 1:2. Cells were left for 48 hours before experiments to allow expression of the construct.

10.2.6 iPSC culture

This project used KOLF2.1S iPS cells, a subclone of KOLF2-C1 (also known as WTSli018-B-1, RRID: CVCL_9S58), which is itself a subclone of KOLF2 (also known as WTSli018-B, RRID: CVCL_AE29). The KOLF2-C1 line, a reference human hiPSC line characterised previously⁶⁸, was corrected for a heterozygous polymorphism at the ARID2 locus, giving rise to KOLF2.1S. CRISPR-Cas9 mediated correction of the polymorphism, which may have affected neuronal development, was performed by the Wellcome Sanger Institute⁶⁹. These iPSC lines, derived from the fibroblasts of a healthy male of white-British descent, were reprogrammed with non-integrating Sendai virus vectors. Cell cultures were maintained in mTeSR1 medium (Stem Cell Technologies, 85850) on 1% Geltrex-coated plates (Gibco, A1413201). Work was covered under REC Reference 09/H0304/77, covered under HMDMC 14/013 and REC reference 14/LO/0345.

For maintenance, cells were dissociated into clumps using 0.5 mM EDTA in D-PBS, at 37°C for 5 minutes. After aspirating PBS-EDTA, cell clumps were washed with growth medium and passaged at a 1:6 ratio. Where necessary, centrifugation of iPSC was performed at 400 x g for 5 minutes at room temperature. Where a single iPSC suspension was required, cells were washed with D-PBS, then washed with TrypLE Express and

incubated at 37°C for 5 minutes before resuspending in the required medium supplemented with 10 µM Rho Kinase Inhibitor Y-27632 dihydrochloride (Abcam, ab120129) 1 hour before, and 24 hours after, dissociation. iPSC were cryopreserved in KnockOut DMEM (Gibco, 10829018) supplemented with 30% ESC-qualified FBS (Gibco, 16141079) and 10% DMSO. Upon banking, the karyotype of all KOLF2.1S-derived clones was assessed using SNP-array based karyotyping on an Illumina GSA Array-24 V2.0 (Life & Brain GmbH, Bonn) and analysed with GenomeStudio (Illumina). Differentiation experiments and CRISPR-Cas9 manipulation were performed within two passages from the parent iPSC stock to minimise aneuploidy.

10.2.7 Generation of co-isogenic *OXR1*^{-/-} iPSC clones

10.2.7.1 CRISPR Knockout Strategy

This protocol was developed by W. S. James and S. A. Cowley⁷⁰. gRNAs were designed with the assistance of CRISPOR⁶⁶ and Cas-OFFinder⁶⁷. Two strategies were used to generate *OXR1* LOF iPSC clones (Figure 10.2).

1. **A splice donor-targeting strategy** resulted in null expression of *OXR1* by inclusion of intronic DNA in a neo-exon. The gRNA sequences were targeted to the splice donor of ENSE00003675755 (exon 14), an exon common to all splice variants of *OXR1* (ENSG00000164830). The gRNA sequences used were:

37/Rev – 5' -GTACCATAAACAAGAGTCCATGG-3'

215/Fw – 5' -CCTTCAAAGGGATTATAGAGTGG-3'

2. **A truncation strategy** to excise the TLDc domain from *OXR1*, resulting in the expression of a TLDc-null *OXR1* protein. The gRNAs were designed flanking the conserved TLDc region. The sequence of these guides is:

24/Rev – 5' -TATTTAGAGCATCGATCCTGCGG-3'

10.2.7.2 RNP Preparation

Alt-R CRISPR-Cas9 custom crRNA oligonucleotides (IDT) were duplexed with tracrRNA (IDT, 1072532) at equimolar concentrations (44 μ M) at 95°C for 5 minutes in Duplex Buffer (IDT, 11-05-01-03). RNP complex was formed by incubating Alt-R S.p. Cas9 Nuclease V3 (IDT, 1081060) with equimolar (20 pmol) gRNA complexes at room temperature for 20 minutes in a total volume of 1 μ L.

10.2.7.3 iPSC Transfection

Transfection of RNP complexes was achieved using the Neon Transfection System Pipette (MPP100), Pipette Station (MPS100) and 10 μ L Kit (MPK1025), all from Thermo Fisher. Cells were treated with 10 μ M Y-27632 1 hour before transfection. iPSC were dissociated with EDTA, centrifuged, and washed with PBS as described above. Following EDTA removal, cells were washed with PBS and then mixed gently to achieve a single-cell suspension in Buffer R at a concentration of 2.2×10^7 cells/mL. 20 pmol of RNP complexes prepared above (1 μ L) were mixed with 200,000 cells in buffer R (9 μ L) along with 2 μ L of 10.8 μ M Alt-R Electroporation Enhancer (IDT, 1075915) diluted in Buffer R. Cells were pulsed once at 1400 V with a 20 ms width in a 10 μ L Neon Tip before incubating in a Geltrex-coated 24-well plate in mTeSR1 supplemented with 10 μ M Y-27632.

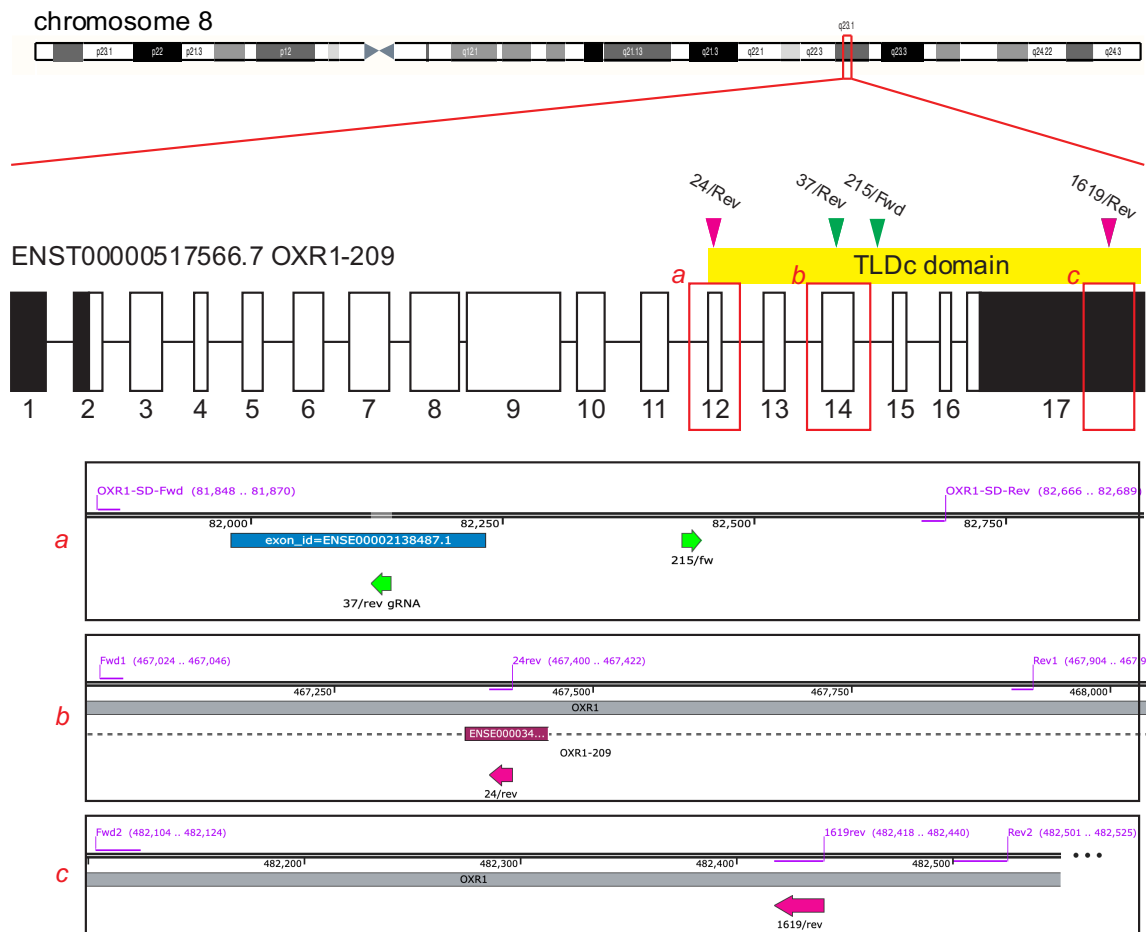


Figure 10.2. OXR1 knock-out strategy in hiPSC.

OXR1 is located on the q-arm of chromosome 8. ENST00000517566.7 (OXR1-209) is used as a representative transcript for OXR1 gene structure as it contains all common exons. gRNAs used to generate OXR1^{-/-} are shown in magenta, with gRNAs used to generate OXR1^{TLDC^{-/-}} shown in green. The exons encoding the TLDC domain are highlighted in yellow.

10.2.7.4 Single-cell clone expansion

Following transfection, cells were dissociated with TrypLE Express and plated at 2,000 cells per well in a 0.1% gelatin-coated 6-well plate on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells (Millipore, PMEF-CFL-P1) in mTeSR1 supplemented with Y-27632. After 7 days of expansion, colonies derived from individual cells were manually picked onto a Geltrex-coated 96-well plate in mTeSR1. Successfully edited clones were expanded, banked, and used for experiments within two passages.

10.2.7.5 Screening for successful genetic modification

After picking colonies into a 96-well plate, the plate was duplicated by dissociating cells with EDTA, as above. Cells of one replica plate were lysed in 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM NaCl, 0.25% Triton X-100 with 1 mg/mL proteinase K (Sigma Aldrich, P6556) at 55 °C in a humidified chamber overnight. Following lysis, genomic DNA (gDNA) was precipitated in 5 M NaCl in ethanol at -80 °C for 2 hours at room temperature. gDNA was washed twice with 70% ethanol and dried before resuspension in water at 4 °C overnight.

Once confluent, cells were dissociated using TrypLE Express and either cryopreserved or pelleted for gDNA extraction. gDNA was extracted as per manufacturer's instructions using the DNeasy Blood and Tissue kit (Qiagen, 69504) and sent for SNP array karyotyping.

10.2.8 Differentiation of iPSC to microglia

iPSC-derived microglia (iPS-microglia) were generated using existing protocols published previously by our lab^{71–73}. 4 E6 hiPSC were seeded in an AggreWell 800 plate (Stem Cell Technologies, 34811) in embryoid body (EB) medium (see Table 10.4 for composition) by centrifuging at 100 x g for 3 minutes. Cells in AggreWell plates were

fed daily with EB medium for 7 days. On day 7, EBs were removed from AggreWells into T175 flasks (factories) in OxM Factory Medium, a macrophage precursor differentiation medium described previously⁷⁴ and in Table 10.4. Factories were fed weekly for a maximum of 12 weeks and once macrophage precursors were produced, the supernatant was harvested through a 40 μ m strainer and resuspended in microglia medium, which has been described previously⁷³ and in Table 10.4. Precursors were plated at high, medium, or low density (1.9×10^5 , 9.4×10^4 , 4.7×10^4 cells/cm², respectively) and differentiated for 14 days before experiments, with a 50% medium change every 3-4 days.

Table 10.4. Differentiation medium composition

Growth Factor	Abbreviation	Supplier	Catalogue number	Concentration
<i>A. Embryoid Body (EB) Medium</i>				
mTeSR1	-	Stem Cell Technologies	85850	Base
Bone morphogenic protein 4	BMP4	Invitrogen	PHC9534	50 ng/mL
Stem cell factor	SCF	Miltenyi	130-096-629	50 ng/mL
Vascular endothelial growth factor	VEGF	Invitrogen	PHC9394	50 ng/mL
<i>B. OxM Factory Medium</i>				
Advanced Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture	ADMEM/F12	Gibco	12634010	Base
GlutaMAX	-	Gibco	35050-038	2 mM
Insulin	-	Sigma	I9278-5ML	5 µg/mL
Interleukin-3	IL-3	Invitrogen	PHC0033	25 ng/mL
Macrophage colony stimulating factor	M-CSF	Invitrogen	PHC9501	100 ng/mL
Tropolone	-	Sigma	T89702-1G	15 µM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (1M, pH 7.4)	HEPES	Gibco	15630080	15 mM
<i>C. Microglia Medium</i>				
Advanced Dulbecco's Modified Eagle Medium/Ham's F12 Nutrient Mixture	ADMEM/F12	Gibco	12634010	Base
GlutaMAX	-	Gibco	35050-038	2 mM
Granulocyte-macrophage colony stimulating factor	GM-CSF	Life Technologies	PHC2013	10 ng/mL
Interleukin-34	IL-34	Peprtech	200-34	100 ng/mL
Macrophage colony stimulating factor	M-CSF	Invitrogen	PHC9501	25 ng/mL
Transforming growth factor β-1	TGFβ	Peprtech	100-21C-250ug	50 ng/mL
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (1M, pH 7.4)	HEPES	Gibco	15630080	10 mM

10.2.9 iPSC-derived microglia treatments

After 14 days of differentiation, iPSC-derived microglia were treated with 100 ng/mL human recombinant IFN- β . To induce a classical M1 phenotype, microglia were treated with 100 ng/mL LPS and 20 ng/mL recombinant human IFN- γ (Gibco, PHC403). Treatments were for 6 hours (transcript quantification) or 24 hours (protein quantification).

10.2.10 Production and purification of lentivirus

293T cells were seeded in a T175 flask at 1.1×10^7 cells per flask the day before transfection. Cells were then transfected with 4.4 μ g, pMD2.G (Addgene, 12259), 5.5 μ g, psPAX (Addgene, 12260) and 9.2 μ g expression vector (pLV-Stuffer or pLV[Exp]-Bsd-SFFV>3xFLAG/3xGS/hOXR1[NM_001198533.2], FLAG-OXR1, VectorBuilder) in 1 mL JetPrime Buffer containing 50 μ L JetPrime Reagent (Polyplus, 101000015). Medium was harvested from cells at 24- and 48-hours post-transfection and stored at 4°C. Harvested medium was filtered through a 0.45 μ m PES filter (Starlab, E4780-1456) and centrifuged at $140,000 \times g$ at 4°C for 2 hours in an Optima XPN-80 ultracentrifuge (Beckman Coulter). The sedimented virus was resuspended in 1.5% bovine serum albumin (BSA) in PBS.

10.2.11 Transduction of microglia with lentiviral vectors

Microglia were co-infected with simian immunodeficiency virus (pSIV) *Vpx*-carrying virion-like particles (*Vpx*-VLP). *Vpx* is a virion-associated protein that targets mammalian SAM and HD domain-containing deoxynucleoside triphosphate (dNTP) triphosphohydrolase 1 (SAMHD1) for degradation, increasing the availability of free dNTPs thus enhancing retroviral integration^{75,76}. A range of *Vpx*-VLP (produced by Biomolecular Core Facility, University of Edinburgh) dilutions were applied to microglia and SAMHD1 protein levels measured by Western blot. The *Vpx*-VLP dilution which

reduced SAMHD1 to non-detectable levels was used (1:200-400). Lentivirus was functionally titrated in microglia using antibiotic selection (1:100-1:200). Macrophage precursors were co-infected with lentivirus and *Vpx*-VLP 24 hours after seeding in a 50% medium change. Selection was started 48 hours after infection, with 5 µg/mL puromycin or 5 µg/mL blasticidin (Invivogen, ant-bl-05), with daily 80% medium changes, until all of the cells in the non-transduced condition were dead (5 days).

10.3 Biochemical methods

10.3.1 RNA isolation, reverse transcription, and quantitative real-time PCR

Tissue was dissected immediately post-mortem and homogenised using 1 mL QIAzol Lysis Reagent (Qiagen, 79306) in Lysing Matrix D tubes and a FastPrep 5G homogeniser (MP Biomedicals, 116913050-CF). RNA was isolated as per QIAzol Lysis Reagent protocol using chloroform extraction and isopropanol precipitation with 70% ethanol wash. DNase I digestion (Qiagen, 79254) was performed on ≤ 20 μ g RNA, followed by purification using RNeasy MinElute Cleanup Kit (Qiagen, 74204) as per manufacturer's protocol.

Confluent cells in 6- or 12-well plates were washed in PBS and scraped into 350 μ L Buffer RLT and processed as per manufacturer's instructions using the RNeasy Mini Kit with on-column DNase I digestion (Qiagen, 74104). For RNA-seq, cells were harvested in buffer RLT and then sent to GENEWIZ (Azenta Life Sciences) for RNA extraction.

RNA from tissues or cells was resuspended in nuclease-free water and [RNA] was estimated by measuring optical density at 230 and 270 nm using a NanoDrop 8000 spectrophotometer (Thermo Fisher).

Reverse transcription of extracted RNA was performed at 42°C for 1 hour using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher, K1632) then terminating the reaction at 70°C for 5 minutes. RNA (2 μ g) was primed for reverse transcription by incubating at 65°C for 5 minutes with 1 μ L oligo(dT) (100 μ M) and 1 μ L random primers (100 μ M) and quenched on ice. cDNA synthesis was performed by adding 1 μ L RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/ μ L), 1 μ L

RiboLock RNase Inhibitor (20 U/ μ L), 2 μ L dNTP Mix (10 mM) and 5X Reaction Buffer in 20 μ L reaction volume.

Quantitative, real-time (q)PCR was performed in technical duplicates using 2X Fast SYBR Green Master Mix (Thermo Fisher, 4385612), 50 ng template, 300 nM sense primer, 300 nM antisense primer in a 20 μ L total reaction volume. qPCR primers were verified for 90-110% efficiency and validated for target specificity using melt curve and gel analysis. qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher) at 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds followed by a melting protocol of 95°C for 15 seconds, then 15 seconds at 0.35°C increments between 60°C and 95°C. No RT and no-template controls were included in each reaction. Relative quantification of transcript was performed using the $2^{-\Delta\Delta C_t}$ method⁷⁷ with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and 40S ribosomal protein S16 (*Rps16*, mouse) or *GAPDH* and Hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*, human) as reference genes. Primers were validated to have an amplification efficiency between 95-105% and products were electrophoresed to confirm the correct size of a single amplicon. Primer probe sequences and species for RT-qPCR are listed in Table 10.5.

Table 10.5. Primer probe sequences and species used for RT-qPCR

Target	Species	Strand	Sequence (5'-3')
Mouse primers (Mmu)			
Gapdh	Mmu	Forward	AGAACATCATCCCTGCATCC
Gapdh	Mmu	Reverse	CACATTGGGGGTAGGAACA
IFNb1	Mmu	Forward	CCAGCACTGGGTGGAATGAG
IFNb1	Mmu	Reverse	TGAGGACATCTCCACGTCA
IL6	Mmu	Forward	AACGATGATGCACTTGCAGA
IL6	Mmu	Reverse	CTCTGAAGGACTCTGGCTTTG
Ncoa7-FL	Mmu	Forward	TGTCGCTACTTCACTGATGG
Ncoa7-FL	Mmu	Reverse	GCGTCTTTTGATCTTCATGTG
Ncoa7-B	Mmu	Forward	GCCCCGAGACATTCAGATT
Ncoa7-B	Mmu	Reverse	CTGTGGGGCTGTAGGATAGG
Oxr1-FL	Mmu	Forward	CAGTCGTGACTGGACAGGTTT
Oxr1-FL	Mmu	Reverse	ATGGGCTACATCTGGAGTCG
Rps16	Mmu	Forward	TTCTGGGCAAGGAGCGATT
Rps16	Mmu	Reverse	GATGGACTGTGCGATGGCA
Human primers (Hsa)			
HPRT1	Hsa	Forward	AGGGTGTTTATTTCCTCATGGACTAA
HPRT1	Hsa	Reverse	TCCTTCATCACATCTCGAGCAA
ISG15	Hsa	Forward	CGCAGATCACCCAGAAGATCG
ISG15	Hsa	Reverse	TTCGTCGCATTTGTCCACCA
NCOA7-FL	Hsa	Forward	TGGAATACACTGCTGGAAACCA
NCOA7-FL	Hsa	Reverse	CCACCAATTTATTGGGAGTGATGTT
NCOA7-B	Hsa	Forward	AGAGAGCCACACTTCTCACTG
NCOA7-B	Hsa	Reverse	TGCGCTTTGCCTCTTCAACA
Oxr1-FL	Hsa	Forward	GCAGTTGTTACTGGACAGGTTCT
Oxr1-FL	Hsa	Reverse	AGCCTCAGATGATGTTGGTGAC
Oxr1-c	Hsa	Forward	CACTGTAGTAAGGCTCTAGTGCTG
Oxr1-c	Hsa	Reverse	TCCCATACCAGAGACGAGACAT

10.3.2 RNA sequencing preparation

Microglia were differentiated at 1 E6 cells per well for 14 days in 6 well plates. After differentiation, microglia were washed in PBS and scraped into 350 μ L Buffer RLT, then sent to GENEWIZ (Azenta Life Sciences) for RNA extraction and Illumina 2x150 bp sequencing with poly(A) selection and 20 million paired-end reads per sample. Four samples from independent factories were sent per *OXRI*^{-/-} hiPSC clone.

10.3.3 RNA sequencing analysis

RNA-seq analysis was performed by Szymon Stodolak in the James lab and the below methods section was authored by him.

Raw reads were pre-processed using fastp 0.20.1 software⁷⁸. In addition to the default filtering parameters, polyN sequences in read tails and adapter sequences were trimmed, and mismatched base pairs were corrected by overlap analysis. Filtered and trimmed reads were mapped to a full decoy-aware transcriptome using the Gencode version 34 reference human transcriptome and GRCh38 primary assembly genome. Salmon 1.2.1 selective alignment was used for transcript mapping and quantification with optional flags correcting for GC and random hexamer priming biases⁷⁹. All downstream analyses were done using the R 4.0 programming language. Transcript abundance estimates in TPMs were summarised to the gene level using the tximport 1.16.0 package to correct for sample-specific transcript length biases⁸⁰. Lowly expressed genes with length-scaled abundance estimates less than 10 in 3 samples were excluded by the default filtering function in the edgeR package⁸¹. The samples were normalised by variance stabilising transformation and projected onto a principal component analysis (PCA) to visualise the level of transcriptomic similarity⁸². Differential expression was tested using limma-voom⁸³. Volcano graphs were plotted to visualise the most significantly altered

genes using the EnhancedVolcano package⁸⁴. Gene Ontology and KEGG analyses were done using the enrichR package. The terms were ranked by an enrichment score which was defined as a harmonic mean of odds ratio and log₁₀ p-value.

10.3.4 Western blotting

Cells or tissue were lysed using the RIPA Lysis Buffer System (Santa Cruz, sc-24948) as per manufacturer's instructions. Lysate was cleared by centrifuging at 20,000 x g, 4°C for 20 minutes. Protein in the supernatant was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher, 23225) as per manufacturer's instructions, using BSA standards. After heat denaturation at 70°C for 10 minutes, samples were mixed with 4X Bolt LDS Sample Buffer and 10X Bolt Reducing Agent and electrophoresed through Bolt Bis-Tris Plus Mini Gels (all from Invitrogen, NW0412B) for the recommended time and voltage. Protein was then transferred to 0.45 µm nitrocellulose at 10 V for 1 hour using the Mini Blot Module (Invitrogen, B1000). Membranes were blocked in 4% milk protein (Millipore, 70166), 0.1% Tween-20 (Sigma Aldrich, P1379) in PBS for 1 hour at room temperature. Membranes were then incubated with primary antibodies in blocking buffer at 4°C overnight, followed by washing with PBS-Tween 20 (0.1%) and incubation with secondary antibodies in blocking buffer in the dark at room temperature for 1 hour. After washing, membranes were imaged using the Odyssey CLx and analysed using Empiria Studio (both from Li-Cor). Catalogue numbers and dilutions for antibodies used in Western blotting are listed in Table 10.6.

Table 10.6. Antibodies used in Western blotting.

Target	Host, clonality	Supplier	Catalogue number	Dilution used
ATP6V1A	Rabbit, polyclonal	ProteinTech	17115-1-AP	1:8,000
α -Tubulin	Mouse, monoclonal	Sigma	T5168	1:5,000
β -actin	Mouse, monoclonal	ProteinTech	66009-1-Ig	1:20,000
CD68	Mouse, monoclonal	ProteinTech	66231-2-Ig	1:1,000
DYKDDDDK (FLAG)	Rabbit, monoclonal	Invitrogen	701629	1:1,000
LC3 A/B	Rabbit, polyclonal	Cell Signalling Technology	4108S	1:1,000
NCOA7	Rabbit, polyclonal	ProteinTech	23092-1-AP	1:1,000
NCOA7	Mouse, monoclonal	Santa Cruz	sc-393427	1:1,000
OXR1	Rabbit, polyclonal	ProteinTech	13514-1-AP	1:1,000
p62	Mouse, monoclonal	Abcam	ab56416	1:1,000
Mouse	Goat, polyclonal	Li-cor	926-32210	1:20,000
Rabbit	Goat, polyclonal	Li-cor	926-68071	1:20,000

10.3.5 Immunoprecipitation

ChromoTek DYKDDDDK Fab-Trap Agarose beads (ProteinTech, ffa) were used to immunoprecipitate FLAG-tagged NCOA7-B as per manufacturer's protocol. Briefly, 1 E7 SIM-A9 cells were harvested and lysed in 200 μ L lysis buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% IGEPAL CA-630 [Sigma, I8896]) on ice for 30 minutes with trituration every 10 minutes. Lysate was centrifuged at 17,000 x g for 10 minutes at 4°C. The cleared lysate (supernatant) was then transferred to a pre-cooled tube, and 300 μ L dilution buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA), supplemented with protease inhibitor (Roche, 04693132001). 50 μ L of the diluted lysate was preserved for subsequent analysis as the input fraction. 25 μ L of bead slurry was

added to diluted lysate and rotated end-over-end for 1 hour at 4°C. Post-binding, beads were washed in wash buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% IGEPAL CA-630, 0.5 mM EDTA) centrifuging the beads at 2,500 x g for 5 minutes at 4°C. The supernatant was saved for further analysis (flow-through fraction). Beads were subsequently resuspended in 500 µL Wash buffer, and the washing process was repeated at least twice, with the beads sedimented at 2,500 x g for 5 minutes at 4°C. During the final washing step, beads were transferred to a new tube. Bound protein was eluted by boiling at 100°C for 5 minutes in 40 µL 4X Bolt LDS Sample Buffer before analysis by SDS-PAGE (see section 10.3.4

10.3.6 Immunocytochemistry

Cells, cultured on coverslips, were incubated with 4% paraformaldehyde (PFA) in PBS (Thermo Fisher, J19943.K2) at room temperature for 20 minutes, followed by washing with PBS. Fixed cells were blocked in 10% BSA, 0.3% Triton X-100 (X100, Sigma Aldrich) in PBS for 1 hour at room temperature then incubated with primary antibodies for 2 hours at RT. After washing with 0.3% Triton X-100 in PBS, cells were incubated with secondary antibodies for 1 hour at room temperature, followed by washing and mounting in ProLong Glass Antifade Mountant with NucBlue Stain (Invitrogen, P36981). To stain for V-ATPase holoenzyme, 1:100 recombinant SidK conjugated to Alexa Fluor 568 was included in the secondary antibody staining. SidK is a *Legionella pneumophila* effector protein that specifically binds the V-ATPase⁵⁵ and was a gift from Dr M. Maxson, The Hospital for Sick Children, Toronto, Canada.

Table 10.7. Antibodies used in immunocytochemistry

Target	Host, clonality	Supplier	Catalogue number	Dilution used
OXR1	Rabbit, polyclonal	ProteinTech	13514-1-AP	1:500-1:50
OXR1	Rabbit, polyclonal	Invitrogen	PA5-82973	1:500-1:50
HA tag	Goat, polyclonal	Novus	NB600-362	1:500
GFP	Mouse, polyclonal	ProteinTech	66002-1-Ig	1:200
Rabbit	Goat, polyclonal	Invitrogen	A21244	1:1,000
Goat	Donkey, polyclonal	Invitrogen	A11058	1:1,000
Mouse	Goat, polyclonal	Invitrogen	A28181	1:1,000

10.3.7 Genotyping PCR

Genomic DNA, extracted using the DNeasy Blood and Tissue Kit, was quantified by measuring optical density at 230 and 270 nm using a NanoDrop 8000 spectrophotometer. PCR of gDNA was performed using Platinum SuperFi II Master Mix (Thermo Fisher, 12368010) as per manufacturer's instructions. Briefly, 5-100 ng gDNA template was amplified with 10 μ M forward and reverse primer at 98°C for 30 seconds followed by 35 cycles of 98°C for 5 seconds, 60°C for 10 seconds (universal annealing temperature) and 72°C for 15 seconds, followed by a final extension at 72°C for 5 minutes. The PCR product was then electrophoresed through 1-3% agarose-tris acetate EDTA gel containing SYBR Safe DNA Gel Stain (Invitrogen, S33102) with appropriate loading controls and DNA marker ladder. Gels were imaged using a Bio-Rad Gel Doc XR+ and analysed using Image Lab software (Bio-Rad). Primers used for genotyping PCR are listed in Table 10.8.

Table 10.8. Primer sequences and species used in genotyping PCR

Target	Species	Use	Notes	Sequences (5'-3')
NCOA7	Mmu	To determine genotype of NCOA7-DEL mice.	WT penultimate coding exon	AGGCAATGCTGGAAATGACAGAGG
			WT 3' UTR and 3' end of deletion	CCCTGGAATGGATCATGTCATGTC
			WT 5' UTR and 5' end of the deletion	GATTGCCTCCCTCTTATGAAAGCC
OXR1	Hsa	Used to screen for splice donor removal of OXR1 in hiPSC	Flanking the splice donor to be excised	CTGTGTCTGGTTCAGGGTGATTC
				AAGTACTTGAAGTCTGCTTTTGA
OXR1	Hsa	Used to screen for TLDC domain excision	Flanking the 5' CRISPR site (exon 5' of TLDC domain exons)	AGACCGTTTAGTTTTTCAGACGTT
				CCAAGAATGCCTCAAGAACAGT
			Flanking the 3' CRISPR site (3' UTR)	AAGTGTGATTGCCAGGTTGC
				TGGTATATGCCACTATGACCTTATT

10.3.8 Sanger sequencing

For PCR product sequencing, PCR reaction was processed using the QIAquick PCR Purification Kit (Qiagen, 28104), eluted in the provided buffer EB and DNA was quantified using the NanoDrop 8000 spectrophotometer. Processed PCR products, along with primers were sent to Source BioScience (Cambridge) for Sanger Sequencing.

10.4 Functional Assays

10.4.1 Ratiometric lysosomal pH measurement using dextran conjugates

Oregon Green (OG) is a fluorescent dye which, when excited at 488 nm, is exquisitely pH sensitive. However, when excited at 425 nm, the emitted fluorescence is much more stable. Hence, using a dextran-conjugated OG, it will be trafficked to the lysosomes and the ratio of intensity at 488 nm to 425 nm can be used to measure lysosomal pH and simultaneously control for lysosomal content and photobleaching⁸⁵. Microglia were differentiated at 3 E4 cells per well in 96-well PhenoPlates (Perkin Elmer, 6055302) for 14 days. Microglia were exposed to 50 µg/mL Oregon Green-conjugated dextran (OG-dextran, 10,000 MW, Invitrogen, D7170) for 2 hours, followed by two washes with warm medium. Microglia were then left for 4 hours or overnight to allow dextran to traffic to lysosomes.

To build a standard curve, pH clamping buffers were used in OG-dextran loaded microglia. These buffers ranged from pH 3.5 to pH 7 at pH 0.5 increments and contained 20 µM nigericin (ApexBio, D7170) or 10 µM monensin (Abcam, AB120499) to allow for isotonic intracellular pH clamping. The buffer composition was 140 mM KCl (all from Sigma, P9541), 1 mM CaCl₂ (C8106), 1 mM MgCl₂ (M2670), 5 mM glucose (G7528). Buffers pH > 6.5 were buffered with 15 mM HEPES (all from Sigma, 54457), buffers pH 5.5-7 were buffered with 15 mM MES (69889) and buffers pH < 5.5 were buffered with potassium acetate (236497). pH was achieved using a pH calibrator and adjustment with 3 M KOH (Sigma, 221473) or 8.2 mM glacial acetic acid (Alfa Aesar, 10994). Cells were incubated in buffers for 5 minutes, before imaging.

Cells were imaged at 37°C, 5% CO₂ automatically using the Opera Phenix High Content Screening System (Perkin Elmer). Analysis was performed using Columbus.

Briefly, the pixel intensities after excitation at 488 nm were divided by the intensities after 425 nm excitation, a threshold was then applied to the resulting image to remove background and the mean intensity per field was measured.

10.4.2 Measuring lysosomal activity using DQ-BSA

Microglia were differentiated at 3 E4 cells per well in 96-well PhenoPlates for 14 days. Cells were treated with the following drugs for 30 minutes (all dissolved in DMSO): 50 nM Concanamycin A (Santa Cruz Biotechnology, sc-202111); 10 μ M Dynasore I (Cayman Chemical, 14062); equimolar (50 μ M) cocktail of pepstatin (Enzo Life Sciences, ALX-260-085-M005), leupeptin (Enzo Life Sciences, ALX-260-009-M005) and E64d (Abcam, Ab144048). Microglia were then incubated with 10 μ g/mL DQ-Red-BSA (Invitrogen, D12051) and 50 μ g/mL Alexa Fluor 488-BSA (Invitrogen, A13100), both dissolved in water, for 1 hour. Cells were then washed thrice with warm medium and incubated for 2 hours before imaging.

Cells were imaged at 37°C, 5% CO₂ automatically using the Opera Phenix High Content Screening System. Images were analysed using Columbus. Briefly, the intensity, area, and integrated density of DQ-Red-BSA and Alexa Fluor 488-BSA were calculated as per Section 10.5.1 and Alexa Fluor 488 signal was used as a normalisation factor for endocytosis.

10.4.3 Lysosomal imaging using Lysosensor and LysoTracker

Microglia were differentiated at 3 E4 cells per well in 96-well PhenoPlates for 14 days. Cells were incubated with 2 μ M Lysosensor Green DND-189 (Invitrogen, L7535) or 50 nM LysoTracker Red DND-99 (Invitrogen, L7528) for 30 minutes and then imaged at 37°C, 5% CO₂ automatically using the Opera Phenix High Content Screening System. For flow cytometry, cells were washed with D-PBS, incubated for 5 minutes with TrypLE

Express, and then resuspended to approximately 1 E6 cells per mL in D-PBS, supplemented with 1 mM EDTA and 1% FBS.

10.4.4 Flow Cytometry

Flow cytometry was performed using an LSRFortessa X-20 (BD) with UV (355nm), Violet (405nm), Blue (488nm), Yellow-Green (561nm) and Red (640nm) lasers. Specific laser-filter configurations are denoted in figure legends.

10.4.5 High-content imaging of hiPS-microglia

For images captured using the Opera Phenix High Content Screening System, a minimum of 10 fields per well were captured using the 40x water (Perkin Elmer, HH14000422, 1.1 NA) or 63x water objective (Perkin Elmer, HH14000423, 1.15 NA) lens. A minimum of 6 planes were captured on the Z-axis at 0.5 μm , the minimum recommended distance between planes required to satisfy Nyquist's sampling theorem⁸⁶.

10.5 Data analysis and statistics

10.5.1 Image analysis

Analysis of images captured using the Opera Phenix High Content Screening System was performed using Columbus Image Data Storage and Analysis System (CambridgeSoft). Generally, analysis pipelines developed in Columbus followed the following process: Z-stacks were processed into maximum intensity projections; nuclei were identified using method B and this was used as a proxy for cell count; image regions were found using an absolute threshold set at the minimum value above background (determined using an unstained sample); intensity and morphology properties were calculated for the regions of interest. Integrated density was defined as the mean intensity for the region of interest multiplied by the sum of pixels in the region.

Images captured using the EVOS M7000 or Zeiss LSM-700 confocal microscope were analysed using FIJI⁸⁷, CellProfiler⁸⁸, ZEN software (Zeiss).

10.5.2 Analysis software

Western blot images were analysed using EmpiriaStudio (Licor). Analysis of flow cytometry data was performed using FlowJo (BD, v10.8.1). Sequence alignment and genome editing design were performed with SnapGene (Dotmatics). Graphs and statistical analysis were performed using GraphPad Prism (Dotmatics).

10.5.3 Statistics

Data are presented as mean $\pm$ SEM, except where stated otherwise. Data were assumed to be normally distributed and analysed with either Student's t-test (two groups) or one- or two-way ANOVA (more than two groups) with Dunnett's post-hoc correction where groups are compared to a single condition (i.e. WT). $P < 0.05$ is considered significant.

11 Identifying and characterising the interferon-stimulated NCOA7-B isoform

11.1 Chapter Abstract

NCOA7-B is a novel ISG that has been demonstrated to be upregulated in immune cells, including macrophages, in response to pro-inflammatory stimuli and potentiates V-ATPase activity and lysosomal function^{7,39,42}. However, the expression level and function of *NCOA7-B* in microglia have not been explored. Here, I have demonstrated stimulation of peripheral inflammation by intraperitoneal injection of LPS in mice also induces *Ncoa7-B* expression in the cerebral cortex, indicating a role in the cerebral response to inflammation. However, localisation of transcript expression *in situ* was not achievable using modified oligonucleotide probes. It was hypothesised microglia would upregulate *NCOA7-B* expression in the brain, based on previous evidence of induction in macrophages^{7,42}. Indeed, TLR3, TLR4 and interferon- α/β receptor (FNAR) agonism caused a robust upregulation of *NCOA7-B* transcript that was conserved in mouse and human microglia. Furthermore, inhibition of STING in poly(I:C)-treated mouse microglia inhibited IFN β expression, thus *Ncoa7-B* upregulation; together indicating an autocrine IFN β signalling loop downstream of TLR agonism drives *Ncoa7-B* upregulation. This study also aimed to tag the N-terminus of NCOA7-B to allow quantification and localisation of NCOA7-B protein, since detection of NCOA7-B with a commercial antibody raised to the C-terminal domain was unable to detect NCOA7-B protein expression on a Western blot. Whilst IFN-induced transcript expression was maintained after the insertion of an N-terminal FLAG tag, FLAG-NCOA7-B protein could not be detected using anti-FLAG antibodies or immunoprecipitated with anti-FLAG agarose

beads. Finally, GFP tagging of NCOA7-B in 293T cells indicated an IFN β -stimulated upregulation of GFP-NCOA7-B protein, however, the protein could not be localised within cells. Thus, this chapter presents novel findings of robust, inflammation-associated upregulation of *NCOA7-B* in microglia. Furthermore, it presents a model – GFP-NCOA7B-293T cells – to interrogate the molecular function and interacting partners of NCOA7-B, including V-ATPase subunits.

11.2 Introduction

11.2.1 NCOA7-B is a widely expressed, interferon-inducible, short isoform of NCOA7

The short C-terminal isoform of *NCOA7* (also known as interferon-inducible NCOA7 or NCOA7 alternative start (-AS)), and hereafter referred to as NCOA7-B to avoid confusion with the antisense transcript of *NCOA7*) is a 219 residue (221 residue in *Mus musculus*) protein expressed from the *NCOA7* locus. NCOA7-B has a short N-terminus, encoded by a unique first exon, that is not shared with the full-length isoform (NCOA7-FL) and includes a putative *cis* interferon-stimulated response element (ISRE) identified in murine bone marrow-derived macrophages⁷. The interferon responsive nature of *NCOA7-B* expression was first demonstrated by exon microarray of peripheral blood mononuclear cells (PBMCs) from multiple sclerosis patients treated with IFN β ; and has since been reported in human and mouse and in different cells, including myeloid immune cells. For example, *NCOA7-B* has been detected by RT-qPCR in PBMCs from healthy individuals and foetal brain cells treated with IFN β *in vitro*, by Northern blot in human fibrosarcoma cells (HT1080), neuroblastoma cells (SH-SY5Y) and murine monocyte-derived macrophages treated with LPS or IFN β as well as human monocyte or iPS-derived macrophages stimulated with LPS^{7,42}. Additionally, NCOA7 enrichment at the protein level has been demonstrated in murine dendritic cells by Western blot and flow cytometry. However, since the details of the antibody used to detect NCOA7 in this study are not given, nor the molecular weight of the detected protein, the specific isoform of NCOA7 that is expressed cannot be inferred⁸⁹. Additionally, it has been demonstrated that LPS upregulates *NCOA7-B* expression. However, this upregulation is likely a result of autocrine IFN β signalling, since LPS stimulation via TLR4 engagement in macrophages induces IFN β expression⁹⁰. In

summary, the interferon-inducible nature of *NCOA7-B* is well conserved across human and mouse cell types. However, demonstration of expression has been limited to transcript only and endogenous *NCOA7-B* protein expression has not been demonstrated or quantified in any experimental system to date.

11.2.2 *NCOA7-B* is a TLDC domain-containing protein with potent oxidative stress resistance properties

The crystal structure of the TLDC domain in zebrafish¹⁸, fly⁹¹ and human⁶³ has been resolved. Whilst the 53-residue N-terminus of *NCOA7-B* is not included in these data, bioinformatic analysis of the human TLDC domain from *NCOA7-B* predicts a disordered structure with two beta-strands at residues 10-15 and 24-28⁶³. Whilst it is difficult to hypothesise the function of *NCOA7-B* from the structure of its TLDC domain, this model is invaluable for comparing TLDC domain conservation and mutation mapping, with predictions of polymorphism effects at the molecular level, all of which are discussed in the general introduction.

NCOA7-B has strong antioxidant properties when overexpressed in the N2a murine neuronal cell line and this is dependent on the conservation of specific residues¹⁵. Arsenite-induced protein S-nitrosylation (a specific marker of nitric oxide-induced oxidative stress), ROS levels and cell death in N2a cells were limited by *NCOA7-B* overexpression¹⁵. Notably, whilst arsenite-induced oxidative stress was sufficient to upregulate *NCOA7-FL* transcript by around 50% in murine primary cortical neurons, *NCOA7-B* transcript was not induced by arsenite treatment¹⁵. Finally, the neuroprotective effects of ectopic *NCOA7-B* expression were abrogated by targeted alanine substitution of G93, S95, and L204; these residues were identified as the most conserved across species, suggesting a central role in stress resistance. Mechanistically, these residues were

predicted to preserve the structural integrity of the TLDc domain by bridging α -helices and maintaining hydrogen bonding¹⁵, supporting the hypothesis that the TLDc domain is essential for the stress resistance properties of TLDc proteins and this is directly related to its structure. Overall, the data indicates a potent neuroprotective role of *NCOA7-B* in oxidative stress, dependent on specific residues to maintain structural integrity and highlights opportunities for exploration of this function in myeloid cells, where *NCOA7-B* is strongly induced by proinflammatory stimuli.

11.2.3 NCOA7-B inhibits endosome-mediated viral entry by promoting vesicular acidification and interacts with the V-ATPase

As an integral gene product induced by the antiviral interferon response, the function of *NCOA7-B* has been investigated in viral infection. Overexpression of *NCOA7-B* in human glioblastoma U87MG cells was observed to restrict viral entry via endosomes and therefore limit nucleoprotein import³⁹. This restriction mechanism functioned by augmenting vesicular acidification, which was determined by Lysosensor staining intensity, and limiting viral escape by preventing endosome-viral membrane fusion. This is hypothesised to be a result of the interaction of *NCOA7-B* with V1-domain V-ATPase subunits A, B2, E1 and/or G2, hence increased lysosomal activity, demonstrated by elevated cathepsin activity and increased DQ-OVA cleavage³⁹. V-ATPase function was not directly measured, nor was vesicular pH quantified, however, it is reasonable to infer elevated V-ATPase activity given the relative increase in vesicular acidity and increased lysosomal degradation of substrates. Thus, akin to other TLDc domain-containing proteins, including *NCOA7-FL*, ectopic *NCOA7-B* has been demonstrated to interact with the V-ATPase. In the aforementioned study, ectopic HA-tagged *NCOA7-B* was demonstrated to interact with V1-domain subunits, which has been hypothesised to be the mechanism of increased vesicular acidification³⁹. However, likely

due to the lack of specific probes for NCOA7-B, interaction of the endogenously expressed protein with the V-ATPase has not yet been explored.

11.2.4 Aims and hypothesis

11.2.4.1 Cellular and subcellular distribution of NCOA7-B remains unresolved

Unlike NCOA7-FL, which contains multiple domains, NCOA7-B consists almost entirely of the TLDc domain. Unfortunately, the short, unique, first exon of the gene has made developing probes for localisation of endogenous *NCOA7-B* (both oligonucleotides and antibodies) challenging. Thus, the distribution of expression of NCOA7-B within tissues and cell types is limited to bulk transcript quantification by RT-PCR and Northern blot. The discovery of a specific probe for *NCOA7-B* localisation would allow us to resolve the spatial distribution of the transcript and/or protein within tissue and at the subcellular level.

11.2.4.2 Endogenous NCOA7-B protein expression has not been demonstrated

Furthermore, the level of endogenous NCOA7-B protein expression has not been demonstrated and data are limited to quantification of transcript. Thus, it is currently unknown whether the endogenous transcript is translated and whether protein expression and stability are maintained after translation.

11.2.4.3 The expression and function of NCOA7-B in microglia remain unexplored

Finally, despite robust transcript expression in both the brain and in macrophages⁷, the expression and function of *NCOA7-B* in microglia have not been delineated. Investigating the biology of *NCOA7-B* presents an opportunity to explore novel gene function in microglia, especially in diseases with a strong interferon component, such as multiple sclerosis and viral infection.

11.2.4.4 Hypotheses

H₁ – *Ncoa7-B* is expressed in microglia within the mouse brain.

H₂ – Endogenous NCOA7-B protein expression is interferon inducible.

H₃ – Flag-tagged NCOA7-B interacts with the V-ATPase on lysosomes.

11.2.4.5 Aims

The aims of this study were to:

1. Localise *Ncoa7-B* transcript in the mouse brain *in situ* using modified oligonucleotide probes with enhanced specificity;
2. Epitope tag the N-terminus of endogenous NCOA7-B with a FLAG (DYKDDDDK) peptide in a mouse microglial cell line to quantify and localise protein expression;
3. Co-localise FLAG-tagged NCOA7-B with lysosomal compartments and the V-ATPase within mouse microglia;
4. Identify interacting partners of NCOA7-B using FLAG-mediated co-immunoprecipitation and time-of-flight mass spectrometry.

11.3 Results

11.3.1 *Ncoa7-B* transcript upregulation is conserved in human and mouse microglia in response to TLR and interferon signalling

To explore the hypothesis that microglia respond to inflammation by expressing *Ncoa7-B*, toll-like receptor (TLR) 3 and 4 agonists, polyinosinic:polycytidylic acid (poly(I:C), 100 µg/mL) and LPS (10 µg/mL) were used to induce an inflammatory response in BV-2 (a commonly used, oncogene-immortalised microglial cell line) and SIM-A9 (spontaneously immortalised) murine microglia. Both cell lines demonstrated stable expression of *Ncoa7-FL* in response to stimulation, however, expression of *Ncoa7-B* was increased 10-fold in BV-2 cells treated with LPS or poly(I:C) (Figure 11.1a). More strikingly, expression increased 100-fold in SIM-A9 microglia stimulated with LPS or poly(I:C) (Figure 11.1b), thus, confirming that *Ncoa7-B* is potentially a critical gene product of the microglial inflammatory response.

Since *Ncoa7-B* enrichment is reported to be dependent on interferon (IFN) stimulation, with a putative ISRE identified proximal to the promoter region of *Ncoa7-B*, it was hypothesised that autocrine IFN β signalling in response to TLR3 stimulation was responsible for the upregulation of *Ncoa7-B*. Indeed, in SIM-A9 cells, expression of IFN β preceded *Ncoa7-B* upregulation in response to poly(I:C) (Figure 11.1c). Interestingly, poly(I:C) induction of both IFN β and *Ncoa7-B* was inhibited by the small molecule stimulator of interferon genes (STING) inhibitor, H-151 (Figure 11.1d).

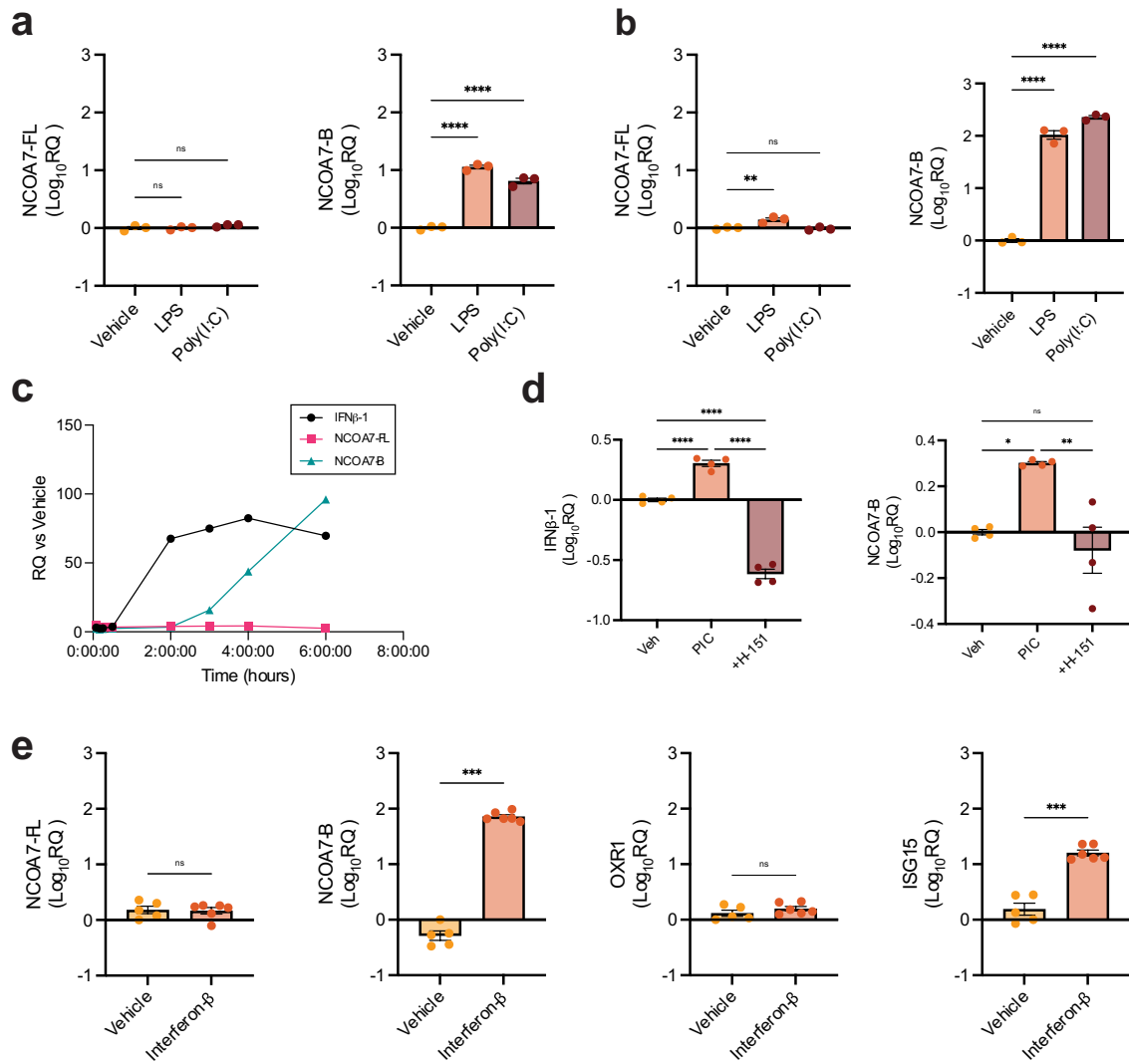


Figure 11.1. Expression of NCOA7-B transcript in microglia.

a,b, Expression of full-length (FL) and interferon-inducible (B) NCOA7 in BV-2 (a) and SIM-A9 (b) microglia treated with 100 µg/mL lipopolysaccharide (LPS) or 10 µg/mL polyinosinic:polycytidylic acid (poly(I:C)) for 4 hours (n = 3 independent experiments using cells at different passages). c, Expression of NCOA7-FL, NCOA7-B and interferon β-1 (IFNβ-1) over time in SIM-A9 cells treated with poly(I:C) (n = 1). d, Expression of IFNβ-1 and NCOA7-B in SIM-A9 cells treated as above with poly(I:C) or co-treated with 10 µM H-151 (n = 4 independent experiments using cells at different passages). e, Expression of NCOA7-FL, NCOA7-B, OXR1 and ISG15 in iPSC-derived microglia (14-16 days post-differentiation) treated with 100 ng/mL recombinant human IFNβ for 4 hours (n = 5 independent experiments/differentiations). RT-qPCR experiments were analysed using the 2^{-ΔΔCt} method⁹² with *Gapdh* and *Rps16* (mouse) or *GAPDH* and *HPRT1* (human) as reference genes (see section 10.3.1). One-way ANOVA with Dunnett's post-hoc correction or Student's unpaired t-test. ns = not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Finally, IFN β -induced expression of *NCOA7-B* was measured in human iPSC-derived microglia to identify the conservation of this induction pathway from the mouse data presented above. Similar to SIM-A9 mouse microglia, hiPSC-microglia, confirmed to be IFN β -responsive with interferon-stimulated gene 15 (*ISG15*) upregulation, showed a 100-fold enrichment of *NCOA7-B* transcript (Figure 11.1e). Meanwhile, the expression of *NCOA7-FL* and its homologue, *OXR1*, remained constant. Together, these data identify a consistent role for *NCOA7-B* in the microglial inflammatory response in human and mouse cells.

11.3.2 *Ncoa7-B* is stimulated in the mouse brain in response to peripheral inflammation

Unpublished data from the Oliver research group demonstrated that the *Ncoa7-B* transcript is induced in mouse hippocampal tissue following peripheral treatment with LPS, implying that *Ncoa7-B* has a yet unexplored function in the brain inflammatory response. To confirm this finding, adult wild-type mice were injected with 250 μ g/kg LPS into the peritoneal cavity for 4 hours; RT-qPCR analysis of brain somatosensory cortical tissue confirmed an approximate 6-fold increase in *Ncoa7-B* expression, whilst expression of the full-length isoform (*Ncoa7-FL*) remained stable (Figure 11.2a). These data indicate that *Ncoa7-B* upregulation is conserved in both the hippocampus and cortex in response to inflammation, however as expression data are from bulk tissue, the cell types responsible for upregulation could not be identified and upregulation in other brain regions is unknown. However, it is hypothesised that upregulation is mediated by microglia as the primary immune cells distributed throughout the brain parenchyma.

11.3.3 *In situ* hybridisation with locked nucleic acid probes fails to localise the *Ncoa7-B* transcript in the mouse brain

Since expression of *Ncoa7-B* in response to proinflammatory signalling has been demonstrated specifically in myeloid cells, it was hypothesised that microglia were the principal cells responsible for upregulation in the brain. Since no commercial antibodies against NCOA7-B are available and previous attempts by the Oliver group to raise an antibody had failed, *in situ* hybridisation (ISH) was employed to localise the *Ncoa7-B* transcript in the brain. Expression of *Ncoa7-FL* has been robustly demonstrated in the Purkinje cell layer of the cerebellum^{15,2}; thus this region was used to demonstrate the specificity of probes for the full-length isoform. The spatial distribution of *Ncoa7-B* has not been characterised, however since microglia are distributed throughout the brain parenchyma it was hypothesised that signal would be detectable in the cerebellum, which has a comparable density of microglia to the cortex, despite an increased neuronal density⁹³. *Ncoa7-B* has a short, unique 5' exon (231 nucleotides) which differentiates it from other *Ncoa7* transcript variants, so to enhance the specificity of ISH, locked nucleic acid (LNA) probes were used initially with designs recommended by the manufacturer's *in silico* pipeline. LNA probes have greater specificity due to a 2'-O, 4'-C methylene bridge in the probes which primes the oligomer for Watson-Crick base pairing. However, these probes were unable to demonstrate sufficient specificity, evidenced by substantial signal in *Ncoa7*^{DEL/DEL} brain tissue from mice treated with LPS, as above (Figure 11.2b). An *Ncoa7-FL* DIG-labelled cDNA probe worked well, demonstrating an absence of transcript in the knockout brain, and displaying a known pattern of enrichment of *Ncoa7* in WT Purkinje cells².

11.3.4 BaseScope *in situ* hybridisation fails to localise *Ncoa7-B* in the mouse brain

BaseScope are proprietary ISH technologies that afford superior specificity and sensitivity. BaseScope employs a shorter probe design than *in vitro* transcribed cDNA probes, consisting of multiple pairs of oligonucleotide probes that bind in tandem on the target RNA, allowing for signal amplification only when all pairs are bound. Thus, a BaseScope probe was designed bespoke to the first, unique exon of *Ncoa7-B* and was employed to detect *Ncoa7-B* in brain sections from LPS-treated mice. As anticipated, the *Ncoa7-FL* probe displayed enrichment in the Purkinje cell layer which has been demonstrated previously². Furthermore, the absence of *Ncoa7-FL* signal in *Ncoa7^{DEL/DEL}* brains demonstrates the suitability of *Ncoa7^{DEL/DEL}* mice as a true, null control for localisation and expression studies. However, the *Ncoa7-B* probe lacked specificity with off-target binding in the *Ncoa7^{DEL/DEL}* tissue (Figure 11.3a).

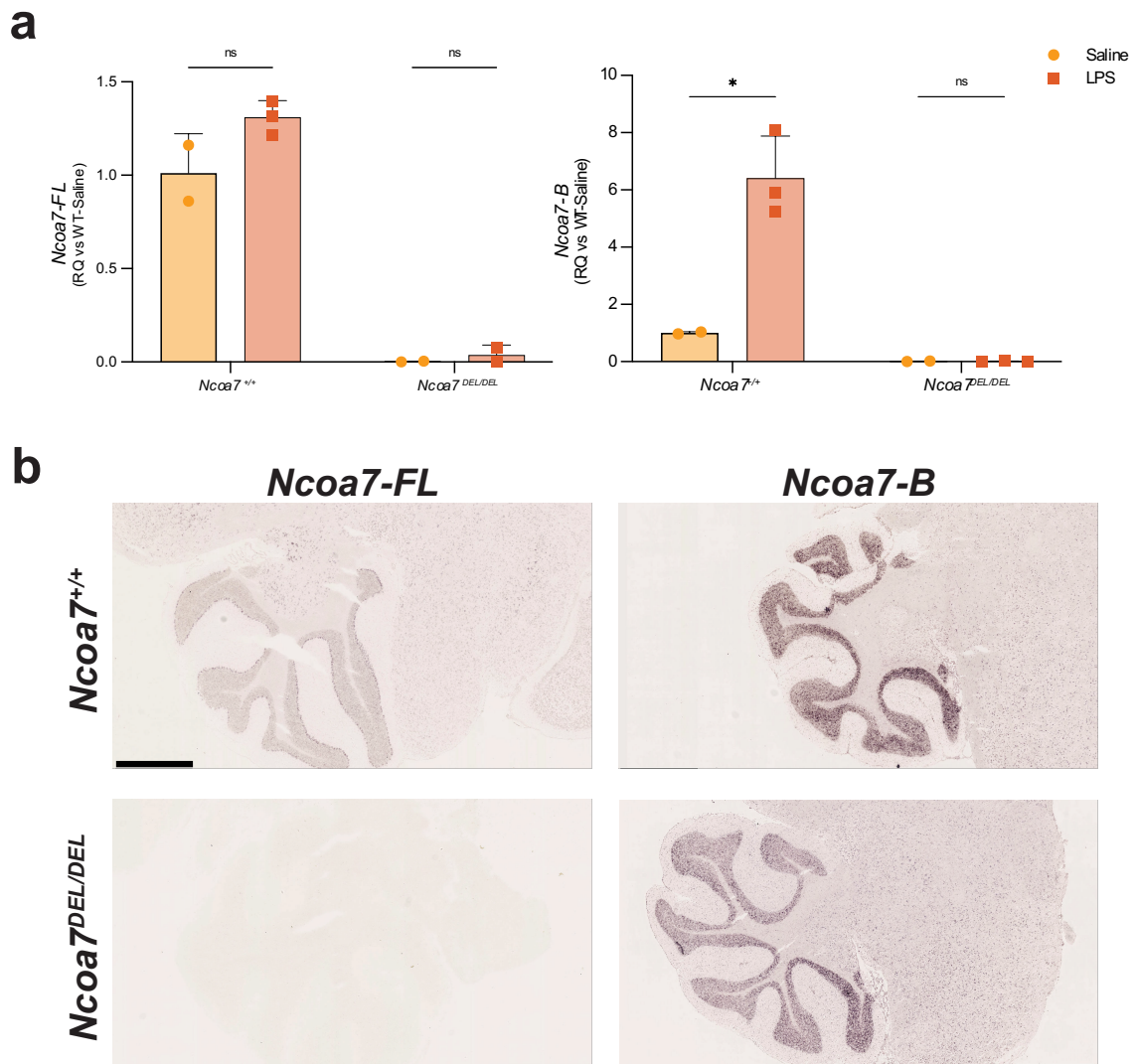


Figure 11.2. Exploring expression of *Ncoa7-B* transcript in mouse brain.

a, RT-qPCR of full-length (NCOA7-FL) and interferon-inducible (NCOA7-B) NCOA7 in somatosensory cortical tissue from mice (wild type, NCOA7^{+/+} and knock-out, NCOA7^{DEL/DEL}) injected i.p. with 250 μ g/kg lipopolysaccharide (LPS) in saline for 4 hours (n = 2-3 adult female mice). RT-qPCR experiments were analysed using the $2^{-\Delta\Delta C_t}$ method⁹² with *Gapdh* and *Rps16* as reference genes (see section 10.3.1). b, *In situ* hybridisation of NCOA7-FL and NCOA7-B transcripts in WT and DEL mouse brain treated with LPS as above. Scale bar is 1 mm. Student's t-test, unpaired. ns = not significant, * P < 0.05.

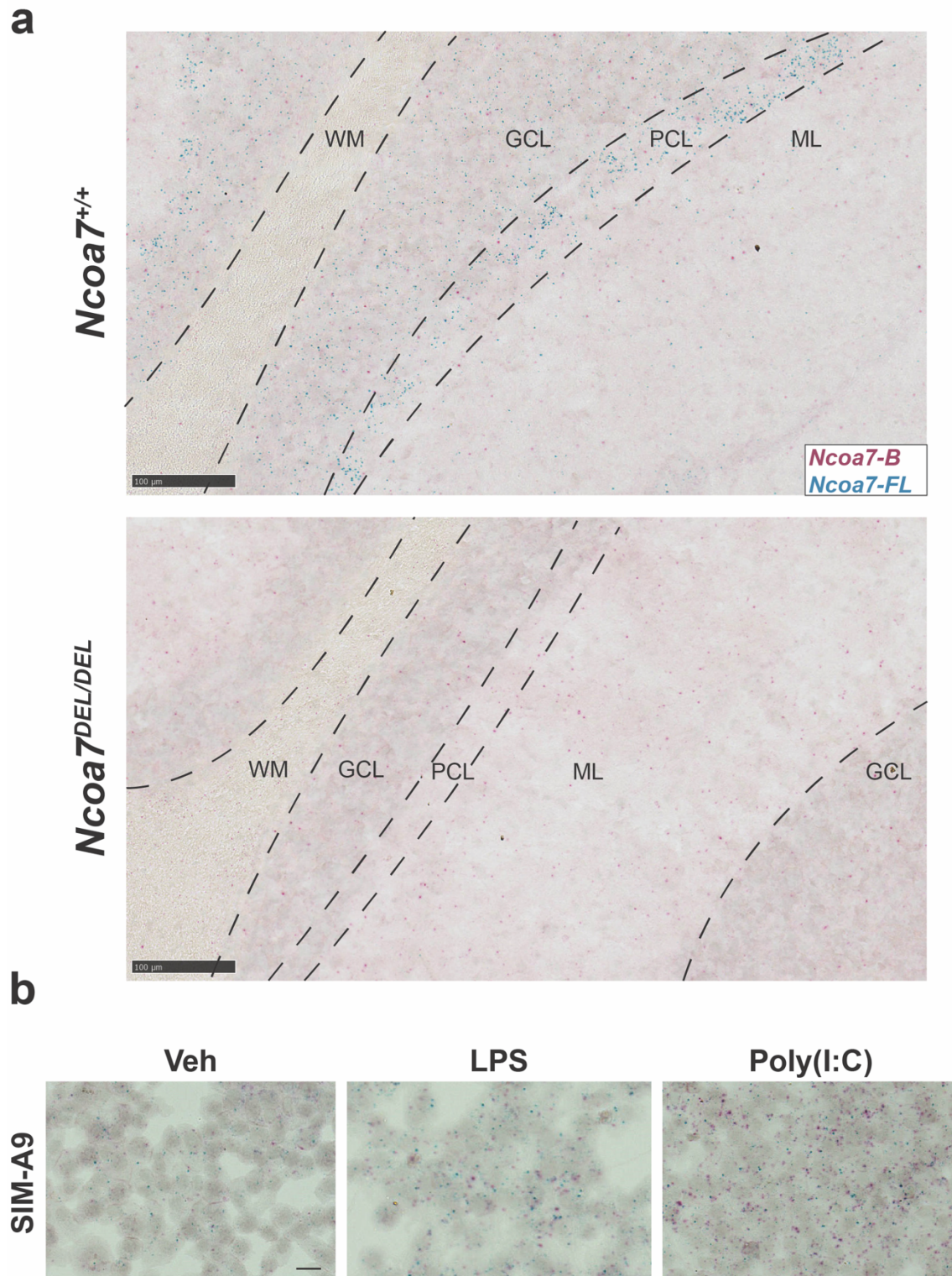


Figure 11.3. BaseScope detection of *Ncoa7-B*.

a, BaseScope *in situ* hybridisation (ISH) of the full-length (NCOA7-FL, blue) and interferon-inducible (NCOA7-B, red) transcripts in the cerebellum of mice (*Ncoa7^{+/+}*, WT and *Ncoa7^{DEL/DEL}*, DEL) injected i.p. with lipopolysaccharide (LPS) in saline (250 μg/kg) for 4 hours. Scale bar is 100 μm. b, BaseScope ISH of

NCOA7-FL and NCOA7-B (as above) SIM-A9 microglia treated with 10 µg/mL LPS or 100 µg/mL polyinosinic:polycytidylic for 4 hours. Scale bar is 50 µm. WM – white matter; GCL – granule cell layer; PCL – Purkinje cell layer; ML – molecular layer.

As I had demonstrated previously a robust upregulation of *Ncoa7-B* transcript in poly(I:C)-stimulated SIM-A9 microglia, these cells were used to demonstrate whether the BaseScope *Ncoa7-B* probe was sufficiently sensitive to detect upregulation. The same BaseScope probes were used to detect transcripts in SIM-A9 microglia. These cells displayed an enrichment of *Ncoa7-B*, suggesting that, whilst the probe lacks specificity it may be sensitive to *Ncoa7-B* enrichment (Figure 11.3b). Furthermore, these cells express *Ncoa7-FL* at a detectable level which does not appear to change with inflammatory stimulation.

11.3.5 NCOA7-B protein is not identifiable by immunoblot

Data supporting upregulation of *Ncoa7-B* is mostly limited to transcriptomic studies from mouse and human cells with novel data presented above indicating upregulation in the mouse brain in response to i.p. injection with LPS. To identify a functional role for *NCOA7-B*, it would be advantageous to identify and characterise NCOA7-B protein. To identify endogenous NCOA7-B protein in model systems used, whole protein extract from SIM-A9 microglia treated with 100 µg/mL poly(I:C) for 24 hours, and various mouse tissues with enriched transcript expression of *Ncoa7-B*³¹ were probed using an NCOA7 antibody raised to the c-terminal domain of human NCOA7 (sc-393427, Santa Cruz). The predicted molecular weight of NCOA7-B is 25.5 kDa. Whilst a candidate band at approx. 25 kDa was identified in mouse tissue, this was demonstrated to be non-specific due to positivity in *Ncoa7*^{DEL/DEL} tissue (Figure 11.4a,b). The specificity of the antibody for longer isoforms of NCOA7 was demonstrated, with positive bands separating between 100 and 150 kDa and lower bands between 50 and 65

kDa. Whilst these bands were present in protein extract from WT tissue, they were not present in *Ncoa7*^{DEL/DEL} tissue (Figure 11.4b), suggesting these proteins derive from the *Ncoa7* locus.

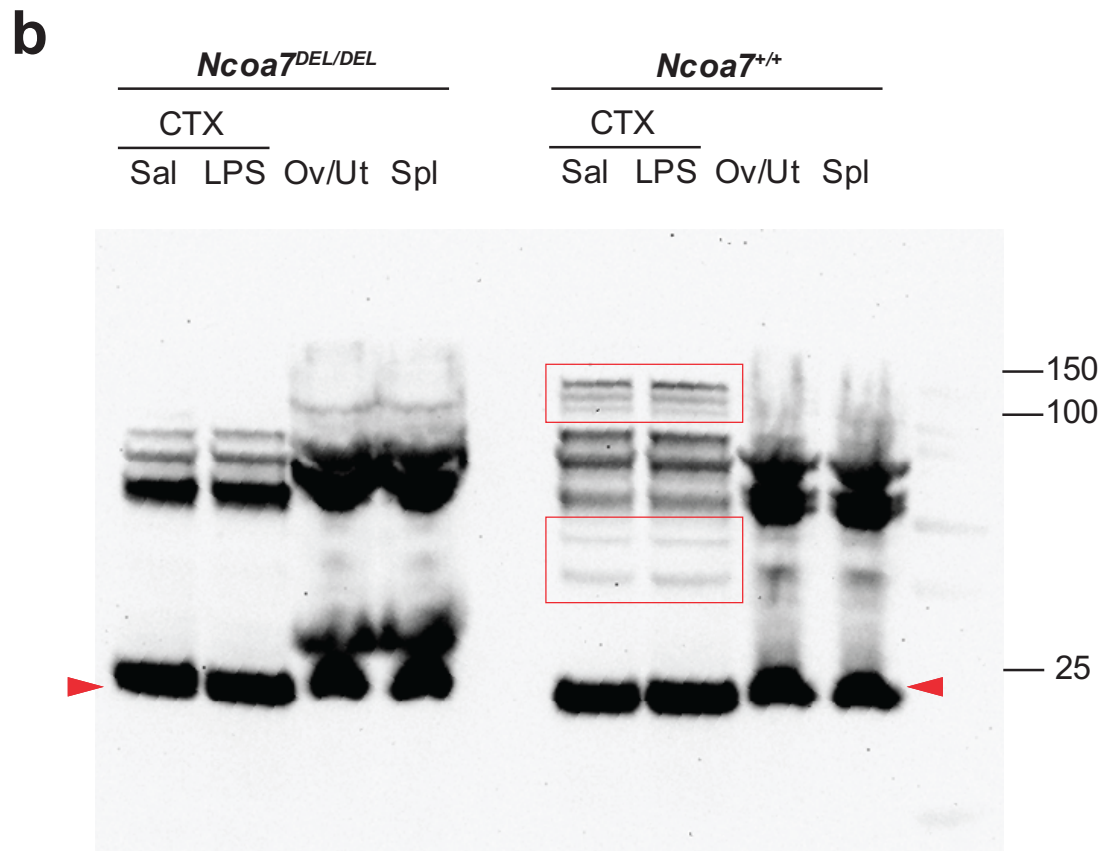
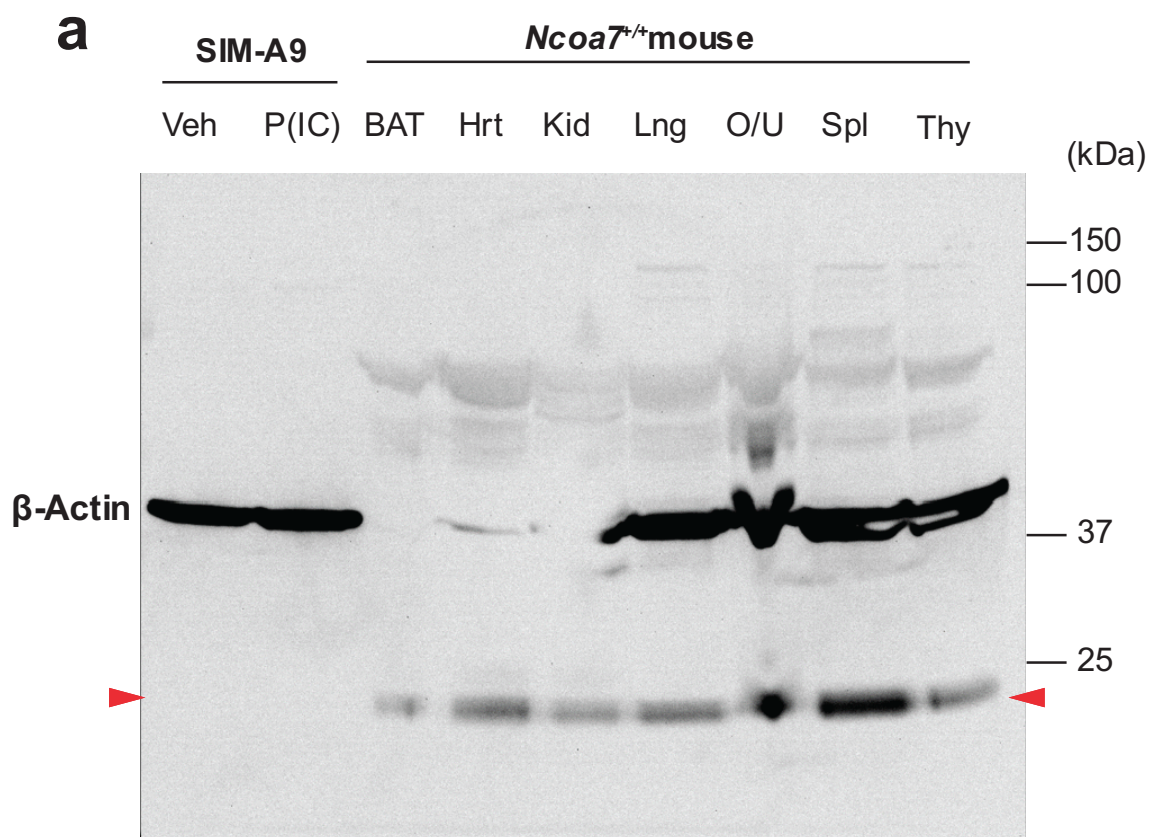


Figure 11.4. NCOA7-B protein is not identifiable by immunoblot with commercial antibody.

a, Whole protein extract (40 μ g) from SIM-A9 microglia treated for 24 hours with 100 μ g/mL polyinosinic:polycytidylic (poly(I:C)) or brown adipose tissue (BAT), heart (Hrt), kidney (Kid), lung (Lng), ovary/uterus (O/U), spleen (Spl) and thymus (Thy) (all tissues with demonstrated *Ncoa7-B* transcript expression³¹) all from wild-type (NCOA7^{WT/WT}) mice were immunoblotted for NCOA7 and β -actin. Red arrows show candidate NCOA7-B band at approximately 25 kDa. b, Whole protein extract from cerebral cortex (CTX) treated with lipopolysaccharide (250 μ g/kg for 4 hours), Ov/Ut and spleen from NCOA7^{DEL/DEL} and NCOA7^{WT/WT} mice were immunoblotted for NCOA7. Red boxes identify NCOA7 isoforms in WT mice. Red arrows demonstrate non-specific antibody binding.

11.3.6 Overexpression of an HA-tagged NCOA7-B in microglia suggests cytoplasmic, aggregated localisation

NCOA7-B has been identified as a microglial response gene to IFN stimulation and TLR agonism with an unexplored function in these cells. To explore the subcellular localisation of NCOA7-B in microglia, SIM-A9 cells were transfected with N-terminal HA-tagged NCOA7-B. Compared to a GFP-expressing vector, exogenous protein levels of HA-NCOA7-B were relatively low, limiting detection capabilities (Figure 11.5a). Orthogonal projection of HA-NCOA7B staining demonstrated diffuse cytoplasmic staining with punctate aggregations (Figure 11.5b,c). Whilst this demonstrates that *NCOA7-B* overexpression is tolerable in microglia and potentially detectable, ectopic overexpression does not reflect the *bona fide* pathways of *NCOA7-B* expression mechanisms and protein levels and may lead to mislocalisation of the protein and aggregation within the cell, as has been described with GFP-tagged proteins that are overexpressed^{94,95}.

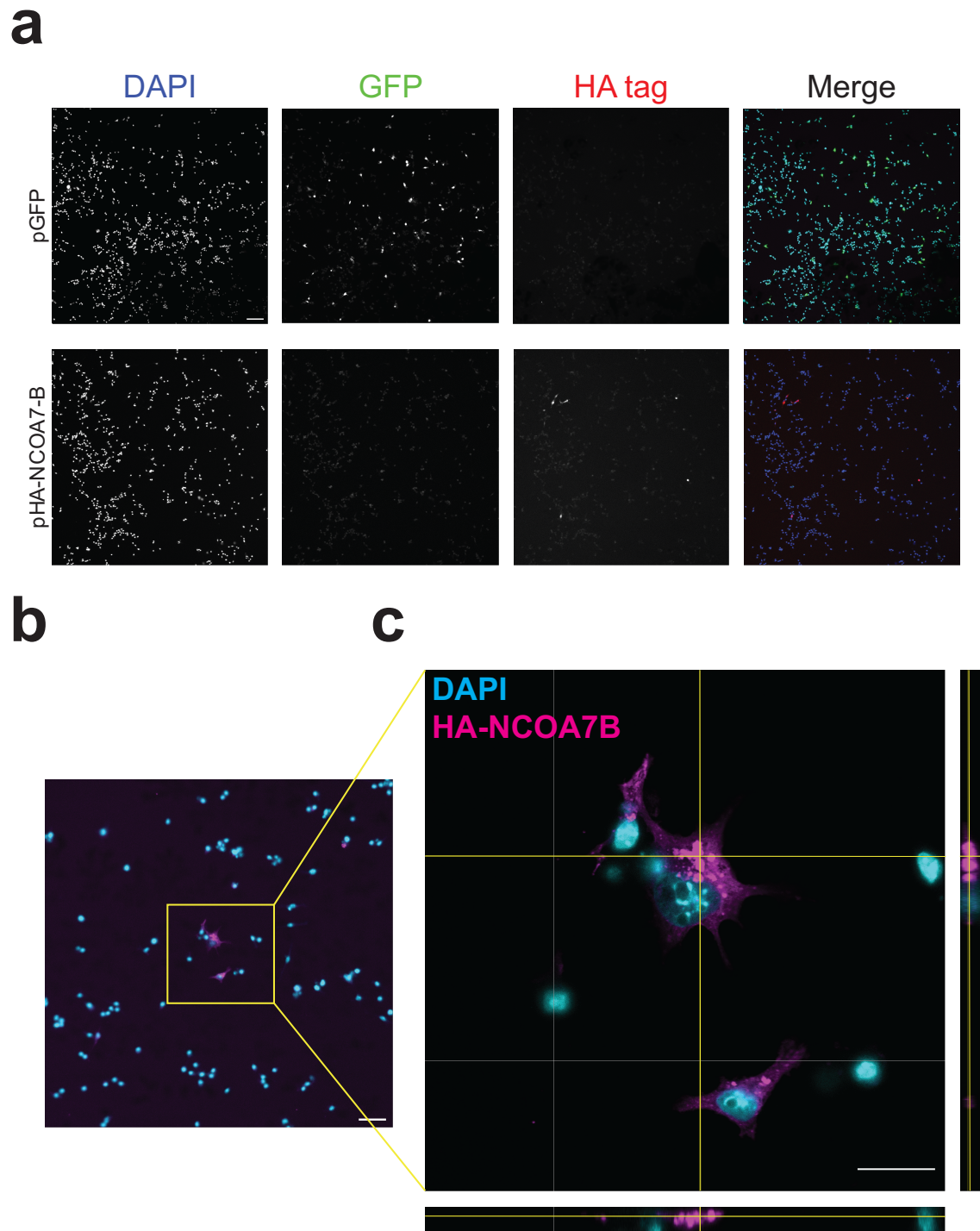


Figure 11.5. HA-NCOA7B overexpression in SIM-A9 microglia.

a, Fluorescent photomicrographs of SIM-A9 cells with a GFP (pGFP) or HA-tagged NCOA7-B (pHA-NCOA7-B) plasmid. Scale bar is 100 μ m. b, Photomicrograph of SIM-A9 cells expressing pHA-NCOA7-B. Scale bar is 50 μ m. c, Confocal photomicrograph orthogonal projection of SIM-A9 cells expressing pHA-NCOA7-B. Scale bar is 10 μ m.

11.3.7 FLAG-epitope tagging of endogenous NCOA7-B in microglia

Given the limitations and low-expression levels of ectopic overexpression HA-NCOA7-B in microglia, to localise and quantify endogenous NOCA7-B protein expression, I aimed to tag NCOA7-B in SIM-A9 microglia with an N-terminal FLAG (DYKDDDDK) epitope appended with a G(6) linker. Unlike a large, fluorescent tag (i.e. GFP), the small FLAG tag was employed to limit post-translational disruption of the protein and interference with the TLDc domain, which is hypothesised to be critical for V-ATPase binding. Briefly, a protospacer site was identified proximal to the start codon of NCOA7-B in its unique first exon and a gRNA-Cas9 RNP was co-delivered with an HDR template containing a FLAG-G(6) coding sequence immediately after the start codon (Figure 11.6a).

Individual SIM-A9 clones were screened for successful integration of the donor template by amplifying gDNA with a forward primer that sits within the insertion sequence (FLAG-G(6)) and a reverse primer that binds downstream of the 3' homology arm to eliminate possible amplification of residual donor sequence (Figure 11.7a). Three clones were identified which had successfully integrated the donor sequence into at least one allele. To determine the zygosity of the tag and confirm the fidelity of the *Ncoa7-B* coding sequence (CDS), the insertion region was amplified, implying all three clones were heterozygous for the FLAG tag (Figure 11.7b). Sequencing of the products confirmed the preservation of the *Ncoa7-B* CDS and insertion at the correct locus (Figure 11.6b).

Additionally, to confirm the insertion had not disrupted the interferon-inducible expression of *Ncoa7-B*, RT-PCR was performed in WT and FLAG-tagged microglia

(Figure 11.7c). The presence of a product at 158 bp that was visibly enriched in interferon-treated cells confirmed that the interferon-inducible expression was not disrupted.

Furthermore, to identify the expression of a transcript encoding a FLAG-tagged NCOA7-B, RT-PCR was also performed using an alternative forward primer that bound to the FLAG-G(6) sequence and a reverse primer binding in exon two of *Ncoa7-B* (Figure 11.7d). This RT-PCR confirmed that transcriptomic expression of FLAG-tagged NCOA7-B was preserved and remained sensitive to IFN β treatment. Despite this, the immunoblot of whole protein extract from IFN β -treated FLAG-NCOA7B cells failed to yield an anti-FLAG positive band at the expected molecular weight of 27 kDa (Figure 11.7e). To investigate whether FLAG-NCOA7-B was expressed below the limit of detection of the Western blot, whole protein extract from IFN β -treated SIM-A9 cells was immunoprecipitated using anti-FLAG agarose beads. However, no FLAG-positive band was detectable in the eluted fraction (Figure 11.7f). A band at 25 kDa, detectable by an anti-Mouse secondary antibody is likely to be an IgG light chain from the agarose beads. To determine whether the detection of NCOA7-B or its interacting partners was limited by the immunoblotting method, time-of-flight mass spectrometry of immunoprecipitated elutes was used to detect protein fragments. However, mass spectrometry was unable to detect NCOA7-B protein in the eluted fraction of the immunoprecipitation, nor any hypothesised protein interacting partners (i.e. V-ATPase subunits) (Appendix 1).

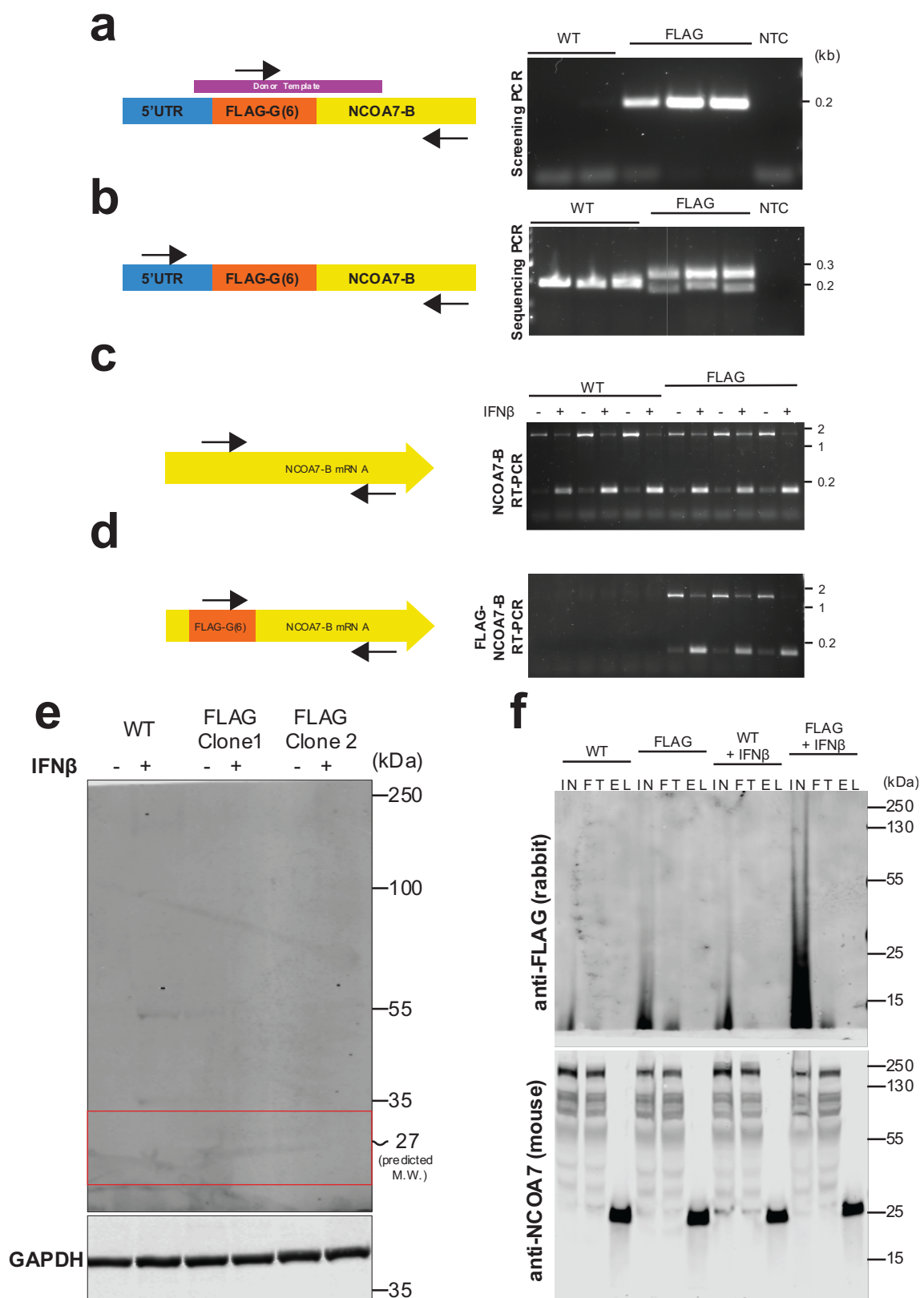


Figure 11.7. Characterising expression of FLAG-NCOA7-B in microglia.

a, gDNA from wild-type (WT) or FLAG-tagged SIM-A9 microglia was amplified in PCR using primers binding to the sequence encoding FLAG-G(6) and downstream of the 3' homology arm of the donor template. b, gDNA, as above, was amplified using primers flanking the insertion site. c, cDNA reverse transcribed from RNA extracted from WT and FLAG-tagged SIM-A9 microglia treated with 100 ng/mL interferon β (IFN β) for 4 hours was amplified (RT-PCR) with primers to detect NCOA7-B transcript, size of expected product is 158 bp. d, cDNA prepared as above was amplified using a forward primer recognising the sequence encoding FLAG(G6) and a reverse primer binding in a downstream exon, size of expected product is 134 bp. e, Whole protein extract (40 μ g) from FLAG-tagged or WT microglia treated with 100 ng/mL IFN β for 24 hours was immunoblotted for FLAG with a GAPDH loading control. Red box indicates expected region of molecular weight of FLAG-G(6)-NCOA7-B. f, Immunoprecipitated protein from SIM-A9 cells treated as in (e), input (IN), flow-through (FT) and eluted (EL) fractions were immunoblotted for FLAG and NCOA7.

11.3.8 Overexpression of FLAG-G(6)-NCOA7-B in 293T cells

To further explore why endogenous FLAG-tagged NCOA7-B was undetectable in SIM-A9 cells, I used a mammalian expression vector encoding FLAG-G(6)-NCOA7-B linked to EGFP by a T2A self-cleaving peptide sequence (pNCOA7-B) (Figure 11.8a). The vector was transfected into 293T cells, which are convenient for ectopic protein production. The presence of a strong GFP signal two days after transfection confirms translation of the transgene since EGFP is downstream of *Ncoa7-B* (Figure 11.8b). Furthermore, quantification of ectopic *Ncoa7-B* transcript by RT-qPCR confirmed a 6-fold increase in expression three days after transfection versus no vector control (Figure 11.8c). Together, these data demonstrate that the transgene is efficiently transcribed, and the transcript is stable for at least 3 days. As an alternative method to identify FLAG-tagged protein expression, 293T cells were co-transfected with pNCOA7-B and pFLAG-FB (FLAG-Frankenbody-mRuby2), the latter of which is a genetically encoded, mRuby2⁺ anti-FLAG antibody, to allow for live fluorescent detection of FLAG-tagged NCOA7-B (Figure 11.8d). Co-transfection and fluorescence microscopy confirmed the presence of colocalised GFP⁺ and mRuby2⁺ signal (Figure 11.8e, red arrows), however

when probed on an immunoblot, no anti-FLAG positive signal is visible (Figure 11.8f). Sanger sequencing of pNCOA7-B confirmed the vector sequence was correct.

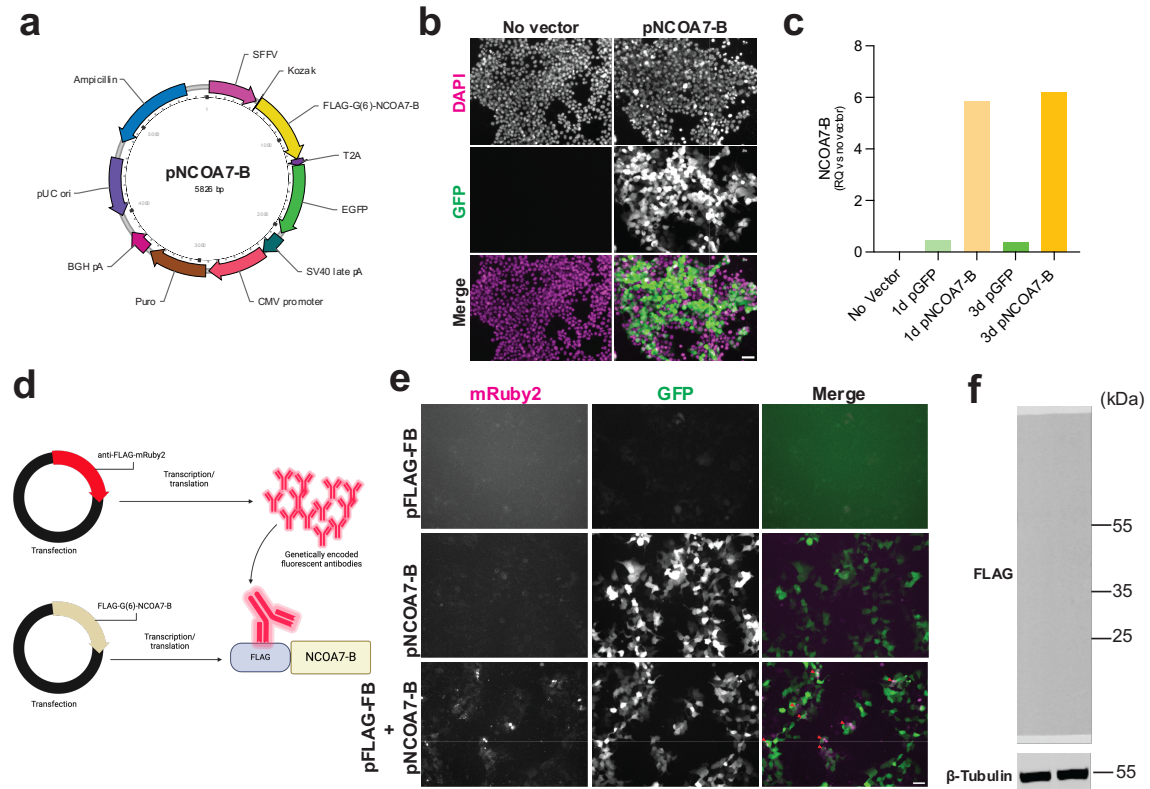


Figure 11.8. Transfection of 293T cells with pNCOA7-B.

a, pNCOA7-B plasmid map. b, Live photomicrographs of 293T cells transfected with pNCOA7-B – T2A – EGFP (green) and stained with DAPI (magenta). c, RT-qPCR expression of Mmu NCOA7-B transcript in 293T cells transfected as above or with pGFP (n = 1). RT-qPCR experiments were analysed using the $2^{-\Delta\Delta C_t}$ method⁹² with *Gapdh* and *Rps16* as reference genes (see section 10.3.1). d, Schematic demonstrating co-expression of pFLAG-FB and pNCOA7-B and detection of FLAG-NCOA7-B by mRuby2⁺ genetically encoded frankenbody (n = 1). e, Live photomicrographs of 293T cells co-transfected with pFLAG-FB (mRuby2⁺, magenta) and pNCOA7-B (T2A – EGFP, green). f, Total protein extract (40 μg) from 293T cells transfected as in (a) immunoblotted with anti-FLAG antibody and β-Tubulin loading control. Scale bars are 50 μm. Schematic produced with Biorender.com.

11.3.9 Attempted detection of endogenous FLAG-tagged NCOA7-B in SIM-A9 cells stably expressing an anti-FLAG genetically encoded antibody

Upon observing the colocalisation of mRuby2 and GFP in 293T cells co-transfected with pFLAG-FB and pNCOA7-B, I next aimed to localise endogenous NCOA7-B within FLAG-tagged NCOA7-B. Stable expression of the anti-FLAG Frankenbody in SIM-A9 cells was achieved by electroporation of FLAG-tagged SIM-A9 cells with pFLAG-FB, containing a neomycin resistance gene, and subsequent maintenance under selection. Successful electroporation was verified using a control pGFP plasmid, and a kill curve determined 800 µg/mL G418 as the minimal effective dose for inducing 100% SIM-A9 cell death after 3 days of selection (Figure 11.9a). Following electroporation, cells underwent 36 days of selective maintenance (Figure 11.9b). PCR analysis post-recovery confirmed the presence of mRuby2-encoding DNA in both WT and FLAG-tagged SIM-A9 cells, indicative of successful integration and stable expression of pFLAG-FB, though the potential amplification of residual plasmid DNA cannot be entirely excluded (Figure 11.9c). Subsequently, confocal microscopy of FLAG-NCOA7-B-expressing SIM-A9 cells, treated with IFN β to induce expression and stably transfected with pFLAG-FB, failed to reveal a specific mRuby2⁺ signal (Figure 11.9d).

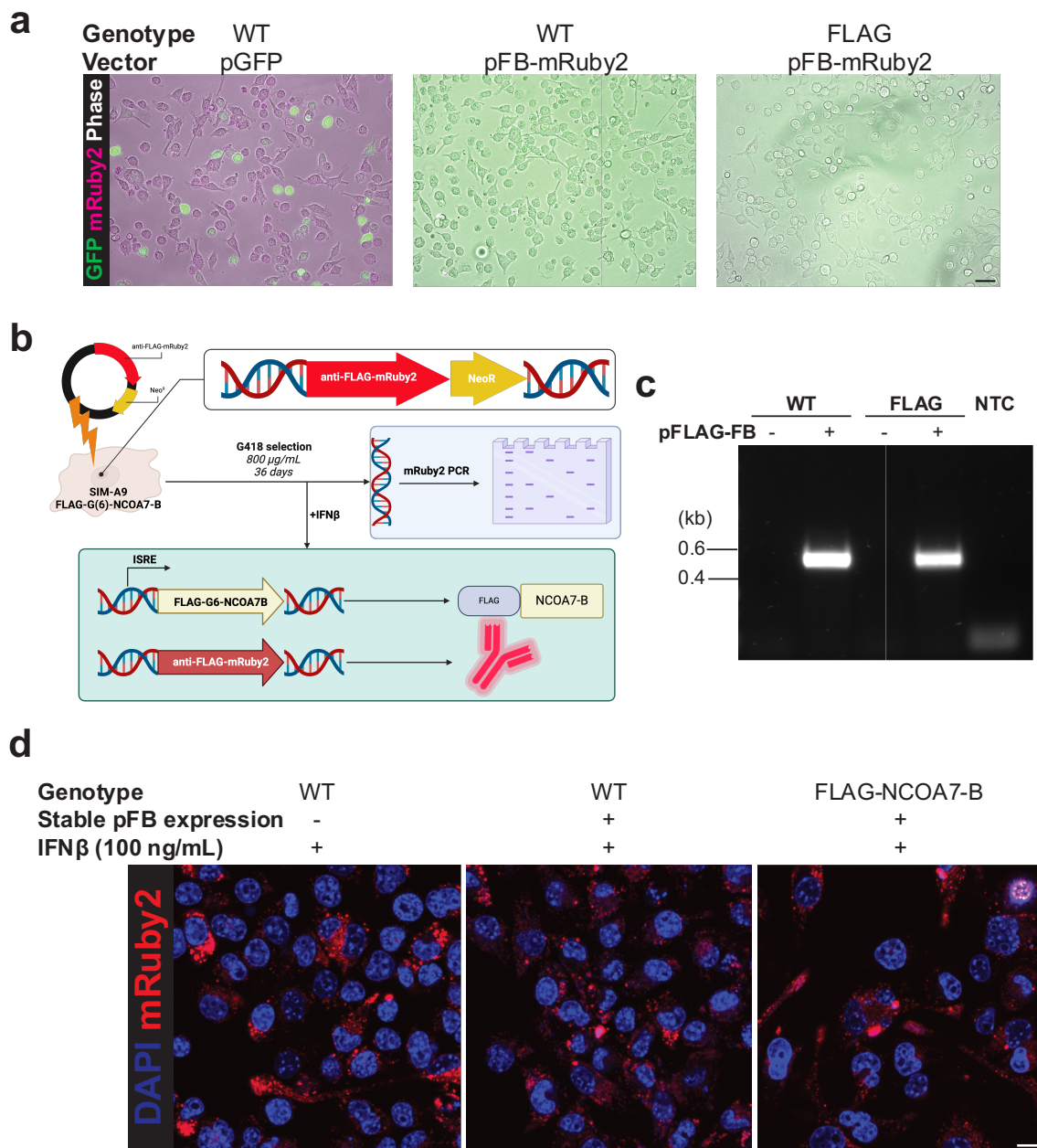


Figure 11.9. Attempted localisation of FLAG-NCOA7-B in SIM-A9 cells with Frankenbody-mRuby2. a, Photomicrographs of wild-type (WT) or FLAG-G6-NCOA7-B (FLAG) SIM-A9 cells transfected with pGFP (green) or pFrankenbody-mRuby2 (pFB-mRuby2, magenta). Scale bar is 50 μ m. b, Schematic demonstrating electroporation and stable integration of pFB-mRuby2 in SIM-A9 microglia; subsequent PCR screening for mRuby2 after 36 days of 800 μ g/mL G418 selection; and treatment with 100 ng/mL interferon (IFN) β to stimulate NCOA7-B expression and identification with anti-FLAG-mRuby2 Frankenbody. c, PCR amplifying mRuby2 in WT or FLAG-tagged SIM-A9 cells transfected with pFLAG-FB. Expected amplicon size is 545 bp. NTC is no template control. d, Confocal photomicrographs of WT or FLAG-tagged SIM-A9 cells, treated for 24 hours with 100 ng/mL IFN β , attempting to localise mRuby2 signal (red) and stained with DAPI (blue). Scale bar is 10 μ m. Schematic produced with Biorender.com

11.3.10GFP-tagging NCOA7-B in 293T cells

Since efforts to measure and localise *Ncoa7-B* in murine myeloid cells with an unintrusive epitope tag were unsuccessful, as a contingency I tagged *NCOA7-B* in human 293T cells with GFP. 293T cells are simple to culture and transfect, allowing efficient genome editing and rapid expansion. GFP tagging was achieved with the Invitrogen TrueTag system (Figure 10.1, page 43) which enables rapid, in-house generation of an HDR template encoding puromycin resistance, conjugated to emerald GFP by a self-cleaving 2A sequence (Puro^R-2A-EmGFP). This allows for fluorescent tagging of the protein of interest and enrichment of edited cells by antibiotic selection (Figure 11.10a).

Since *NCOA7-B* interferon responsiveness was previously demonstrated in myeloid cells, I next determined whether this response is conserved in 293T cells. Indeed, RT-qPCR demonstrated a 10-fold enrichment in *GFP-NCOA7-B* transcript after treatment with 100 or 200 ng/mL IFN β for 6 hours, though the response is weaker than in microglia (Figure 11.10b). Furthermore, since 293T cells are aneuploid and genetically variable, to ensure the expected protospacer sequence and protospacer adjacent motif (PAM) were conserved, Sanger sequencing of the region was performed. This confirmed the sequence is as anticipated (Figure 11.10c). This validates 293T cells as a model to investigate the interferon stimulation of *NCOA7-B*.

The TrueTag HDR template was appended with 25-base pair homology arms directed to the locus surrounding the start codon of *NCOA7-B* by PCR. This PCR product was purified and delivered to cells with Cas9-gRNA RNP. Screening of successful editing was performed using junction PCR, with a forward primer located in the EmGFP sequence and a reverse primer located in the host genome, downstream of the homology arm to prevent amplification of residual donor template. The presence of non-specific

amplicons was observed, however a unique amplicon to cells delivered with RNP with or without HDR enhancer indicated successful integration (Figure 11.10d). After expansion, flow cytometry revealed 1.53% GFP⁺ cells in the edited population versus 0.16% in the no RNP control. The minimum effective dose for puromycin selection in 293Ts was determined to be 1 µg/mL over 7 days and, after selection, GFP⁺ cells were enriched to 37.5% (Figure 11.10e). This relatively low selection efficiency may have been due to the absence of interferon-induced expression of *NCOA7-B* in unstimulated 293T cells. Thus, cells were treated with IFNβ for 24 hours and maintained in selection for a further 2 days before analysis by flow cytometry. A small but significant increase in the % GFP⁺ cells after IFNβ treatment suggests a small subpopulation of cells may not express *NCOA7-B* at sufficient levels to confer puromycin resistance (Figure 11.10f, first panel).

To demonstrate interferon-induction of NCOA7-B protein, puromycin-selected GFP-293T cells were treated with 100 ng/mL IFNβ for 24 hours and the mean fluorescence intensity of GFP was measured. This demonstrated an approximate 25% increase in GFP signal, indicating the expression of NCOA7-B protein is interferon-inducible (Figure 11.10f, second panel). Finally, to localise the GFP-tagged protein, immunocytochemistry was performed with an anti-GFP antibody. Confocal photomicrographs of tagged 293T cells after 24 hours of stimulation with 100 ng/mL IFNβ were unable to demonstrate convincing colocalisation of native GFP signal with anti-GFP antibody (identified with Alex Fluor 647 secondary antibody), thus subcellular localisation of the protein was not determinable (Figure 11.10g). Of note, sequencing of the insert was not performed as attempts to amplify the insertion resulted in poor yield of the PCR product.

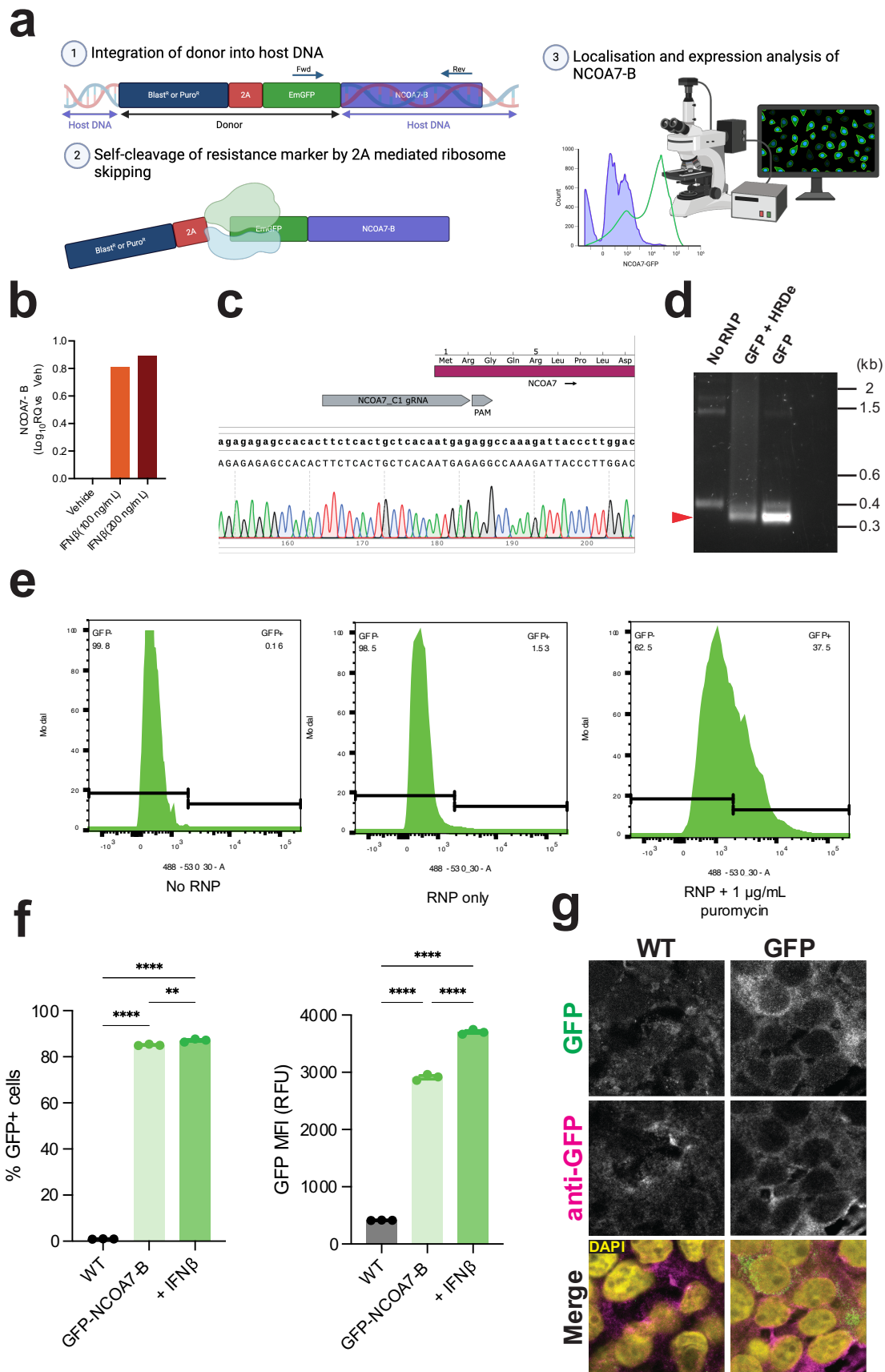


Figure 11.10. Tagging of NCOA7-B with GFP in 293T cells.

a, Schematic demonstrating the Invitrogen TrueTag system which was used to append a puromycin resistance – 2A self-cleaving peptide – Emerald GFP (Puro^R-2A-EmGFP) sequence to the N-terminus of NCOA7-B. **b**, Expression of NCOA7-B transcript in 293T cells treated for 6 hours with 100 or 200 ng/mL IFN β determined by RT-qPCR with relative quantification (RQ) to vehicle-treated cells (n = 3 wells treated independently with IFN β). RT-qPCR experiments were analysed using the $2^{-\Delta\Delta C_t}$ method⁹² with *GAPDH* and *HPRT1* as reference genes (see section 10.3.1). **c**, Sanger sequencing of the protospacer sequence and protospacer adjacent motif (PAM) in 293T cells. **d**, Junction PCR of 293T cells transfected with Cas9-gRNA ribonucleoprotein (RNP) targeted to NCOA7-B with the forward primer binding in the EmGFP sequence and the reverse primer binding in the host genome, downstream of the homology arms (see **a**, 1). Red arrow indicates a band unique to 293T cells transfected with GFP homology-directed repair (HDR) donor and with IDT HDR enhancer (20 μ M). **e**, Gating of 293T cells transfected with or without RNP as in (**d**) and selected with 1 μ g/mL puromycin for 7 days. Analysis of GFP signal was performed with flow cytometry. **f**, Change in % GFP⁺ cells before and after treatment with 100 ng/mL IFN β for 1 day (n = 3 passages) **g**, Confocal photomicrographs of WT or GFP-tagged 293T cells stained with anti-GFP-Alexa Fluor 647 and DAPI. Scale bar is 10 μ m. Schematic produced with Biorender.com.

11.4 Discussion

11.4.1 NCOA7-B is a novel ISG in microglia

Consistent with previous findings in peripheral macrophages^{7,42}, a robust upregulation of *NCOA7-B* was demonstrable in both human and mouse microglia in response to a variety of inflammatory stimuli, but likely underpinned by IFN β signalling. Recent research has highlighted the importance of the type I interferon response in Alzheimer's disease, where nucleic acid-containing amyloid fibrils induce a type I interferon transcriptomic signature in the brain and specifically in microglia surrounding amyloid plaques⁴⁸. Furthermore, IFN β -mediated activation of microglia resulted in C3-dependent synapse elimination, contributing directly to neurodegeneration⁴⁸. Since the V-ATPase is crucial for the proper degradation of phagocytosed substrates⁹⁶, further investigation into the role of *NCOA7-B* in plaque-associated microglia is warranted.

11.4.2 Paradoxical inhibition of NCOA7-B transcription by STING inhibitor, H-151

Poly(I:C) is a synthetic analogue of double-stranded RNA (dsRNA) and is recognised by TLR3 on the cell surface and by melanoma differentiation-associated protein 5 (MDA5) and Retinoic Acid Inducible Gene I (RIG-I) receptors in the cytosol⁹⁷. These receptors initiate signalling cascades leading to the recruitment of pro-inflammatory transcription factors, notably IRF3 and NF- κ B, which upregulate the expression of type I interferons, including IFN β ⁹⁸. STING is a critical component of the cytosolic dsDNA-sensing pathway, which leads to the production of type I interferons in response to the detection of cytosolic DNA but is typically independent of dsRNA stimulation⁹⁹. The inhibition of IFN β and NCOA7B upregulation by H-151 could be by indirect activation of the STING pathway by dsRNA. Potentially by the generation of cytosolic DNA during the cellular or mitochondrial stress in response to poly(I:C)

stimulation. Indeed, TLR3 agonism has been demonstrated to induce mitochondrial stress in human hepatocytes¹⁰⁰ and may lead to the release of mtDNA and secondary activation of the cGAS-STING pathway¹⁰¹. Alternatively, a model has been purported whereby engagement of RIG-I by dsRNA indirectly induces STING activation via the mitochondrial antiviral signalling (MAVS) protein¹⁰². Further experiments to elucidate the exact mechanism include investigating whether blocking TLR3, MDA5, or RIG-I affects the Poly(I:C)-induced IFN β upregulation and how this relates to STING activity. It may also be pertinent to explore whether Poly(I:C) treatment leads to the generation of cytosolic DNA or the induction of proteins known to interact with the STING pathway.

11.4.3 FLAG-NCOA7-B protein was not detectable in the model system used

To further investigate the role of *NCOA7-B* in the IFN response in microglia, I attempted to generate a FLAG-tagged *Ncoa7-B* microglial cell line. However, efforts to detect the FLAG-tagged protein failed. As an alternative model, *NCOA7-B* was tagged in 293T cells, where IFN β -stimulation upregulated both transcript levels and GFP mean fluorescence intensity (MFI). Multiple reasons may explain why FLAG-NCOA7-B was undetectable in SIM-A9 microglia in this study. Any mechanism limiting expression is likely post-transcriptional since the transcript encoding FLAG-NCOA7-B is detected at relatively high levels up to 6 hours following IFN β treatment. Firstly, NCOA7-B might be rapidly degraded post-translation. Treatment of cells with proteasome inhibitors (e.g. MG132) or lysosome inhibitors (e.g. concanamycin A) to block protein degradation pathways could test this hypothesis¹⁰³. This hypothesis may be supported by the relatively low expression of GFP-NCOA7-B in 293T cells, versus transcript expression. GFP-NCOA7-B may remain detectable by limiting the degradation kinetics or GFP may persist after NCOA7-B protein degradation. Secondly, since relative quantification was used to

quantify NCOA7-B transcript expression in RT-qPCR, the protein itself may be expressed at insufficient levels to be detected by immunoblotting techniques. However, this hypothesis may be less likely since immunoprecipitation with anti-FLAG agarose beads was still unable to allow detection of the FLAG-NCOA7-B protein. Furthermore, transcript levels can be incongruent with protein levels and can be affected by delayed synthesis and post-translational modifications (PTM)¹⁰⁴. Viable mechanisms for the lack of detectable protein include translational regulation, such as ribosome stalling, initiation issues, or post-translational modification (i.e. ubiquitination, hence degradation). Ribosome profiling or polysome profiling could help identify these mechanisms, as has been described with ribosome stalling of mutant huntingtin in Huntington's disease¹⁰⁵. However, again this hypothesis may be less likely since a strong GFP signal is present and expressed downstream of a 2A self-cleaving sequence and FLAG-NCOA7-B in ectopic expression, implying the transcript is efficiently translated, at least in 293T cells. Furthermore, the FLAG tag could be cleaved by a protease. This could be a cellular protease recognising a specific sequence in the FLAG tag or the junction between the FLAG tag and the protein. Whilst analysis of the sequence with ExPASy Peptide Cutter¹⁰⁶ failed to identify any mammalian protease sites proximal to the FLAG-NCOA7-B appendage, the use of protease inhibitors would also eliminate this hypothesis. However, TLDC proteins are likely subject to stringent cellular quality control mechanisms. Patient mutations, even at the single nucleotide level, can cause reduced or null expression of TLDC proteins and this is observable in patients with mutated TLDC proteins, OXR1 and TBC1D24^{3,107}. Furthermore, attempts by the Oliver lab to HA-tag TBC1D24 in mice resulted in an approximate 90% reduction in cortical expression of TBC1D24 [unpublished].

11.4.4 Potential function and application of NCOA7-B in microglia

Since ectopic *NCOA7-B* has been demonstrated to enhance vesicular acidification and interacts with V-ATPase subunits in various cell lines³⁹, it would be advantageous to determine whether this function translates to microglia and macrophages, where expression of *NCOA7-B* is strongly induced by TLR agonism via IFN β autocrine signalling. This could have ramifications for enhancing phagocytic degradation of disease-relevant substrates, particularly misfolded and aggregated proteins in neurodegenerative diseases (i.e. amyloid- β and hyperphosphorylated tau in Alzheimer's disease). This has previously been demonstrated where stimulation of primary microglia with M-CSF leads to enhanced lysosomal acidification and increased degradation of fibrillar amyloid- β ¹⁰⁸. Furthermore, disruption of V-ATPase subunit expression in mice genetically modified to overexpress P301S mutant tau impairs lysosomal acidification in microglia and increases levels of hyperphosphorylated tau in the brain¹⁰⁹. If such a functional link between NCOA7-B and V-ATPase expression can be demonstrated, due to the small size of NCOA7-B and tolerable overexpression in cells, it may be beneficial to explore the effects of NCOA7-B delivery on neurodegenerative pathologies.

shRNA-mediated knockdown of *Ncoa7-FL* (NM_172495.5) was insufficient to enhance arsenite-mediated murine N2a cell death¹⁵, however, it remains to be determined whether interrupting endogenously upregulated *Ncoa7-B* worsens cell death in a model of oxidative stress. This could be achieved by *Ncoa7-B* knockout, RNAi, or mutation of the putative ISRE in IFN-stimulated myeloid cells under oxidative stress. A potential link to *NCOA7-B* stress resistance and ROS production by phagocyte-specific NADPH oxidase-2 (Phox/NOX2) has been alluded to and is an avenue for further research since

both proteins are upregulated in inflammatory conditions and regulate cellular redox processes¹¹⁰.

Whilst *NCOA7-B* is sufficient to limit endosome-mediated viral entry in human glioma cells, the necessity of *NCOA7-B* in viral defence has not been investigated. Since *NCOA7-B* transcript is strongly enriched in respiratory tissue from patients infected with SARS-Cov-2¹¹¹, it would be beneficial to investigate whether interferon-stimulation, hence endogenous *NCOA7-B* upregulation is necessary for vesicular acidification and limiting viral entry. Nonetheless, these studies demonstrate an important role for *NCOA7-B* in viral defence and the small size of the protein makes it an attractive therapeutic strategy for direct protein delivery. As a concept, this has been demonstrated by the delivery of modified, cell-permeable, low molecular weight parkin protein in models of Parkinson's disease¹¹². This has potential applications for *NCOA7-B* delivery in viral infection or diseases with a strong oxidative stress component, such as Parkinson's disease.

12 Maternal Immune Activation in *Ncoa7^{DEL}* mice

12.1 Chapter Abstract

The *NCOA7* locus has been identified as a potential gene hub in autism spectrum disorder (ASD) and LOF mutations have been found in ASD patients^{12,29}. Furthermore, deletion of the *Ncoa7* locus in mice (*Ncoa7^{DEL}*) has been demonstrated to cause social interaction deficits, anxiety-like behaviour and reduced inhibitory synaptic outputs². Simultaneously, activation of the maternal immune system (MIA) during gestation is a significant risk factor for the development of ASD in the foetus¹¹³. Whilst behavioural and microglial dysfunction in MIA are closely related to the interferon response¹¹⁴, the influence of the novel ISG *NCOA7-B* is unknown. To explore the interaction between *Ncoa7* deletion and MIA in the pathogenesis of ASD, poly(I:C), a viral mimetic, was used to induce MIA in pregnant dams carrying foetuses from an *Ncoa7^{DEL}* intercross. Quantification of *Ncoa7-B* transcript and the inflammatory regulator interleukin-6 (IL6) in the foetal head demonstrated both genes are induced in the acute response to MIA. Furthermore, the characterisation of behavioural endophenotypes revealed anxiety-like behaviours in the light-dark box and reduced motor learning in the rotarod test in *Ncoa7^{DEL/DEL}* MIA offspring. This novel study highlights the interaction between *Ncoa7* and MIA in the development of ASD-associated endophenotypes and warrants further investigation into neuropathological changes governing these behavioural changes.

12.2 Introduction

12.2.1 The emerging role of prenatal inflammation and the foetal microglial response in autism spectrum disorder

Animal models to investigate gene-environment interactions in ASD are guided by the frameworks of the diathesis-stress and differential-susceptibility models¹¹⁵. The

diathesis-stress model posits that genetic predispositions in individuals may lead to ASD when combined with adverse environmental triggers, emphasising the critical role of negative environmental factors¹¹⁵. Conversely, the differential-susceptibility model suggests that these genetic factors can modulate responses to both deleterious and beneficial environments, offering a comprehensive approach to better understand and model ASD in animal studies¹¹⁶. Thus, ASD is increasingly recognised as a complex neurodevelopmental disorder (NDD) influenced by intricate gene networks and environmental factors. Recent large-scale genomic studies highlight the role of gene clusters – particularly those related to synaptic function and neurodevelopment – in the aetiology of ASD^{117,118}. Concurrently, research on activation of the maternal immune system during early gestation (Maternal Immune Activation, MIA) suggests external factors also contribute to ASD pathogenesis, potentially through modulation of these genetic networks^{118,119}. MIA is commonly achieved by inducing a “sterile” immune response in a pregnant dam, often using the viral mimetic poly(I:C) or the bacterial mimetic LPS and investigating changes in the offspring¹¹³. These studies highlight a strong immune component to ASD, with microglia increasingly implicated in the shaping of neuronal circuits during critical development periods^{120,121}. Together, these studies underscore a multifactorial model of ASD, where both genetic susceptibilities and environmental exposures during early development influence cortical circuitry. An experimental model of this paradigm is a “two-hit” model whereby gene perturbation (e.g. haploinsufficiency of a risk locus) is coupled with MIA to recapitulate the multifactorial nature of ASD¹²².

Activation of the murine maternal immune system is sufficient to cause ASD-associated behavioural endophenotypes (including the typically associated triad of

behaviours, comprising social interaction deficit, communication impairment and repetitive behaviours¹²³), neuronal circuit malformation and microglial dysfunction in the offspring^{124–126}. The behavioural and neuropathological changes in Poly(I:C)-MIA are dependent on IL6 signalling along the maternal-placental-foetal axis, where IL6 is necessary and sufficient to produce gene expression changes in the frontal cortex and social deficits exhibited in MIA offspring¹²⁷. The cell type responsible for IL6 production and the IL6-responsive cells in the foetal MIA brain have not been identified, however, microglia have been identified as the sole cells expressing both transcripts encoding the IL6 receptor (IL6R and IL6ST) in human foetal neocortical cells¹²⁸. In the mouse, poly(I:C)-MIA increases microglial transcript expression of various proinflammatory cytokines, including IL6 and IL1 β , with concomitant increases in foetal microglial motility in acute brain slices¹²⁹. Furthermore, poly(I:C)-MIA has been found to diminish the proinflammatory transcriptomic response to LPS in postnatal microglia, indicating an inflammatory deficiency in microglia persisting into adulthood¹²⁶. Elimination and subsequent repopulation of embryonic myeloid cells in the MIA model with CSF1R antagonist, PLX5622, ameliorated this blunted LPS response. Crucially, this repopulation also restored aspects of astrocytic and neuronal function. For example, microglial repopulation in MIA normalised IL6 expression in astrocytes and restored MIA-induced excitatory post-synaptic current frequency in dopamine receptor type 2 medium spiny neurons in the ventral striatum of adult offspring¹²⁶. Together, these data indicate a novel cell-non-autonomous role of microglia in MIA-associated NDD, as well as a deficient inflammatory response in adult microglia.

The MIA model is closely linked to the IFN-response with viral infection during pregnancy a strong correlate of ASD in offspring¹¹³. Recently, MIA induced with

poly(I:C) at E14.5 in pregnant dams was found to reduce microglial proliferation-associated gene signatures in the offspring and this was dependent on IFN-I signalling¹¹⁴. Induction of MIA with IFN β was sufficient to reduce proliferation markers and antibody-mediated blockade of IFNAR restored expression, demonstrating that IFN-I is both necessary and sufficient for impaired microglial proliferation marker expression in poly(I:C)-induced MIA¹¹⁴. Furthermore, IFN-I was sufficient to induce reduced social preference, increased repetitive behaviour in the marble burying test and a higher degree of spontaneous alternation in females and, in males, less time spent in the open arm of an elevated plus maze. This indicates a degree of sexual dimorphism in the response to IFN-I-mediated MIA and identifies a link between the IFN-I response, MIA-associated changes to behaviour and microglial function in the pathogenesis of ASD.

12.2.2 Exploring the role of microglial NCOA7 in ASD

NCOA7 is an ideal candidate to explore the interaction between MIA and risk loci in the pathogenesis of ASD. Firstly, the full-length variant of the *NCOA7* locus, *NCOA7-FL*, exhibits broad expression across the brain, with peak transcript expression at P14 in mice and during early adulthood in humans^{2,130}. Additionally, *Ncoa7* is enriched in cerebellar Purkinje cells in the mouse² and Purkinje cells are absent in MIA offspring in lobule VII of MIA offspring¹³¹, suggesting a specific vulnerability of this cell type. This pattern of expression suggests a critical function in neuronal development, especially in Purkinje cells. In a study using the BrainSpan human transcriptome database to explore the genetics of ASD, *NCOA7* emerged as a potential central gene with a significant correlation to the condition, marking it as a putative node within the ASD network²⁹. This discovery was further substantiated by subsequent exome sequencing in the Autism

Sequencing Consortium, which identified a unique recessive ASD case linked to a homozygous nonsense mutation in *NCOA7*¹².

To investigate this association further, a mouse model was created by the Oliver group where the entire *Ncoa7* coding locus was deleted (*Ncoa7*^{DEL})². Behavioural examination of *Ncoa7*^{DEL/DEL} mice revealed a social interaction deficit and anxiety-like behaviours, commonly associated with ASD. Despite the absence of *Ncoa7*, no marked brain pathology or neuronal death was observed, even in aged animals. However, a reduction in inhibitory VGAT-gephyrin⁺ synapse formation was observed in primary neurons, hypothesised to be due to defective lysosomal-mediated pruning of neurite outgrowths. Crucially, lysosomal defects and inhibitory synapse density were rescued by transfection of *Ncoa7-FL* *in vitro*. However, the influence of microglia and astrocytes were not considered in this monoculture system nor was the function of the short, interferon-inducible isoform of *NCOA7*, *NCOA7-B*. Indeed, it is unknown whether rescue with *NCOA7-B* is sufficient to restore synapse development and behavioural changes in *Ncoa7*^{DEL} mice. Together, this evidence indicates that the *Ncoa7* locus is important in supporting neurodevelopment, with disruptions possibly leading to ASD-associated behaviours through adverse effects on lysosomal activity and synapse formation.

At the transcriptomic level, *NCOA7* is enriched in human microglia and an interferon-inducible isoform, *NCOA7-B*, can augment V-ATPase activity and lysosomal acidification in viral defence in human glioma cells, as discussed previously (Section 11)^{34,39}. Given the relationship outlined above between the antiviral response in pregnancy, interferon signalling and microglial dysfunction, this chapter aims to examine the interplay between *NCOA7-FL* and interferon-inducible *NCOA7-B* in microglia in the pathogenesis of ASD.

12.2.3 Aims & Hypotheses

12.2.3.1 Hypotheses

H₁ – *Ncoa7-B*, a novel ISG, is upregulated in the foetal brain in response to MIA induced with poly(I:C).

H₂ – Deletion of the *Ncoa7* locus augments MIA-induced neuro-behavioural deficits.

12.2.3.2 Aims

To address these hypotheses, the aims of this study were to:

1. Measure changes to *Ncoa7-B* expression in the foetal brain in response to MIA;
2. Generate a viable MIA model in an *Ncoa7^{DEL}* intercross breed to produce MIA-*Ncoa7^{DEL}* mice;
3. Identify behavioural impairments in MIA-*Ncoa7^{DEL}* mice associated with ASD.

12.3 Results

Phenotyping data is presented as mean $\pm$ 95% confidence intervals. Phenotyping was performed across 3 cohorts of young adult (10-20 weeks old) female mice. Statistical tests and post-hoc corrections used in analyses are denoted in the figure legends. Despite higher rate of ASD diagnosis in males¹³², male mice were excluded from behavioural analysis to ensure that: (i) the study maintained sufficient statistical power to detect significant differences attributable to genotype and MIA; (ii) behavioural deficits had previously been demonstrated in adult, female *Ncoa7*^{DEL/DEL} mice²; and (iii) NCOA7 contains an oestrogen-binding domain and has been shown to bind oestrogen receptors, which may be more active in females¹³³.

12.3.1 Deficient LPS-induced CD68 upregulation in *Ncoa7*^{DEL/DEL} mice

Involvement of the *Ncoa7* locus in immune regulation has been demonstrated previously where *Ncoa7-B* is upregulated by proinflammatory stimulation of various cell types and *Ncoa7-FL* is expressed in murine dendritic cells⁸⁹. To explore the potential function of the *Ncoa7* locus in microglial activation *in vivo*, adult, female mice were injected with 250 mg/kg LPS i.p. and brains were stained for microglial marker Iba1 and the activation marker CD68¹³⁴ (Figure 12.1a). The number of Iba1⁺ and CD68⁺ cells were counted in the somatosensory cortex of parasagittal sections, where *Ncoa7-B* upregulation has previously been demonstrated in response to i.p. treatment with LPS (Section 11.3.2). There was a trend of increasing Iba1 immunoreactivity in LPS treatment in both genotypes, however, this comparison lacked significance (Figure 12.1b). The number of CD68⁺ cells was significantly increased in *Ncoa7*^{WT/WT} mice (Figure 12.1c). This effect was not seen in *Ncoa7*^{DEL/DEL} mice, which appeared to have more CD68⁺ cells irrespective of LPS treatment. Elevated CD68 expression in *Ncoa7*^{DEL/DEL} brains suggests

microglial activation when the *Ncoa7* locus is deleted, confirming a role for the locus in immune regulation in the brain.

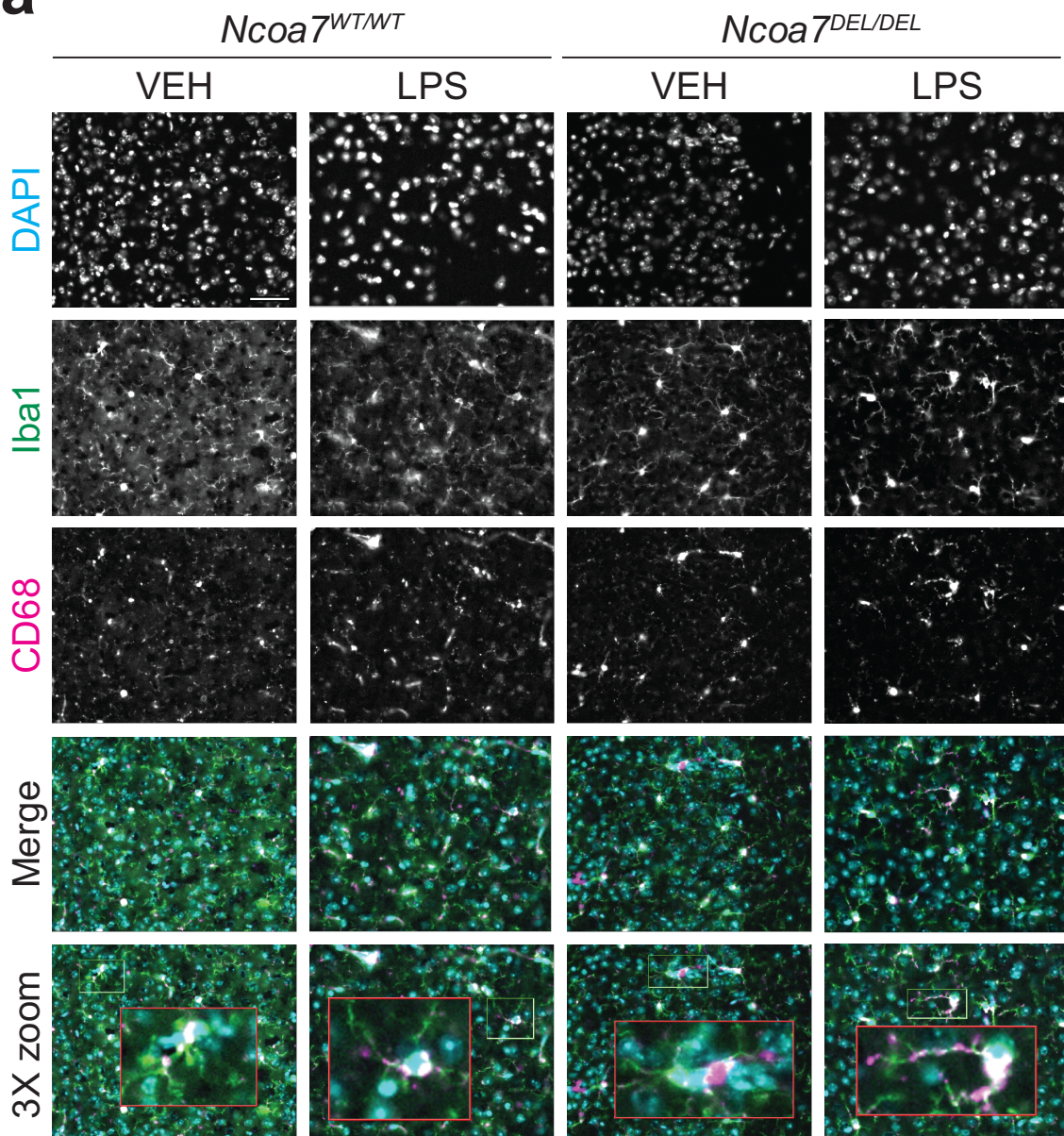
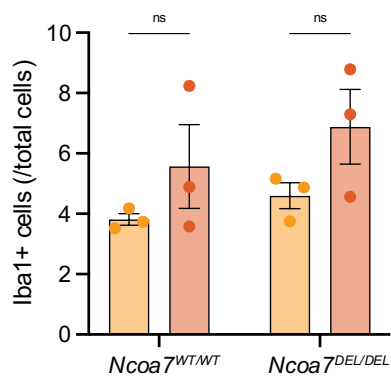
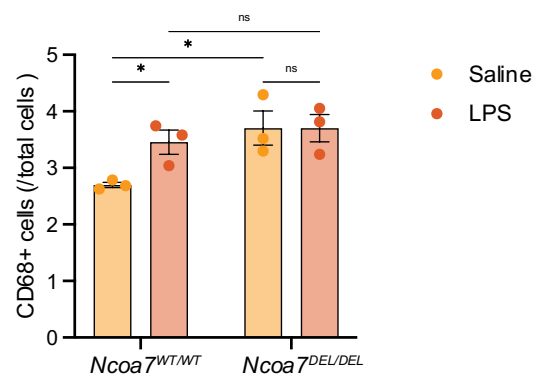
a**b****c**

Figure 12.1. Deficient LPS-induced CD68 upregulation in *Ncoa7^{DEL/DEL}* mice.

a, Representative confocal photomicrographs of DAPI, Iba1 and CD68 stained parasagittal somatosensory cortex of adult, female *Ncoa7^{WT/WT}* and *Ncoa7^{DEL/DEL}* mice. A 3x-zoom inset displays CD68⁺ microglia. Scale bar is 50 μ m. b, The number of Iba1⁺ cells as a proportion of total cells in confocal photomicrographs of the somatosensory cortex. c, Number of CD68⁺ cells as a proportion of total cells in confocal micrographs. N = 3 mice (average of 4 images plotted per mouse). Images were blinded for quantification. Two-way ANOVA with Tukey's post-hoc correction (c). ns = not significant, * P < 0.05.

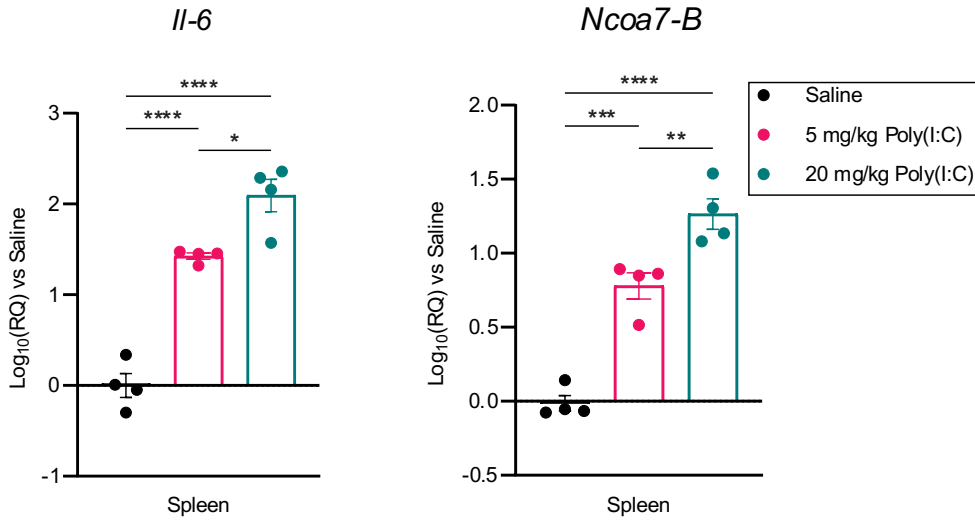
12.3.2 Determining an appropriate dose of poly(I:C) to induce maternal immune activation in *Ncoa7* intercross litters

To explore the role of *Ncoa7* in development during MIA, a pilot study was performed to determine a safe and effective dose to induce poly(I:C)-MIA in *Ncoa7^{+ /DEL}* pregnant dams. Commonly, doses of poly(I:C) in MIA studies range from 5 - 20 mg/kg^{135,136}, thus pregnant dams were injected i.p. on E12.5 with either 5 or 20 mg/kg poly(I:C). IL6 expression along the maternal-placental-foetal axis has been demonstrated to be necessary and sufficient for poly(I:C)-mediated MIA induction in wild-type mice¹³⁷. Therefore, the transcript expression of IL6 in the maternal spleen was measured at both doses by RT-qPCR 4 hours post-dose to validate a maternal IL6 response to poly(I:C) in *Ncoa7^{+ /DEL}* dams. A dose of 5 mg/kg poly(I:C) increased IL6 expression 30-fold, whilst 20 mg/kg increased expression approximately 100-fold (Figure 12.2a). This suggests both doses of poly(I:C) are effective in stimulating an acute IL6-responsive immune reaction in the *Ncoa7^{+ /DEL}* pregnant dam.

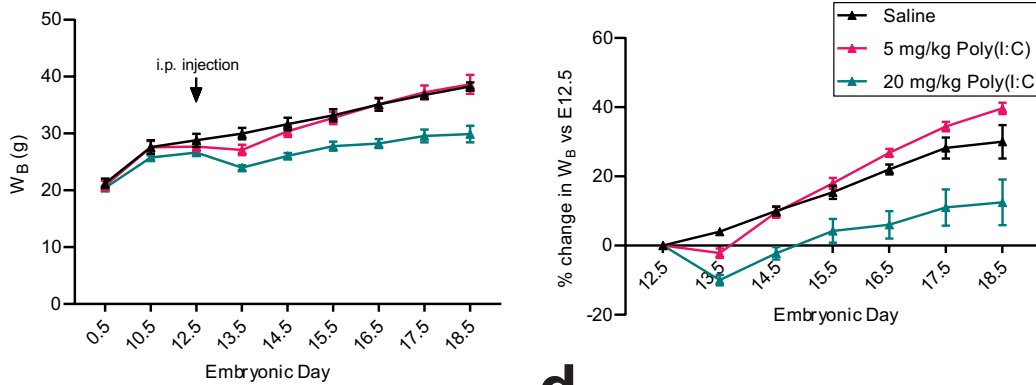
As the dams in this study are heterozygous for deletion of the *Ncoa7* locus, to ensure an *Ncoa7-B* response to MIA is preserved, the transcript expression of *Ncoa7-B* was also measured in the maternal spleen. Again, whilst 20 mg/kg poly(I:C) elicited a stronger response (approx. 20-fold increase), a 5.7-fold induction of *Ncoa7-B* was observed with 5 mg/kg poly(I:C) (Figure 12.2a). This suggests that, despite the

heterozygous genotype of the dams, *Ncoa7-B* is still induced in the maternal immune response.

a



b



c

	% change 1 day post-dose		
	Saline	PIC (5 mg/kg)	PIC (20 mg/kg)
Average	4%	-2%	-10%
Diff	-	**	***
SEM	0.011	0.015	0.015
SD	0.025	0.034	0.029
P value	-	0.009	0.000

d

	Litter size P4		
	Saline	PIC (5 mg/kg)	PIC (20 mg/kg)
Average	5.6	6.4	1.5
Diff	-	-	*
SEM	0.93	1.08	0.96
SD	2.07	2.41	1.91
P value	-	0.589	0.019

Figure 12.2. Validation of Poly(I:C) dose in MIA.

a, *Ncoa7*^{+/DEL} pregnant dams from intercross breeds were injected with 5 or 20 mg/kg poly(I:C) on E12.5 and spleens were extracted 4 hours post-dose. Expression of interleukin 6 (Il-6) and *Ncoa7-B* in the maternal spleen was determined by RT-qPCR. Experiments were analysed using the 2^{-ΔΔCt} method⁹² with *Gapdh* and *Rps16* as reference genes (see section 10.3.1). b, Pregnant dams treated as per (a) were weighed on E0.5, E10.5 and daily from E12.5 to E18.5 and per cent change in body weight from E12.5 was calculated. c, Statistical analysis of per cent change in body weight on E13.5 (one-day post-dose) in

pregnant dams. d, Litter size from pregnant dams treated as per (a) at P4. Unpaired Student's t-test. N = 4-6 dams. - = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

To assess the welfare of injected dams, body weight was measured each day post-dose until E18.5. Dams injected with both doses of poly(I:C) lost more weight than saline-injected dams the day after the injection, however, the body weight of dams injected with 5 mg/kg poly(I:C) recovered to saline controls, whilst body weight of dams injected with 20 mg/kg poly(I:C) did not, indicating a more severe response that may include spontaneous abortion of fetuses (Figure 12.2b,c). *Post-partum*, the litter size at P4 was counted, and where the average litter size from 20 mg/kg injected dams was 1.5, there was no significant difference between litters from saline and 5 mg/kg poly(I:C) injected dams (Figure 12.2d). This evidence indicates that 5 mg/kg is a safe and effective dose to use for this MIA study and limits distress to pregnant dams and pup mortality.

12.3.3 Acute changes in IL6 expression in the foetal brain in MIA

Based on the results of the pilot study, pregnant dams were injected i.p. on E12.5 with 5 mg/kg poly(I:C). 4 hours post-dose, embryos were dissected to measure *Ncoa7-B* and IL6 expression in foetal brain or allowed to reach parturition, aged to early adulthood (P56 – P81) and their behaviour was characterised in several tests (Figure 12.3a). Firstly, in the brain of *Ncoa7^{+/+}* embryos *Ncoa7-B* transcript increased 1.5-fold, suggesting MIA in this model was sufficient to induce *Ncoa7-B* upregulation in the foetal brain that cannot occur in *Ncoa7^{DEL/DEL}* littermates (Figure 12.3b). Finally, there were trends of increasing IL6 in the brain of *Ncoa7^{+/+}* and *Ncoa7^{DEL/DEL}* embryos which may have been diminished in *Ncoa7^{DEL/DEL}* embryos, though these comparisons lack statistical significance (Figure 12.3b). Overall, whilst these data demonstrate an increase in *Ncoa7-B* expression in

Ncoa7^{+/+} embryo brains in response to MIA, an increase in IL6 expression could not be determined, perhaps due to lack of statistical power.

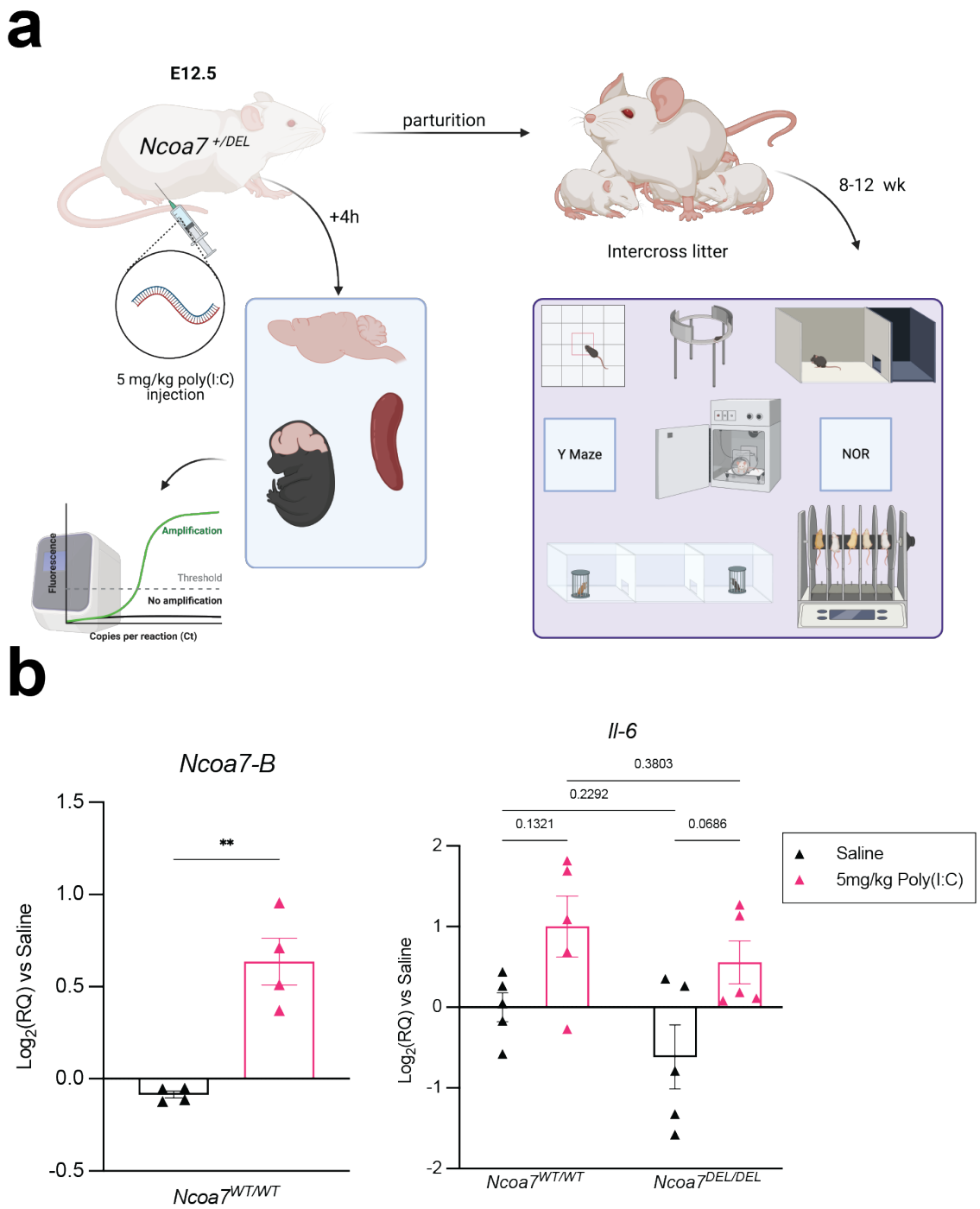


Figure 12.3. Experimental design and expression changes in foetal brain of MIA offspring.

a, Schematic outlining the experimental set-up of the study. b, Changes in gene expression (Log_2 fold-change versus WT) measured by RT-qPCR in foetal heads derived from mothers exposed to 5 mg/kg poly(I:C) for 4 hours intraperitoneally. $n = 4 - 5$ foetal heads from different litters. RT-qPCR experiments were analysed using the $2^{-\Delta\Delta C_t}$ method⁹² with *Gapdh* and *Rps16* as reference genes (see section 10.3.1). Student's t-test (*Ncoa7*-B, left) or two-way ANOVA with Tukey's post-hoc correction and comparison of all means. ** $P < 0.01$.

12.3.4 General activity in the open field maze is unchanged in MIA mice

A comprehensive behavioural pipeline was performed to characterise behaviour in adult mice lacking *Ncoa7* that have been exposed to MIA. Whilst *Ncoa7* knockout mice have demonstrated anxiety-like behaviours and social interaction deficits, the interaction of this locus with environmental perturbation has not been characterised. Firstly, to assess general locomotor activity and exploratory behaviour, mice were tracked in an open field maze for 20 minutes. The amount of time spent in the inner zone (11 cm² central square) and the remaining outer zone of the open field maze were unchanged between groups indicating similar levels of exploration and activity (Figure 12.4a). The total distance moved in the open field maze was also unchanged between the groups, suggesting no locomotor dysfunction or reduced exploratory behaviour resulting from genotype or treatment. The thigmotaxic preference of the mice was measured by calculating the preference ratio for the outer zone (outer zone duration / duration in maze). As expected, all groups showed a greater than chance preference for the outer zone (i.e. > 0.5 preference ratio), however, there were no differences between the groups in outer zone preference indicating no difference in anxiety-like behaviour.

12.3.5 Thigmotaxic behaviour is unchanged in the elevated zero maze

Similar to a preference for the walled, outer zone in the open field maze, thigmotaxic behaviour was also assessed in the elevated zero maze, where a greater than normal preference for the closed arms may indicate anxiety-like behaviour. Whilst

genotype had a significant impact on distance moved in the elevated zero maze, with heterozygous mice exploring less of the maze, no groups displayed an increased preference for closed arm (duration in closed arm / duration of test) versus control *Ncoa7^{+/+}* mice, concordant with open field results (Figure 12.4b). Additionally, all groups showed similar preference for entries into the closed arm (entries into arm / total entries).

preference ratio (frequency) = frequency of entries into closed arm / total entries. c, Mice were automatically tracked in a light-dark box. Dark zone preference ratio (frequency) = frequency of entries into dark zone / total entries). Dark zone preference ratio (duration) = time in the dark zone / duration of test. n = 10 – 16 female mice. Two-way ANOVA with Tukey's post-hoc correction and comparison of all means. * $P < 0.05$. † No statistically significant comparisons on graph. Table indicates significant sources of variation for the graph above.

12.3.6 MIA-*Ncoa7*^{DEL/DEL} mice show greater preference for the dark zone in the light-dark box

To assess anxiety-like behaviour independent of thigmotaxis, mice were scored on preference for the dark zone of a light-dark box. Deletion of the *Ncoa7* locus causes a preference for the dark zone of the light-dark box², however, the interaction of MIA on an *Ncoa7*^{DEL/DEL} background has not been explored. Mice in all groups showed a similar preference for dark zone entries, however, MIA-*Ncoa7*^{DEL/DEL} showed a greater preference for the dark zone than any other group (duration in dark / duration of the test) indicating anxiety-like behaviour resulting from MIA on an *Ncoa7* knockout background (Figure 12.4c). In a two-way ANOVA, genotype was primarily responsible for the variation (6.48% of variation, $P = 0.036$) whilst the effect of treatment was statistically insignificant (3.5%, $P = 0.056$). The interaction effect of both genotype and treatment was responsible for the most variation (8.04%, $P = 0.017$), indicating a cumulative effect of *Ncoa7* deletion and MIA on anxiety-like behaviour in the light-dark box.

12.3.7 *Ncoa7*^{DEL/DEL} mice have reduced preference for the novel arm in the Y-maze forced alternation test

In the forced alternation Y-maze test, mice were initially habituated to the maze for 10 minutes with one novel arm blocked, followed by a 2-minute inter-trial interval in their home cage (Figure 12.5a). Subsequently, they were reintroduced into a clean maze with free access to all arms, including the previously blocked novel arm, to evaluate spatial memory and exploratory behaviour. The total distance traversed in the test was

not different between the groups, indicating comparable levels of exploration (Figure 12.5b).

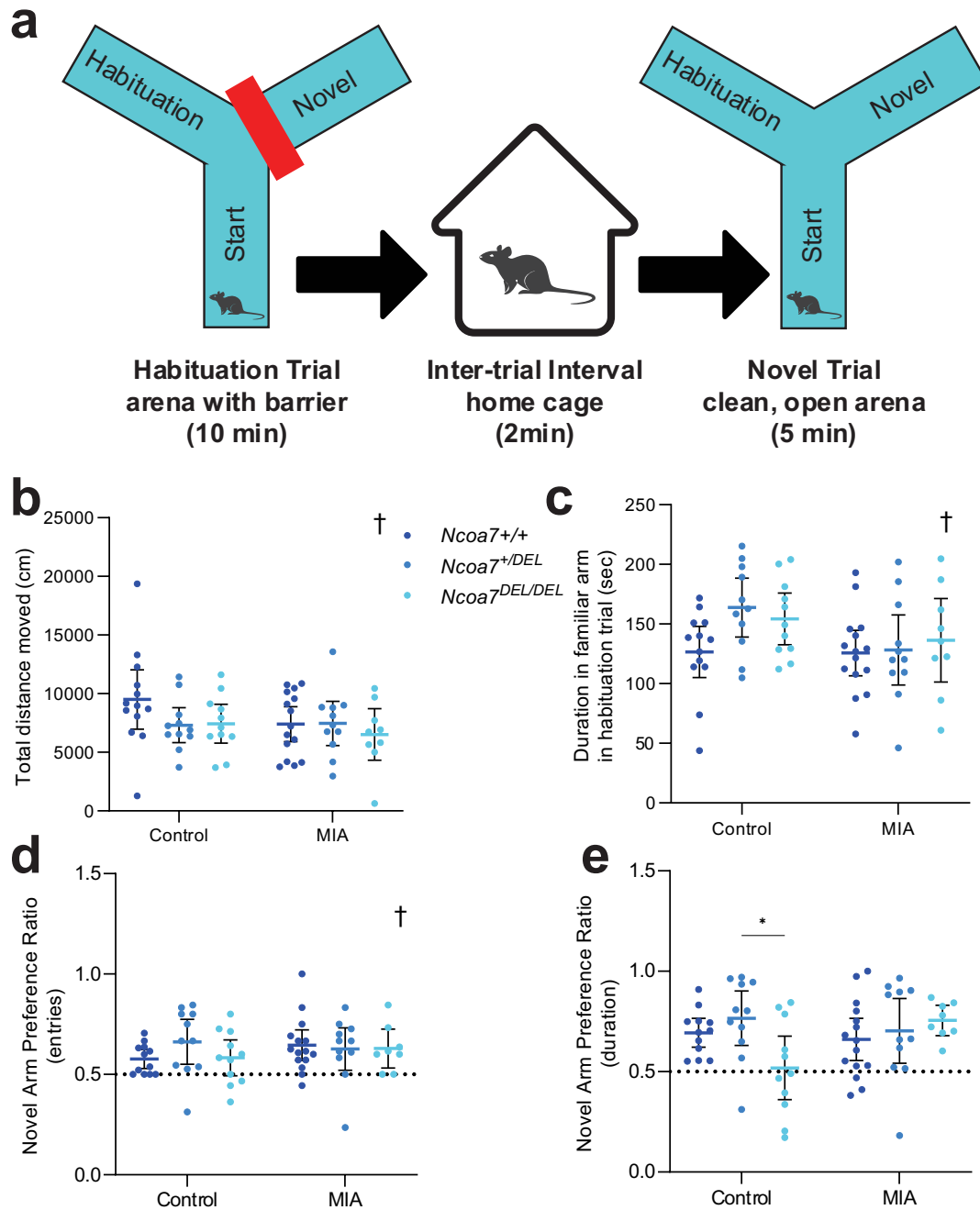


Figure 12.5. Assessment of spatial working memory by forced alternation in the Y-maze.

a, Schematic of the experimental procedure. b, Total distance moved by mice during the test. c, Duration of time spent by mice in the familiar arm during the habituation trial. d, Preference ratio of entries into the novel arm during the novel trial (frequency of entries into novel arm / frequency of entries into novel and familiar arm). e, Preference ratio of the duration of time spent in the novel arm (duration of time in novel

arm / duration of time in the novel arm and familiar arm). n = 10 – 16 female mice. Two-way ANOVA with Tukey's post-hoc correction and comparison of all means. * $P < 0.05$. † No statistically significant comparisons on graph. Table indicates significant sources of variation for the graph above.

Furthermore, the time spent in the familiar arm during the habituation trial was unaffected by genotype or treatment, suggesting no impairment to exploratory drive (Figure 12.5c). In the novel trial, entry frequency into the novel arm was higher than into the familiar arm for all groups, demonstrating consistent exploration (Figure 12.5d). However, *Ncoa7*^{DEL/DEL} mice had a significantly reduced preference for exploration time in the novel arm, indicating possible impairment in spatial working memory due to *Ncoa7* deletion (Figure 12.5e). Notably, this effect was mitigated in MIA-*Ncoa7*^{DEL/DEL} mice.

12.3.8 No change to implicit memory in cue- or context-dependent fear conditioning

To assess implicit memory fear conditioning was performed. Context and cue-dependent fear conditioning is used to assess how animals learn to associate specific environmental contexts or discrete cues (audible tone) with aversive stimuli (foot shock). This method tests the animal's ability to acquire and retrieve fear memories, providing insights into learning and memory function. All groups demonstrated significantly increased freezing on context and cue exposure after conditioning and a discrimination index >1 (% baseline freezing behaviour / % stimulus-dependent freezing behaviour), indicating functional implicit memory. Furthermore, baseline freezing was not significantly different between groups. However, there was no difference in context or cue discrimination between any group suggesting no implicit learning and memory deficits in response to *Ncoa7* deletion, MIA, or the interaction of both (Figure 12.6a-d).

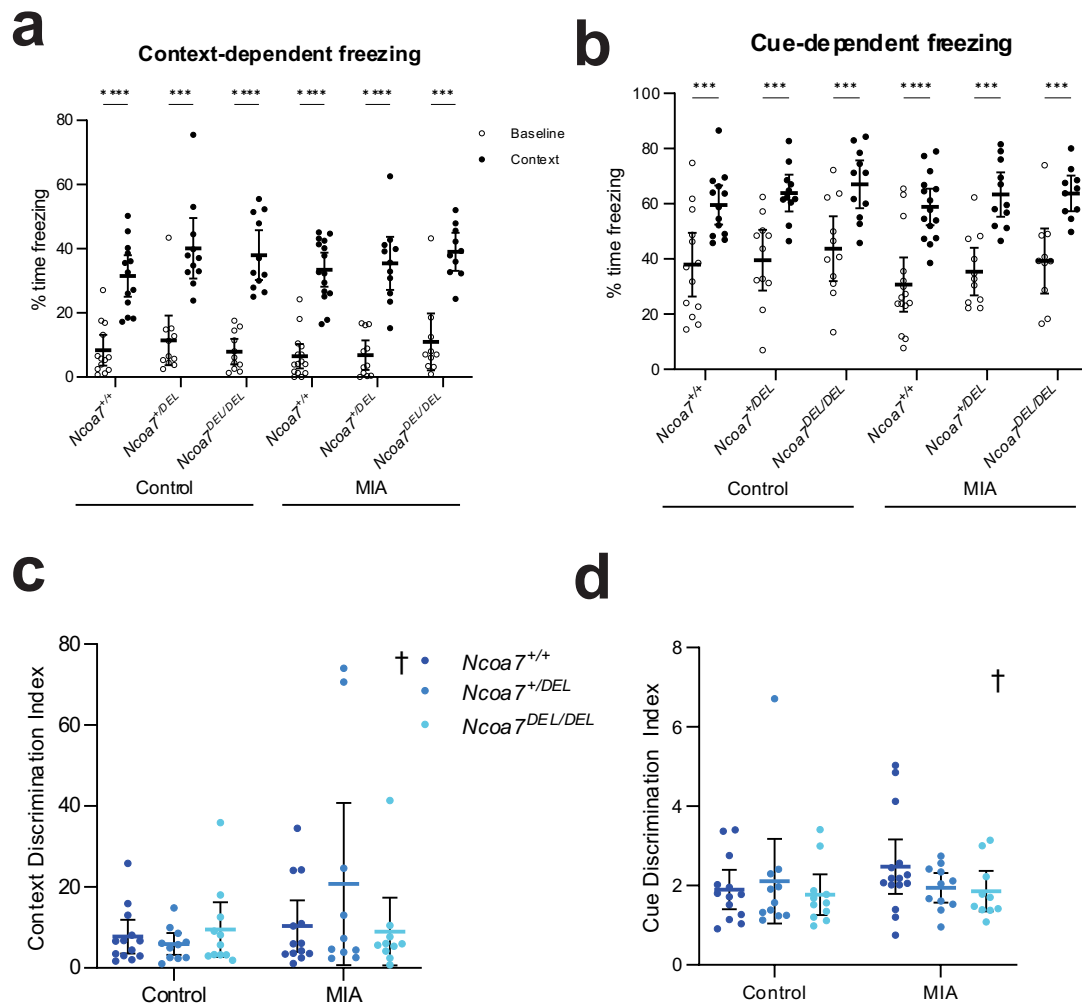


Figure 12.6. Context- and cue-dependent fear conditioning.

a, % time freezing at baseline and after exposure to context in control and maternal immune activation (MIA) offspring. b, % time freezing at baseline and after exposure to cue in control and MIA offspring. c, d, Context (c) and cue (d) discrimination index (% freezing at baseline / % freezing post-exposure to stimulus). n = 10 – 13 female mice. Student's independent t-test (a, b) or two-way ANOVA with Tukey's post-hoc correction and comparison of all means (c, d). *** P < 0.001. **** P < 0.0001. † No statistically significant comparisons on graph.

12.3.9 No change to social novelty preference in the three-chamber test

In the three-chamber social novelty test, mice were subjected to a 10-minute habituation phase with two empty cages positioned in separate chambers, followed by a 2-minute inter-trial interval. Subsequently, during a 10-minute exposure trial, mice were presented with a choice between an empty cage and a cage containing a novel conspecific mouse for a 10-minute recording period. This test was used to evaluate social preference which has been found to be deficient in MIA models of ASD and in *Ncoa7^{DEL/DEL}* mice^{2,138}. Overall, the total distance travelled in this test was significantly affected by treatment (6.67% variation, $P < 0.05$), however, no specific comparison reached significance. Whilst there was a trend of reduced interaction in control *Ncoa7^{DEL/DEL}* mice and MIA mice on an *Ncoa7^{+/+}* background, these comparisons did not reach statistical significance.

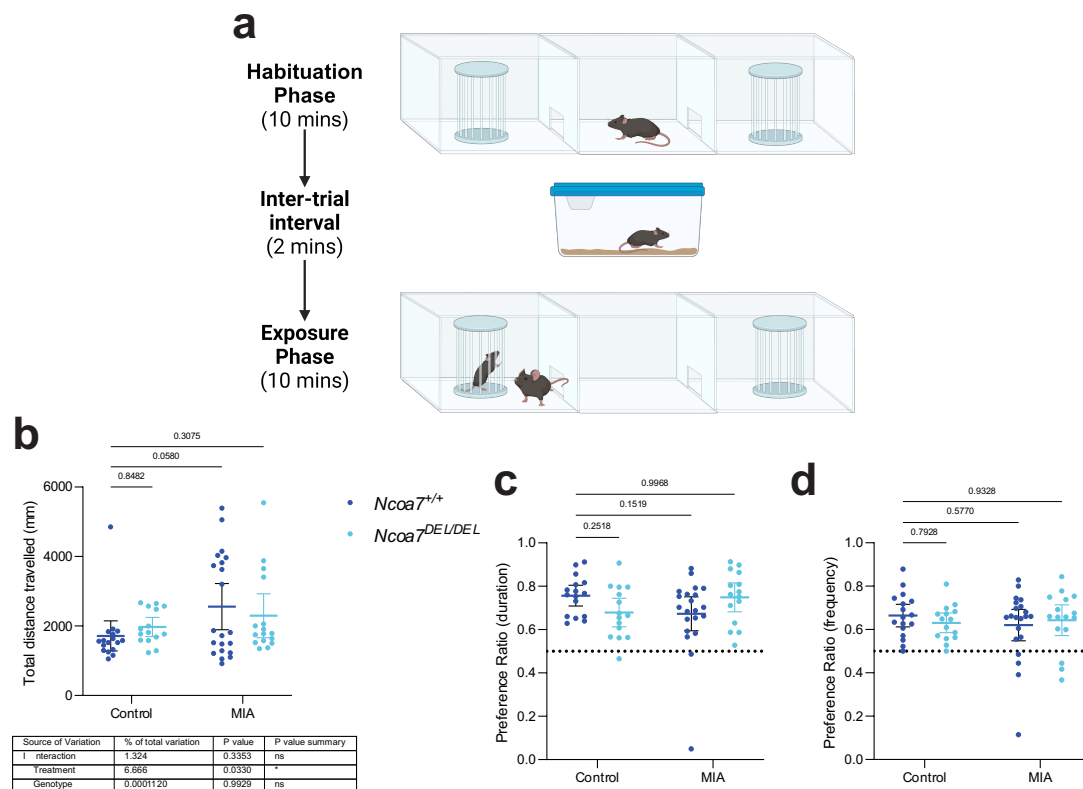


Figure 12.7. Three-chamber social novelty preference test.

a, Schematic outlining the experimental protocol. b, Total distance travelled during the exposure phase of the experiment. c, d, Preference ratio for the novel mouse based on duration of interaction and frequency of interactions (interaction with novel mouse / [interaction with novel mouse + interaction with empty cage]). $n = 15 - 21$ female mice. Two-way ANOVA with Tukey's post-hoc correction and comparison of all means against Control-*Ncoa7*^{+/+}. Table indicates significant sources of variation for the graph above.

12.3.10 MIA-*Ncoa7*^{DEL/DEL} mice are deficient in motor learning

Disruption of the *Ncoa7* homologue, *Oxr1*, in mouse, causes ataxia, hypothesised to result from cerebellar granule cell death¹. Furthermore, *Ncoa7* is enriched in cerebellar Purkinje cells which play a pivotal role in coordinating motor control by modulating the timing and precision of signal transmission within the cerebellum^{2,139}. Thus, to assess motor coordination and learning, the time taken to fall from an accelerating rotarod apparatus was recorded on three consecutive trials with a 24-hour interval between each trial. All groups except MIA-*Ncoa7*^{DEL/DEL} mice displayed a trend of increasing the time

to fall over three trials, and whilst in the initial trial the time to fall was not different between the groups, on the final day MIA-*Ncoa7*^{DEL/DEL} mice had a significantly shorter time to fall versus Control-*Ncoa7*^{+/+} mice. This implies that whilst motor coordination may not be impaired by MIA with deletion of *Ncoa7*, this intervention may cause a deficit in motor learning.

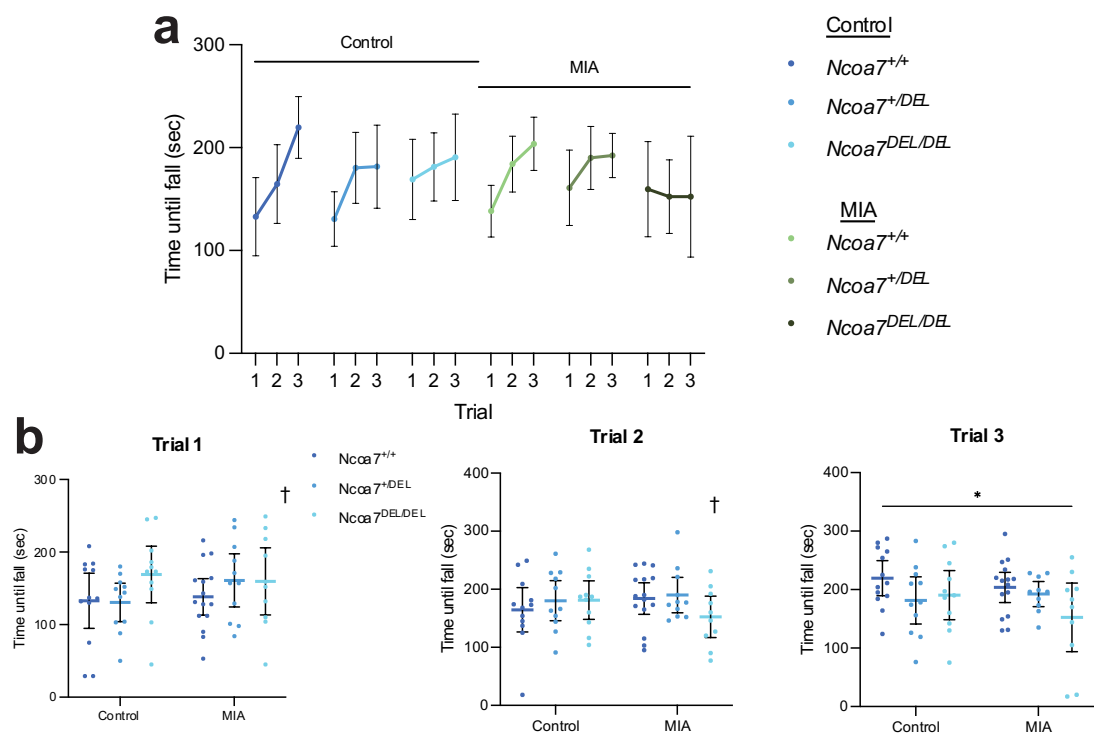


Figure 12.8. Motor coordination and learning on the rotarod apparatus.

a, time to fall (seconds) on an accelerating rotarod across three consecutive trials with a 24-hour interval.
b, Time to fall on trials one, two and three. n = 10-15 female mice. Two-way ANOVA with Tukey's post-hoc correction and comparison of all means. * P < 0.05. † No statistically significant comparisons on graph.

12.4 Discussion

The findings presented here have begun to explore the interaction between MIA, the *Ncoa7* gene locus and their impact on neurodevelopmental outcomes in offspring. Specifically, the investigation into the *Ncoa7* deletion through the *Ncoa7^{DEL}* mouse model offers novel insights into how genetic susceptibilities can modulate the effects of environmental triggers such as MIA, potentially modifying cytokine production and behavioural changes.

12.4.1.1 Interpretation of Findings

These results demonstrate that deletion of *Ncoa7* alters the response to MIA, leading to some significant behavioural changes across a battery of tests, such as anxiety-like behaviours in the light-dark box and reduced motor learning in the rotarod test. The observed deficiency in LPS-induced CD68 upregulation in *Ncoa7^{DEL/DEL}* mice suggests a compromised microglial activation pathway, which is critical given the role of microglia in synaptic pruning and neuroinflammation—processes known to be perturbed in ASD^{120,140}, although it remains to be determined how MIA may affect this process.

Furthermore, these findings indicating altered IL6 signalling across the maternal-placental-foetal axis in response to MIA provide insights into the molecular cascades that may underlie behavioural phenotypes associated with ASD. IL6 is a known mediator of inflammation and is necessary and sufficient for behavioural and cortical transcriptomic changes in MIA offspring¹²⁷. Its reduced expression in *Ncoa7^{DEL/DEL}* MIA offspring suggests the IL6 secretion, signalling and potentially microglial function is disrupted in LOF of *Ncoa7*. Based on a reduced IL6 response, *Ncoa7* deletion might be hypothesised to mitigate the behavioural endophenotypes and transcriptomic changes observed in MIA. However, this hypothesis is reductionist and disregards other molecular modulators of

MIA, such as IFN-I¹¹⁴. Furthermore, *Ncoa7* LOF mutations have been described in ASD patients¹² and, whilst a significant social interaction deficit could not be identified in this study, a consistent anxiety-like phenotype in the light-dark box was observable in MIA-*Ncoa7*^{DEL/DEL} mice that warrants further investigation. Finally, the dose of poly(I:C) used to induce MIA in this study (5 mg/kg) was lower than doses routinely used in literature, which are often ≥ 10 mg/kg and sometimes coupled with additional environmental stressors, such as maternal separation¹¹³. However, this study demonstrated that a 20 mg/kg dose of MIA in *Ncoa7* intercross litters was intolerable and results in high rates of embryonic lethality (Figure 12.2), thus the lower tested dose was used. Further studies should investigate the effects of intermediate doses (e.g. 10 mg/kg) on embryonic lethality and offspring behaviour.

12.4.1.2 Limitations

Whilst the current study is a novel model of MIA, several limitations should be acknowledged. First, ensuring sufficient statistical power in this study is challenging since behavioural changes, especially in mice, may be subtle and can be affected by multiple biological factors both *in utero* and postnatally. For example, in a study using C57BL/6 pregnant dams treated with poly(I:C) on E12.5 it was possible to stratify the offspring into “susceptible” and “resilient” based on the presence or absence of behavioural dysfunctions in adulthood, such as impaired social preference and reduced pre-pulse inhibition¹⁴¹. This apparent susceptibility was correlated with transcriptomic profiles, for example, more genes associated with oxidative phosphorylation and demyelination were downregulated in the medial prefrontal cortex and amygdala of susceptible mice, respectively. Additionally, mice could be stratified based on peripheral inflammatory cytokine levels, with increased peripheral IL1 β , IL6, IL10 and TNF α

correlated with reduced social preference and pre-pulse inhibition, whereas MIA offspring with no changes to their cytokine levels were classed as being resilient to behavioural changes¹⁴¹. This study demonstrates an inherent, and potentially classifiable, variability in the immunological and behavioural outcomes of MIA which could be applied to this model. This approach, whilst informative, requires detailed characterisation of markers in every experimental animal.

Furthermore, metrics of early life events were not included in this study, for example, measuring key developmental behavioural and neuropathological milestones. Whilst, standardised, robust methods to track development across life are currently limited¹⁴², automated home-cage analysis, ultrasonic vocalisation and longitudinal magnetic resonance imaging (MRI) could have been adopted to achieve these aims^{143,144}. Longitudinal studies that track neurodevelopmental and immunological outcomes from perinatal stages through to adulthood should provide invaluable data on the progression of ASD-like phenotypes and the long-term impacts of interventions targeting the immune system or specific genetic pathways, including IL6, IFN-I and NCOA7 rescue. Such studies would be crucial in aligning mouse model findings more closely with the human condition, potentially guiding therapeutic developments.

Additionally, some factors affecting responsivity to MIA can be partially mitigated by averaging measured outcomes from a litter, thus treating the entire litter as a single biological unit¹⁴¹. This can counterbalance differences in embryonic poly(I:C) exposure that may occur depending on placental blood flow and proximity to the site of Poly(I:C) injection^{145–147}. This does not, however, mitigate for different litter sizes, which have been demonstrated to be affected by LPS-MIA and also affect behavioural and neuroanatomical outcomes in the offspring¹⁴⁸. Nonetheless, it has been recommended to

report on the litter as a biological unit to avoid these confounds^{146,147}. Whilst not possible in this study due to litters of mixed genotypes, this would be possible in a model of haploinsufficiency where wild-type and heterozygous litters are compared.

Moreover, the interpretation of immune response data, particularly relating to cytokine levels, is complex and can be influenced by numerous factors, including the gestational stage of MIA, dose of poly(I:C), sex of the offspring, tissues, brain regions or cells analysed^{114,129}. For example, males have been found to display reduced motor activity and repetitive behaviour in the marble burying test¹⁴⁹. Whilst female mice were found to exhibit social interaction deficits and impaired working memory in IFN β -mediated MIA¹¹⁴, suggesting a degree of sexual dimorphism in the pathogenesis of ASD. ASD is more commonly diagnosed in males, thus many studies use exclusively male offspring in behavioural evaluation¹⁵⁰, which makes analysis of sexual dimorphism in the pathogenesis of NDD in females difficult to ascertain, thus raising problems in the diagnosis of ASD in girls¹⁵¹.

12.4.1.3 Suggestions for Future Research

Given the limitations and findings of this study, several avenues for future research are suggested. It would be beneficial to explore the interaction of *Ncoa7* deletion-mediated inhibitory synapse dysfunction with MIA. Whilst *Ncoa7* deletion was found to decrease the number of cortical neuron inhibitory contacts *in vitro*², MIA has been demonstrated to enhance GABAergic transmission in pyramidal neurons of the hippocampal CA1 in adult offspring¹²⁵. The histopathological outcome of the interaction of these factors could be studied in MIA-*Ncoa7*^{DEL} brain regions with impaired connectivity in ASD, such as the hippocampus, frontotemporal lobe and amygdala¹⁵².

Experimentally, further studies could investigate the microglia-specific roles of IL6, IFN-I and NCOA7-B in mediating the effects of MIA. Investigating microglial changes postnatally could be achieved by FACS sorting of microglia and performing RNA-seq to identify disrupted gene expression pathways, as has been demonstrated previously, where the microglia of offspring exhibited IFN-induced expression of TNF α and CD45¹¹⁴. A useful tool in the delineation of the effect of NCOA7-B in MIA is the *Ncoa7^{tm1.1}* mouse. In this model, *Ncoa7* expression is disrupted by the insertion of a LacZ/NeoR cassette targeted to exons 2 and 3 of *Ncoa7*. Since NCOA7-B is encoded downstream of this mutation with its unique alternative promoter, it is hypothesised expression of NCOA7-B would be preserved in this model, thus providing a useful model to define the effects of specific *Ncoa7* isoforms *in vitro*.

12.4.1.4 Conclusion

In conclusion, this study highlights the potential role of the *Ncoa7* locus in modulating the neurodevelopmental impacts of MIA, especially in anxiety-like behaviour. The interaction between genetic predispositions and environmental factors, such as MIA, provides a multifaceted model for understanding complex NDD, like ASD. This study provides a framework for continued investigation of the gene-environment interaction with specific consideration to ASD risk loci, such as *Ncoa7*. Continued exploration of these pathways holds promise not only for elucidating the underlying mechanisms of ASD but also for developing targeted interventions that could mitigate its impacts. Further studies should explore the changes to microglial function since a reduced IL6 expression was observed in MIA-*Ncoa7^{DEL/DEL}* mice, the gene expression changes and neuropathology underlying observed anxiety-like responses and the effect of

additional post-natal ASD-related stressors, such as maternal separation or postnatal immune challenge.

13 Investigating the function of Oxidative Stress Resistance 1 (OXR1) in human iPS-microglia

13.1 Chapter Abstract

OXR1 is a conserved gene expressed throughout the brain that is crucial for neuronal survival. Knockout of *Oxr1* in mice causes neuronal death, accompanied by activation of microglia and astrocytes before neuronal death, whilst overexpression of *Oxr1* in ALS model mice limits neuroinflammation and preserves motor function and lifespan. The molecular mechanisms underpinning these observations have yet to be elaborated, but OXR1 has been demonstrated to interact with the V-ATPase, a vesicular proton pump crucial for intraluminal acidification. Consistent with this hypothesis, patients with LOF mutations in *OXR1* show lysosomal accumulation with abnormal content. Whilst *OXR1* is highly expressed in human microglia, the principal immune cells of the brain, its function in these cells is unexplored. Thus, I generated *OXR1* knockout iPSC and differentiated them into microglia. I demonstrated reduced density of *OXR1* knockout cells *in vitro* and increased density of the V-ATPase. This was accompanied by lysosomal accumulation and reduced endocytic uptake but no changes to lysosomal pH. Furthermore, RNA-seq of *OXR1*^{-/-} microglia demonstrated upregulation of transcripts encoding MHC Class II receptors, suggesting a novel role for *OXR1* in antigen presentation to CD4⁺ T cells.

13.2 Introduction

13.2.1 OXR1 is a highly conserved NCOA7 paralogue important in neurological disease

Initially discovered in a screen for antioxidant proteins, OXR1 is a conserved eukaryotic gene and is most closely related to its paralogue, NCOA7^{13,14,22}. OXR1 and NCOA7 share similar domain structure and a degree of residue sequence homology (38% in human); both can protect against oxidative stress and cause neuronal dysfunction when disrupted^{14,1,153,17,2}. Furthermore, both genes are highly expressed throughout the brain, with *OXR1*-enriched in cerebellar granule cells and *NCOA7* in cerebellar Purkinje cells^{1,153,2}.

Within the context of human neurological disease, perturbation of *OXR1* has recently been identified as a factor that may influence lysosomal dysfunction³. Our research group has substantiated these findings by demonstrating the disruption of lysosomal enzyme expression and immune signalling pathways in the brain of *Oxr1*^{-/-} mice⁶. Notably, these disturbances manifest before the onset of significant cerebellar neuronal death. Furthermore, and reviewed below, the TLDc domain is reported to engage with the V-ATPase in multiple systems^{154,155,8,49}. Given that the V-ATPase mediates lysosomal acidification, hence degradation of phagocytosed material, a mechanistic relationship between *OXR1* disruption, V-ATPase activity, and lysosomal dysfunction is a plausible hypothesis for further research. Especially given the impact of V-ATPase activity on immune regulatory processes and cellular responses within phagocytic immune cells. Consequently, this chapter will focus on the regulation of lysosomal function and immune responses by *OXR1* specifically within brain parenchyma macrophages, or microglia.

13.2.2 *OXR1* loss-of-function mutations cause severe neurodevelopmental phenotypes in human and mouse

Bi-allelic LOF of *OXR1* in the human population is rare, likely in part due to the severity of the resulting phenotype³. The few homozygous LOF mutations in *OXR1* described to date are highly penetrant in patients and strongly associated with neurodevelopmental disease.

For example, a missense recessive mutation (ENST00000517566.7:c. 233A > G, p.Lys78Arg) is associated with severe, bilateral sensorineural hearing loss but not intellectual disability nor ataxia⁴. This substitution is not predicted to change the sequence of the short isoforms of *OXR1* (*OXR1D*, ENST00000449762.6 and ENST00000297447.10), which consist almost entirely of the TLDc domain, suggesting a degree of functional rescue by these proteins.

However, reported polymorphisms resulting in frame-shifts and truncations around, or close to, the TLDc domain (ENST00000531443.6:c.2082+1G>T;c.1100C>G, p.Ser367* and ENST00000531443.6:c.1324delA, pSer442Valfs*2 and ENST00000531443.6: c.2236-1G>C) result in developmental delay, hypotonia, seizures and cerebellar atrophy (Figure 13.1). These mutations were reported to result in complete abrogation of *OXR1* expression, determined by immunoblotting patient tissue with two antibodies upstream of the mutations, and a more severe phenotype. Of note, the shorter isoforms of *OXR1* (*OXR1D1* and *OXR1D2*) were not measured, thus their expression cannot be excluded. Crucially, Yang et al.²³ demonstrated by Northern blot that, whilst *OXR1A* is the dominant transcript in the human brain, *OXR1D* is still reasonably detected.

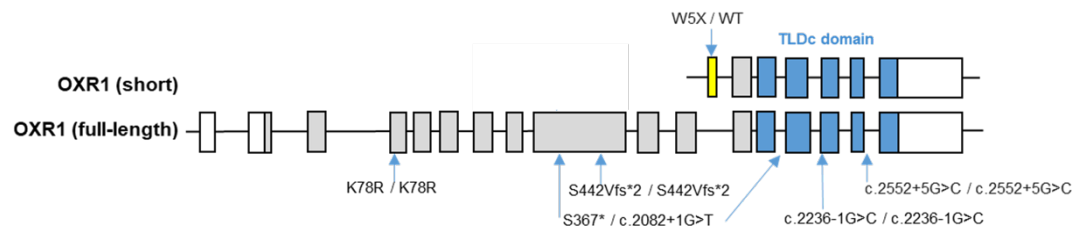


Figure 13.1. Diagram of published OXR1 variants in full length (NM_001198533.2) and short (NM_001198534.1) isoforms.

Blue indicates the position of the TLDC domain and yellow is the unique first coding exon of the short isoform. Variants are K78R / K78R⁴, S367* / c.2082+1G>T, S442Vfs*2 / S442Vfs*2 and c.2236-1G>C / c.2236-1G>C³, W5X¹⁵⁶, c.2552+5G>C / c.2552+5G>C (Lin et al., 2023)⁵. Not to scale. Reproduced from Bucknor et al. (2024)³⁸ under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

In summary, these patient mutations emphasise the functional significance of the TLDC domain, since mutations affecting this common sequence, hence multiple *OXR1* isoforms, are associated with a more severe phenotype. Measuring the expression of distinct *OXR1* isoforms at both the tissue and cellular levels holds promise for elucidating the functional redundancy apparent in *OXR1* isoforms. This approach may also provide insights into the structural interactions critical for *OXR1*'s function, i.e. by comparing the consequences of specific isoform or domain disruption and facilitating a more comprehensive understanding of molecular mechanisms.

13.2.3 OXR1-knockout mice recapitulate human loss of function and show neuroinflammation and lysosomal dysfunction

In *Oxr1*^{-/-} mice, a degree of the clinical presentation and neuropathology of human patients is recapitulated. Whilst heterozygous knockout mice show no overt gait or neuropathology and live as long as wild-type counterparts, *Oxr1* disruption renders cerebellar granule cells susceptible to oxidative stress contributing to neurodegeneration, ataxia and death at around P25¹. This is comparable to human patients detailed above with reported null expression of OXR1, who display reduced cerebellar volume, due to

atrophy or hypoplasia. Furthermore, the rescue of neuronal death in the mouse is possible with the murine orthologue of *OXRID*, which further highlights the functional redundancy of *OXRI* isoforms in the brain and the TLDc domain in general¹. This also suggests patients with *OXRI* frame-shift mutations and a severe phenotype do not express any *OXRI* isoform since, if expressed, *OXRID* could be expected to limit or prevent neuronal dysfunction³.

At the cellular level, the mechanisms underpinning the neurodegenerative pathology caused by loss of *OXRI* are poorly understood, although oxidative damage in granule cells¹ and lysosomal accumulation³ have been proposed. In addition, the Oliver group demonstrated a striking infiltration of reactive astrocytes in the granule cell layer of *Oxr1*^{-/-} mice in the cerebellum before neuronal death, accompanied by bushy and amoeboid microglia surrounding the region, a morphological hallmark of neuroinflammation [doctoral thesis, unpublished]⁶. Bulk RNA-seq analysis of the *Oxr1*^{-/-} cerebellum revealed a significant increase in the expression of genes encoding immune signalling and lysosomal proteins before the onset of neuronal death (P19)⁶. For example, complement cascade components (*Clqa*, *Clqb*, *Clqc*), pattern recognition receptors, (*Fcrls*, *Trem2*), chemokine receptors (*Cx3cr1*), cathepsins (*Ctss*, *Ctsz*, *Ctsd*) and phagocytosis-associated lysosomal glycoproteins (*Cd68*, *Laptn5*). Collectively, these transcriptomic and histopathological data reveal the initiation of neuroinflammation preceding cell death in *Oxr1*^{-/-} mice.

Whilst the molecular mechanism remains unclear, this inflammatory response is concomitant with lysosomal dysfunction, both processes dependent on the V-ATPase, a conserved *OXRI* interactor⁸. These findings propose an unexplored role for *OXRI* in microglial phagocytosis and the transition of microglia to a neurotoxic phenotype.

Exploring lysosomal biology and inflammatory pathways in *OXRI*^{-/-} human microglia emerges as an advantageous model system, offering insights into the cellular mechanisms of neuroinflammation in *OXRI* LOF. Manipulation of *OXRI* in human cells with a euploid genetic background (i.e. iPSC) represents an ideal strategy to fulfil this aim. Such an investigation not only contributes to understanding the pathophysiology but also validates the conservation of neuroinflammation across mouse and human systems.

13.2.4 Overexpression of OXR1 is protective against inflammation

Invertebrate studies have begun to link TLDC domain-containing proteins to innate immune function. In the silkworm, *P. aeruginosa* and *S. aureus* infection upregulated *OXRI* transcript expression and pharmacological inhibition of the Jun kinase (JNK) pathway limited this response, which reduced the viability of bacteria²¹. These data suggest that infection may be a stimulus for OXR1 upregulation, possibly via the JNK pathway. Furthermore, gain-of-function (GOF) of the OXR1 homologue, *mustard* (*mtd*), in *Drosophila* are more resistant to *V. cholerae* infection, suggested to be a consequence of NFκB regulation²⁰ or reduced mitosis of intestinal stem cells¹⁵⁷. Of note, *mtd* is considerably shorter than mammalian *OXRI*, consisting almost entirely of the TLDC domain, and thus is more accurately described as an orthologue of *OXRID*.

In a mouse model of autoimmune glomerulonephritis, systemic delivery of mesenchymal stem cells transformed to overexpress *Oxr1* limited the extent of renal damage and reduced macrophage and T cell infiltration by inhibiting expression of chemokines, interleukins and NFκB, however, the mechanism remains unexplored¹⁵⁸. Moreover, there is genetic evidence that *OXRI* overexpression has specific anti-neuroinflammatory effects in mouse models of neurodegenerative disease. *OXRI* is upregulated in sporadic patient amyotrophic lateral sclerosis (ALS) and mouse *SOD1*^{G93A}

spinal cord tissue, indicating a conserved role in the pathophysiology of the disease. In *SOD1^{G93A}* mice, neuronal overexpression of a full-length *OXRI* isoform delays motor neuron death in vivo and extends lifespan, whilst inhibiting non-cell autonomous inflammatory activation and complement cascade in the spinal cord³⁷. Specifically, glial fibrillary acidic protein (GFAP)-positive astrogliosis and the expression of CD68, a marker of proinflammatory, phagocytic microglia are reduced in spinal cords across the lifespan of *SOD1^{G93A}* mice with ectopic *OXRI*. This protective effect was recapitulated in an independent, humanised *TDP-43^{M337V}* model of ALS, where a reduction of Iba1 and GFAP immunoreactivity was also seen with the same genetically driven neuronal overexpression of *OXRI*³⁵. Finally, delivery of an *OXRI* plasmid vector by liposomes to rats with experimental thoracic spinal contusion improved hindleg coordination and strength, reduced CD68 expression and mitigated neuronal apoptosis.

Together, these data provide further proof-of-concept for the application of *OXRI* gene therapy in CNS injury¹⁵⁹. However, the mechanisms that underlie the apparent neuroprotective role of *OXRI* are poorly understood. Interestingly, a very recent study has focussed on one aspect of *OXRI*-mediated neuroprotection in age-related neurodegeneration and the role of dietary restriction. In a screen of genetic variants in *Drosophila* that confer neuroprotection under dietary restriction, the fly *OXRI* orthologue, *mtl*, was found to stabilise the retromer to delay brain ageing¹⁶⁰. The retromer is a conserved protein complex involved in endosomal trafficking, specifically responsible for the retrograde transport of cargo from endosomes to the trans-Golgi network, thereby regulating the sorting and recycling of membrane receptors in eukaryotic cells¹⁶¹. Further, restoration of retromer function by gene or drug therapy rescued endolysosomal function in human *OXRI* patient fibroblasts and

neurodegeneration in *mta^{-/-} Drosophila*. Crucially, this mechanistic link combines the protective effect of *OXR1* with its association with lysosomal dysfunction, since the retromer is crucial for endolysosomal trafficking. In this study levels of inflammation were not measured, thus it is unclear whether restoring retromer stability, hence vesicular trafficking, also limits inflammation that may be induced by loss of *OXR1*.

Taken together, these data suggest a broad, protective, anti-inflammatory role for *OXR1* that is poorly understood but may involve regulation of vesicular trafficking regulated by retromer stabilisation. Furthermore, the combined findings of upregulation in ALS and its protective effect when delivered to the CNS highlight *OXR1* as a promising disease-modifying therapeutic target to limit neuroinflammation. Correlation of the level of *OXR1* expression with disease progression or severity in ALS would bolster this hypothesis and is an avenue for further research. This collective evidence underscores the need for further exploration of the underlying molecular interactions and gene-regulatory mechanisms of *OXR1* in neuroinflammation. Indeed, the role of *OXR1* in human microglia, the principal immune cells of the central nervous system (CNS), remains unexplored. Crucially, *OXR1* is lowly expressed in mouse microglia whereas in humans microglia and neurons have the highest level of *OXR1* expression among the principal brain cell types^{33,34}. This apparent relative difference in cell-type distribution suggests the function of *OXR1* in microglia may be a species-specific context that is yet understudied.

13.2.5 OXR1 is a novel interactor of the V-ATPase with unknown consequence

Evidence suggests *OXR1* has an important role in protecting cells from oxidative stress and influencing inflammation, however, the molecular mechanisms are poorly described. More recently, the TLDc domain was identified as a novel V-ATPase

interacting peptide, as described in the general introduction. Whether this interaction is related to the anti-inflammatory property of *OXR1* is unclear, but it may provide a functional connection between endolysosomal defects, retromer stabilisation and neuroprotection. A combination of structural and biochemical studies have begun to predict the physiological consequences of TLDc protein binding to the V-ATPase, although the directionality of effect is suggested to vary between TLDc proteins. For example, TLDc domain binding during viral infection is suggested to lower vesicular pH³⁹, whereas *in vitro* biochemistry and structural analysis predict that binding inhibits V-ATPase holoenzyme formation⁴⁹, which would be expected to raise vesicular pH.

At the cellular level, limited insight into the effect of *OXR1* LOF has suggested lysosomal accumulation in patient fibroblasts and the presence of atypical vesicles with membranous content, which may be autolysosomes and/or endo-lysosomes³. These findings would be consistent with V-ATPase dysregulation, since the proton pump is crucial in lysosomal acidification and thus may affect degradative processes, including autophagy and phagocytosis, with implications for neuroinflammation⁶¹. It is now crucial to identify how *OXR1* regulates neuroinflammation in humans and how this relates to conserved V-ATPase binding and lysosomal function.

13.2.6 Aims and hypotheses

Ultimately, *OXR1* is inherently pleiotropic, exhibiting diverse cellular functions, that are likely contingent on cell- and species-specific and contextual factors. Cumulative evidence supports the following assertions (a) *OXR1* is anti-inflammatory in models of neurodegeneration; (b) *OXR1* LOF correlates with prevalent lysosomal deficits; and (c) via its TLDc domain, *OXR1* interacts with the V-ATPase, a protein crucial for endo-lysosomal acidification and trafficking. As mentioned previously, *OXR1* is highly

expressed in human microglia, which regulate inflammation in the brain and contribute to neuronal death but remains unexplored in these cells. Thus, the specific hypothesis and aims guiding the exploration of *OXR1* in this model are:

Hypotheses

H₁: *OXR1* is expressed in human microglia, and via its interaction with V-ATPase limits lysosomal acidification, and therefore phagocytic degradation.

H₂: *OXR1* negatively regulates the expression of phagocytosis-associated and pro-inflammatory genes in human microglia.

Aims

1. To determine the level of OXR1 protein expression in iPS microglia;
2. To generate co-isogenic *OXR1*^{-/-} and *OXR1*^{TLDc^{-/-}} (collectively, *OXR1*-LOF) human iPS cells using CRISPR-Cas9 editing;
3. To differentiate *OXR1*-LOF iPS cells into microglia;
4. To measure lysosomal accumulation, function, and pH in *OXR1*-LOF microglia;
5. To measure genome-wide transcript expression in *OXR1*-LOF microglia without bias.

13.3 Results

13.3.1 OXR1 is expressed in iPSC-microglia

To ascertain the level of expression in model human systems, I first measured OXR1 protein in iPSC-derived brain cells. To achieve this, KOLF2.1S iPSC were infected with lentiviral vectors carrying either *NFIB* or *NGN2* to induce an astrocytic or neuronal lineage, respectively (**Figure 13.2a**). Additionally, microglia were derived from the same parent iPSC using a primitive haematopoiesis protocol, faithful to the ontogenetic origin of microglia⁷⁰.

Whole protein extract from these three cell types was probed for OXR1 by Western blot. A loading control protein was not used, due to inconsistent expression between the cell types, however Coomassie staining for total blotted protein confirmed <10% variation between total protein loaded. Expression of OXR1 was highest in microglia with lower expression in neurons and astrocytes (**Figure 13.2b**). Furthermore, whilst astrocytes, microglia and neurons appear to share the same isoforms of *OXR1*, at least one distinct isoform or modified protein of a lower molecular weight is expressed in undifferentiated iPSC. This confirms relatively high OXR1 expression in microglia, supporting the use of this system as a valid experimental model.

13.3.2 OXR1 knock-out in iPSC

To explore the molecular function of *OXR1* in human microglia, I intended to knock out *OXR1* in iPSC. To this end, the knock-out strategies outlined in Section 10.2.7 were applied to iPSC, and *OXR1*^{-/-} and *OXR1*^{TLDC/-} clones were generated using CRISPR-Cas9 editing, clonal selection and expansion (**Figure 13.2c**). This was achieved through two independent LOF strategies:

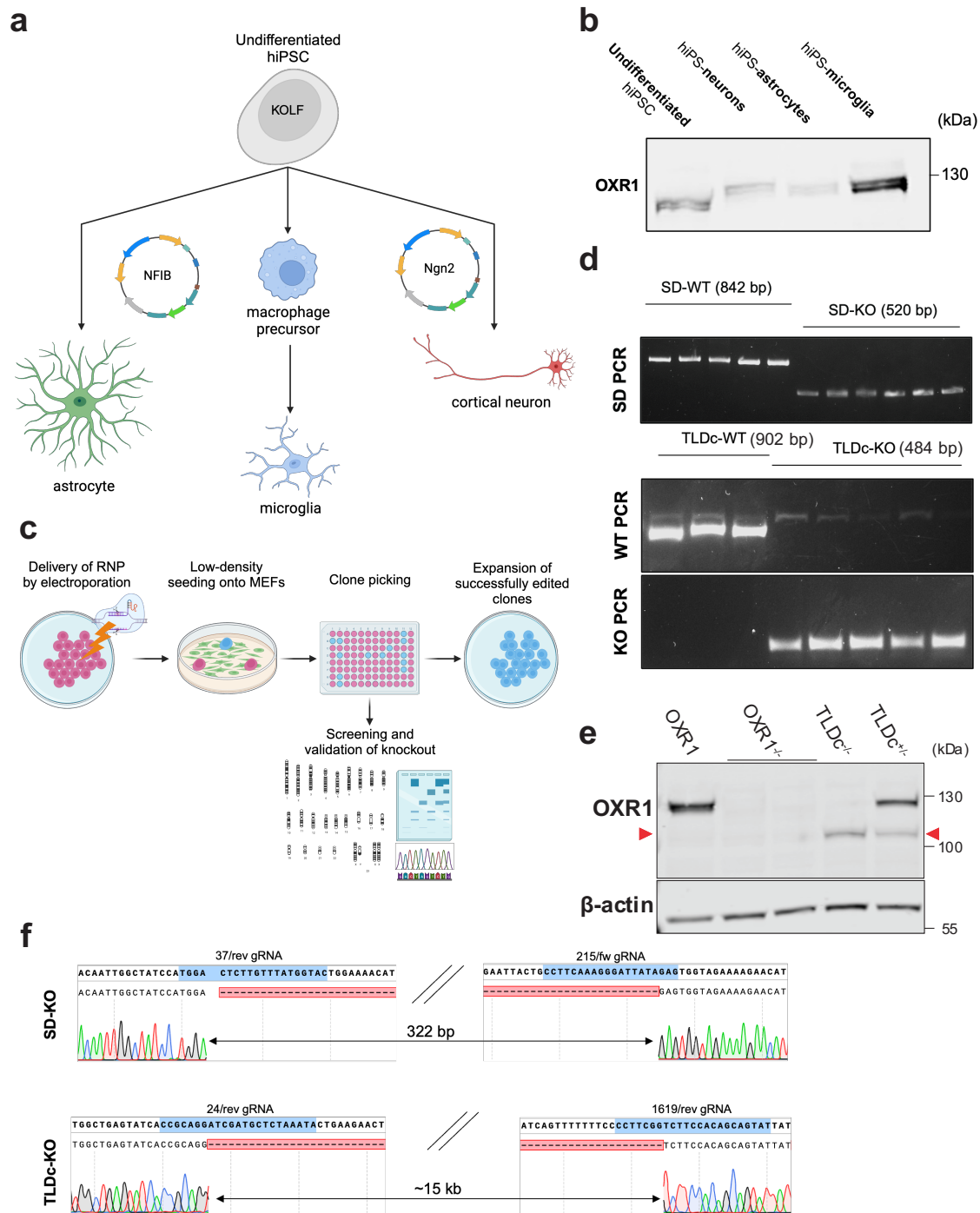


Figure 13.2. Generation of OXR1^{-/-} hiPS cells.

a, Schematic of astrocyte and neuron differentiation from iPS using NFIB and Ngn2 lentiviral vectors, respectively. Microglia were differentiated using a primitive hematopoietic differentiation protocol b, OXR1 expression in whole protein extract (40 μ g) in different iPS-derived cells. c, Workflow schematic of OXR1^{-/-} iPS cell cloning and genomic validation of knockout (RNP – ribonucleoprotein, MEFs – mouse embryonic fibroblasts). d, Polymerase chain reaction (PCR) amplification of gDNA from hiPS clones determined as wild-type (WT) or knock-out (KO). The reactions screen for splice donor (SD) removal or truncation of OXR1 by removal of the TLDc domain. e, OXR1 expression in whole protein extract (10 μ g)

in WT, *OXR1*^{-/-}, *OXR1*^{TLDC-/-}, and *OXR1*^{TLDC+/-} iPSC. Removal of the TLDC domain results in a novel OXR1 peptide lacking the TLDC domain (red arrows). f, Representative sequence alignment of SD- or TLDC-KO iPSC cell clones in the edited portion of each knockout strategy.

- SD-KO (*OXR1*^{-/-}) excision of a splice donor site in an exon common to all *OXR1* transcript variants, resulting in null expression;
- TLDC-KO (*OXR1*^{TLDC-/-}) excision of the portion of the genome encoding the TLDC domain of *OXR1*.

Targeting a splice site sequence in CRISPR editing can result in the expression of aberrant proteins due to skipping of the targeted exon¹⁶², in this case, expression of the TLDC domain may remain intact, with potential consequences for V-ATPase activity. So, to test the hypothesis that the TLDC domain is responsible for V-ATPase interaction, thus regulation of lysosomal activity, the TLDC-KO strategy was also employed.

Successful deletion of the splice donor region, to abrogate *OXR1* expression, was screened for using primers flanking the splice donor site of exon 14. A removal of 322 bp from the PCR product indicated successful removal of the target locus (Figure 13.2d). To screen for the removal of all exons encoding the TLDC domain, an amplification strategy was designed with primer sequences flanking each cut site (TLDC-Fwd-1/TLDC-Rev-1 and TLDC-Fwd-2/TLDC-Rev-2), with TLDC-Fwd-1 situated upstream of the 5' cleavage site and TLDC-Rev-2 situated downstream of the 3' cleavage site (Figure 10.2, page 47). Amplification with either primer pair (i.e. TLDC-Fwd-1 + TLDC-Rev-1 or TLDC-Fwd-2 + TLDC-Rev-2) producing an amplicon at 902 bp or 422 bp, respectively, indicated the presence of a WT allele in three clones. A faint, higher band can be seen in all screened clones and is likely indicative of off-target primer binding. Amplification using TLDC-Fwd-1 and TLDC-Rev-2 yielded a 484 bp amplicon indicating successful deletion of the

region in five clones and the absence of the WT allele (Figure 13.2d). Generated amplicons were sequenced and confirmed the removal of either a 322 bp or ~15 kb sequence (Figure 13.2f). Finally, I measured OXR1 protein levels by Western blot, confirming no detectable OXR1 protein in clones generated using the SD-KO method with an antibody that binds upstream of the edit site (*OXR1*^{-/-}, Figure 13.2e). In clones edited with the TLDc-KO method (*OXR1*^{TLDc-/-}), a novel protein is present (red arrows, Figure 13.2e). The observed distance between the larger, WT band and the lower, novel band approximates to the theoretical molecular weight of the cleaved portion of OXR1, which is 24.9 kDa. Thus, this novel band is assumed to be a truncated species of OXR1, lacking the TLDc domain.

The karyotype of edited clones was assessed using a SNP array to ensure euploidy had persisted through the cloning process. The karyograms for all clones used in this chapter can be found in Appendix 2. The SNP array analysis unveiled euploid cells exhibiting loss of heterozygosity at chr3p26.3-12.1, encompassing approximately 85 mb, across all clones. This is denoted by normal log R ratios (LRR = 0), indicative of normalised signal intensity for each SNP and consequently normal copy numbers at individual loci (i.e., two copies). However, the B allele frequency consistently registered as 0 or 1 throughout the entire locus, signifying a complete absence of one of the two alleles. Whilst gene dosage remains typical, this suggests that the loss of heterozygosity likely persists from a dominant population existing below the 20% threshold of detection in the parent line, as verified by karyotyping three passages prior. The utilisation of SNP array karyotyping is advantageous, as it enables the characterisation of such aberrations, a capacity not afforded by G-banding techniques.

13.3.3 *OXR1*^{-/-} and *OXR1*^{TLDc^{-/-}} iPS cells are capable of differentiating into microglia lacking, or expressing a truncated form of *OXR1*

iPSC derived from five knockout clones: *OXR1*^{-/-} (SD-KO-A1, SD-KO-A7), *OXR1*^{TLDc^{+/-}} (TLDc-HET-C2), and *OXR1*^{TLDc^{-/-}} (TLDc-KO-A12, TLDc-KO-D4) were successfully expanded and quality controlled. Throughout the remainder of this chapter *OXR1*^{-/-} and *OXR1*^{TLDc^{-/-}} genotypes are referred to collectively as *OXR1* loss-of-function (*OXR1*-LOF). These clones were differentiated into microglia as per standard protocol. Briefly, iPSC were aggregated in embryoid bodies with growth factors to stimulate the production of primitive hemogenic endothelium for seven days, embryoid bodies were then lifted into T175 flasks and long-term, primitive myelopoiesis was stimulated using IL-3 and M-CSF, resulting in the production of primitive macrophage precursors which were harvested and finally differentiated into microglia with IL-34, TGF-β, M-CSF and low-dose GM-CSF⁷³. *OXR1*-LOF iPSC differentiated at a similar rate, and with similar yield to, WT co-isogenic iPSC. The production of macrophage precursors was lower (~40%) in *OXR1*-LOF factories compared to WT factories initially, however, this difference was reduced by week eight (Figure 13.3a).

To characterise the nature of *OXR1* expression in KO microglia, protein levels were analysed by western blot, which indicated a lack of *OXR1* expression in *OXR1*^{-/-} microglia and expression, albeit reduced, of a truncated isoform in *OXR1*^{TLDc^{-/-}} microglia (Figure 13.3b). This confirms that iPS-derived microglia can differentiate and survive without *OXR1* or its TLDc domain (Figure 13.3b,c).

13.3.4 Subcellular localisation of *OXR1* is not possible using antibodies at tested dilutions

Endogenous *OXR1* protein has not been localised within myeloid cells and evidence of localisation in other cells lacks stringent null-expression controls.

Furthermore, colocalisation of OXR1 with the V-ATPase within the cell would be a novel finding and a compelling addition to the body of evidence suggesting interaction. Thus, I aimed to test two candidate antibodies for the detection of OXR1 in microglia using *OXR1*^{-/-} as a control for specific antibody binding (Figure 13.3d,e). Staining demonstrated that non-specific binding was present in all dilutions tested (1:50, 1:250 and 1:500) with both antibodies. Whilst a dilution of 1:500 with antibody 13514-1-AP (ProteinTech) initially appeared to show specific staining, there is significant background staining (cyan outline, Figure 13.3e). Hence these antibodies were demonstrated to be non-specific and not used further. Due to the high degree of sequence homology between *OXR1* and *NCOA7*, it is possible that the antibodies cross-react. Indeed, a search using BLAST confirms that the antigen 13514-1-AP was raised to shares 57% sequence identity with *NCOA7*, whilst PA5-82973 shares 53% identity.

13.3.5 OXR1 knockout increases V-ATPase holoenzyme assembly

To assess the extent of V-ATPase holoenzyme expression and assembly in microglia with *OXR1* LOF, I employed Alexa Fluor 568-labeled SidK for cellular staining. SidK is a *Legionella pneumophila* effector protein, provided by M. Maxson, that specifically binds the V-ATPase⁵⁵. Quantitative analysis of SidK staining intensity and area revealed an approximately two-fold increase in SidK integrated density in *OXR1*-LOF microglia suggestive of elevated V-ATPase assembly and/or expression in the absence of *OXR1* (Figure 13.4a,b). Additionally, *OXR1*-LOF microglia exhibited prominent V-ATPase-positive vesicles, characterized by a rounded, open morphology (red arrows, Figure 13.4a). Interestingly, images from this study were also suggestive of a morphological change in *OXR1*-LOF microglia, characterised by rounder, larger cells as indicated by phalloidin staining for F-actin.

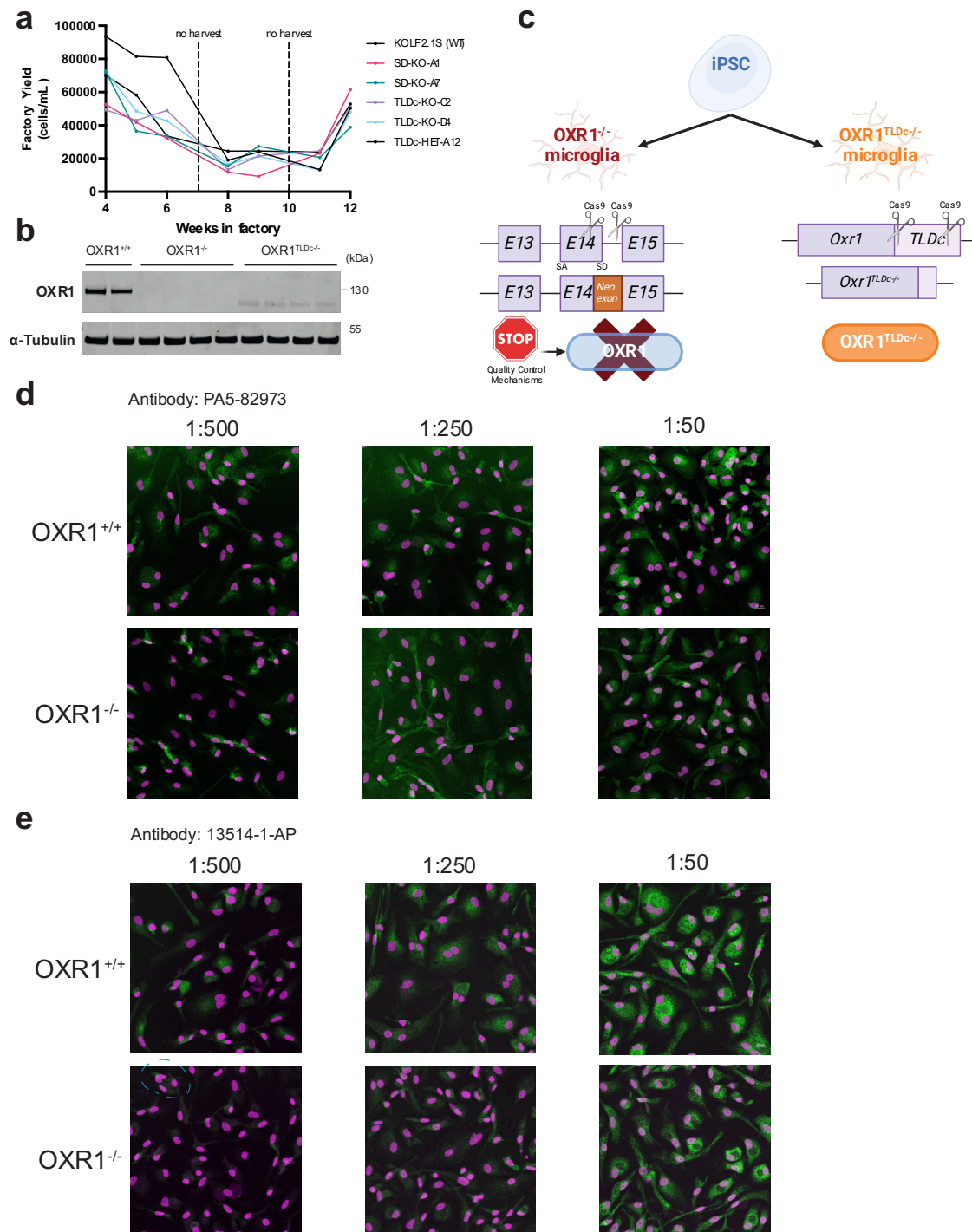


Figure 13.3. Characterising OXR1-KO microglia.

a, Macrophage precursor yield from hiPSC clone-derived factories. b, Western blot of OXR1 expression in OXR1^{+/+}, OXR1^{-/-}, and OXR1^{TLDC-/-} microglia. c, Schematic hypothesising loss of OXR1 protein due to cellular quality control mechanisms and the expression of truncated OXR1 lacking a TLDC domain. d, e,

Immunocytochemistry of OXR1^{+/+} and OXR1^{-/-} microglia with Invitrogen anti-OXR1 antibody (d, PA5-82973) and ProteinTech anti-OXR1 antibody (e, 13514-1-AP). Cyan outline indicates non-specific antibody binding. Scale bar is 20 μ m.

Since SidK specifically binds the A subunit of the V₁ multimeric domain, expression was measured by Western blot to establish the degree to which increased staining was due to elevated expression of ATP6V1A. Quantification of expression suggests a modest (~50%) increase in subunit A expression compared to a two-fold increase in the integrated density of SidK (Figure 13.4c). This implies that, in part, the increase in SidK binding is due to upregulated ATP6V1A expression. However, other factors, such as holoenzyme stabilisation, may be influencing increased signal.

13.3.6 OXR1 LOF causes reduced final density of microglia

Comparison of nuclei counts demonstrated that, despite seeding the same number of macrophage precursors after harvesting, there were consistently fewer microglia in *OXR1*^{-/-} and *OXR1*^{TLDC^{-/-}} cultures than WT at the end of 14 days' differentiation (Figure 13.4d). This phenomenon could have been caused by several possibilities over the 14-day differentiation period from macrophage precursor to microglia, which are examined in this section's discussion.

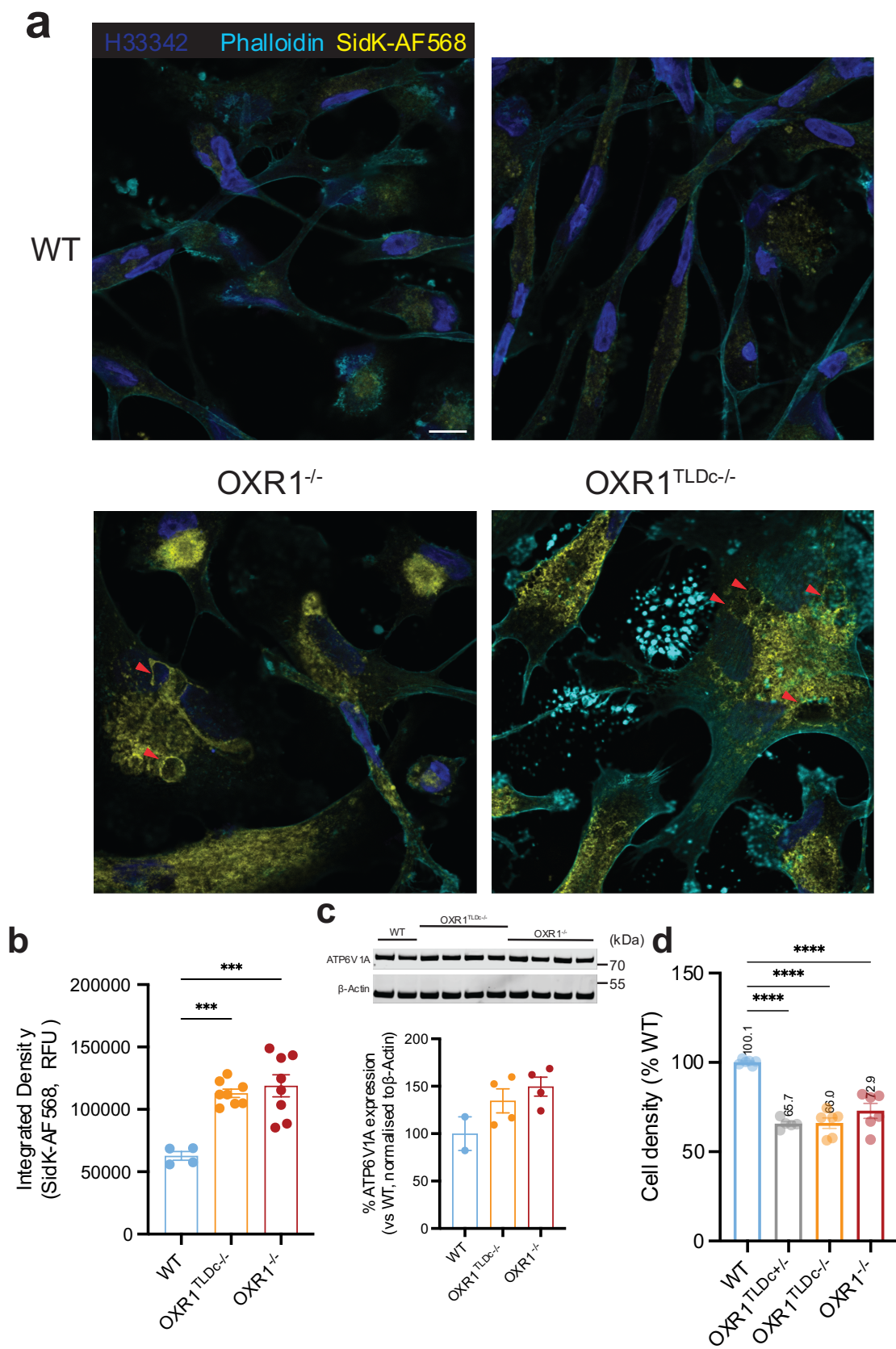


Figure 13.4. V-ATPase expression in OXR1-LOF microglia.

a, Representative confocal photomicrographs of wild-type (WT, top) *OXR1*^{-/-} (bottom, left) and *OXR1*^{TLDC}^{-/-} (bottom, right) microglia stained with Hoechst 333342 (blue), phalloidin (cyan) and SidK conjugated to Alexa Fluor 568 (yellow). Red arrows indicate large, V-ATPase-positive vesicles. b, Integrated density in the 568 nm channel was measured for photomicrographs (n = 4 or 8 coverslips, mean of 3 fields of view from a coverslip repeated over two independent experiments) c, Total protein (10 µg) from WT or *OXR1* loss-of-function (LOF) microglia immunoblotted for ATP6V1A and β-actin and signal quantified and normalised to β-actin. d, Cell density of microglia, after 14 days' differentiation, derived from nuclei counts (n = 4-6 independent experiments). Scale bar is 10 µm. One-way ANOVA with Dunnett's post-hoc correction. *** P < 0.001, **** P < 0.0001.

13.3.7 *OXR1* knockout microglia show lysosomal accumulation

Since TLDC proteins, including *OXR1*, have previously been demonstrated to interact with the V-ATPase, I aimed to identify changes to lysosomal characteristics in *OXR1*-LOF microglia. Using LysoTracker Red DND-99 to label acidic compartments, I was able to count the total number of lysosomes and the mean area of each lysosome per field of view. Since iPS-microglia are highly vesicular cells, I also segmented vesicles by size into small (< 25 px², mean area of vesicles in WT cells = 0.4 µm²), medium (25-150 px², mean area in WT = 1.7 µm²) and large (> 150 px², mean area in WT = 11.1 µm²) to increase the sensitivity of the assay to detect abnormally sized lysosomes (Figure 13.5a). Since lysosomes in macrophages are typically 0.5-1 µm in diameter¹⁶³ (≈0.8-3.1 µm², assuming a circular morphology), most normal lysosomes should fall in the 'medium' category. Overall, there were more lysosomes present per cell in *OXR1*-LOF and *OXR1*^{TLDC}^{+/-} microglia compared to WT (Figure 13.5b,d). This appears to be driven by a large increase in small lysosomes and a modest increase in medium lysosomes. Finally, there were fewer large vesicles per cell in *OXR1*-LOF cells.

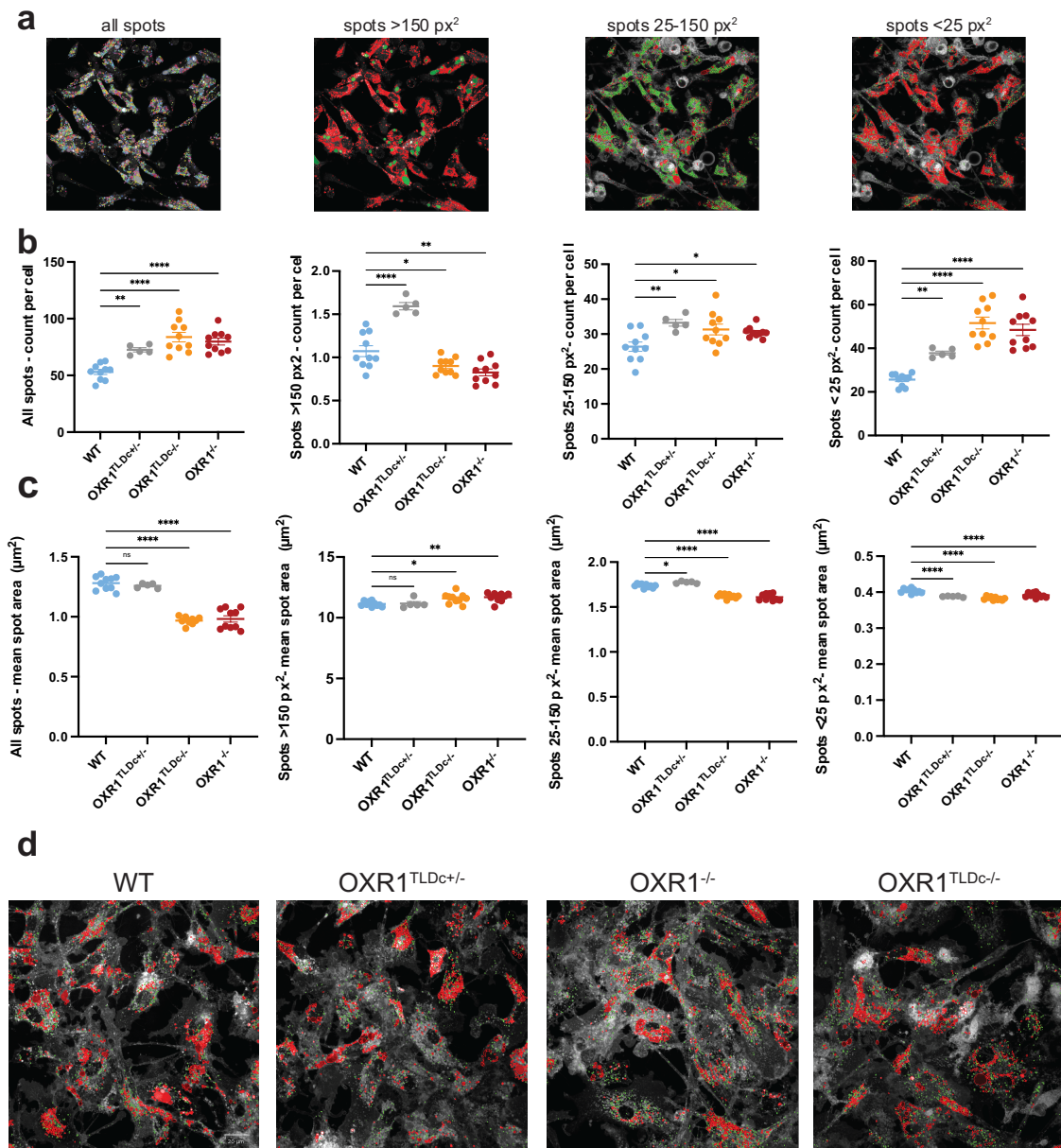


Figure 13.5. Small- and medium-sized lysosomes accumulate in OXR1-LOF microglia.

a, Representative images of binning of detected lysosomes (all spots) into small, medium and large (< 25 μm², 25-100 μm² and >100 μm², respectively). b, Number of detected lysosomes (all, large, medium, and small) in OXR1-LOF microglia. c, mean lysosome area of all, large, medium, and small lysosomes in OXR1-LOF microglia. d, Representative images of small lysosomes (green) vs medium and large lysosomes (red) per genotype. Scale bar is 20 μm. One-way ANOVA with Dunnett's post-hoc correction, n = 5-10 wells (mean of 4 fields of view per well), over 2 independent experiments. ns = not significant, * P < 0.05, ** P < 0.01, **** P < 0.0001.

Additionally, the average area of a lysosome in *OXR1*-LOF was reduced in microglia and this was not replicated in *OXR1^{TLDc+/-}* cells (Figure 13.5c). Again, this was likely driven by the significant increase in small lysosome count, as there were no large changes in lysosome area in each category. There does, however, exist a small but significant increase in the size of larger lysosomes and a small, significant decrease in the size of medium and small lysosomes. Taken together, this data indicates the accumulation of small- and medium-size lysosomes in *OXR1*-LOF microglia.

13.3.8 Endocytic uptake of *OXR1*-LOF microglia is reduced whilst lysosomal degradation is slightly increased

Since *OXR1*-LOF display lysosomal accumulation and enrichment of V-ATPase around vesicles, which could be expected to decrease organelle pH and increase degradation of endocytosed material, I next measured the degradative capacity of *OXR1*-LOF microglia using DQ-Red-BSA. DQ-Red-BSA is heavily labelled with BODIPY TR-X, a pH-insensitive fluorophore, which emits fluorescence only after hydrolysis of BSA to single, dye-labelled peptides by lysosomal proteases. Microglia were loaded with DQ-Red-BSA and AF-488-BSA simultaneously. This allows measurement of both endocytic uptake and degradative activity of lysosomal enzymes. Combining the substrates effectively controls for uptake, enabling a normalised readout of proteolytic cleavage of DQ-Red-BSA taken into the endolysosomal system.

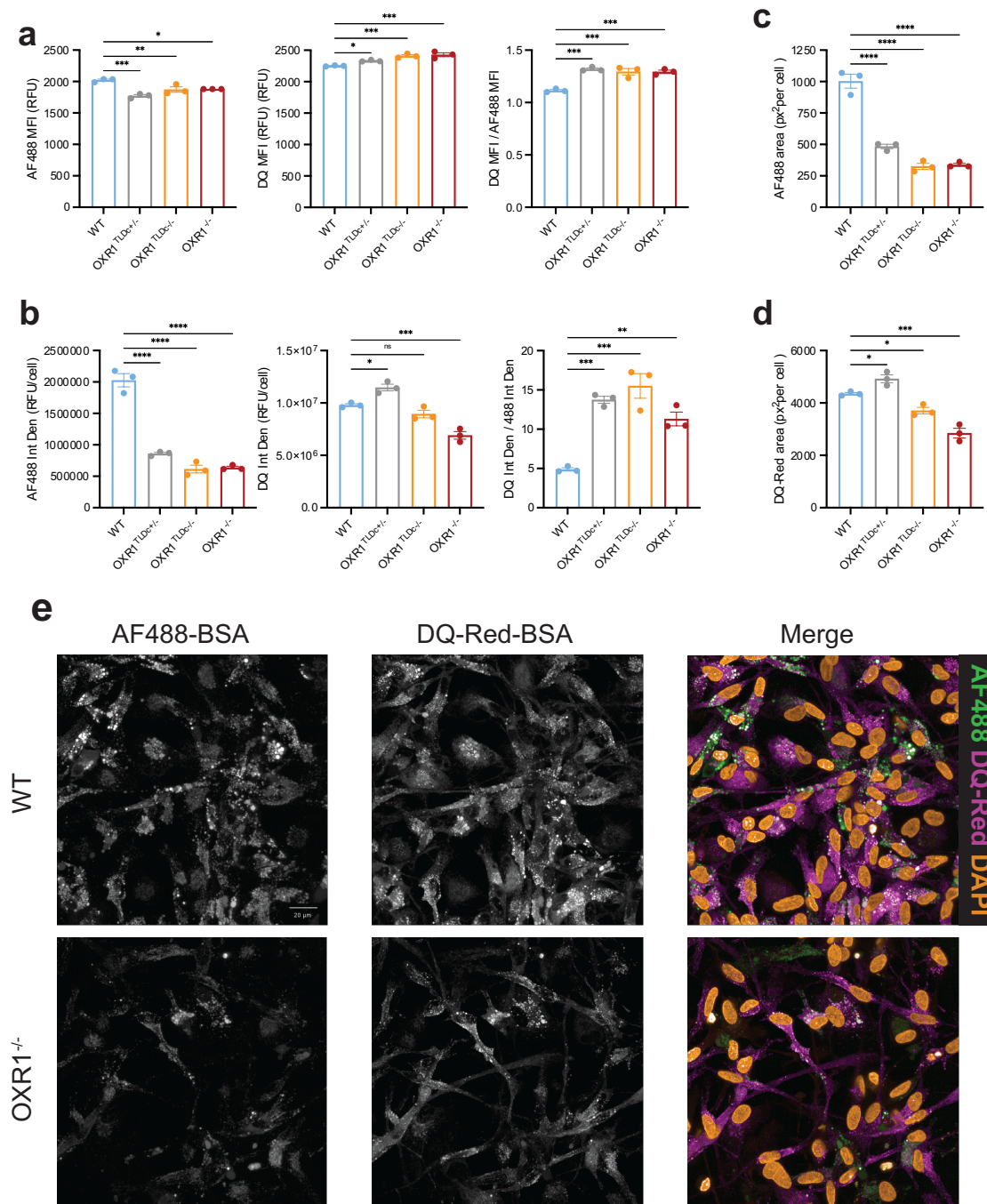


Figure 13.6. Degradative and endocytic capacity of OXR1-LOF microglia.

a,b, mean fluorescence intensity (MFI, a) and integrated density (Int Den, b) of microglia loaded with Alexa Fluor 488 conjugated to bovine serum albumin (AF488-BSA) and DQ-Red-BSA. c,d, Signal area of AF488-BSA (c) and DQ-Red-BSA (d). e, Representative photomicrographs of microglia stained, as above with, AF488-BSA (green), DQ-Red-BSA (magenta) and DAPI (orange). f, g, Integrated density per cell (f) and signal area per cell (g) of AF488-BSA in microglia transduced with a control vector or OXR1. Scale bar is 20 μ m. One-way ANOVA with Dunnett's post-hoc correction, n = 3 independent experiments (mean of 3-4 wells per experiment, 4 fields of view per well). ns = not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

There was an observed reduction of approximately 75% in AF488 integrated density, partly attributed to a decrease in intensity but predominantly influenced by a reduction in the AF488 area (Figure 13.6c,d; approximately 25% of wild-type). This substantiates a significant reduction in the endocytic capacity of *OXR1*-LOF cells. Despite a diminished uptake in *OXR1*-LOF, an augmented mean fluorescence intensity (MFI) ratio of DQ-BSA to AF488-BSA signifies an increased degradative capacity across all *OXR1*-LOF genotypes (Figure 13.6a). This augmentation is driven by an elevation in DQ-Red intensity, indicative of heightened degradative capability, coupled with a reduction in AF488 intensity, suggesting a diminished lysosomal content. Given that intensity alone does not encapsulate the total signal area, the integrated density was also analysed (mean pixel value multiplied by signal area). A noteworthy increase in the integrated density ratio of DQ-Red to AF488 corroborates an elevated lysosomal protease activity in *OXR1*-LOF cells, despite a marked reduction in DQ integrated density in *OXR1*^{-/-} microglia, attributable to reduced uptake as evidenced by diminished DQ-Red area (Figure 13.6b).

Overall, a substantial decrease in AF488 integrated density indicates that *OXR1*-LOF in microglia impairs endocytic uptake of proteinaceous interstitial material, hence reducing lysosomal content. However, augmented lysosomal protease activity, substantiated by an increased mean fluorescence intensity ratio of DQ-BSA to AF488-BSA, suggests a compensatory enhancement in the degradative pathway, delineating a distinctive phenotype in *OXR1*-LOF microglia with implications for endo-lysosomal homeostasis and phagocytosis.

13.3.9 Qualitative changes in lysosomal pH in OXR1-LOF microglia

Since OXR1 interacts with the V-ATPase via its TLDc domain, and knockout of *OXR1* in iPS-microglia causes vesicular accumulation and elevated V-ATPase expression, I next sought to determine whether KO of *OXR1* would affect lysosomal pH in iPS-microglia.

As an initial measurement of lysosomal pH, Lysosensor Green DND-189 ($pK_a \sim 5.2$) was used, which is a fluorogenic dye that increases intensity in acidic compartments. These acidotropic dyes accumulate non-selectively within acidic organelles (including lysosomes and Golgi) and are subject to modulation based on the size, quantity, characteristics, and contents of the vesicular structures⁸⁵. As such these dyes are not appropriate for use with drugs that affect lysosomal content, e.g. chloroquine, which increases lysosomal pH by quenching protons but induces a paradoxical increase in Lysosensor intensity¹⁶⁴.

Nonetheless, treatment of WT microglia with 400 nM Torin1 (an mTORC1/2 inhibitor that lowers lysosomal pH¹⁶⁵) seemed to increase the frequency and intensity of Lysosensor-positive lysosomes, whereas treatment with 10 nM Concanamycin A (a V-ATPase inhibitor that raises pH¹⁶⁶) seemed to cause dispersion of the dye throughout the cytoplasm with no bright puncta, as seen in other conditions (Figure 13.7a). Confirming the dyes used in gauging lysosomal pH. Quantitatively, a significant increase in Lysosensor intensity was seen only in *OXR1*^{-/-} microglia (Figure 13.7b).

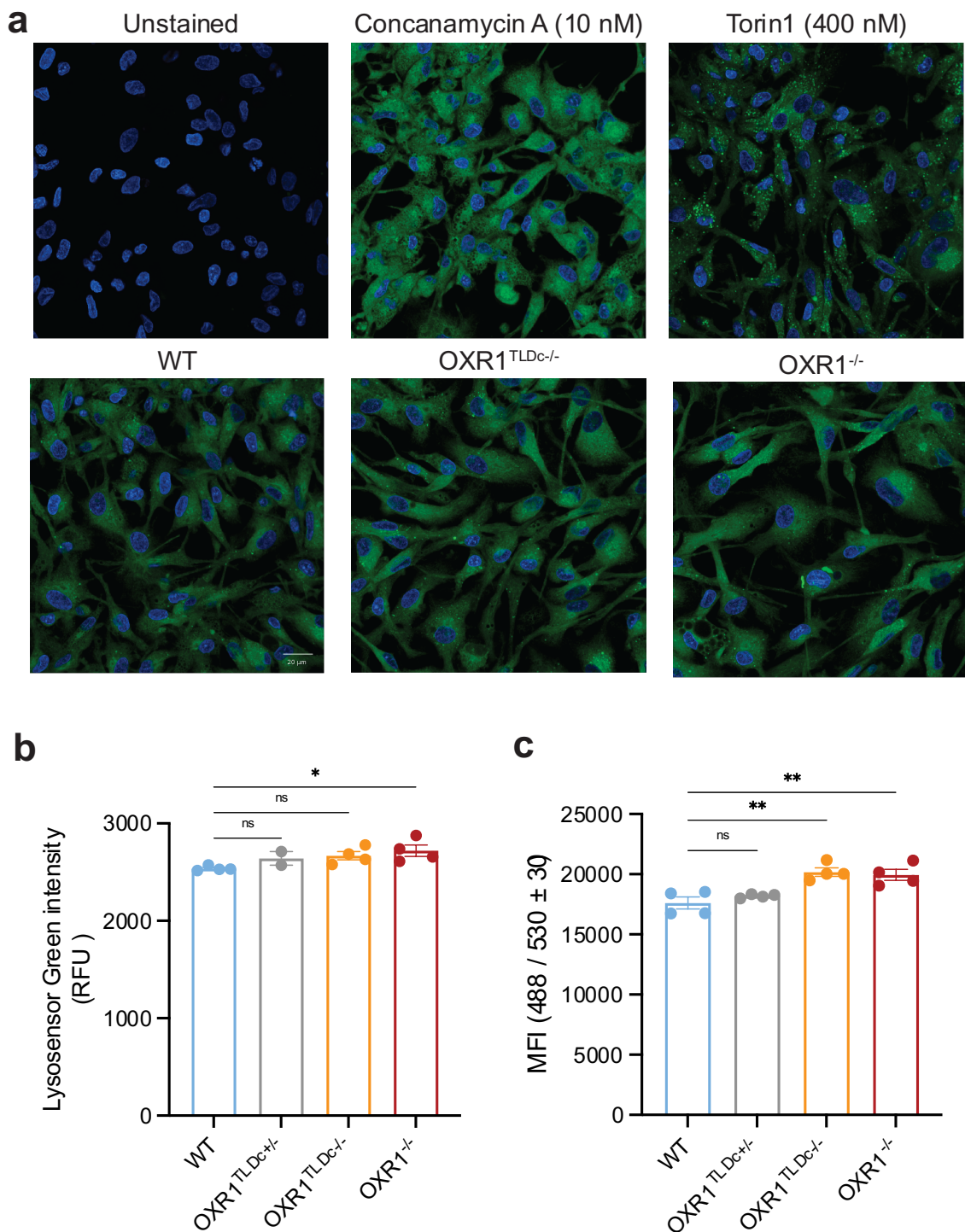


Figure 13.7. Quantification of lysosomal acidity with Lysosensor.

a, Representative images of iPSC-derived microglia treated with Concanamycin A (10 nM), Torin 1 (400 nM) or untreated (genotypes) for 4 hours and stained with Lysosensor Green DND-189. b, Analysis of mean intensity of images taken as per a (n = 2-4 wells from 2 independent experiments, 4 fields of view per well). c, Cells treated and stained as per a were analysed by flow cytometry, excited at 488 nm with emission measured at 530 ± 30 nm, and mean fluorescence intensity calculated (n = 4 measurements from

2 independent experiments). Scale bar is 20 μm . One-way ANOVA with Dunnett's post-hoc correction, n = 3 independent experiments. ns = not significant, * $P < 0.05$, ** $P < 0.01$.

Since the sensitivity of the assay seemed to be hindered by a high degree of background staining, the same experiment was performed with analysis of fluorescence by flow cytometry. In these experiments, both *OXRI*^{TLDc-/-} and *OXRI*^{-/-} had significantly higher MFI for Lysosensor green (Figure 13.7c). Together these results suggest a lower pH in *OXRI*^{-/-} microglia, though the biological significance of this change is difficult to judge since the assay does not measure quantitative changes in lysosomal pH.

13.3.10 Lentiviral rescue of OXR1-LOF

To comprehensively address the hypothesis regarding the effect of *OXRI* expression on lysosomal acidification, I adapted a quantitative lysosomal pH assay in conjunction with lentiviral-mediated *OXRI* rescue in iPS-microglia. This experimental setup facilitates the examination of whether bidirectional changes in *OXRI* expression influence lysosomal pH, thereby accounting for potential gene dosage effects. Furthermore, demonstrable phenotypic rescue of *OXRI* would mitigate confounding factors associated with karyotypic variations (i.e. loss of heterozygosity) in iPSC clones. The lentiviral rescue was achieved with a lentiviral vector expressing *OXRI*Δ1 with an N-terminal 3xFLAG tag (DYKDHDG-DYKDHDG-DYKDDDDK) appended with a 3xGS linker, to allow for detection of the transgene (pLV[Exp]-Bsd-SFFV>3xFLAG/3xGS/hOXRI[NM_001198533.2], pLV-OXR1). High-efficiency transduction was achieved by co-infecting pLV-OXR1 (or pLV-Stuffer) with Vpx-VLP to enhance transgene integration in microglia, which otherwise transduce with poor efficiency due to inherent innate immune restrictions.

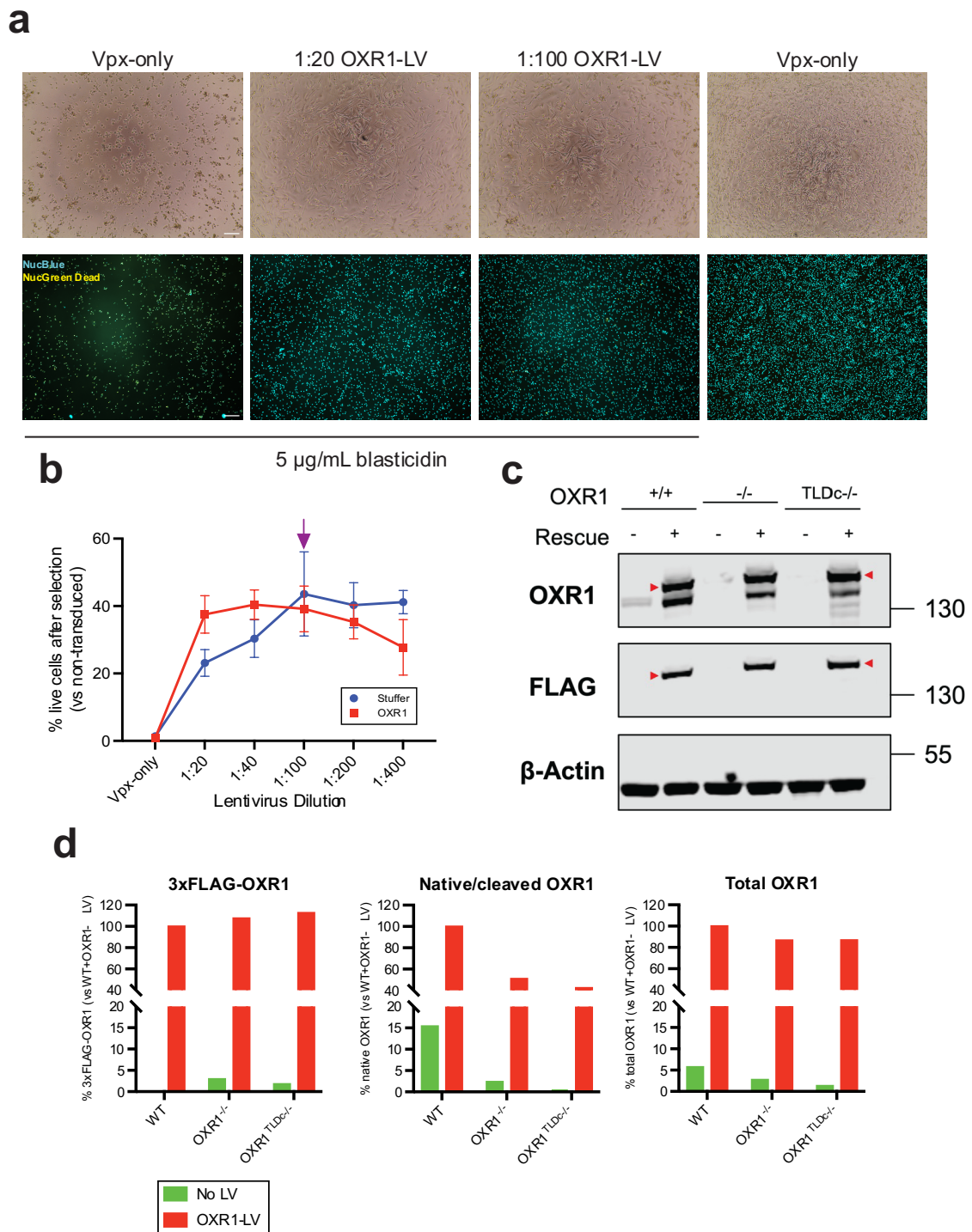


Figure 13.8. Lentiviral rescue and overexpression of OXR1 in iPS-microglia.

a, Photomicrographs of microglia after selection blasticidin (scale bar is 200 µm). Cells were transduced with Vpx-expressing virion-like particles or OXR1 encoding lentivirus (LV) 48 hours before selection. b, The number of live cells determined by NucBlue (cyan) and NucGreen Dead (yellow) were quantified and 1:100 (purple arrow) was determined to be the optimum dose for pLV-OXR1 (red) and pLV-Stuffer transduction (blue) (n = 4 wells in 1 experiment). c, Total protein was blotted for OXR1 (top) and FLAG (bottom) peptide in cells that had been infected with OXR1 LV (rescue). Red arrows indicate overlapping

FLAG/OXR1 dual positive bands. d, Protein was quantified using OXR1 signal normalised to β -actin (n = 1).

First, a kill curve determined the optimum dose of blasticidin for 100% selection in iPS-microglia to be 5 μ g/mL over 5 days. To titre the lentivirus, live/dead staining after infection with different dilutions of virus and subsequent selection suggested 1:100 to be the optimum dilution of virus for efficient transduction based on cell viability (**Figure 13.8a,b**). Then, to assess rescue at the molecular level, cells were transduced without selection and immunoblotting of total protein extracts for OXR1 and FLAG epitope revealed an approximate 20-fold increase in total OXR1 expression (**Figure 13.8c,d**).

Notably, 3xFLAG-OXR1 in microglia was expressed not only as full-length OXR1 with FLAG attached but approximately half of it was processed within the cells to the size of native OXR1 without the FLAG tag, thus this lower band was only detected with anti-OXR1 antibody and not anti-FLAG. This implies an endogenous mechanism for cleaving the 3xFLAG tag (Figure 13.8c, red arrows), however, analysis of the protein sequence at the N-terminus of 3xFLAG-OXR1 with the ExPASy PeptideCutter tool (https://web.expasy.org/peptide_cutter/) failed to reveal any targets of mammalian proteases. The proposed removal of the epitope tag has consequences for protein localisation, as the tag may be lost in a subset of proteins derived from the transgene.

13.3.11 Quantitative analysis of lysosomal pH in OXR1-LOF microglia indicates acidity is unchanged

To quantitatively determine lysosomal pH in *OXR1*-LOF and *OXR1*-rescue microglia, Oregon Green 488 conjugated to dextran (OG-dextran) was used. OG-dextran overcomes the limitations of Lysosensor since it is endocytosed and trafficked to lysosomes specifically and has a pH-sensitive emission at ex488 nm but a pH-insensitive

emission at ex425 nm, allowing for ratiometric measurement. This allows experimental control for size, content of lysosomes and photobleaching since the rate of photobleaching at ex488nm is equivalent to that at ex425 nm. Furthermore, the pK_a of OG is 4.8, meaning it is particularly sensitive in the range of lysosomal pH⁸⁵. FITC, from which OG is derived, also has the same excitation-dependent, pH-dependent properties but has a greater pK_a at 6.5¹⁶⁷, meaning it should be less sensitive at the expected pH range of lysosomes.

To determine the most suitable dye for lysosomal pH determination, I incubated FITC, OG and a pH-insensitive green dye, AF488, in a range of isotonic pH buffers from pH 3.5 to pH 7.0 and measured emission at 525 nm (Figure 13.9a-c). Excitation of the dyes at 488 nm confirmed that both FITC and OG increased in fluorescence intensity with OG having a steeper slope in the pH range of lysosomes, confirming greater sensitivity, whilst the emission of AF488 was stable. The emission of all three dyes was relatively stable at ex425 nm, e.g. em525 nm of OG at pH 4.0 vs pH 5.0 increased approximately 5-fold when at ex488 nm, versus a 1.5-fold increase at ex425 nm. This difference is reflected in the ratio of em525 nm at ex488 nm to ex425 nm, which demonstrates OG is most sensitive in the expected lysosomal pH range whereas AF488 is stable across the pH range.

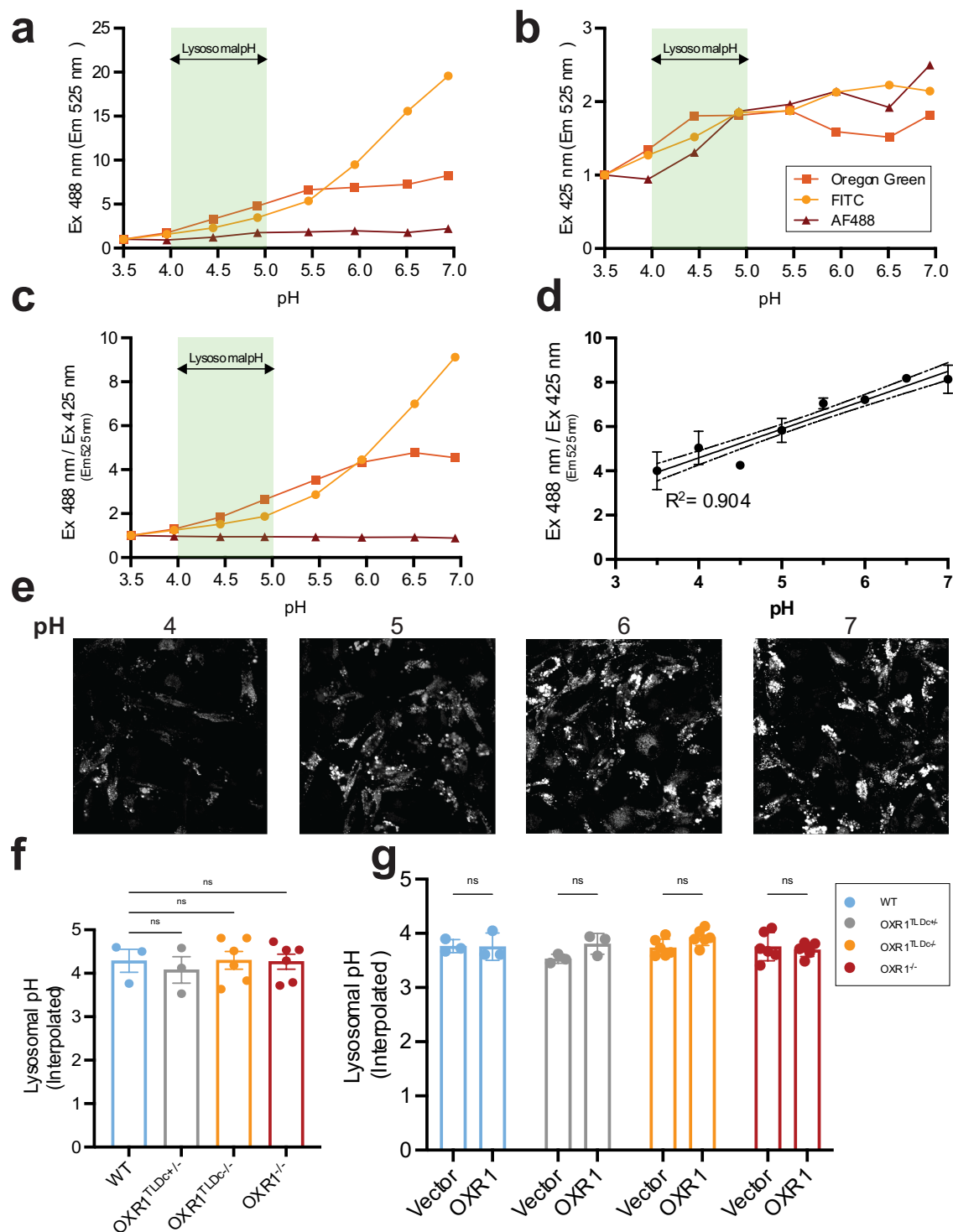


Figure 13.9. Lysosome pH is unaffected by OXR1-LOF.

a, b, The fluorescent emission of Oregon Green 488, fluorescein isothiocyanate (FITC) and Alexa Fluor 488 (AF488) at 525 nm after excitation at 488 nm (a) and 425 nm in a range of pH buffers. c, The ratio of emission of the three dyes in pH buffers at 525 nm after excitation at 488 nm and 425 nm. d, The ratio of fluorescence intensity of Oregon Green conjugated to dextran (OG-dextran) in microglial lysosomes

incubated with isotonic pH buffers showing a linear relationship between (Ex488, Em525) / (Ex425, Em525) and pH ($n = 4$ wells in 1 experiment). e, Representative images [(Ex488, Em525) / (Ex425, Em525)] of microglial lysosomes loaded with OG-dextran in buffers of increasing pH. f, Lysosomal pH interpolated from a standard curve in OXR1-LOF microglia. One-way ANOVA with Dunnett's post-hoc correction, $n = 3-6$ wells from 2 independent experiments. g, As f, but in microglia transduced with a control or OXR1 lentiviral vector. Two-way ANOVA comparing the means within each group (i.e. control vector versus OXR1) and Šídák post-hoc correction. Scale bar is 20 μm . ns = not significant.

Thus, to determine lysosomal pH microglia were fed OG-dextran, washed, and incubated to allow OG-dextran to traffic to the lysosomes. Before imaging, cells were treated with a series of K^+ isotonic pH standard buffers from pH 2.5 to pH 7.0, containing the ionophores nigericin and monensin to augment K^+ and H^+ ion exchange, thus isotonic pH equilibration in lysosomes. Further, Na^+ was excluded from the buffers to minimise the interference of Na^+/H^+ exchanger channels, which would oppose pH buffering. Rapid confocal microscopy of these cells with lasers exciting at 425 nm and 488 nm allowed me to build a standard curve from which the pH of OXR1-LOF microglial lysosomes could be extrapolated (Figure 13.9d,e).

Analysis of lysosomal pH in OXR1-LOF microglia confirmed no change compared to WT (Figure 13.9f). Furthermore, rescue or overexpression of OXR1 does not alter pH (Figure 13.9g). Thus, whilst OXR1 may interact with the V-ATPase, its abrogation does not appear to alter lysosomal pH in this system. Hence, the increase in the degradative capacity of OXR1-LOF microglia may be independent of lysosomal pH changes.

13.3.12 Pilot data suggests a limited impact of OXR1-LOF on autophagic flux

A common functional outcome of lysosomal impairment is impaired autophagic flux. To measure autophagy in OXR1-LOF in a preliminary experiment ($n = 1$), microglia

were treated with Concanamycin A, a V-ATPase inhibitor which prevents lysosomal acidification and fusion of autophagosomes with lysosomes, and Torin1, an inhibitor of the mTORC1/2 which promotes autophagy in part by enhancing V-ATPase activity.

To measure the degree of autophagic degradative activity, p62 was measured (Figure 13.10a). A cargo adapter protein that delivers autophagy substrates to autophagosomes for degradation, p62 itself is degraded in autophagy and accumulates when autophagy is inhibited, thus it is a proxy measure of autophagic degradation. Additionally, microtubule-associated protein light chain 3 (LC3) was measured. LC3 exists in two forms: LC3-I (18 kDa), which is cytoplasmic and is associated with phosphatidylethanolamine to form LC3-II during phagophore formation. Thus LC3-II/I ratio is a measure of autophagosome formation and degradation. Finally, to explore the potential rescue of lysosomal degradation deficits by *NCOA7*, a homologue of *OXR1*, its expression was also measured.

In WT microglia, the level of p62 appeared to increase with concanamycin A treatment, reflective of the reduced autophagic flux caused by impediment of autophagosome-lysosome fusion via V-ATPase inhibition (Figure 13.10b). With Torin1 treatment, thus stimulation of autophagy by inhibition of the mammalian target of rapamycin complex (mTORC), p62 appeared to increase, indicating a functional autophagic pathway in WT microglia^{2,39}. This trend is reflected in *OXR1*-LOF microglia, suggesting, concordant with previous evidence, that degradative capacity is not impaired by *OXR1* perturbation.

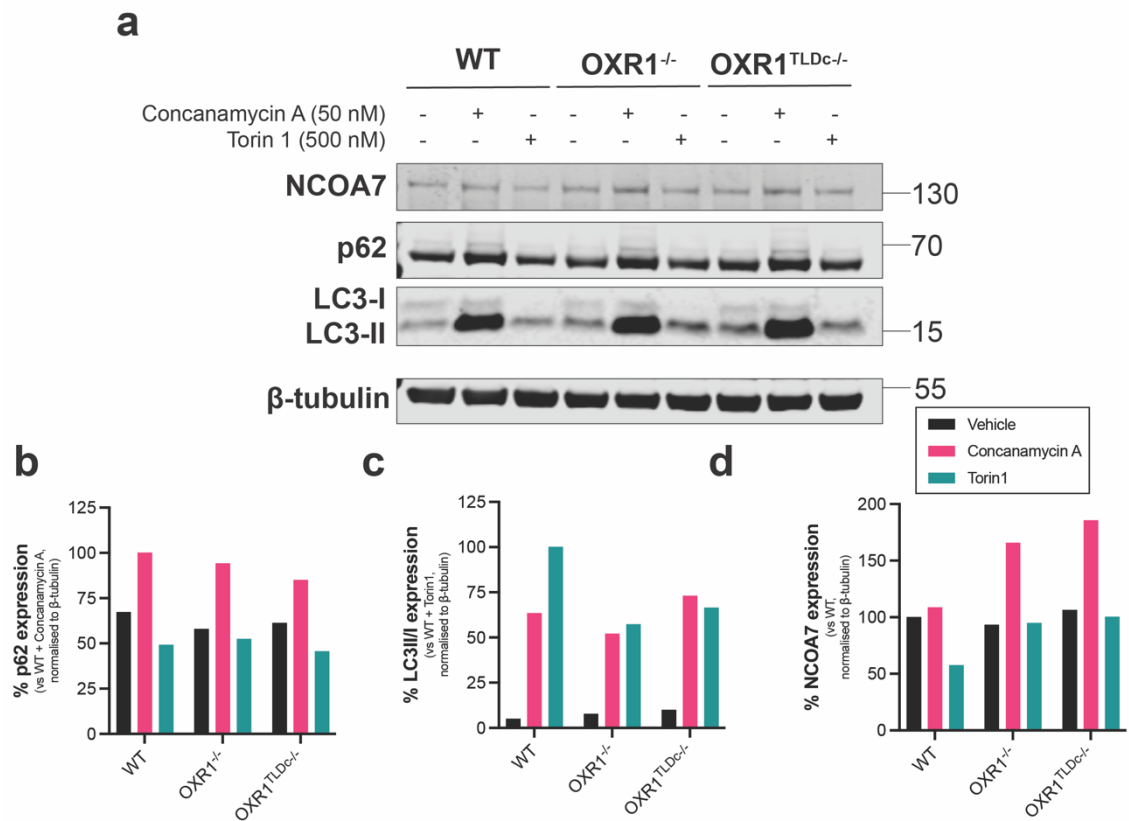


Figure 13.10. Quantification of autophagy substrates in OXR1-LOF microglia.

a, Western blot of total protein extract (10 μ g) from iPSC-microglia treated with 50 nM Concanamycin A or 500 nM Torin1 for 4 hours. Nuclear receptor coactivator 7 (NCOA7), p62 and microtubule-associated light chain 3 (LC3, isoforms I and II) were blotted for with β -tubulin as a loading control. b, Relative quantification of p62 expression as a percentage of expression in WT microglia treated with Concanamycin A. c, Relative quantification of LC3II/I expression as a percentage of expression in WT microglia treated with Torin. d, Relative quantification of NCOA7 expression as a percentage of expression in vehicle-treated WT microglia. n = 1 experiment.

In WT microglia LC3-II accumulates with concanamycin A treatment, as a result of autophagosome accumulation (Figure 13.10c). An increase in LC3-II expression is also seen with stimulation of autophagy by Torin1, likely resulting from increased autophagosome formation. However, in *OXR1*-LOF microglia, this increase is around 50% of the Torin1-induced LC3-II increase. This suggests that either autophagosome synthesis in stimulated autophagy is impaired in *OXR1*-LOF microglia or the fusion of autophagosomes with lysosomes with impaired.

Finally, in WT microglia, *NCOA7* appears to be downregulated by 50% when autophagy is induced (Figure 13.10d). Since *NCOA7* is also reported to enhance V-ATPase activity, this may be a result of inhibition due to Torin1-dependent augmentation of V-ATPase activity in autophagy. Interestingly, whilst concanamycin A treatment seems to have no effect on *NCOA7* in WT microglia, in *OXR1*-LOF microglia inhibition of the V-ATPase seems to increase expression almost two-fold. This suggests an unexplored function of *NCOA7* in microglia deficient in *OXR1* where V-ATPase activity is inhibited.

13.3.13 RNA-seq of OXR1-LOF microglia shows upregulation of transcripts related to the MHC Class II pathway

Unpublished RNA-seq data from the Oliver lab has demonstrated upregulation of transcripts associated with microglial activation and lysosomal cathepsins in bulk *Oxr1*^{-/-} mouse cerebellum tissue. To identify expression changes in *OXR1*-LOF microglia, RNA-seq was performed on human iPS-microglia derived from *OXR1*-LOF clones. Whilst a comparison of analysis of the RNA-seq data including and excluding the genes at chr3p26.3-12.1 showed no substantive differences, the locus was excluded from the

final analysis to limit confounds due to loss of heterozygosity at this locus (see section 13.3.2).

As expected, *OXRI* transcript expression was not completely abrogated by the knockout strategies used in iPSC (Figure 13.11a). This has been demonstrated previously in mice, where targeted mutation of the *Oxr1* TLDC domain results in a comparable cerebellar degenerative phenotype to deletion of the *Oxr1* locus, but with residual transcript expression and abrogated protein expression^{8,26}. Nonetheless, analysis of protein expression in this study confirmed knockout or truncation of OXR1 in each LOF iPSC line. Reductions in transcript expression in *OXRI*-LOF microglia likely represent decreased stability of *OXRI* transcripts or impaired transcription of the gene.

It was also observed that wild-type (WT) microglia expressed approximately 150 fewer genes compared to *OXRI*-LOF microglia (Figure 13.11b). Interestingly, the number of genes expressed among *OXRI*-LOF microglia remained consistent across the knockout strategy employed, indicating that the alterations in gene expression could be attributed to the disruption of *OXRI*. To discern the extent of variation among samples, a comparative analysis of gene expression and variability was conducted via Principal Component Analysis (PCA). PCA of the top 10% of differentially expressed genes (DEGs) revealed a distinct clustering of *OXRI*-LOF clones separate from WT microglia along the first principal component (PC1) (Figure 13.11c). This suggests that *OXRI*-LOF is the primary variable driving the observed variation within the top 10% of DEGs. However, when the analysis was expanded to the top 90% of DEGs, the distinct clustering of *OXRI*-LOF clones was represented along PC2 (Figure 13.11d). This indicates that while *OXRI*-LOF accounts for the most variation among the most variable genes, it only accounts for the second most variation across the wider transcriptome. This further

underscores the similarity of gene changes among *OXRI*-LOF microglia in the genes most differential from WT but suggests other factors may influence expression when this analysis encompasses the wider transcriptome.

Finally, when comparing common DEGs vs WT among *OXRI*-LOF clones, 1,244 DEGs were shared between all clones (Figure 13.11e). In contrast, when comparing significant DEGs between clones the number of genes was much lower, i.e. in the tens of genes. Taken together, these data suggest that *OXRI*-LOF is driving gene changes to a similar degree independent of the knockout strategy employed.

Considering expression changes to single transcripts in LOF vs WT microglia, the top three up- and down-regulated genes common across the samples are summarised in Table 13.1. Common processes involving these genes include inflammatory and metabolic regulation, cell cycle progression and mitochondrial ROS activity. Unexpectedly, transcripts encoding V-ATPase subunits, lysosomal membrane-associated proteins (*LAMP* genes), endosome-associated Rab GTPases (*RAB5*, *RAB7*), or lysosomal cathepsins (*CTS* genes) were not found within the top 5% up- or down-regulated genes. Next, the top 20 DEGs from each knockout clone versus WT microglia were identified in a volcano plot of magnitude and significance of differential expression. This analysis demonstrated a similar pattern of gene expression in *OXRI*-LOF microglia with common genes and similar changes in magnitudes of expression comparable between clones (Figure 13.12).

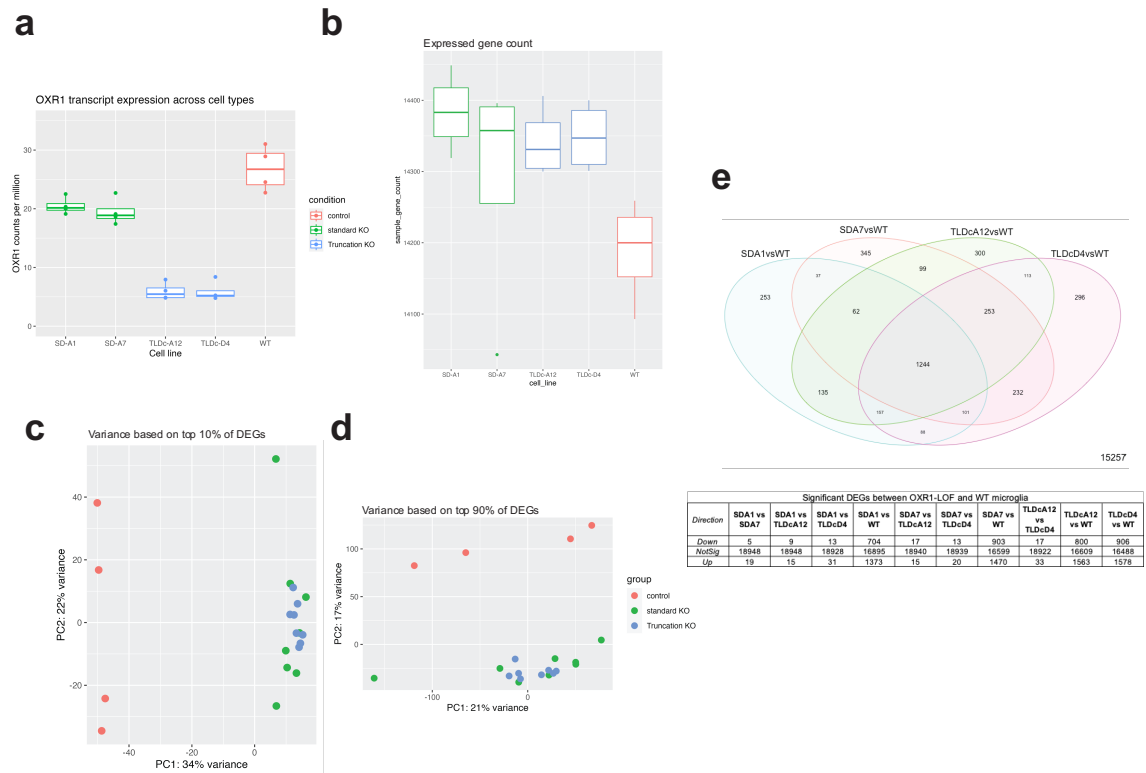


Figure 13.11. RNA-seq sample comparison. a, OXR1 transcript expression in OXR1-LOF microglia (n = 4 per LOF strategy).

Control = WT, standard KO = OXR1^{-/-}, truncation KO = OXR1^{TLDc-/-}. b, the number of expressed genes per sample (n = 4). c, principal component analysis (PCA) of the top 10% differentially expressed genes (DEGs). d, PCA of the top 90% of DEGs. e, Venn diagram demonstrating the number of common DEGs vs WT between samples and a table outlining the number of significant DEGs between different samples.

Table 13.1. List of top up- and down-regulated three DEGs in OXR1-LOF microglia

Gene symbol	Gene name	Direction of change in OXR1-LOF	Gene function
<i>GPAT2</i>	Glycerol-3-Phosphate Acyltransferase 2, Mitochondrial	Up	Spermatogenesis ¹⁶⁸ ; Tumour development ¹⁶⁸ ; GPATs associated with glycerolipid biosynthesis and lipid droplet formation in M1 macrophages ¹⁶⁹ .
<i>TEAD1</i>	TEA domain family member 1	Up	Vascular smooth muscle differentiation and proliferation ^{170,171} ; limiting mitochondrial ROS in cardiomyocytes ¹⁷² ; cytoskeleton dynamics in PSCs ¹⁷³ .
<i>LILRB4</i>	Leukocyte Immunoglobulin Like Receptor B4	Up	Antigen processing and presentation; inhibition of T cell activation and iNOS production ¹⁷⁴ .
<i>TRIM4</i>	Tripartite motif containing 4	Down	E3 ubiquitin ligase; modulation of type I interferon response ^{175,176} ; sensitisation to mitochondrial ROS ¹⁷⁷ .
<i>RYBP</i>	RING1 and YY1 binding protein	Down	Regulation of histone ubiquitination, including via regulation of histone E3 ubiquitin ligase activity of PRC1 ^{178,179} .
<i>TIMP3</i>	Tissue Inhibitor of Metalloproteinases-3	Down	Resolution of inflammation, including metabolic-related inflammation ¹⁸⁰ ; suppression of neutrophil chemotactic activity ¹⁸¹ .

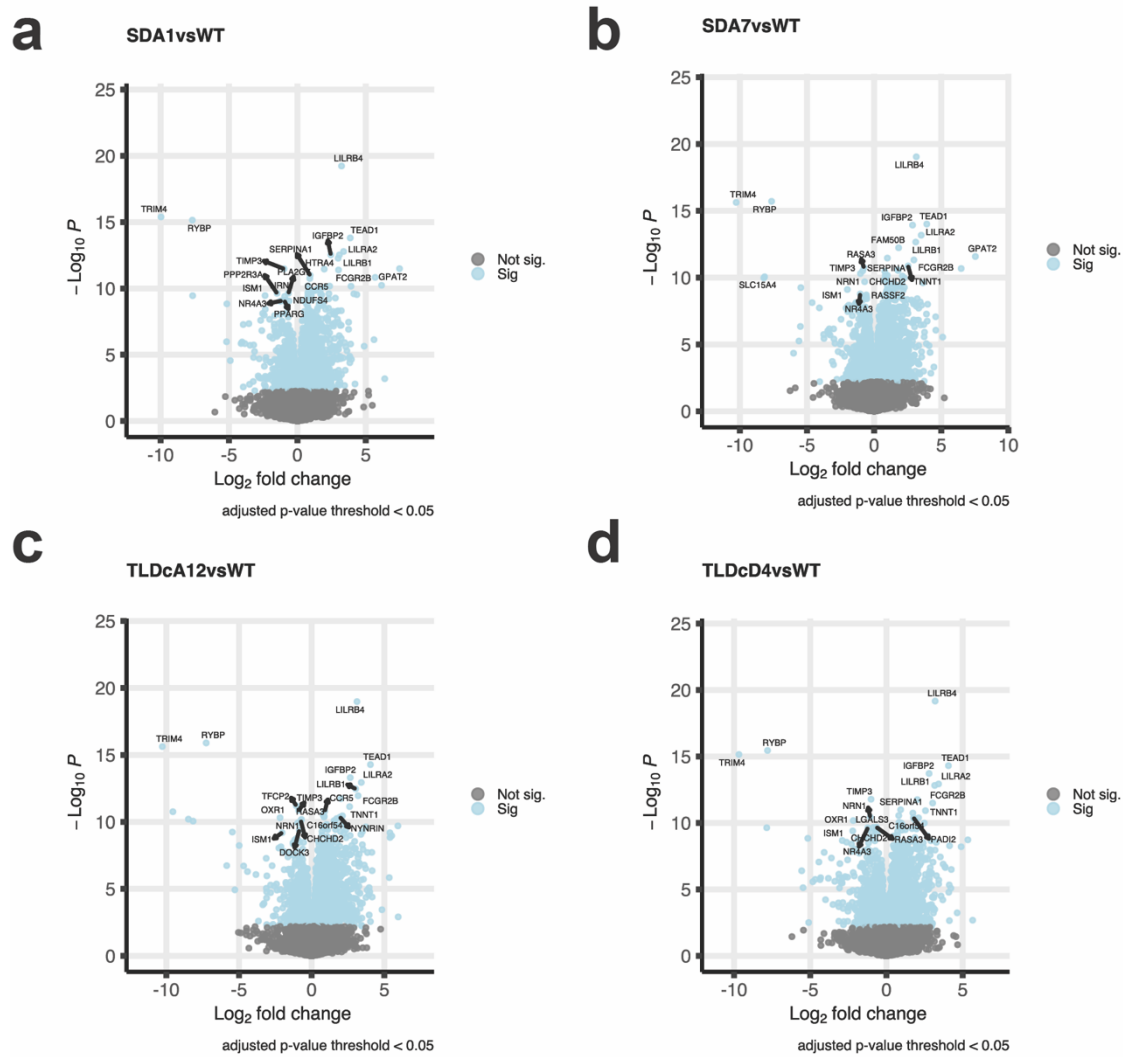


Figure 13.12. Volcano plots highlighting the top 20 DEGs in each sample.

a, SD-A1 clone vs WT. b, SD-A7 clone vs WT. c, TLDc-A12 clone vs WT. d, TLDc-D4 clone vs WT.

Beyond the expression of individual genes, to further delineate altered gene expression pathways and biological processes with consideration to the top 10% of DEGs, gene ontology (GO) analysis was performed¹⁸². GO analysis revealed enrichment of transcripts from genes associated with major histocompatibility complex (MHC) class II, transport vesicle membrane, negative regulation of cell adhesion and chemokine-mediated signalling pathway (Figure 13.13). These pathways demonstrate upregulation of an array of genes predicted by GO analysis to be involved in MHC Class II function, including *HLA-DQ* and *HLA-DR* genes, *CD74*, as well as a multitude of chemokine ligands and receptors. The top 10 gene ontology pathways associated with *OXR1*-LOF in microglia are detailed in Table 13.2. These data outline increased expression of transcripts encoding MHC Class II endogenous peptide presentation (e.g. *HLA-DQ*, *HLA-DR*), receptor cycling associated with MHC Class II peptide engagement and disengagement via the endoplasmic reticulum and Golgi apparatus (e.g. *RAB1A*, *CD74*) and chemokine signalling (e.g. *CX3CL1*, *CXCL10*, *CCL20*).

Table 13.2. Top ten gene ontology pathways.

Gene ratio – the proportion of differentially expressed genes (DEGs) associated with the GO term relative to the total number of DEGs. Background ratio – the proportion of all expressed genes associated with the GO term relative to the total number of genes expressed.

GO Term	Gene Ratio	Background Ratio	p value	Adjusted p value	Gene IDs
MHC class II protein complex	12/1211	14/12993	3.11E-11	4.00E-09	CD74/HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-DMB
peptide antigen assembly with MHC class II protein complex	11/1163	13/12455	2.94E-10	1.36E-07	HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-DMB
peptide antigen binding	16/1191	28/12830	2.86E-10	1.41E-07	CD209/TAP1/HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-E/HLA-F/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-B/HLA-DMB
MHC class II protein complex binding	13/1191	23/12830	1.68E-08	4.15E-06	CD4/CD74/HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-DMB
positive regulation of immune response	11/1163	18/12455	7.72E-08	2.04E-05	HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-DMB
luminal side of endoplasmic reticulum membrane	13/1211	23/12993	1.77E-08	1.70E-06	CD74/HM13/HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DRA/HLA-E/HLA-F/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-B
antigen processing and presentation of exogenous peptide antigen via MHC class II	14/1163	27/12455	2.22E-08	6.85E-06	CD74/FCGR2B/CTSV/HLA-DQB1/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DOA/HLA-DMA/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2/HLA-DMB
negative regulation of growth	9/1163	15/12455	1.56E-06	2.40E-04	PTK6/MT1G/MT2A/GPC3/MT1E/ALOX15B/MT1X/MT1F/MT1H
transport vesicle membrane	14/1211	30/12993	1.21E-07	7.74E-06	CD74/CEACAM1/CPE/RAB11FIP5/ITPR1/HLA-DQB1/RPH3AL/HLA-DRB1/HLA-DQA1/HLA-DRB5/HLA-DRA/HLA-DPB1/HLA-DPA1/HLA-DQB2

13.4 Discussion

13.4.1 Summary of findings

I have demonstrated that *OXR1* is highly expressed in iPSC-derived human microglia and LOF of *OXR1* in this model results in a loss of microglial density, potentially as a consequence of phagocytosis of apoptotic cells. LOF microglia also display lysosomal accumulation, a reduction in endocytic capacity and upregulation of MHC Class II components and chemokine ligand and receptor pathways (Table 13.3). Concurrently, I have established that in microglia, whilst lysosomal pH and degradative capacity are unaffected by *OXR1* perturbation, there is evidence for increased V-ATPase density.

Table 13.3. Summary of findings from OXR1-LOF human iPS-microglia.

This table provides a semi-quantitative, subjective summary of changes in OXR1^{-/-} microglia. 0 – indicates no change from wild-type condition, (+/-)1 – indicates a mild increase or decrease in the metric and (+/-)2 indicates a greater increase or decrease in the metric.

Phenotype	Assay	OXR1 ^{+/-} +	TLDc ^{-/-}	OXR1 ^{-/-}	LV- OXR1
V-ATPase density	SidK binding	0	2	2	n/a
V-ATPase subunit expression	ATP6V1A Western Blot	0	1	1	n/a
Lysosomal accumulation	Lysotracker	0	1.5	1.5	n/a
Lysosomal degradation	DQ-BSA	0	1	1	n/a
Lysosomal pH [qualitative]	Lysosensor	0	1	1	n/a
Lysosomal pH [quantitative]	OG-dextran	0	0	0	0
Endocytic uptake	BSA-488 uptake	0	-2	-2	n/a
Cell Density	Nuclei count	0	-2	-2	n/a
MHC Class II upregulation	RNA-seq	0	2	2	n/a

13.4.2 Human microglia strongly express OXR1

The results presented here suggest that, out of the major human brain cell types, *OXR1* is most strongly expressed in microglia. However, this is based on differentiated cells cultured in isolation. Despite this, the finding is in general agreement with currently available transcriptomic data from human brains^{33,34}, wherein *OXR1* expression is only

higher in neurons. However, the apparent relatively low level of *OXRI* in iPSC-derived neurons and astrocytes presented here may be an artefact of the relatively rudimentary neuronal and astrocyte differentiation protocols, whereas the differentiation protocol for microglia was faithful to their haematopoietic ontogeny, representing yolk sac-derived, tissue-resident macrophages⁷⁰. Additionally, the expression of *OXRI* is age-dependent. In transcriptomic data from developing human and mouse brains, expression rises steeply from P3 to P28 in mice and humans between 7 and 32 years of age¹³⁰, by which point microglia have populated the brain parenchyma from the yolk sac. Functionally, this is reflected in *Oxr1* knockout mice which are relatively healthy until early adolescence (P19) at which point neuronal death and motor impairment worsen¹. Since iPSC-derived neurons and astrocytes in this study were differentiated and maintained for 30 days *in vitro*, these cells have likely not developed the complexities of *OXRI* transcriptional control that occur *in vivo*. Finally, *NGN2*-derived neurons are most similar to cortical neurons¹⁸³, where *OXRI* has previously been demonstrated to be enriched in the cerebellum and hippocampus¹. Thus, expression may be limited further by the lineage of iPS-neurons used in this study.

13.4.3 Loss of OXR1 does not impair lysosomal acidification

Here, I found an accumulation of lysosomes in *OXRI*-LOF microglia coupled with a deficiency in endocytic capacity, indicating impairments in the endolysosomal system. Whilst lysosomal pH was unchanged the degradative capacity of *OXRI*-LOF microglia was slightly, but significantly, increased. Furthermore, elevated SidK staining suggested the expression and/or assembly of the V-ATPase was increased when *OXRI* is perturbed, indicative of a potential functional connection between OXR1 and the V-ATPase that warrants further investigation. It is now crucial to determine whether the

interaction between OXR1 and V-ATPase subunits is conserved in microglia, as it has been demonstrated in other cell types. This is achievable through co-immunoprecipitation of V-ATPase subunits using OXR1 antibodies and has been demonstrated previously in mouse kidney tissue but not in brain tissue⁸. Furthermore, whilst there is no indication that OXR1 impacts V-ATPase homeostasis, it remains to be demonstrated whether *OXR1*-LOF affects stimulation or inhibition of V-ATPase activity, for example via concanamycin A or Torin1 treatment, respectively.

13.4.4 OXR1-LOF may disrupt autophagy in microglia

Lysosomal accumulation in *OXR1*-LOF microglia is comparable with observations in fibroblasts derived from patients with *OXR1* mutations³. Lysosomal accumulation is often a result of reduced degradative capacity and can be a downstream result of defective autophagy, especially in lysosomal storage disorders¹⁸⁴. Thus, to investigate this further, autophagic flux was measured in *OXR1*-LOF microglia. Whilst *OXR1*-LOF does not appear to impact autophagic degradation in microglia, under stimulated autophagy with mTORC inhibition, LC3II was shown to be reduced compared to WT in preliminary data. This suggests that either autophagosome synthesis in autophagy is impaired in *OXR1*-LOF microglia or the fusion of autophagosomes with lysosomes with compromised. The former is likely true since p62 degradation was equivalent to WT microglia, pH is unchanged and lysosomal degradation of DQ-BSA is not impaired. These data contradict previous evidence, which found increased p62 expression in *Drosophila* brains with disrupted *mtl* expression; this finding was attributed to reduced autophagic degradation³. It is, however, noteworthy that these data from fly brain tissue that lack microglia and the *OXR1* homologue *mtl* lacks the N-terminal domains of full-length, human OXR1. Additional studies are required to confirm these

findings and understand the effect of *OXRI* on phagosome formation. Concurrently stimulating phagosome production with Torin1 and inhibiting phagosome-lysosome fusion using concanamycin A presents an avenue for elucidating the causal factors contributing to the observed decline in LC3II levels. This experimental approach facilitates the discrimination of diminished LC3II levels due to a reduction in phagosome formation or an augmented degradation.

Moreover, the upregulation of *NCOA7* observed in *OXRI*-LOF microglia where V-ATPase activity is inhibited, implies a potential functional redundancy with *OXRI* in relation to the V-ATPase. Given that, in *Drosophila*, *mtd* stands as the sole homologous counterpart to both *NCOA7* and *OXRI*³, it is reasonable to infer the absence of such functional rescue in *Drosophila*. Notably, in mammalian systems, disruption of both *OXRI* and *NCOA7* leads to embryonic lethality², demonstrating that expression of at least one of these homologues is necessary for survival. A more in-depth exploration into the intricacies of the interrelated functions of these closely associated TLDC proteins is warranted. Achieving this could involve the inducible knockdown or overexpression of *NCOA7* or *NCOA7-B* in *OXRI*-LOF microglia, presenting an avenue for unravelling the nuanced functional relationships between these proteins.

13.4.5 OXR1 may regulate LC3-associated endocytosis

Whilst crucial in autophagy, LC3 is also associated with a non-canonical mechanism of endocytosis (LANDO) and phagocytosis (LC3-associated phagocytosis, LAP), especially in microglia¹⁸⁵. LAP is a protective clearance mechanism associated with neuronal phagocytosis after injury and β -amyloid clearance in Alzheimer's disease^{186,187}. Dysregulation of LAP is associated with neuroinflammation and degeneration^{186,187}. Whilst crucial in autophagy, LC3 is also associated with a non-

canonical mechanism of endocytosis (LANDO) and phagocytosis (LC3-associated phagocytosis, LAP), especially in microglia¹⁸⁵. LAP is a protective clearance mechanism associated with neuronal phagocytosis after injury and β -amyloid clearance in Alzheimer's disease^{186,187}. Dysregulation of LAP is associated with neuroinflammation and degeneration^{186,187}. Since *OXR1* perturbation in the mammalian brain results in excessive inflammation and neuronal death, this may be a consequence of impaired LAP in microglia¹. Furthermore, the accumulation of vesicles in *OXR1*-LOF microglia, along with increased MHC Class II regulation and loss of microglial density, is indicative of phagocytosis of neighbouring cells¹⁸⁸. Since LANDO is associated with the clearance of cytotoxic insults in microglia, whilst impaired LANDO results in neuronal death¹⁸⁷, measuring the expression of LANDO-associated proteins such as ATG5 and Rubicon, along with characterising LC3-associated endosomes and their contents would provide evidence for this hypothesis.

13.4.6 RNA-seq identified dysregulation of gene expression pathways involved in MHC class II peptide presentation

Since hypothesis-driven investigation of lysosomal activity found no change to lysosomal pH and limited impact on lysosomal function in response to *OXR1*-LOF, RNA-seq was employed as an unbiased method to explore changes to gene expression pathways in *OXR1*-LOF microglia. Pathway analysis of DEGs demonstrated a striking upregulation of transcripts associated with MHC class II peptide presentation and receptor recycling. To validate these findings, it would be pertinent to determine whether transcriptomic changes translate to the protein level, e.g. by flow cytometry quantifying MHC class II-expressing cells and whether reconstitution of *OXR1* by lentivirus restores transcript levels to WT. Concurrently, transcript associated with endo-lysosomal function and V-ATPase subunits displayed only minor changes in expression. This unexpected finding is

nonetheless intricately related to endolysosomal function, especially in the context of MHC class II receptor recycling, as discussed below.

13.4.7 OXR1 loss of function may impair peptide presentation by destabilisation of the retromer

OXR1-LOF microglia have enriched expression of transcripts encoding MHC Class II receptors (*HLA-DQ* and *HLA-DR*) and trafficking proteins involved in receptor cycling (*CD74*, *RAB1A*, *VAMP7*)^{189–191}. MHC class II-mediated peptide presentation is highly dependent on the endo-lysosomal system and trafficking of vesicles for cleavage of endocytosed peptides and receptor cycling¹⁹². Whilst OXR1 has previously been demonstrated to interact with and stabilise retromer complex proteins to maintain endolysosomal trafficking¹⁶⁰, proper regulation of the retromer by the actin-regulator protein complex, WASP and SCAR homologue (WASH), facilitates both MHC class II recycling and helper T cell priming in dendritic cells¹⁹³. Furthermore, the retromer-associated WASH complex is required for retrieval of the V-ATPase from degradative organelles in phagocytosis and dysfunction of WASH-mediated retrieval leads to an accumulation of phagolysosomes and failure of exocytosis of indigestible dextran¹⁹⁴. This phenotype is similar to *OXR1*-LOF microglia, where endosomal accumulation is observable. Therefore, future experiments could focus on determining the stability of the retromer and the levels of actin polymerisation and V-ATPase retrieval by WASH.

Regulation of MHC Class II by OXR1

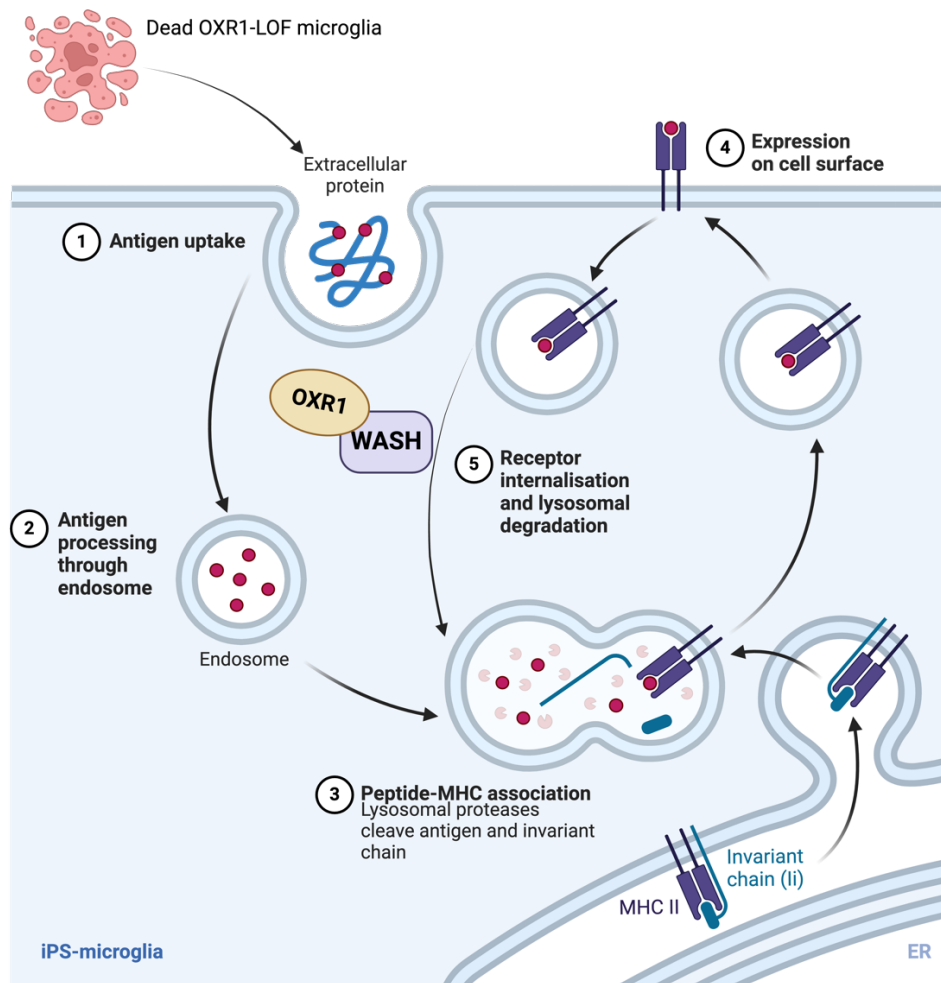


Figure 13.14. A potential mechanism of OXR1 mediated upregulation of MHC Class II in iPS-microglia. This model proposes that OXR1 LOF causes microglial death, leading to endocytosis of extracellular proteins which are processed through the endolysosomal system and presented with MHC Class II receptors. OXR1 may also regulate receptor internalisation and recycling and LOF of OXR1 could impair this process. Adapted from Roche & Furuta, 2015¹⁹².

13.4.8 Loss of OXR1 may induce a cytotoxic microglial state *in vitro*

During the 14-day differentiation period of macrophage precursors to microglia, the density of cells was consistently lower by approximately 40% in *OXR1*-LOF cultures. This could reflect increased sensitivity to stress, causing cell death in the differentiation process, or a shift in the microglial phenotype to a pro-phagocytic, cytotoxic state, in

which case *OXR1*-LOF microglia could phagocytose or otherwise (e.g. by release of cytotoxic cytokines) contribute to cell death in culture. This hypothesis is supported by the increased expression of inflammatory cytokines such as CX3CL1 in *OXR1*-LOF microglia. In addition, reduced density may be a consequence of increased cell death or detachment of microglia from culture plates at a greater rate. As well as a potential cause of cell loss itself, either scenario is likely to result in phagocytosis of dead and dying neighbouring cells. This is supported by MHC Class II upregulation in *OXR1*-LOF microglia, since as professional antigen-presenting cells, microglia can present peptides of engulfed neighbouring cells to regulate CD4⁺ lymphocyte recruitment and activation¹⁹⁵.

Under physiological conditions, T cells are mostly absent from the brain parenchyma, given that their origin in the thymus requires entry to the brain through the blood-brain barrier¹⁹⁶. However, recent evidence has demonstrated the infiltration of circulating haematopoietic cells in neuroinflammatory conditions, specifically regulatory T cells, which are mediated by microglial antigen presentation^{197,198}. Additionally, MHC Class II-positive microglia are associated with chronic neurodegeneration in conditions including multiple sclerosis and Alzheimer's disease^{199,200}, supporting a neurotoxic function of *OXR1*-LOF microglia.

To identify whether microglia are contributing to neuronal loss in *OXR1* several experiments would provide useful information. Firstly, in the mouse brain, identification of T cells by IHC or flow cytometry in the brain parenchyma would confirm infiltration of circulating lymphocytes and the priming of the adaptive immune system to self-peptides expressed in the brain. Treatment with suppressive cytokines (such as TGFβ or IL4) or inhibition of IFNγ in organoids or *in vivo*, to ameliorate the T cell response could

provide a mechanistic link between T cell activation and neuronal death^{197,201}. Finally, the culture of *OXR1*-LOF microglia with neurons, with healthy or perturbed *OXR1*, would be helpful to identify direct phagocytosis of neurons.

14 Discussion

14.1 Summary of findings

The primary aim of this thesis was to investigate the roles of the TLDC proteins NCOA7 and OXR1 in neuroinflammation and microglial function. This was pursued through three models: (1) determining the molecular function and subcellular localisation of the interferon-inducible NCOA7-B in microglia via epitope tagging; (2) measuring Autism Spectrum Disorder (ASD)-associated behavioural endophenotypes in a mouse model of Maternal Immune Activation (MIA) and characterising the gene-environment interaction at the *Ncoa7* locus in *Ncoa7^{DEL}* mice; and (3) measuring lysosomal pH and quantifying gene expression changes in *OXR1* loss-of-function (LOF) human iPS-microglia.

14.1.1 Investigating the expression and subcellular localisation of NCOA7-B protein in microglial cells

I have shown that *Ncoa7-B* is upregulated in response to pro-inflammatory stimuli across various models. *NCOA7-B* expression in human iPS-microglia is stimulated by IFN β , aligning with previous findings in human peripheral myeloid cells such as macrophages and PBMCs, where a putative ISRE has been identified⁷. In mice intraperitoneally injected with LPS, *Ncoa7-B* transcript levels increase in the cerebral cortex, although the cell-type specific expression levels remain unquantified. Additionally, treating murine immortalised microglia with LPS or poly(I:C) *in vitro* triggers a robust *Ncoa7-B* response, correlating with IFN β expression and possibly mediated by an autocrine loop initiated by TLR-stimulated IFN β secretion. This points to a potential novel role for NCOA7-B in the microglial response to inflammation, particularly in the interferon response.

Despite multiple studies suggesting the importance of NCOA7-B, no endogenous protein data has been shown to date. Therefore, to determine NCOA7-B protein levels, localise it within subcellular compartments, and identify its interacting partners, I engineered an endogenous FLAG-tagged version of the protein in murine immortalised microglia. Although the tag was validated at the genomic and transcriptomic levels, efforts to detect the FLAG-tagged protein failed, likely due to post-translational degradation of the epitope tag. As an alternative, I tagged NCOA7-B with GFP in 293T cells, enabling quantification of GFP-NCOA7-B expression via flow cytometry and revealing an IFN β -dependent increase in GFP mean fluorescence intensity (MFI), indicative of a rise in NCOA7-B protein levels. These findings require further validation, including verifying the insertion of GFP at the correct locus and excluding off-target integrations. Nonetheless, this approach may facilitate the identification of endogenous NCOA7-B interacting partners through co-immunoprecipitation using the GFP tag, potentially including V-ATPase subunits. For example, it would be interesting to determine whether tagged NCOA7-B protein could be detected after immunoprecipitation using a V-ATPase subunit antibody.

14.1.2 Measuring ASD-associated behaviours in Maternal Immune Activation (MIA) on an *Ncoa7^{DEL}* genetic background

Microglia have an increasingly recognised role in CNS development, notably in synapse engulfment during critical developmental periods²⁰². Recent studies have highlighted their role within the MIA model of ASD, given the strong immune component and the coincidence of microglial precursor migration from the yolk sac to the brain (E10.5) with the administration of immune activators in the MIA model²⁰³. Considering that *NCOA7* LOF is implicated in ASD and this locus has a specific immune-related regulatory mechanism, this thesis investigated the influence of loss of the *Ncoa7* locus on

ASD-related endophenotypes associated with MIA. Utilising a well-studied paradigm, poly(I:C) was administered to pregnant dams from *Ncoa7^{DEL}* intercross breeds on E12.5 and inflammatory gene expression and behavioural outcomes were measured in the offspring. These data revealed a trend towards a diminished IL6 response in the maternal-foetal immune axis and increased *Ncoa7-B* expression in the brains of wild-type embryos, suggesting that *NCOA7-B* may play a role in the maternal-foetal immune response and cytokine expression may be affected by loss of *Ncoa7*. Behaviourally, the interaction of MIA and *Ncoa7* deletion elicited some evidence of anxiety-like behaviours and impaired motor learning in the rotarod test.

This initial exploration into the interaction between the ASD-associated *Ncoa7* locus and immune activation during development demonstrates that the interaction between the *Ncoa7* locus and MIA does result in ASD-associated behavioural abnormalities, as hypothesised. However, further investigation identifying neuropathological changes in these mice is warranted. Such studies would deepen our understanding of the molecular mechanisms underlying both *Ncoa7* LOF in ASD and microglial involvement in NDD.

14.1.3 Measuring lysosomal function in human OXR1-LOF microglia

OXR1 has previously been reported to interact with the V-ATPase; however, the implications of the full-length isoform's binding within a human cell system remain unexplored. Here, to investigate lysosomal dysfunction, including a quantitative analysis of lysosomal pH, I employed knockout and TLDC truncation strategies on a human iPSC line, differentiating these into microglia to measure OXR1 protein levels compared to those in iPS-neurons and iPS-astrocytes. Consistent with transcriptomic data, *OXR1* was found to be highly expressed in human microglia. Disruption of *OXR1* led to increased

V-ATPase expression, lysosomal accumulation, and reduced endocytosis. Surprisingly, neither LOF nor overexpression of *OXR1* resulted in significant changes to lysosomal pH. This was contrary to expectations, as OXR1 was hypothesised to stabilise the V-ATPase, based on existing *NCOA7* studies outlined below, with its LOF expected to increase lysosomal pH by perturbing normal V-ATPase assembly.

In parallel, to understand molecular pathways of *OXR1* disruption in non-neuronal cells, RNA-seq analysis of *OXR1* LOF microglia was performed. This indicated upregulation of gene expression pathways associated with MHC Class II antigen presentation, but not transcripts associated with lysosomal function or cathepsins, in contrast to bulk RNA-seq from mouse cerebellum tissue⁶. Together, this research suggests that *OXR1* does not influence lysosomal pH in cultured human microglia, yet it does result in endocytosis deficiencies, lysosomal accumulation, and enhanced gene expression pathways associated with MHC Class II. Future work should focus on determining whether OXR1 interacts with the V-ATPase in microglia, potentially through co-immunoprecipitation, and if changes to transcripts associated with MHC Class II are reflected at the protein level. Furthermore, the functional consequences or mechanisms leading to the upregulation of the MHC Class II pathway in *OXR1*-LOF microglia remain to be elucidated.

14.2 Contextualisation of findings

14.2.1 The consequence of TLDC protein binding remains to be determined in mammalian cells

Numerous studies, including those modelling mammalian systems, have established TLDC proteins as *bona fide* V-ATPase-interacting proteins^{50,39,2,8,10}. Furthermore, computational prediction studies, *in vitro* biochemical assays and yeast cell

models have identified modulation of V-ATPase activity upon TLDC protein binding^{16,49,204,205}. A recent study explored the effect of incubating TLDC proteins and recombinant TLDC domains on V-ATPase activity, in which oxidation of NADPH was measured as a proxy for ATP hydrolysis. Here they found that the TLDC domains alone of OXR1 and NCOA7, expressed and isolated as recombinant proteins, promoted holoenzyme disassembly thus reducing V-ATPase activity⁹. The mechanism for V-ATPase inhibition was proposed to be conserved from yeast Oxl1p, a TLDC protein consisting almost entirely of the TLDC domain⁴⁹. In yeast, the cryo-EM structure revealed that Oxl1p inhibits V-ATPase by binding to the B subunit of an open catalytic site, subunit C, and peripheral stalk EG2 in yeast V₁ during an ATP-induced rotary state⁴⁹. This interaction induced structural rearrangements in the C subunit and peripheral stalk EG2, destabilising the V₁-V₀ interface and leading to enzyme disassembly.

Whilst the TLDC domains of NCOA7 and OXR1 inhibited V-ATPase activity, recombinant mEAK7, which possesses a TLDC domain that is not fully C-terminal, was found to increase ATP hydrolysis⁹. Here, the authors hypothesise that mEAK7 binding facilitates the opening of an additional catalytic site, potentially increasing the enzyme's turnover rate. It was suggested that this interaction, unlike interactions observed with yeast Oxl1p, does not distort the subunits at the V₁-V₀ interface, thus maintaining V-ATPase assembly and enhancing its activity under turnover conditions^{9,49}.

While these studies offer valuable insights into the binding kinetics and mechanisms of TLDC protein binding, their fundamentally reductionist approach excludes non-TLDC residues for OXR1 and NCOA7 and may reflect functions specific to the known short isoforms, such as NCOA7-B. Furthermore, these data point towards an inhibitory role of OXR1, whereas existing research manipulating TLDC protein

expression in mammalian systems suggests that OXR1 and NCOA7 binding enhances V-ATPase activity^{2,3,10,39,50}. For example, NCOA7 in the mouse brain was found to interact with the V₁ domain of V-ATPase through co-immunoprecipitation and colocalised immunofluorescence in primary neurons². Primary neurons from mice lacking the *Ncoa7* locus exhibited reduced expression of V₁ regulatory subunits, increased lysosomal pH, and diminished degradative capacity for endocytosed substrates, highlighting a decrease in V-ATPase activity with functional consequences for lysosomal degradation². Additionally, overexpression of NCOA7-B in glioma cells was observed to decrease vesicular pH, associated with V-ATPase interaction³⁹. Furthermore, in the kidney, both OXR1 and NCOA7 were identified as key V-ATPase interactors in a renal interactome study; in *Ncoa7*^{Tm1.1} mice, the loss of full-length NCOA7 was associated with elevated urine pH and partial distal renal tubular acidosis, phenocopying LOF in renal V-ATPase subunit isoforms⁵⁰.

Together, this highlights an incongruence between computational, biochemical, and yeast studies—which predict a predominantly inhibitory function of TLDc proteins upon V-ATPase binding—and mammalian studies which confirm V-ATPase binding but suggest inhibited V-ATPase activity upon LOF of TLDc proteins and enhanced activity upon overexpression. A crucial gap in these studies is demonstrating that TLDc protein binding stabilises the V-ATPase complex and enhances activity in a cellular context, a challenge that may prove difficult in more complex mammalian systems. However, this could be achieved by targeted mutation of key residues responsible for V-ATPase binding, as has been demonstrated previously in NCOA7-B¹⁵.

Although *OXR1* LOF or overexpression showed no effect on lysosomal pH in this study, a clear endolysosomal phenotype with endocytic dysfunction, lysosomal

accumulation, and elevated V-ATPase assembly was observed in *OXR1*-LOF microglia, comparable to fibroblasts derived from *OXR1* LOF patients³. Therefore, whilst *OXR1* may not directly control lysosomal pH it may influence other V-ATPase factors such as enzyme localisation, which has been demonstrated in yeast²⁰⁵. Furthermore, the interaction between *OXR1* and V-ATPase in microglia was not determined in this study, nor has the interaction between *NCOA7* isoforms and other TLDC proteins been specifically examined concerning V-ATPase function. Given their coincident expression in microglia and the ability of both *NCOA7* and *OXR1* to bind V-ATPase, it is plausible to hypothesise functional redundancy between *OXR1* and its homologue *NCOA7*, which may compensate for changes to lysosomal pH due to the loss of *OXR1*. This interaction could be further explored by knockdown of *NCOA7* in microglia.

In conclusion, although substantial evidence exists for the interaction of TLDC proteins with the V-ATPase, and initial studies have examined the functional consequences of TLDC domain binding, the impact on V-ATPase activity within complex mammalian systems remains undefined. The directionality of these effects is likely specific to cell type and context, influenced by competitive binding and compensatory mechanisms between TLDC proteins. Consequently, sophisticated cellular models are essential to elucidate the physiological regulation of V-ATPase by TLDC proteins effectively.

14.2.2 Clinical applications of V-ATPase regulation by TLDC proteins in neuroinflammation

Along with patient mutations, there is evidence of involvement of TLDC protein in idiopathic diseases, such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS).

14.2.2.1 *Elucidating a potential therapeutic role of NCOA7-B and OXR1 in neurodegenerative diseases*

In MS, IFN β is used therapeutically to modulate the immune system, reducing inflammation and the frequency of relapses in patients with multiple sclerosis by inhibiting immune cells that attack the myelin sheath and preserving the blood-brain barrier's integrity⁴⁵. Thus, the myeloid response to IFN-I signalling is a crucial aspect of current treatment. Whilst microglia are present in active and mixed active/inactive lesions, their contribution to the pathology of MS remains to be determined²⁰⁶. Understanding the gene expression response to IFN β treatment in the context of MS is an important aspect of further elucidating the role of microglia in the pathogenesis of MS. Additionally, the IFN-I response is becoming recognised as a mediator of the microglial response to amyloid- β (A β) in Alzheimer's disease. Exposure of primary microglia to A β induces levels of bioactive IFN-I and expression of ISGs⁴⁷. Whilst microglia from *Ifnar1*^{-/-} mice display phagocytic deficits of A β and increased morphological radius and area of microglia *in vivo*, which is correlated with an anti-inflammatory phenotype^{47,207}. In contrast, treatment of mice with recombinant IFN β resulted in elevated expression of CD68, increased microglial density, reduced dendrite length and C3-dependent synapse elimination and blockage of IFN signalling dampens the microglial response⁴⁸. Thus, the IFN-I response is being recognised as playing an important role in the phagocytic response to A β in Alzheimer's disease and, given the demonstrated interactions of NCOA7-B with the V-ATPase and consequently elevated vesicular pH³⁹, which is crucial in phagocytosis, an understanding of the role of this novel ISG in microglial biology is crucial to understanding the IFN response in Alzheimer's disease.

Similarly, *OXR1* is upregulated in the spinal cord of ALS patients and has been shown to mitigate the inflammatory response in ALS model mice, thereby improving

motor outcomes and extending lifespan (as discussed in Section 13.2.4). The cellular mechanisms underlying *OXR1* rescue could be further explored using iPSC models, which have advanced recent understanding of the role of microglia in ALS pathogenesis^{208–210}. In neuron-microglia co-cultures derived from C9orf72-ALS patients, microglia exhibit a pro-inflammatory, neurotoxic phenotype, primarily through the secretion of neurotoxic metalloproteinase-9 (MMP9)²¹⁰. Although overexpression of *OXR1* has been shown to improve outcomes in TDP-43 and SOD1^{G93A} mouse models, its applicability to C9orf72-ALS remains unexplored. This model offers a valuable opportunity to investigate the disease-modifying potential of *OXR1* in human cells and to explore the molecular mechanisms of rescue, particularly whether these are dependent on neuronal or microglial expression of *OXR1*.

Beyond direct delivery of small TLDC protein isoforms (NCOA7-B and OXR1-C) potential druggability of the TLDC domain has been demonstrated very recently using a computational approach in pharmacophore modelling and virtual small molecule screening²¹¹. Here, the authors were able to identify and manufacture a compound to maintain increased levels of NCOA7 in murine pulmonary arterial endothelial cells by small molecule binding to its TLDC domain. This molecule was demonstrated to increase NCOA7-ATP6V1B2 interaction in a proximity ligation assay and significantly increase Lysosensor intensity, indicating lower lysosomal pH²¹¹. The potential application of such compounds to investigate the influence of TLDC binding on the V-ATPase in mammalian systems is extensive.

14.3 Conclusion

This thesis has examined some of the multifaceted roles of the TLDC proteins, NCOA7 and OXR1, in neuroinflammation and microglial function, illustrating their

potential significance in CNS development and disease modulation. By investigating their interactions in diverse experimental models, it has been demonstrated that OXR1 influences endo-lysosomal function, V-ATPase expression, and MHC Class II expression, with possible consequences for neurological disease mechanisms. Particularly, this study has shown that while loss of *OXR1* does not directly regulate lysosomal pH, its putative interaction with the V-ATPase complex plays a critical role in modulating microglial functions that are crucial in neuroinflammatory pathways. Conversely, NCOA7 interactions appear to actively modulate V-ATPase activity, suggesting a protective role against neurodegeneration by enhancing lysosomal degradation capacity; here I have presented a model where this role can be elucidated further in an immortalised human cell line. Concurrently, I have demonstrated an interaction between *Ncoa7* locus deletion and behavioural outcomes in the MIA model of ASD, where anxiety-like behaviours and a motor learning deficit were observable. These findings further support an important role for TLDc proteins where microglial activity and inflammation are pivotal; yet further exploration into the molecular mechanisms of TLDc proteins is needed if they can be harnessed successfully as therapeutic targets.

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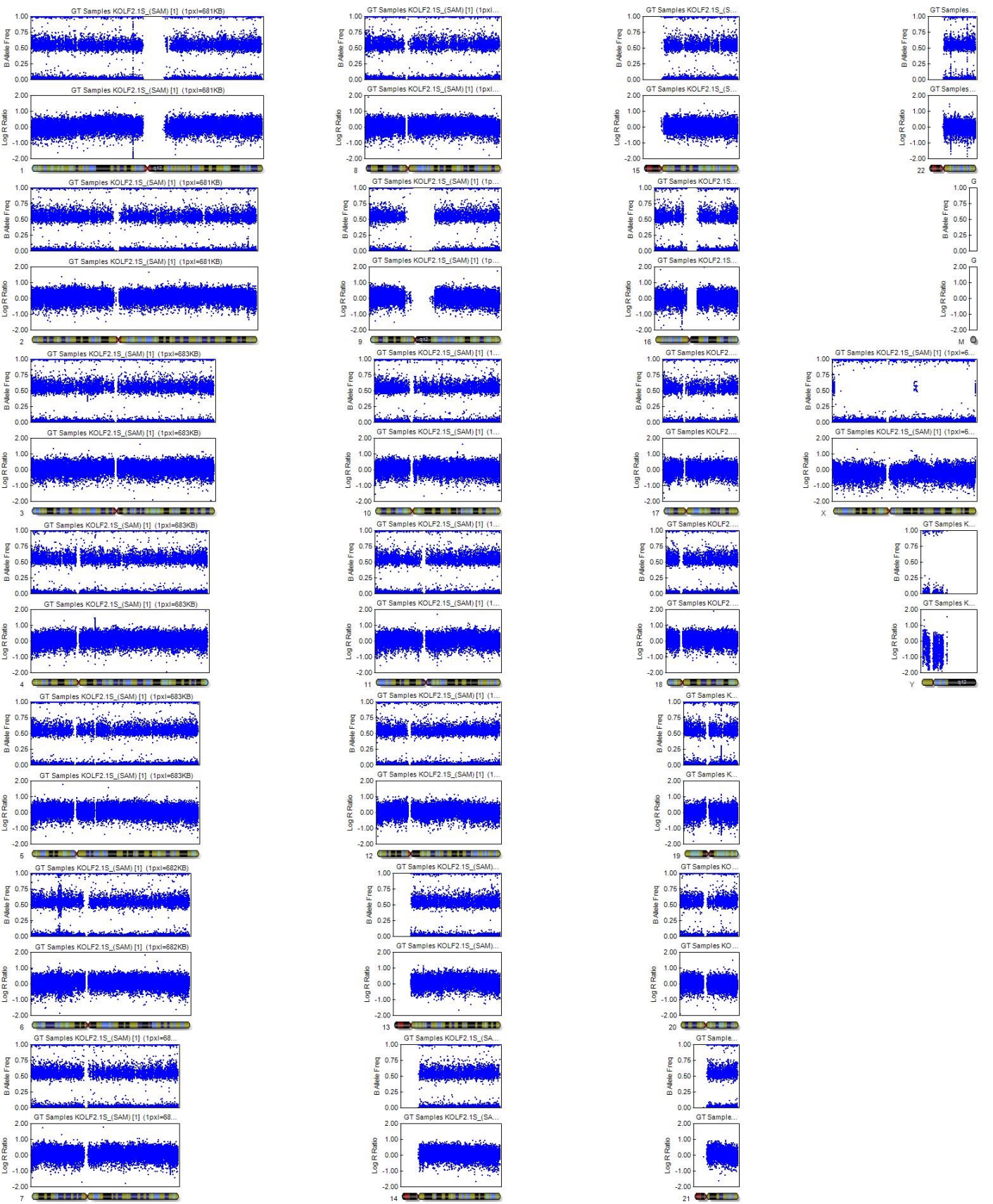
16 Appendices

Appendix 1. First 100 most abundant targets detected my mass spectrometry of FLAG immunoprecipitated protein elute.

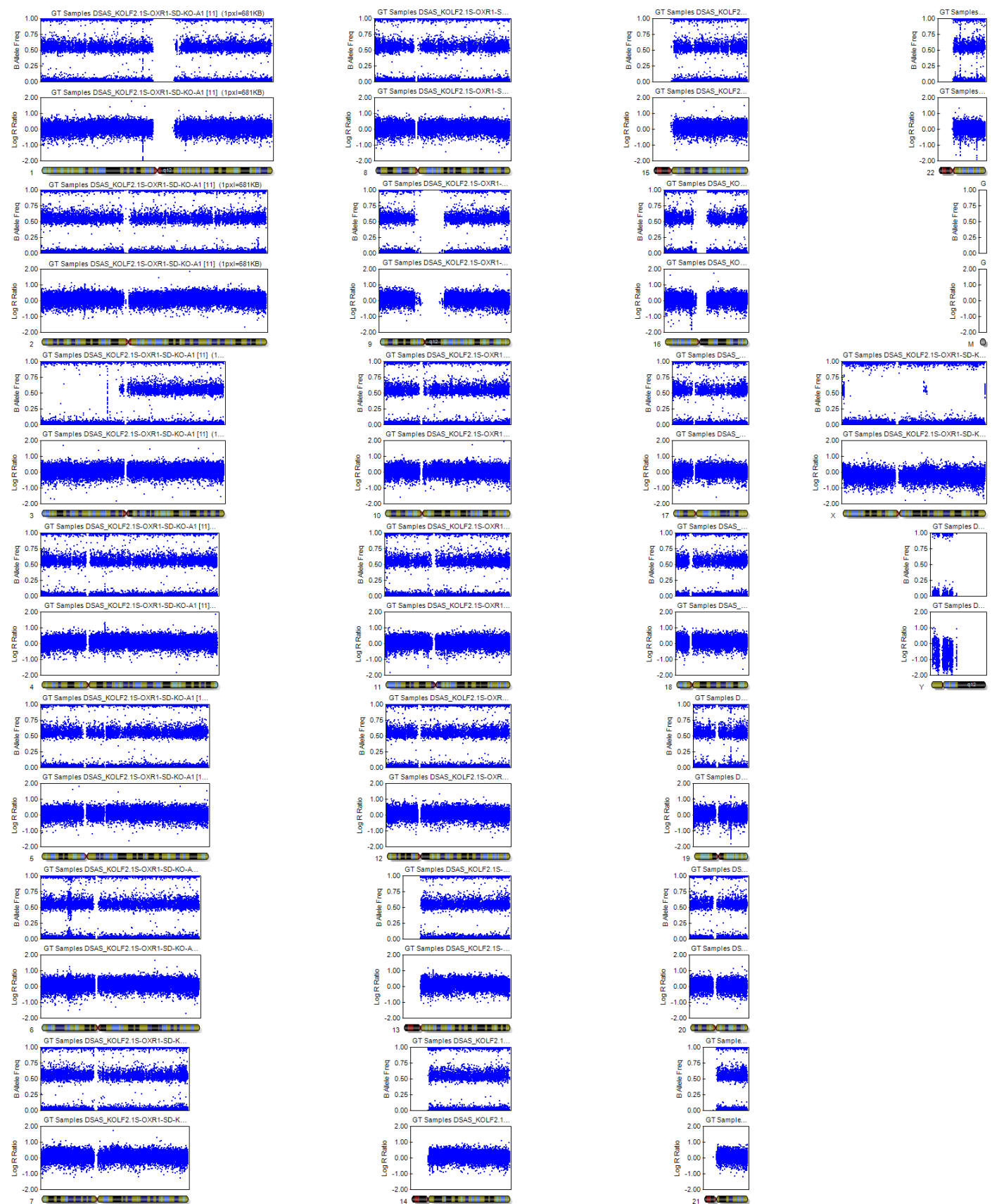
Accession	# AAs	MW [kDa]	calc. pI	Description	A5: Area	B5: Area	C5: Area	Score A2	average	# Peptides	# PSM A2	Score B2	average	# Peptides	# PSM B2	Score C2	average	# Peptides	# PSM C2
Q9JMH9	2050	232.6	6.28	Unconventional myosin-XVIIIa OS=Mus musculus OX=10090 GN=Myo18	2.383E10	3.949E10	2.617E10	106706	62.00	134	301	1158.03	60.54	135	325	1096.32	60.44	133	299
Q9VDD5	1960	226.2	5.66	Myosin-9 OS=Mus musculus OX=10090 GN=Myh9 PE=1 SV=4 - [MYH_9]	2.683E9	4.367E9	1.036E10	693.21	57.96	123	220	736.42	57.04	126	222	936.61	61.43	148	274
Q9J128	1271	144.7	6.06	Protein flightless-1 homolog OS=Mus musculus OX=10090 GN=Flil PE=1 SV=1	2.295E10	2.503E10	1.952E10	67801	60.42	80	198	749.56	61.45	82	206	669.93	58.30	84	187
P60710	375	41.7	5.88	Actin, cytoplasmic 1 OS=Mus musculus OX=10090 GN=Actb PE=1 SV=1	6.427E10	8.339E10	9.477E10	442.32	73.07	29	152	502.61	76.53	31	166	504.52	77.07	32	166
Q9J104	1853	215.4	8.63	Unconventional myosin-Va OS=Mus musculus OX=10090 GN=Myo5a PE=1	1.813E9	4.196E9	2.420E9	296.28	43.44	75	103	372.55	46.36	82	126	240.81	36.43	59	81
Q3J239	729	79.2	4.42	Leucine-rich repeat flightless-interacting protein 1 OS=Mus musculus O	2.024E10	2.192E10	1.467E10	496.42	64.61	36	141	411.72	46.50	30	120	389.85	45.13	29	112
Q9JH00	352	39.5	5.14	Tropomodulin-3 OS=Mus musculus OX=10090 GN=Tmod3 PE=1 SV=1 -	4.003E10	4.428E10	3.746E10	260.88	66.76	31	106	299.30	65.63	30	108	267.92	62.78	28	102
Q9JWK0	415	47.1	5.68	Leucine-rich repeat flightless-interacting protein 2 OS=Mus musculus O	1.274E10	1.109E10	7.140E9	391.47	66.02	36	121	362.57	55.90	31	105	267.21	54.46	25	85
P68033	377	42.0	5.39	Actin, alpha cardiac muscle 1 OS=Mus musculus OX=10090 GN=Actc1	4.951E10	5.253E10	7.061E10	204.55	33.16	18	100	217.40	43.77	21	104	248.62	44.83	22	112
P20152	466	53.7	5.12	Vimentin OS=Mus musculus OX=10090 GN=Vim PE=1 SV=3 - [VIME_M	3.494E9	3.019E9	4.238E9	280.01	81.12	47	92	286.46	81.12	48	85	251.00	79.61	46	80
P10126	462	50.1	9.01	Elongation factor 1-alpha 1 OS=Mus musculus OX=10090 GN=Elf1a1 PE	1.178E10	1.273E10	2.592E10	196.70	58.23	22	61	244.43	51.30	22	72	255.99	61.26	26	75
P99024	444	49.6	4.89	Tubulin beta-5 chain OS=Mus musculus OX=10090 GN=Tubb5 PE=1 SV=	4.585E9	3.919E9	8.701E9	239.89	83.11	30	76	223.32	77.25	29	66	204.91	81.31	29	59
P13020	780	85.9	6.18	Gelsolin OS=Mus musculus OX=10090 GN=Gsn PE=1 SV=3 - [GELS_MO	4.851E9	5.721E9	5.226E9	205.11	46.03	30	64	218.22	46.03	31	64	209.02	47.44	31	67
P68372	445	49.8	4.89	Tubulin beta-4B chain OS=Mus musculus OX=10090 GN=Tubb4b PE=1	4.679E9	3.549E9	8.062E9	230.76	82.92	30	74	204.76	73.71	27	61	189.98	81.12	28	56
P11499	724	83.2	5.03	Heat shock protein HSP 90-beta OS=Mus musculus OX=10090 GN=Hsp5	1.368E9	1.678E9	2.383E9	144.24	45.03	36	51	176.24	50.55	37	61	156.86	38.81	33	52
P52480	531	57.8	7.47	Pyruvate kinase PKM OS=Mus musculus OX=10090 GN=Pkm PE=1 SV=	2.458E9	5.473E9	9.936E9	150.63	64.78	28	39	234.93	65.73	35	61	269.09	70.43	32	71
P63017	646	70.8	5.52	Heat shock cognate 71 kDa protein OS=Mus musculus OX=10090 GN=H	4.633E9	3.842E9	4.311E9	182.20	54.49	34	61	183.16	54.95	33	59	191.07	51.55	38	61
P36993	390	42.8	5.19	Protein phosphatase 1B OS=Mus musculus OX=10090 GN=Ppm1b PE=1	2.961E9	4.743E9	7.226E9	171.03	71.79	23	54	181.55	73.33	24	59	196.60	78.72	25	61
P01837	107	11.9	5.59	Immunoglobulin kappa constant OS=Mus musculus OX=10090 GN=Igkc	2.337E10	1.232E10	2.566	78.50	50	9	71	202.11	88.79	10	59	180.99	88.79	10	50
Q8BF73	376	42.0	5.49	Beta-actin-like protein 2 OS=Mus musculus OX=10090 GN=Actb2 PE=1	3.577E10	4.339E10	9.317E10	107.48	35.11	12	55	121.66	33.51	11	58	120.50	36.70	12	60
P71782	434	47.1	6.80	Alpha-enolase OS=Mus musculus OX=10090 GN=Enol PE=1 SV=3 - [EN	3.162E9	5.485E9	9.379E9	148.54	64.52	31	55	160.24	63.59	31	58	203.23	65.90	35	74
Q61879	1976	228.9	5.54	Myosin-10 OS=Mus musculus OX=10090 GN=Myh10 PE=1 SV=2 - [MYH	1.504E9	1.651E9	4.192E9	102.18	15.38	28	36	168.62	26.77	44	58	204.83	30.16	49	69
P07901	733	84.7	5.01	Heat shock protein HSP 90-alpha OS=Mus musculus OX=10090 GN=Hsp	1.386E9	2.351E9	2.760E9	129.31	50.61	34	47	149.01	45.29	36	56	161.03	44.07	35	57
P63242	154	16.8	4.84	Eukaryotic translation initiation factor 5A-1 OS=Mus musculus OX=100	9.124E9	1.307E10	9.618E9	158.57	56.49	18	48	165.96	56.49	17	54	152.03	56.49	17	46
Q7TMM9	445	49.9	5.29	Tubulin beta-2A chain OS=Mus musculus OX=10090 GN=Tubb2a PE=1	1.418E9	3.553E9	8.062E9	185.49	55.51	24	64	167.78	54.16	22	53	149.35	61.80	23	47
P16858	333	35.8	8.25	Glyceraldehyde-3-phosphate dehydrogenase OS=Mus musculus OX=100	7.794E9	1.395E10	1.798E10	148.79	71.17	18	42	195.80	72.67	20	53	246.35	72.67	21	66
Q9JH44	4644	531.7	6.42	Cytoplasmic dynein 1 heavy chain 1 OS=Mus musculus OX=10090 GN=	2.778E9	3.108E9	6.510E9	145.26	13.48	49	52	143.30	13.72	50	52	216.06	20.24	67	73
P48678	665	74.2	6.92	Protein-A/C OS=Mus musculus OX=10090 GN=Lmna PE=1 SV=2 - [LH	1.244E9	7.284E9	5.323E9	172.62	60.15	43	60	139.13	56.69	39	50	106.70	46.17	31	39
Q3V3R1	977	105.7	7.08	Monofunctional C1-tetrahydrofolate synthase, mitochondrial OS=Mus m	1.827E9	1.178E9	1.774E9	202.62	50.15	42	60	178.31	47.70	38	50	167.70	45.04	38	49
Q9JLX0	189	20.0	6.07	Parkinson disease protein 7 homolog OS=Mus musculus OX=10090 GN=	1.283E10	9.761E9	1.439E10	171.50	78.31	16	53	167.38	78.31	16	50	176.89	79.37	17	53
P50564	364	39.3	8.09	Fructose-bisphosphate aldolase A OS=Mus musculus OX=10090 GN=Alk	1.824E9	3.785E9	7.283E9	108.91	75.55	23	33	169.27	85.99	29	48	187.96	82.97	25	50
Q9W177	1063	121.9	9.35	Unconventional myosin-Ic OS=Mus musculus OX=10090 GN=Myo1c PE=	6.151E9	6.188E9	1.293E9	92.60	32.08	28	31	153.64	46.28	42	48	129.62	41.49	35	39
Q9J1V4	465	54.1	7.34	Serine/threonine-protein kinase 38 OS=Mus musculus OX=10090 GN=Stl	3.913E9	5.515E9	4.969E9	126.35	47.96	23	47	124.57	53.33	25	47	105.91	49.68	26	40
Q6IRU2	248	28.5	4.68	Tropomyosin alpha-6 chain OS=Mus musculus OX=10090 GN=Tpm4 PE=	7.874E9	3.439E9	3.748E9	96.78	60.48	16	20	155.36	68.95	26	47	149.40	67.34	25	41
Q922V5	1149	125.7	5.70	Histone deacetylase 4 OS=Mus musculus OX=10090 GN=Hdac5 PE=1 S	1.130E9	1.853E9	2.973E9	66.36	22.89	20	35	122.66	29.07	22	42	95.47	24.89	19	29
P68369	451	50.1	5.06	Tubulin alpha-1A chain OS=Mus musculus OX=10090 GN=Tuba1a PE=1	6.308E9	4.805E9	7.627E9	143.32	60.98	23	42	153.38	74.94	25	41	151.31	72.73	23	42
P50213	451	50.1	5.06	Tubulin alpha-1B chain OS=Mus musculus OX=10090 GN=Tuba1b PE=1	6.308E9	4.805E9	7.627E9	143.72	60.98	22	42	153.41	75.17	25	41	154.55	72.95	23	43
P63085	358	41.2	6.98	Mitogen-activated protein kinase 1 OS=Mus musculus OX=10090 GN=M	1.524E9	2.121E9	1.201E9	63.69	23	35	138.62	57.26	22	41	124.37	73.10	22	36	
P68373	449	49.9	5.10	Tubulin alpha-1C chain OS=Mus musculus OX=10090 GN=Tuba1c PE=1	6.308E9	4.805E9	7.431E9	134.61	56.57	22	41	136.10	70.60	23	38	143.24	73.05	23	42
P20029	655	72.4	5.16	Endoplasmic reticulum chaperone BiP OS=Mus musculus OX=10090 GN=	1.810E9	1.495E9	2.072E9	140.97	45.34	31	48	121.53	38.17	26	38	163.33	41.30	36	53
P47941	303	33.8	6.24	Crk-like protein OS=Mus musculus OX=10090 GN=Crkl PE=1 SV=2 - [C	1.162E10	1.331E10	1.056E10	112.58	61.39	18	38	169.81	50.83	15	38	111.73	50.83	16	35
Q5SNZ0	1873	215.8	6.74	Griin OS=Mus musculus OX=10090 GN=Cdc88a PE=1 SV=2 - [GRDIN	1.025E9	1.803E9	6.734E9	106.50	5.82	8	9	108.08	21.09	34	38	125.48	21.52	33	42
Q8BYC6	898	105.3	7.36	Serine/threonine-protein kinase TAO3 OS=Mus musculus OX=10090 GN=	1.180E9	1.453E9	8.959E9	126.39	37.42	33	42	126.60	24.94	26	38	61.51	19.04	19	21
P62631	463	50.4	9.03	Elongation factor 1-alpha 2 OS=Mus musculus OX=10090 GN=Elf1a2 PE	4.284E9	6.128E9	4.775E9	68.96	23.54	9	25	105.54	31.10	11	37	77.83	23.97	9	27
Q61233	627	70.1	5.53	Plastin-2 OS=Mus musculus OX=10090 GN=Lpl1 PE=1 SV=4 - [PLSL_M	5.719E9	6.447E9	1.490E9	89.73	46.89	21	28	117.75	55.18	28	36	111.25	44.66	23	32
Q9V0N2	1023	112.9	5.48	Protein/potassium-transporting ATPase subunit alpha-1 OS=Mus muscu	8.634E9	6.432E9	1.258E9	106.98	34.41	30	35	94.55	33.14	29	35	106.23	31.77	29	34
Q9EQ64	1107	126.7	9.07	Unconventional myosin-Ie OS=Mus musculus OX=10090 GN=Myo1e PE=	1.174E9	6.221E9	6.698E9	143.50	35.68	37	51	100.69	30.62	31	35	62.43	22.58	20	22
Q922F4	447	50.1	4.89	Tubulin beta-6 chain OS=Mus musculus OX=10090 GN=Tubb6 PE=1 SV=	3.394E9	6.328E9	7.561E9	117.93	60.85	22	41	93.43	62.19	21	34	105.08	63.76	20	32
P09411	417	44.5	7.90	Phosphoglycerate kinase 1 OS=Mus musculus OX=10090 GN=Pgk1 PE=1	1.148E9	1.751E9	5.528E9	82.35	53.48	18	24	114.89	56.83	20	34	147.33	59.95	21	47
Q68FD5	1675	191.4	5.95	Clathrin heavy chain 1 OS=Mus musculus OX=10090 GN=Cltc PE=1 SV=	3.203E9	2.586E9	1.314E9	116.45	27.16	35	36	112.71	24.36	31	34	195.18	36.00	47	59
P21107	285	33.0	4.67	Tropomyosin alpha-3 chain OS=Mus musculus OX=10090 GN=Tpm3 PE=	7.185E9	2.379E9	1.682E9	71.43	37.54	16	23	102.65	40.00	19	34	101.53	43.16	19	34
Q8BCD9	611	68.8	5.62	Eukaryotic translation initiation factor 4B OS=Mus musculus OX=10090	2.891E9	3.050E9	2.093E9	115.27	35.68	22	47	80.00	32.08	16	33	82.74	29.13	18	35
P42932	548	59.5	5.62	T-complex protein 1 subunit theta OS=Mus musculus OX=10090 GN=Cc	7.666E9	8.480E9	1.071E9	78.55	43.80	23	28	87.79	49.64	25	32	97.51	49.09	26	36
P63038	573	60.9	6.18	60 kDa heat shock protein, mitochondrial OS=Mus musculus OX=10090	1.829E9	1.137E9	2.362E9	128.38	53.05	27	37	114.39	49.04	24	32	128.90	49.04	27	36
Q04447	381	42.7	5.67	Creatine kinase B-type OS=Mus musculus OX=10090 GN=Ckb PE=1 SV	2.431E9	2.738E9	4.400E9	84.22	39.63	11	33	79.58	37.01	9	31	82.94	39.63	10	32
Q9JFK1	1657	188.6	6.48	Ras GTPase-activating-like protein IQGAP1 OS=Mus musculus OX=100	5.744E9	6.233E9	1.707E9	115.33	29.39	35	37	91.93	21.91	28	31	105.72	27.10	32	36
P17751	249	26.7	7.30	Triosephosphate isomerase OS=Mus musculus OX=10090 GN=Tpi1 PE=	7.624E9	6.822E9	9.430E9	101.05	82.33	16	31								

Appendix 2. Karyograms of OXR1-LOF iPSC

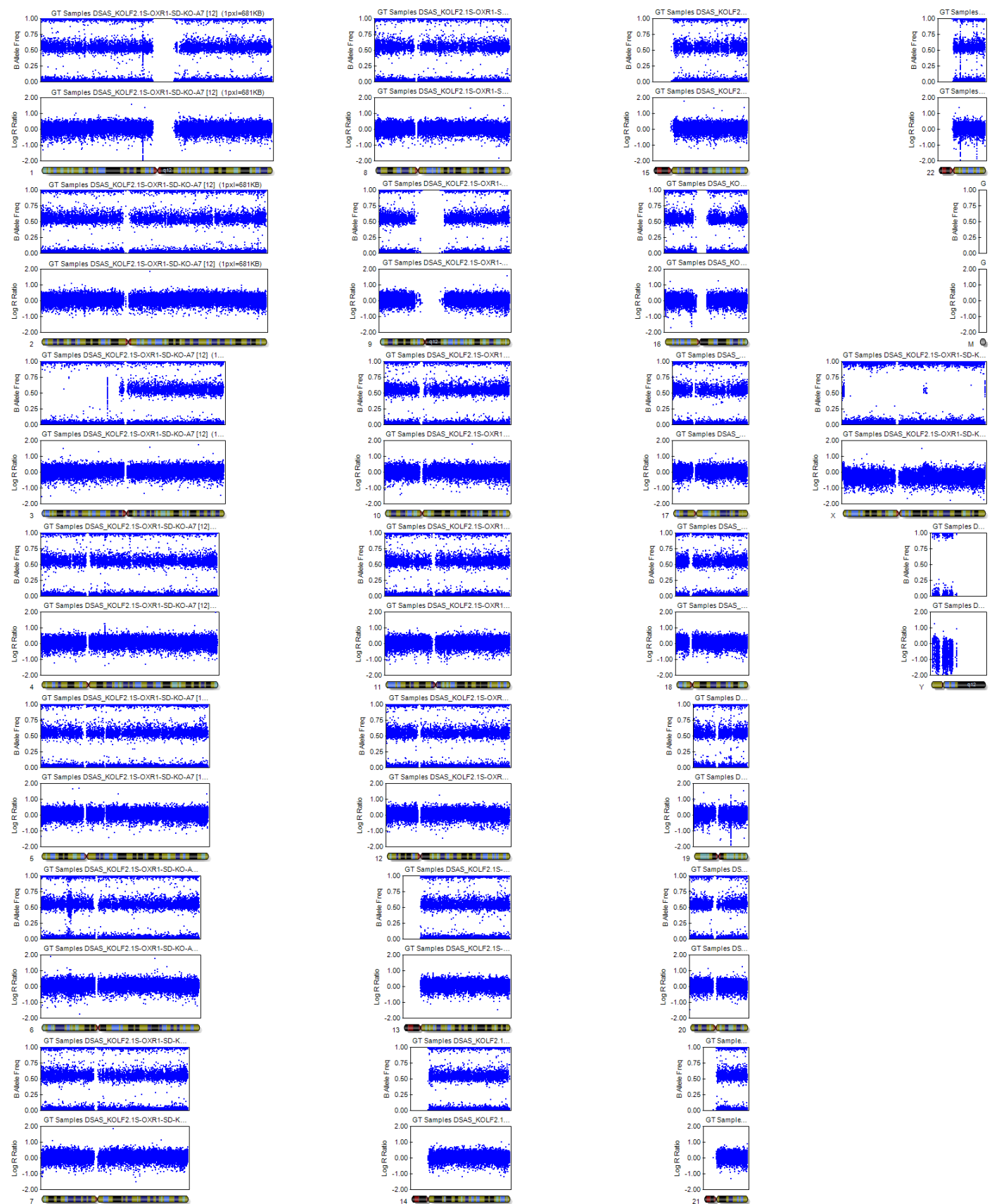
KOLF2.1S (Wild-type)



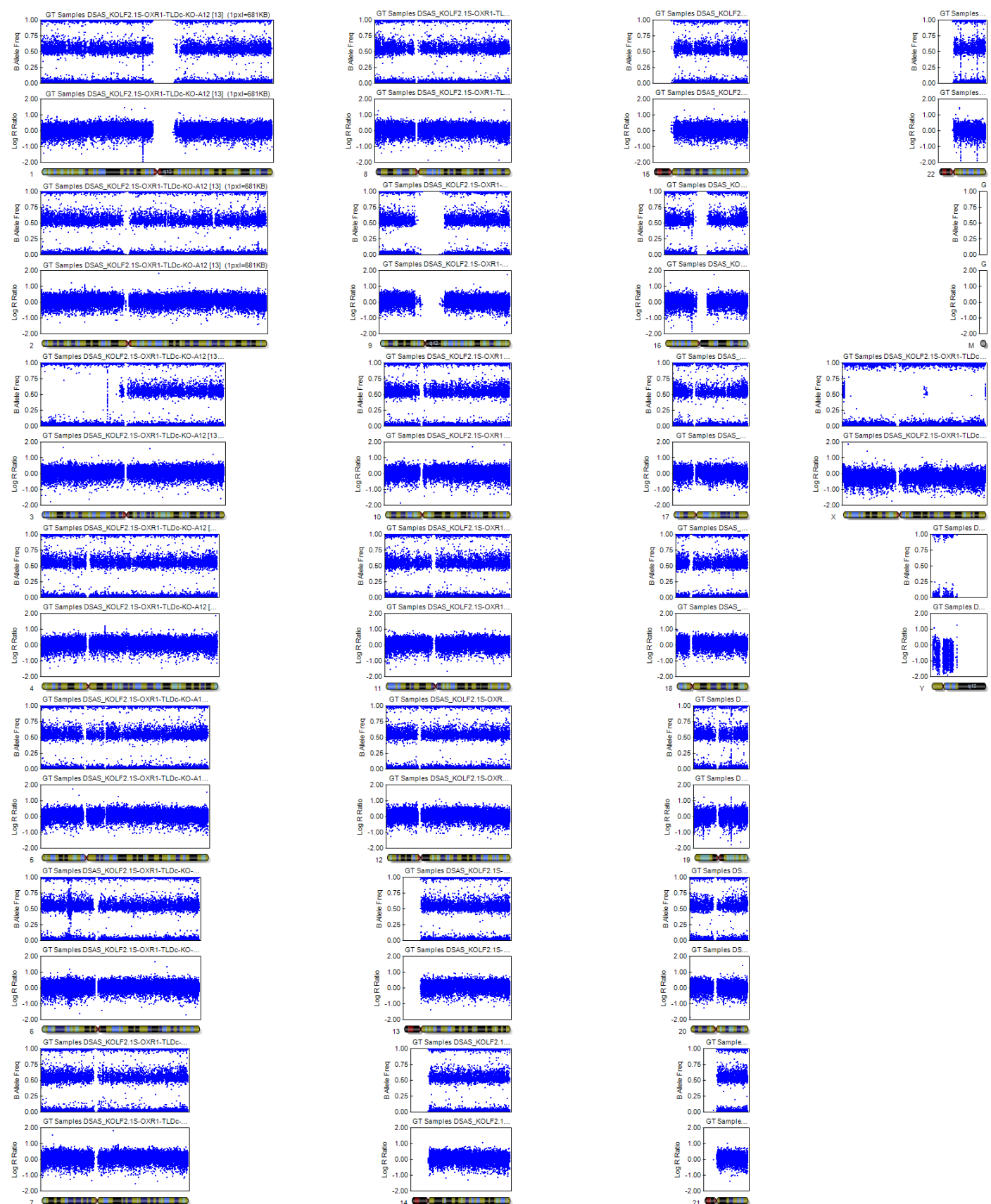
OXR1-SD-KO-A1



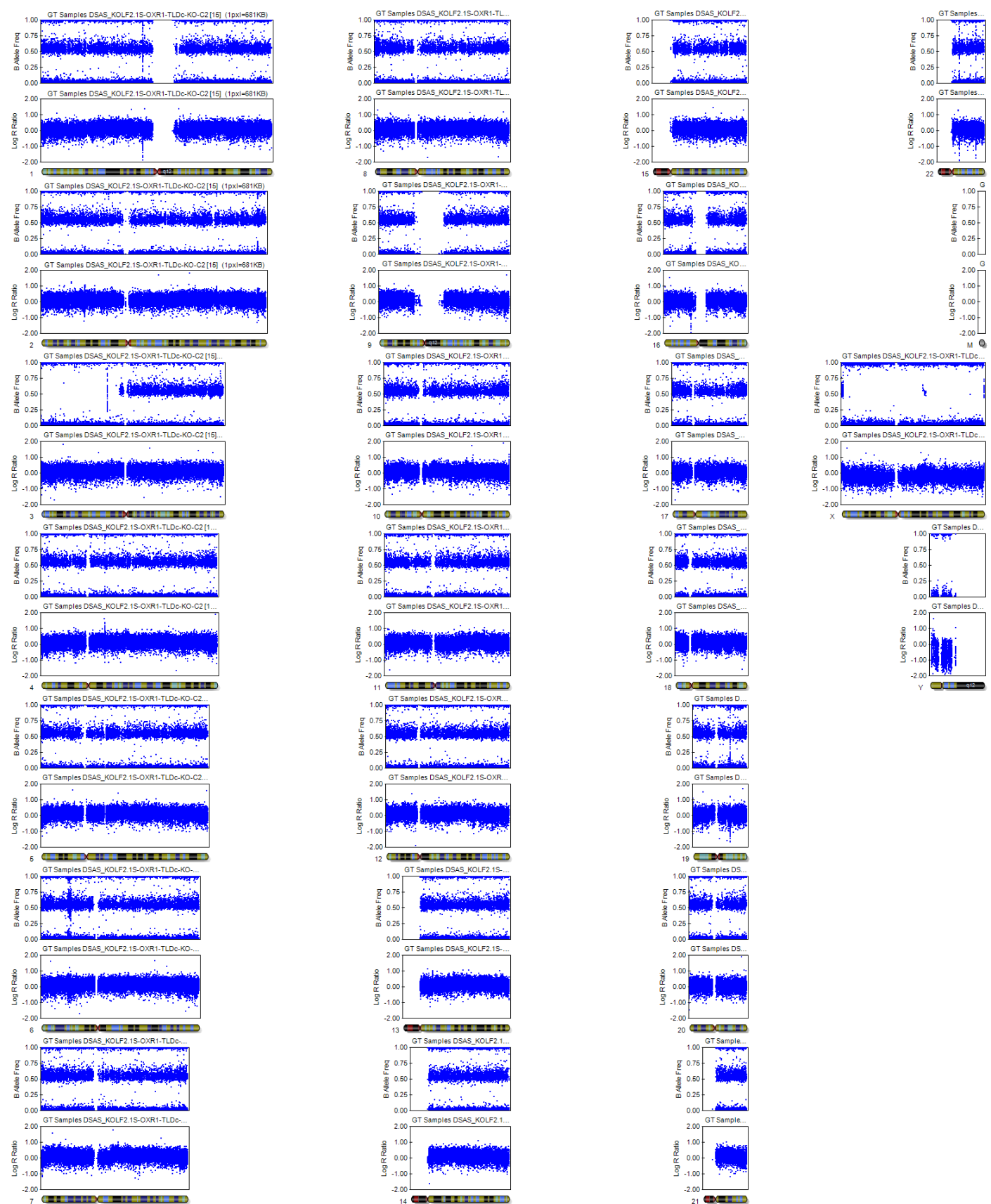
OXR1-SD-KO-A7



OXR1-TLDC-KO-A12



OXR1-TLDC-KO-C2



OXR1-TLDC-KO-D4

