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The Anaphylatoxin Receptors in Neural Progenitor Cell Physiology

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BSc (Hons)

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland
2015

School of Biomedical Sciences

Abstract

The complement cascade is phylogenically ancient pathogen recognition and response system that forms the initial frontier of mammalian innate immunity. Complement forms the bridge between humoural and cellular immunity through the generation of the anaphylatoxins, C3a and C5a, to recruit leukocytes to a pathogenic insult. However, in recent years, novel roles for the complement system outside of innate immunity have been demonstrated, especially in the developing embryo. To add to these novel roles, this thesis explores the role of the complement anaphylatoxin receptors in neural development.

Previous studies in our laboratory have indicated a novel role for C5aR1 in the prevention of neural tube defects in folate-deficient dams. In these studies, C5aR1 was localised to the neuroepithelium throughout the period of neural tube closure and the precursor to C5a, C5, was also shown to be present. It has previously been unclear, both in this study and others, what other components of the complement system are present in the developing embryo that may lead to the generation of anaphylatoxins. Here we demonstrate that the classical and alternative pathways, at the time of murine neural tube closure, lack expression of key propagators. Our data demonstrate the C2/4-independent and extrinsic pathways remain patent as a plausible means of C5a generation.

In order to trace C5aR1-expressing cells within the developing embryo, a transgenic mouse was created using a modified bacterial artificial chromosome approach. A *Cre*^{Ert2}*-IRES-EGFP* transgene was inserted at the second exon of *C5ar1* in order to induce expression from the C5aR1 promoter region. Resulting litters demonstrated transgene expression that was responsive to LPS stimulation and similar to previously reported C5aR1 expression within the CNS. However, crossing with a cre-reporter line demonstrated that the protein produced from the transgene was non-functional.

In separate studies, both C3aR and C5aR1 were shown to be expressed by neural progenitor cells from neural tube closure to birth, and both receptors localised to the apical attachment of progenitor cells. This thesis demonstrated C5a is a constituent of embryonic cerebrospinal fluid and that C5a-C5aR1 interaction activates aPKC to promote neural progenitor proliferation. Blockade of C5aR1 during development leads to behavioural abnormalities in the resulting litters and microstructural brain abnormalities on MRI. C3aR, conversely, promotes differentiation of neural progenitors.

The functions of C5aR1 and C3aR seem to be somewhat redundant during development as anaphylatoxin knockout animals are not known to exhibit any gross neurological abnormalities.

However, here we demonstrate subtle deficits in memory and anxiety in these animals – perhaps resulting from impaired neuroplasticity or development.

In summary, this thesis demonstrates the presence and function of the complement anaphylatoxin receptors in neural progenitor cells. As complement becomes a more viable target for the treatment of inflammatory disorders of pregnancy, this work provides a caution for clinicians by elucidating an important neurodevelopmental role for C3aR and C5aR1.

Declaration by Author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications During Candidature

Journal Articles

Coulthard LG*, Jeanes A*, Mantovani S, Markham K & Woodruff TM (2015) Co-ordinated expression of innate immune molecules during mouse neurulation *Molecular Immunology* **68** (2); 253-260
*Authors contributed equally to the work

Denny K, **Coulthard LG,** Mantovani S, Simmons DG, Taylor SM & Woodruff TM (2015) The role of C5a receptor signaling in endotoxin-induced miscarriage and preterm birth *American Journal of Reproductive Immunology* **74** (2); 148-155

Coulthard LG & Woodruff TM (2015) Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth *Journal of Immunology* **194** (8); 3542-3548

Hawksworth OA, **Coulthard LG,** Taylor SM, Wolvetang EJ & Woodruff TM (2014) Brief Report: Complement C5a promotes human embryonic stem cell pluripotency in the absence of FGF2 *Stem Cells* **32** (12); 3278-3284

Denny KJ, **Coulthard LG,** Jeanes A, Lisgo S, Simmons DG, Callaway LK, Wlodarczyk B, Finnell RH, Woodruff TM & Taylor SM (2013) C5a receptor signaling prevents folate deficiency-induced neural tube defects in mice *Journal of Immunology* **190** (7); 3493-3499

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Coulthard LG*, Li R*, Wu MC, Taylor SM & Woodruff TM (2012) C5L2; a controversial receptor of complement anaphylatoxin, C5a *FASEB Journal* **27** (3); 855-864
*Authors contributed equally to the work

Coulthard LG, Costello J, Robinson B, Shiels I, Taylor SM & Woodruff TM (2011) Comparative efficacy of a secretory phospholipase A_2 inhibitor in a rat model of antigen-induced arthirtis *Arthritis Research and Therapy* **13** (2); R42

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Coulthard LG, Hawksworth OA & Woodruff TM (2013) Complement peptide receptors: C5a receptor. *International Union of Basic and Clinical Pharmacology Database. http://www.iuphar-db.org/database/familymenuforward?familyid=5*

Hawksworth OA, **Coulthard LG** & Woodruff TM (2013) Complement peptide receptors: C3a receptor. *International Union of Basic and Clinical Pharmacology Database. http://www.iuphar-db.org/database/familymenuforward?familyid=5*

Conference Presentations

Coulthard LGJ, Li, R, Hawksworth OA, Kurniawan N, Sepehrband F, Jeanes A, Simmons DG & Woodruff TM (2015) Complement C5a Receptor Directs Neural Stem Cell Proliferation *Australian Society of Medical Research Postgraduate Student Conference, Brisbane, Australia*
*Second Prize – Best oral presentation

Coulthard LGJ, Li R, Hawksworth OA, Jeanes AJ, Simmons DG & Woodruff TM (2014) The Innate Immune Complement C5a Receptor Controls Neural Stem Cell Proliferation *University of Queensland Medical Society Research Conference, Brisbane, Australia*

*Winner – School of Medicine Dean's commendation for best oral presentation

Coulthard LGJ, Hawksworth O, Costantini K, Jeanes A, Taylor SM & Woodruff TM (2011) Complement in Development <u>Invited talk</u>, *Unversity of Otago Biomedical Science Symposium, Dunedin, New Zealand*

Coulthard LGJ, Markham K, Simmons D, Taylor SM & Woodruff TM (2011) Complement anaphylatoxin receptors C3aR and C5aR direct embryonic neural precursor differentiation <u>*Invited poster*</u> *University of Otago Biomedical Science Symposium, Dunedin, New Zealand*

Coulthard LGJ, Ryan S, Lee P, Taylor SM & Woodruff TM (2011) The anaphylatoxin receptors, C3aR and C5aR, direct embryonic neural precursor proliferation *Annual Meeting of the Australian Neuroscience Society, Auckland, New Zealand*

Coulthard LGJ, Costantini KJ, Lor M, Simmons D, Taylor SM & Woodruff TM (2010) Complement expression in mice throughout neural tube closure *XXIII International Complement Workshop, New York*

Coulthard LGJ, Costantini, K, Simmons, D, Finnell, RH, Woodruff, TM & Taylor, SM (2010) Complement factor 5a: A novel mediator of folate-deficient neural tube closure *Annual Meeting of the Australian Neuroscience Society and the 50th Anniversary Meeting of the Australian Physiological Society, Sydney*

Hawksworth OA, **Coulthard LGJ,** Taylor SM, Wolvetang EJ & Woodruff TM (2012) Expression of complement factors and functional C5a recetors in human embryonic stem cells and induced pluripotent stem cells *24th International Complement Workshop, Chania, Crete, Greece*

Denny KJ, **Coulthard LG**, Dekker Nitert M, Barrett HL, Foxcroft K, Woodruff TM, Taylor SM, and Callaway LK (2012) Maternal and Umbilical Cord Plasma C5a in Hypertensive Disorders of Pregnancy *21st Annual RBWH Health Care Symposium*, Brisbane, Australia

Denny KJ, **Coulthard LG**, Dekker Nitert M, Barrett HL, Foxcroft K, Woodruff TM, Taylor SM, and Callaway LK (2012) Maternal and Umbilical Cord Plasma C5a in Hypertensive Disorders of Pregnancy *School of Biomedical Sciences Research Colloquium*, Brisbane, Australia

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Costantini KJ, **Coulthard LGJ**, Woodruff TM, Callaway LK & Taylor SM (2010) The role of complement-mediated inflammation in miscarriage and preterm birth *XXIII International Complement Workshop, New York*

Publications Included in this Thesis

Chapter 1

Coulthard LG, Li R, Wu MCL, Taylor SM & Woodruff TM (2012) C5L2: A Controversial Receptor of Complement Anaphylatoxin, C5a. *FASEB Journal.* 27(3):855-864

Contributor	Statement of contribution
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Author RL	Wrote the paper (20%) Researched the paper (60%)
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Author TW	Edited the paper (5%)

Coulthard LG & Woodruff TM (2015) Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth. *Journal of Immunology* 194(8):3542-3548

Contributor	Statement of contribution
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Author TW	Wrote and edited the paper (10%)

Chapter 2

Coulthard LG*, Jeanes A, Mantovani S, Markham K & Woodruff TM (2015) Co-ordinated expression of innate immune molecules during mouse neurulation *Molecular Immunology* **68** (2); 253-260

Contributor	Statement of contribution
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Author AJ	Wrote and edited the paper (20%), Performed the experiments (40%)
Author SM	Performed the experiments (5%)
Author KM	Performed the experiments (5%)
Author TW	Edited the paper

Contributions By Others to this Thesis

In addition to the contributions detailed above, I would like to also acknowledge the contribution that A/Prof Trent Woodruff has made in guiding the preparation and production of this thesis. I would also like to extend my gratitude to the technical staff of SBMS and UQBR, specifically Dr. Shaun Walters, for his assistance with microscopy, and Trish Hitchcock for her assistance with animal work. In addition, I would also like to acknowledge the generous loan of equipment and mice from A/Prof Linda Richards and her laboratory staff.

Statement of Parts of this Thesis Submitted to Qualify for the Award of Another Degree

No portions of the present thesis have been used, in whole or in part, for the attainment of another qualification.

Acknowledgements

I am forever grateful for the opportunity to undertake a PhD. This has been a labyrinth of learning from the outset; an intense and, uniquely, both a satisfying and infuriating experience. I would do it all again, but maybe with a different approach – but hey, that means I have learnt something, *right?*

This PhD has taken 5½ years to complete. Two years of full-time study and the remainder part-time whilst concurrently completing an MBBS. In this time I have been aided and encouraged by a number of wonderful people, without which this thesis would never have eventuated.

Firstly, thanks to my primary supervisor, A/Prof. Trent Woodruff. I have benefited immensely from your trust and guidance, and from your treatment of me more as a peer, than a student. Your enthusiasm and encouragement in science is valued.

Dr. David Simmons has been my associate advisor throughout this period, and a great friend. Thank you for your always-open door and willingness to talk through any problems. Your integrity, both as a scientist and person, is an aspiration.

Prof. Stephen Taylor was an advisor, until his retirement in 2013. Steve, you got me into this – then escaped out the other side to grow epically hot chillies and brew high IBU beer. One day, I'll also get there! Thankyou, so much, for your support and advocacy, I'm lucky to have been one of your last students.

Thanks also to the wonderful post-docs in the Woodruff lab, Dr Angela Jeanes and Dr Susanna Mantovani. Thanks for your valuable technical assistance, willingness to listen and discussion of problems.

To those in the school of medicine who have sympathised with my position and generously allowed for me to take hospital rotations close to the laboratory, thank you. Specifically, thanks to A/Prof Diann Eley for the advocacy on behalf of medical students undertaking research. Thanks to A/Prof Mark Coulthard (of no relation) and Virginia Whittle who were particularly kind to me in the paediatrics rotation during which I was preparing this thesis.

To the students that have made this journey with me, and mostly left me behind whilst I've done the MBBS. I'm lucky to have had such wonderful companions within the lab throughout these last seven years. Peppermint and Alex, who began the honours year with me and continued through to their own PhDs. Owen and Patrick (for whom I supervised honours projects ...and yet we still remain my friends?); thankyou for time away from science, for greater persuits (particularly the premier league football highlights). Simon, thanks for being there, for a beer and a chat about lab work or otherwise. And to all past and present students of the Woodruff lab and wider SBMS community - thanks for making it exciting.

To my friends outside of science; sorry – I'll be back soon.

To my family; Mam, Dad, Hannah and Christian – thank you for your support. Thank you for the home-cooked dinners, for lending an ear, for being there. Thanks for the laughs and the distractions.

Finally, to Miranda, who bore the brunt of this PhD and who also contributed the emotional and personal sacrifice necessary in the preparation of a thesis. Thank you for your understanding, love, and kindness. Thank you for being there through everything. Thank you for your endless sense of fun and adventure. We'll get away soon xxx.

Keywords

complement, c5a, c5ar1, development, neural progenitor, neural stem cells, neurogenesis, brain development

Australian and New Zealand Standard Research Classifications (ANZSRC)

110707 Innate Immunity, 25%

110903 Central Nervous System, 50%

060103 Cell Development, Proliferation and Death, 25%

Fields of Research (FoR) Classification

1107 Immunology, 25%

1109 Neurosciences, 50%

0601 Biochemistry and Cell Biology, 25%

Spelling and Referencing in this Thesis

Spelling conforms to the standards set out in British English, with the exception of portions published by, or submitted to, journals that required American spelling. Additionally, references in this thesis are in the format of the journal *Nature*, for ease of reading and text flow. Exceptions occur in previously published portions, where references conform to the guidelines of the publishing journal.

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List of Abbreviations

AD	Alzheimer's disease
AD	Axial diffusivity
APS	Antiphospholipid syndrome
AR	Acrosomal reaction
Asp	Acetylating stimulating protein
BAC	Bacterial Artificial Chromosome
BM	Bone marrow
C	Complement
C5aR1	Complement factor 5a receptor 1
C5aR2	C5a receptor 2; C5L2; GPR77
CD	Cluster of differentiation
CFU-GM	Colony forming unit – granulocyte /macrophage progenitor
CNS	Central nervous system
CR	Complement receptor
cRNA	Complementary ribonucleic acid
CSF	Cerebrospinal fluid
DEPC	Diethylpyrocarbonate
EAE	Experimental autoimmune encephalitis
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ES	Embryonic stem (cell)
FA	Fractional anisotropy
FGF	Fibroblastic growth factor
FPR	Formyl peptide receptor
G-CSF	Granulocyte-colony stimulating factor
GFAP	Glial fibrillary acid protein
GLAST	Glutamate-aspartate transporter
GPCR	G-coupled protein receptor
HD	Huntington's disease
HMGB1	High mobility group box-1
HSPC	Haematopoetic stem/progenitor cell
ICH	Intracerebral haemorrhage
IRES	Internal ribosome entry site
IUGR	Intrauterine growth restriction
IVF	In vitro fertilisation
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPK	Mitogen associated protein kinase
MASP	Manosse associated serine protease
MCAO	Middle cerebral artery occlusion
MD	Mean diffusivity
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
NODDI	Neurite orientation and density diffusion imaging
PBMC	Peripheral blood mononuclear cell
PKC	Protein kinase C
PTX	Pertussis toxin
RAGE	Receptor for advanced glycation end-products
RD	Radial diffusivity
SCI	Spinal cord injury
SLE	Systemic lupus erytematosus
SP	Seminal plasma
SVZ	Subventricular zone
TLR	Toll-like receptor
UTR	Untranslated region
VD	Vascular dementia
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone

Chapter 1

Introduction to the Complement Anaphylatoxin Receptors and Neural Progenitor Physiology

1.1 Introduction

This chapter presents an overview of the topics relevant to the thesis. This chapter is structured as a series of papers, as the relevant topics have either previously been published, or are being prepared for publication. In addition, specific notes on the key features of this thesis, C5aR1 and embryonic neurogenesis, are covered in sections 1.2 and 1.3, respectively.

Section 1.4 outlines the state of knowledge on the complement system in development. It has a wide scope, from fertilization to birth, in order to provide a context for the research chapters of this thesis. Sections 1.5 and 1.6 represent previously published reviews relevant to this thesis. Section 1.5 is a review published in *FASEB Journal* on the roles and signaling of the secondary C5a receptor (C5aR2, C5L2). Although C5aR2 is not a focus of this thesis, an understanding of its signaling and actions in disease models is essential as it shares the C5a binding capacity of C5aR1. Section 1.6 is a review of C3a published in *The Journal of Immunology*. This review highlights some of the emerging studies that are prompting a relook at the textbook definitions of the roles of complement proteins. The importance of this section lies in the story of the entrenchment of the canonical roles of complement, that of a potent pro-inflammatory mediator, despite evidence of complex, multifaceted actions in various situations. This is pertinent as this thesis presents a novel role for the complement system in the building of the embryonic brain, far from its accepted (and destructive) roles in the immune system.

In addition, Appendix A contains literature review covering the roles of C3aR in health and disease, prepared for submission in the near future. This section provides background and further reading for the research chapters examining aspects of C3aR physiology.

The introduction is structured in such a way to address the separate, but important, facets of developmental biology and immunology that contribute to the understanding of the proceeding chapters. The complement system has been well studied for its immunological roles over the past century, however the previous decade has demonstrated novel and unexpected roles in developmental biology. An understanding of both roles is essential to form a global appreciation of anaphylatoxin receptor impact on the fetomaternal environment.

1.2 A Note on C5aR1

C5aR1 is an evolutionarily-ancient receptor that is highly conserved throughout multiple species[1,2]. It has a well-defined role in inflammatory disease as a mediator of leukocyte chemotaxis and degranulation[3]. In this section the genetics, structure and ligands of the receptor are discussed in order to provide context to the proceeding chapters of the thesis.

Genetics

The genetic locus for C5aR1 is located on chromosome 7 in the mouse (chromosome 19 in humans) and consists of two exons separated by a large 10kb intron. The first exon, the shorter of the two, contains the 131bp 5' untranslated region (UTR) and the start codon (ATG) of the C5aR1 sequence. Exon 2 contains the remaining coding sequence and a large, 1.5kb 3' UTR region.

The important promotor regions of *C5ar1* occur within 500bp of exon 1, and this region is notable for containing a CCAAT enhancer box sequence (C/EB), AP-4 and NFκB binding sites[4]. The C/EB region is the recognition site for transcriptional activity in cells of the myeloid lineage, such as macrophages and microglia. In the human genome these regions have been demonstrated to be conserved and important for expression of human C5aR1[5]. However, the wider promotor region (~2kb 5' to exon 1) of C5aR1 is not responsible for the upregulation of C5aR1 with differentiation of the U937 monocyte cell line, suggesting more complex regulation of the gene is still yet to be discovered[5]. Additionally, the expression of C5aR in non-myeloid lineages is dependent on other motifs within the C5aR1 promotor. Astrocytic *c5ar1* expression has been shown to be independent of the C/EB motif used for myeloid expression, and instead employs regulatory elements either upstream of this site, or perhaps unknown elements within the large intronic sequence[6]. Highlighting this lineage-specific difference, the NFκB site within the C5aR1 promotor is a suppressor site in astroglia, but not microglia[6]. Whilst both cell types are located within the same tissue and perform a supportive role in brain function, microglia are of the myeloid lineage, whereas astroglia are of the neuroectodermal lineage.

There is a paucity of reports within the available literature on the functions of the intron and 3' UTR in C5aR1 expression and function. The length and phylogenic conservation of the intronic sequence has led to suggestions that it may be involved in the transcriptional regulation of the gene[7]. Interestingly, a recent knockin mouse generated to insert an IRES-EGFP sequence after the second exon of C5ar1 led to increased protein production and impaired trafficking[8]. In the homozygous knockin animal, C5aR1 protein was confined to the intracellular compartment, and was not

detectable within the cellular membrane. It was suggested that the disruption of the 3' UTR caused the dysregulated production and impaired trafficking of the protein product, although this was not demonstrated within the study[8].

Expression

Originally thought to be restricted to cells of the myeloid lineage, C5aR1 is expressed throughout a wide variety of tissues and cell types [9]. However, notably, it is absent from cells of the lymphoid lineage. There has been recent debate as to the status of C5aR1 expression in this lineage, which appears to have been settled in the negative through the recent development of a C5aR1-EGFP reporter mouse[8]. Expression of C5aR1 also occurs within the CNS, which forms the heart of this thesis. Both cells of the neuroectodermal lineage (neurons, astrocytes, oligodendrocytes) and microglia express C5aR1 at detectable levels[10,11]. In the adult brain, neuronal expression of C5aR1 is restricted to specific areas, namely the pyramidal neurons of the neocortex, neurons of the dentate gyrus and Purkinje cells of the cerebellum[11]. Additionally, neural progenitor cells of both the embryo and the adult mouse have also been demonstrated to express C5aR1[12,13]. In particular, C5aR1 expression at the point of neural tube closure within the neuroepithelium has been demonstrated by our laboratory and, here, the receptor is localised to the apical surface of the cell[12]. Additionally, we have also demonstrated C5aR1 expression and function on embryonic stem cells, which is to our knowledge the earliest expression in development[14].

Structure and signalling

C5aR1 is a 7-transmembrane G-protein coupled receptor and is grouped conceptually with the other anaphylatoxin receptors, C3aR1 and C5aR2 (C5L2, GPR77) (reviewed in 1.5/Appendix A and 1.6, respectively). The extracellular N-terminus of C5aR1 and the three extracellular loops participate in the binding of C5a[3]. C5aR1 demonstrates some promiscuity, both in its G-protein coupling, and recruitment of downstream effector pathways. Again, these interactions appear to be dependent on cell-line and state, for instance, in the human monocyte line U937 and macrophages, *in vivo,* C5aR1 has previously been shown to couple to the pertussis toxin (PTX) sensitive G-protein $G_{\alpha i2}$, however in the MONO-MAC-6 human monocyte line C5aR1 signaling is PTX-insensitive[15-17].

The picture become more complex as the focus moves toward second messenger signals, where contradictory results exist depending on the cell line of interest. In macrophages, C5aR1 signals via protein kinase C (PKC) δ to induce NFκB activation and translocation to the nucleus, whereas in neutrophils C5aR induces activation of IκB, the NFκB inhibitor[18]. In mesenchymal stem cells C5a induces chemotaxis that is pertussis toxin sensitive, indicating a G_i mediated response, and

signalling via MAPK and Akt pathways[19]. Additionally, other G-protein independent signaling processes have been shown for C5aR. Yeast-2-hybrid studies have revealed that C5aR1 has also been shown to associate with Wiskott-Aldrich Syndrome Protein (WASP), to directly participate in cell directional migration in neutrophils[20] and C5aR1 has been implicated in angiogenesis as stimulation via C5a causes transactivation of the epidermal growth factor (EGF) receptor[21].

Agonists and Antagonists

Studies into C5aR1 have utilised pharmacological tools to elucidate function from experiments. Our laboratory contributed to the development of the most widely used small molecule C5aR1 antagonist, PMX53[22]. In characterising the antagonist activity of PMX53 it was found to have high affinity and activity at the human, rat and dog C5aR1, but low affinity and activity at the mouse C5aR1 *in vitro*[23]. This is supported by our experience with the antagonist on multiple mouse cell types in the *in vitro* environment. However, *in vivo* use of the antagonist appears to be without the limitations of the *in vitro* work, as often the use of PMX53 recapitulates the data generated in knockout studies[12]. This perhaps suggests that some biological modification of the molecule is required for adequate receptor binding.

The structure of PMX53 has since been used to develop several other C5aR antagonists with favourable properties. For instance, PMX205 has greater lipophilicity and therefore is more suited for use in studies that require passage of the drug through the blood-brain barrier, whilst retaining similar efficacy (pIC_{50} $3.1x10^{-8}$ M vs. $9.0x10^{-8}$ M for PMX53)[24]. A linearised version of PMX53, JPE1375, has also been developed and demonstrates potent *in vitro* activity on mouse C5aR[25]. Staphylococcus aureus express CHIPS (chemotaxis inhibitor protein of S. aureus), which is the only described biologically derived inhibitor of C5aR1. This protein likely plays a role in bacterial evasion of immune surveillance during pathogen colonisation[26,27]. PMX53 and JPE1375 are the frequently used antagonists in this thesis, however a comprehensive list of receptor antagonists can be found in table 1.2.1.

Table 1.2.1; Antagonists of C5aR1

Name	Type	Action	Affinity	Reference
CHIPS	Bacterial-derived biologic	C5aR1 antagonist, FPR antagonist	K_d = 1.1nM (human)	27
W54011	Synthetic organic	C5aR1 antagonist	K_i = 2.2nM (human) Reportedly similar activity for gerbil and monkey.	28
NDT9520492	Synthetic organic	C5aR1 antagonist	K_i = 4.4nM (human) K_i = 13.2nM (Gerbil) K_i = >10,000nM (mouse)	29
AcPhe-Orn-Pro-D-Cha-Try-Arg	Synthetic organic	C5aR1 antagonist	IC_{50} = 12nM (human)	30
PMX205	Synthetic organic Cyclic molecule modelled from tail of C5a	C5aR1 antagonist	IC_{50} = 31nM (human) Reported activity at mouse C5aR	24
PMX53	Synthetic organic Cyclic molecule modelled from tail of C5a	C5aR1 antagonist	IC_{50} = 21nM (human) Reported activity at mouse C5aR1 *in vivo*	23, 24
JPE1375	Synthetic organic Modelled from PMX53 structure	C5aR1 antagonist	IC_{50} = 111nM (human) Reported activity on mouse C5aR1	25
C089	Synthetic organic	C5aR1 antagonist	IC_{50} = 100nM (human)	31
L-156,602	Streptomyces sp. isolate	C5aR1 antagonist	IC_{50} = 2000nM (human)	32
$A8^{\Delta 71-73}$	Synthetic organic Modelled from tail of C5a	C5aR1 antagonist, C5aR2 antagonist	IC_{50} = 27nM (human) Reported activity on mouse C5aR1/C5aR2	33

There are fewer synthetic agonists of C5aR1 and, none that are highly selective for C5aR1 and which exhibit the potency of endogenous C5a. C5a is available commercially in a recombinant form produced in bacteria, or isolated from donor serum. The major difference between these two forms is the glycosylation of endogenous C5a that may contribute to increased affinity for C5aR1. Experiments in our laboratory have shown that isolated human C5a is ~3 fold more potent than the recombinant form for inducing calcium mobilisation in isolated peripheral polymorphonuclear leukocytes (Owen Hawksworth, *personal communication*). Serum carboxypeptidases metabolise C5a to the less potent $C5a_{desArg}$, which retains some affinity for C5aR1, but has greater affinity for the second C5a receptor C5aR2.

Other biological agonists of C5aR1 that have been identified include dimerised ribosomal protein S19, a cell death signal released during membrane breakdown, and the bacterial chaperone protein Skp[34-36]. Synthetic agonists are not widely used in the literature as they have been reported to have off-target effects on the other anaphylatoxin receptors. Perhaps the best studied is the synthetic peptide YSFKPMPLaR (EP54), which has EC_{50} values in the micromolar range for both C5aR1 and C3aR1 in multiple species[23,37]. The available agonists for studies into C5aR1 are detailed in table 1.2.2. Due to the lack of selective and potent C5aR1 agonists, this thesis utilised bacterial recombinant mouse C5a for all *in vivo* and *in vitro* work.

Table 1.2.2; Agonists of C5aR1

Name	Type	Action	Affinity	Reference
C5a	Endogenous ligand	C5aR1 agonist, C5aR2 agonist	EC_{50} = 8nM (human)	38
$C5a_{desArg}$	Metabolite of C5a	C5aR1 agonist, C5aR2 agonist	EC_{50} = 21nM (human)	38
YSFKPMPLaR	Synthetic peptide	C5aR1 agonist C3aR1 agonist	EC_{50} = 3240nM (human) EC_{50} = 600nM (mouse) EC_{50} = 50nM (rat) Also active in dog, sheep, rabbit, guinea pig and pig	23
Skp	Bacterial protein	C5aR1 agonist	Not calculated	36

N-methyl-Phe-Lys-Pro-D-Cha-Cha-D-Arg-CO_2H	Synthetic peptide	C5aR1 agonist	IC_{50} = 25nM (human) Derived from competitive binding assay	31

Conclusion

In summary, C5aR1 is a highly conserved and widely expressed G-protein coupled receptor that exhibits multiple signalling and expression patterns dependent on cell type and tissue location. This thesis utilises the well-characterised specific antagonists, PMX53 and JPE1375, to determine function in the context of development.

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1.3 A Note on Neural Development

Neural development requires the generation of neurons and astrocytes from a self-renewing population of progenitor cells. This population of cells borders the ventricular system in the developing mammalian embryo and is derived from induced ectodermal tissue overlying the notochord early in development. The structure and composition of the progenitor population changes as development proceeds. The present section presents a broad overview of this cell population to provide background for the role of the anaphylatoxin receptors on neural progenitors outlined later in this thesis.

Anatomy of the developing brain

The vertebral central nervous system has its embryological origins after gastrulation. Signals from the notochord induce the formation of a neural plate on the dorsal surface of the embryo. The neural plate then undergoes morphological changes to form the neural tube, through apical constriction the neural plate elevates laterally whilst invaginating centrally and allowing the lateral edges to meet[1]. At this stage there is also neural crest cell migration from the lateral edges, which contribute to the formation of craniofacial structures, cardiac, endocrine and peripheral nervous system tissues[2].

The neural tube is generally closed by 10dpc (Theiler stage 15) in the mouse embryo[3]. This event marks the beginning of neurogenesis and differentiation of the brain and spinal cord. The neural tube is subdivided into aneurysmal areas termed (rostral to caudal) the prosencephalon, mesencephalon and rhombencephalon (figure 1.3.1). The prosencephalon gives rise to the development of the telencephalon (forebrain) and diencephalon (thalamic tissues). The mesencephalon is the precursor to the midbrain, whist the rhombencephalon further divides into the metencephalon (pons and cerebellum) and the myelencephalon (medulla oblongata). Each of these areas is evident by gross observation at 10.5dpc. This thesis focuses on the progenitors of the dorsal telencephalon, which give rise to the cerebral cortex.

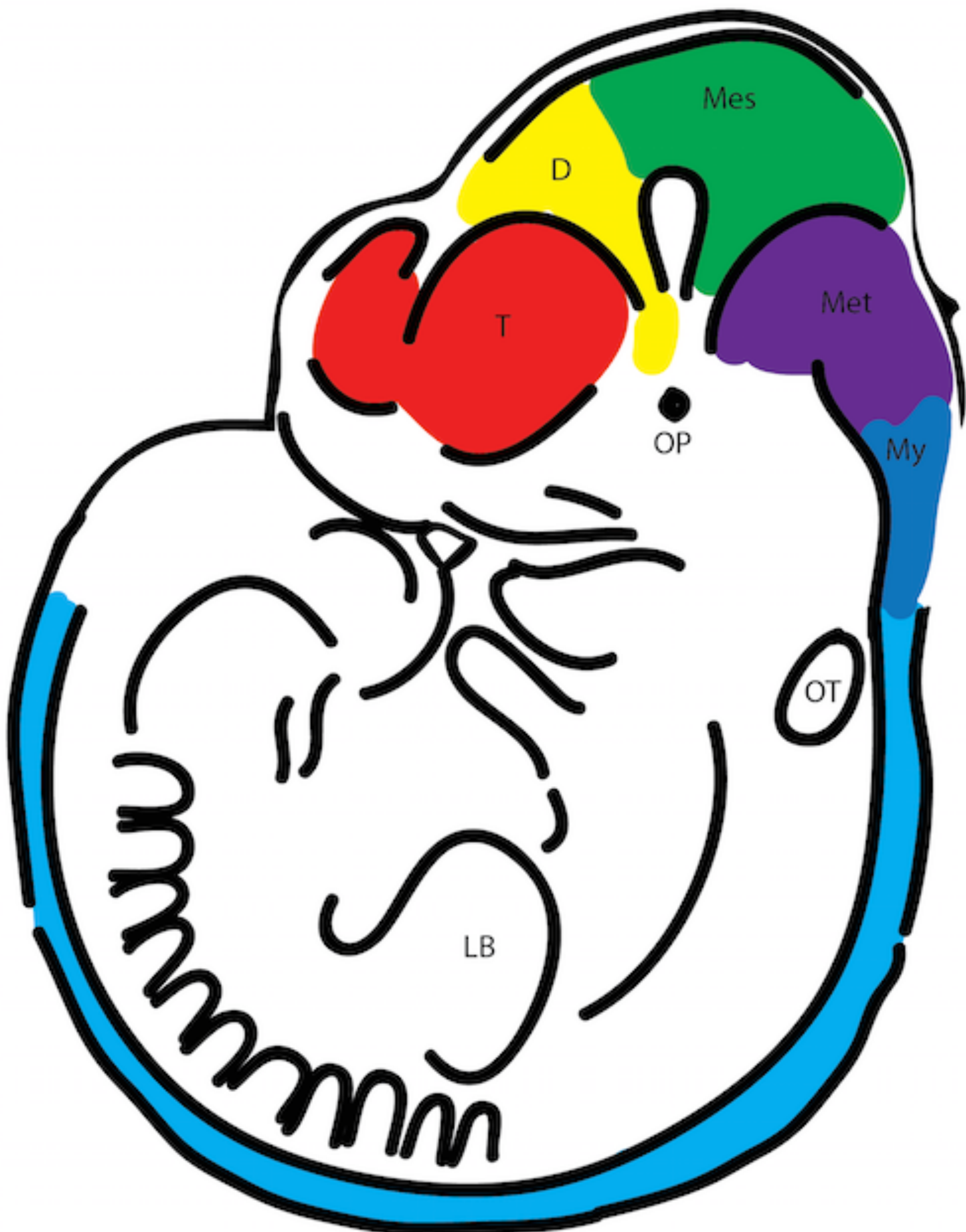

Figure 1.3.1: The anatomical regions of the developing brain. *Stylised mouse embryo at 10.5dpc (Theiler stage 17), Colours represent the different regions of the developing brain. The procencephalon comprises the telencephalic vesicles (T, red) and the diencephalon (D, yellow). The mesencephalon (Mes) is outlined in green. The rhombencephlon comprises the metencephalon (Met, purple) and the myelencephalon (My, blue). Additional features are also labeled; OP, optic vesicles; OT, otic vesicles; LB, limb bud.*

Structure of the cerebral neocortex

The cerebral neocortex comprises six distinct neuronal layers that are numbered from the pial surface to the ventricular zone (VZ). The VZ of the dorsal telencephalon in rodents contributes to the pyramidal neuron populations of these layers, whereas interneurons are a product of neuroblast migration from the ventral telencephalon[4]. The histogenesis of the cerebral cortex begins post-neural tube closure, with the earliest born neurons forming a preplate above the VZ during 11-12dpc[5]. There follows the development of a subventricular zone (SVZ), comprised of basal progenitor cells, between the newly formed preplate and the progenitors of the VZ. From 14dpc to birth the VZ population reduces in number as neurogenic divisions exceed proliferative divisions, migrating neuroblasts split the preplate into a basal marginal zone and a apical subplate, located above the SVZ. This population of newly born neurons is termed the cortical plate and forms the majority of layers within the mature cerebral cortex.

Timing of neuronal birth determines the position of a neuron in the mature cortex. The cells of layer VI are the first to migrate from the ventricular zone and later born neurons form layers V to I sequentially[6]. The specific location and projections of newly born neurons is thought to be guided by the differential expression of adhesion molecules by the neuroblast on leaving the VZ, rather than as a product of the layer environment[7,8]. Hence, the timing of neuronal birth determines both the position and fate of the neuron within the mature neocortex.

Neural progenitor cells and neurogenesis

Neural progenitors in the developing rodent brain comprise two major groups, those with an attachment at the ventricular surface, termed apical progenitors, and those appearing later, undergoing mitosis within the SVZ, termed basal progenitors.

The apical progenitors are of two types. The neuroepithelial cells form the early neural structures of the embryo and give rise to later progenitors and neurons. These cells exhibit epithelial characteristics, such as the presence of apical tight junctions[9]. By 13.5dpc, the apical progenitors consist solely of radial glia, so named due to their astroglial structural and molecular properties; they can be easily labeled through their expression of GLAST and GFAP[9]. Both types of apical progenitors demonstrate nuclear interkinetic migration, whilst maintaining both an apical and basal attachment, the nucleus undergoes S-phase at the basal surface and M-phase apically[10]. Whereas neuroepithelial cells extend processes to the apical surface and the basal lamina of the ventricular zone, radial glia maintain their basal attachment at the pial surface. This extension towards the pial

surface acts as a scaffold for the migrating neuroblast to follow, and allows for the lamination of the neocortex[10].

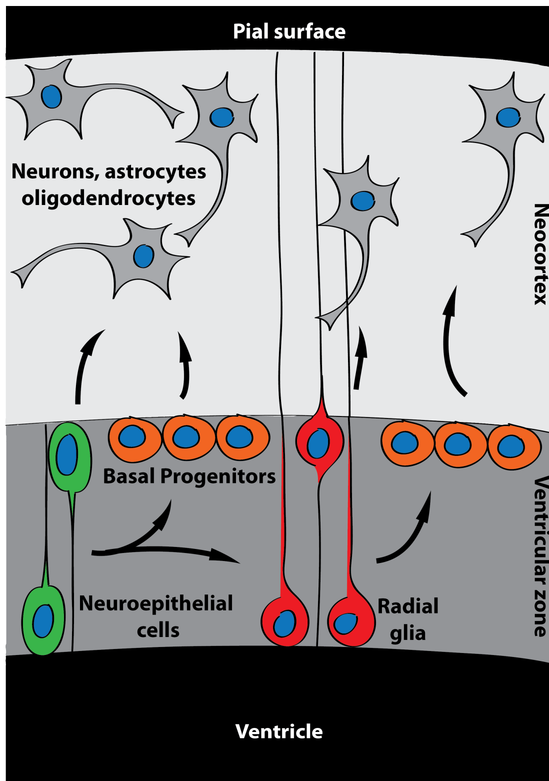

Figure 1.3.2; Cell types of embryonic neurogenesis. *Stylised diagram of the embryonic neocortex. The apical progenitors of the ventricular zone (neuroepithelial cells, radial glia) undergo interkinetic nuclear migration and divide at the apical surface. Neuroepithelial cells are the least lineage restricted cell type and can give rise to radial glia, basal progenitors or post-mitotic cell types. Radial glia are the most abundant progenitor in the mouse and extend their basal process to the pial surface. This process aids in neuroblast migration. The basal progenitors responsible for the majority of newly born neurons in the murine embryo and are located within the subventricular zone. Each of these progenitor types has the potential to generate the cells of the neuroectodermal lineage; neurons, oligodendrocytes and astroglia.*

Division within the apical progenitor population defines the fates of the daughter cells. Apical progenitors undergo three types of division; symmetric proliferative, asymmetric and symmetric neurogenic division. The two former types of division are well recognized and the inheritance of the apical membrane attachment by both (symmetric) or one (asymmetric) of the daughter cells specifies which remains as a progenitor cell[9,11]. In asymmetric division the loss of the apical attachment by one daughter cell leads to differentiation and migration of the cell into the SVZ or cortical plate. Recently, a novel type of symmetric division was identified whereby the apical attachment is expulsed into the embryonic ventricle and neither daughter cell remains as a progenitor, this has been termed symmetric neurogenic division[12,13]. It is suggested that these divisions account for ~20% of the symmetrically dividing cells, but this figure is dependent on embryonic age as division of this type are not observed in the early neuroepithelium[13].

The factors underlying the type of division an apical progenitor cell undergoes are varied and it has been difficult to experimentally differentiate cause and effect. It is suggested that signals from the cortical plate determine the ultimate productive capacity of the cells within the VZ, however how this occurs is still not entirely clear[6]. There is experimental and observational evidence that the

length of the cell cycle is the determining factor for whether a progenitor undergoes symmetric or asymmetric division. Specifically, the length of G_1 phase has been shown to positively correlate with neurogenic division. Ectopic expression of the cylin dependent kinase 1/cyclin D1 complex in neural progenitor cells prevents results in a shortened G_1 phase and a delay in neurogenesis, both *in vitro* and *in vivo*[14]. Additionally, the neurogenesis marker gene, Tis21, causes an increased G_1 phase when transfected into progenitors and results in premature neurogenesis[15-17].

Interestingly, whist inheritance of the apical attachment itself appears to be a major contributor to the fate of the daughter cells, the proximity of the nucleus to signals arising from this attachment may also be a determinant of cell fate. The apical attachment contains a cluster of pro-proliferative, pro-polarising factors, such as the Par2/Par6/aPKC complex, that are clustered around the primary cilium[18]. It has previously been noted that the daughter cell nuclei fated to remain as a progenitor remains at the apical surface for a relatively longer period than its other-fated sister[16,19]. Hence, it has been suggested that this extended time allows for increased signaling to the nuclei from factors on the apical surface that promote maintenance of a progenitor state[9].

What remains an unknown however, are the factors that push an apical progenitor cells towards symmetric or asymmetric division. The mechanics of mitosis within progenitor cells suggest that the orientation of the mitotic spindle will dictate whether the apical attachment is transected during division. Interestingly, at the point of mitosis there is down-regulation of the polarity promoting moieties within the cell membrane, perhaps promoting a variability of orientation that results in both types of division[20]. Loss of mitotic spindle orientation in mice results in a microcephalic phenotype through a premature reduction in the progenitor pool[21]. Indeed, genes that control the orientation of the mitotic spindle at mitosis have been implicated, evolutionarily, in allowing for the cerebral expansion evident in mammalian, and particularly primate, brains[21,22].

At the point of differentiation, most apical progenitors give rise to basal progenitor cells. Basal progenitors lack a distinct polarity and remain within the subventricular zone throughout the cell cycle, in contrast to the animated migration of apical progenitors through the pseudostratified ventricular zone[23]. This cell population is the major contributor to the neurons of the mammalian cortex, as over 90% of neurons are derived from basal progenitors despite the ability of apical progenitor cells to contribute directly[24]. Basal progenitors are commonly demarked by their expression of the transcription factor, Tbr2, which gives way to expression of Tbr1 with the onset of differentiation[25].

In the context of brain evolution, the basal progenitor cells of the subventricular zone appear to be the major embryonic contributors to cortical size. In the mouse, the basal progenitor cell population is relatively modest, accounting for 25-30% of mitotic cells in the developing brain. In humans and other primates, the basal progenitor population of the subventricular zone becomes the predominant progenitor region of the brain, with 80-90% of mitotic cells located in this area[10]. This observation has been suggested to account for the difference in brain size and complexity between rodents and primates, as an expansive basal progenitor population allows for greater neuron generation. Additionally, the basal progenitor population of mice is also a more homogenous cell population than that of the primate, which is composed of multiple subtypes of basal progenitor. For instance, the recognized outer subventricular zone progenitors (basal radial glia) of the primate brain have only recently been shown to occur in mouse development[26]. However, these account for only 3-5% of the basal mitoses in the mouse, whereas in the developing human cortex this region dwarfs both the VZ and SVZ and is the major contributor to neurogenesis[27].

Conclusion

In conclusion, embryonic brain development relies upon the generation of the cortical layers from a distinct and self-renewing population of progenitor cells at the ventricular surface. The factors that govern the proliferation and differentiation of the progenitor population are not well defined in the literature, but observational and experimental studies demonstrate that the cell cycle, polarity and fate of the progenitor cell are intricately linked.

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1.4 Complement In Development

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In preparation for submission as a review article.

Introduction

The complement system has long been associated with innate immunity, and the names and functions of its components have reflected this historical catagorisation[1,2]. However, despite entrenched historical bias towards complement's role in innate immunity, in recent decades, new roles have emerged for this group of proteins in the scope of development. These roles extend from the gamete to post-natal stage and, in instances, are somewhat detached from the traditional role for complement as an innate sensor for pathogens. Given the emergence of these novel roles, and the focus of this thesis on the anaphylatoxin receptors in neural development, this section aims to provide context by describing the wider importance of the complement system in the process of reproduction.

From Gametes to Fertilisation

Complement, both in its physiological roles, and in the pathogen response, is a delicate balance[1,3,4]. Fertilisation is no exception. Complement factors are present on both the sperm and oocyte, as well as secreted into the female reproductive tract by the epithelial lining. Within the female reproductive tract, complement acts as the first line of defence against ascending or introduced pathogens[5]. Both spermatozoa and oocytes express regulators of the complement system. Oocytes express CD59, CD46, CR1 and CR3 on the membrane, whilst CD55 is expressed on the zona pellucida[6,7]. However, expression of these regulators may be dependent on age and status of the oocyte[7].

C3 expression within the female reproductive tract and ovarian follicle is under hormonal control and can be modulated by exogenous administration of oestrogen or progesterone. C3 expression is stimulated by oestrogen and the effect of oestrogen is inhibited by the presence of progesterone[8,9]. Additionally, other important members of the complement system have been demonstrated to be present in the glandular epithelium, in a similar temporal expression pattern to C3, perhaps suggesting similar hormonal control. CD55 and factor B demonstrate a similar expression pattern to C3, whereas CR1 is under similar hormonal control, but localizes to the stroma[10].

The expression of complement factors in the female reproductive tract is not unexpected given the potential for exposure to pathogens. As previously mentioned, an active complement system in the reproductive tract may also aid in eliminating dysfunctional spermatozoa, as healthy spermatozoa express regulators to prevent MAC formation[11]. A third, and perhaps most interesting, role for complement in fertilization is to mediate the interaction between spermatozoa and the oocyte. The presence of iC3b in follicular fluid increases the rate of oocyte maturation[12], whilst this discovery has origins in the investigation of IVF, it is interesting to note the increase in C3 expression at estrus.

In the male reproductive tract, complement cascade proteins, such as C3 and C4, are present in the seminal plasma (SP) ejaculate that accompanies spermatozoa[13]. Overall, SP has been shown to have an inhibitory effect on complement activation[14] and contains specific 'prostasome' complexes that concentrate complement regulators CD55, CD59, and CD46. It is hypothesised that the role of prostasomes is to provide an immediate anti-complement defence for spermatozoa deposited into the female reproductive tract[15]. Upon migration through the female tract, the sperm will leave the SP along with the prostasomes, leaving a requirement for the sperm to possess their own

complement regulators. For this, a number of complement proteins have been identified on mammalian spermatozoa, including CD55, CD59, CD46, C1-INH, C1qR. These proteins have been implicated in their canonical functions, protecting sperm from complement mediated attack; however, their expression is unique, with isoforms specific to the testes reported to be involved in facilitating fertilisation via effects on sperm motility, activation, and sperm interactions with the egg.

After migration from the relative safety of the SP, spermatozoa have to run the gauntlet of the female reproductive tract for successful fertilization to occur and, accordingly, express complement regulatory proteins to prevent complement-mediated lysis. CD55 and CD59a are expressed on the plasma membranes of spermatozoa, whilst CD46 is strongly localized to the inner acrosomal membrane[16]. CD59a and CD55 expression provide a functional defense against MAC-mediated lysis[11]. Additionally, a complement 1 inhibitor-like protein (C1-INH) has been shown to protect spermatozoa from complement mediated lysis, and the dysfunction of this factor is linked to male infertility[17]. In addition, the presence of autoantibodies against C1-INH in human populations is also associated with male infertility[18]. Complement expression in spermatozoa may also be modulated through epigenetic mechanisms as, in folate deficient conditions, *C3ar1* demonstrates altered methylation[19]. However, it is unclear whether other factors in the complement system are under similar epigenetic control.

Components of the complement cascade are found in the follicular fluid of women undergoing ovarian stimulation for *in vitro* fertilization. There is evidence of independent regulation of complement in the follicular fluid when compared to serum, with tighter regulation of complement activation in follicular fluid[20]. Complement factors are expressed by the granulosa cells of the ovarian follicle for secretion into the follicular fluid. However, there is notable absence of the central complement factor, C5, perhaps indicating that MAC formation is not the central aim of expression in this environment[21].

Despite the role of complement as a spermicidal factor in the migration of sperm through the female reproductive tract, the interaction between spermatozoa and oocyte is enhanced by the presence of complement factors on the interacting gametes. Interestingly, whilst CD46 is abundant on the membranes of somatic cells, it is present only on the inner acrosomal membrane of spermatozoa, and hence, only available for interaction with the surrounding environment after the acrosomal reaction[6,16]. Additionally, oocytes express CR1/CR3 on the cellular membrane, and both AR-

spermatozoa and zona-free oocytes bind iC3b strongly *in vitro*[6]. Hence, it has been suggested that iC3b may act as a linkage factor allowing membrane opposition during fertilization (figure 1.4.1).

The importance of CD46-iC3b linkage in fertilization is highlighted by the observation of increased fertilization success in cultures supplemented with additional iC3b. Generation of iC3b at the local level may be achieved through the release of acrosin as a consequence of the acrosomal reaction, as acrosin has been demonstrated to have complement-lytic activity *in vitro*[6]. The C3 fragment, C3b, can then deposit on the inner acrosomal membrane leading to increased adherence of sperm and penetration of hamster oocytes[22]. Accordingly, abnormalities in CD46 have also been associated with human infertility[23]. Interestingly, however, the lack of C3 in *C3* knockout mice has not resulted in sterile animals, suggesting that the CD46-iC3b-CR interaction has some redundancy in the fertilization process or a surrogate linking factor may also assume this role.

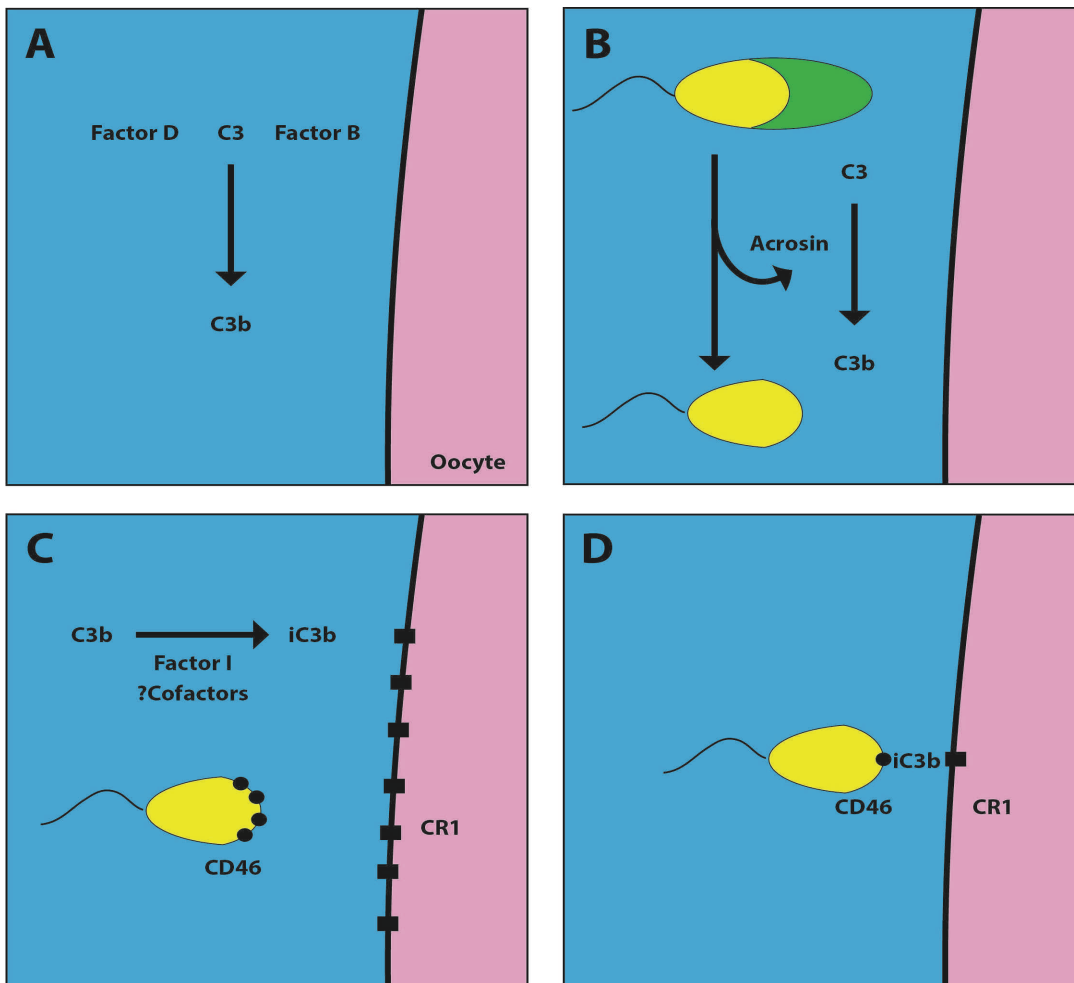

Figure 1.4.1; Diagrammatic representation of complement involvement in the sperm-oocyte interaction. A) Factor B, factor D and C3 are all secreted by oviductal cells, which allows for alternative pathway activation and generation of C3b. B) Acrosin released by the spermatozoa during the acrosomal reaction can cleave C3 to C3b. C) Factor I is present in the oviductal fluid, but may require cofactors for generation of iC3b. The inner acrosomal membrane of the spermatozoa expresses CD46. The oocyte demonstrates expression of complement receptor 1 (CR1). D) Binding of iC3b to CD46 of the inner acrosomal membrane and CR1 of the oocyte allows for opposition of the gamete membranes, and successful fertilization.

It is unclear whether the literature describing a function for CD46 on the AR of mouse spermatozoa is translatable to humans, as several expression differences exist between the two species. Murine expression of CD46 is testis specific, with crry performing the systemic functions that CD46 performs in humans[24]. However, the actions of CD46 in the inflammatory and reproductive scenarios may be mutually exclusive, and whereas the systemic actions of CD46 may have been redundant in the presence of murine crry, the reproductive actions are not. Support for an exclusive action of CD46 in reproduction has been demonstrated in a study utilising new world monkeys. Profiling of CD46 in these animals found a loss of expression of an extracellular domain, CCP1, in all tissue, except the testis, where CCP1 expression was preserved. This site is a preferential binding site for a number of viruses, with evolutionary pressure for pathogen resistance likely the cause for the loss of CCP1 expression[25]. CCP1 does not interact with C3b, therefore loss of expression of this domain would not affect the canonical function of CD46. The preservation of the CCP1 domain in the testis of these animals suggests an importance for this region in reproduction, and an action of CD46 on sperm outside of C3b binding, explaining the continued fertility of C3 knockout mice, but a reduced fertility when CD46 is targeted. In support of this, antibodies directed against CCP1 are able to reduce human spermatozoa-egg binding, whereas an antibody blocking the C3b binding site of CD46 had no effect[26]. Interestingly, CD46 knockout mice have been reported to have increased fertilisation rates due to increased spontaneous acrosome reaction[27], suggesting primate-rodent variations in CD46 function, or multiple functions of this protein.

CD55 and CD59 also display expression patterns unique to the testis with evidence suggesting biological roles beyond protection from complement lysis. Expression of CD55 in the transmembrane form only occurs during a transient stage of spermatid development (stages 11-14), with a GPI-anchored form then expressed on the acrosomal membrane of mature spermatozoa[28]. Whilst it has been demonstrated that CD55 is involved in protection of spermatozoa from complement-mediated lysis in the female genital tract[29], this stage-specific expression of CD55 isoforms in sperm development is suggestive of functions separate to complement protection. This may include signalling roles of transmembrane-CD55 during spermatogenesis and a more fine-tuned regulation of the acrosome reaction by a GPI-anchored CD55. In mice, expression of a CD59 isoform, CD59b, has been reported to be unique to the testis, with a strong localisation to the sperm head[30]. Whilst CD59b knockout mice being are initially fertile, they experiencing a sudden decline in sperm quality with an associated decrease in fertility, suggesting an intriguing role for CD59 in fertility[31].

As with CD46, C1q receptors have also been implicated in sperm-oocyte interactions. Receptors for the collagen-like and globular head regions of C1q, cC1qR and gC1qR respectively, have been detected on human spermatozoa[32], with C1q itself present in follicular fluid[21]. Following capacitation, membrane localisation of gC1qR increases with exogenous C1q increasing sperm agglutination and adhesion to C1q receptor expressing zona-free hamster eggs[33 34].

Blastocyst Stage

At the blastocyst stage complement factors contribute to development of both the trophectoderm and inner cell mass. The complement regulators CD55, CD59a, Crry and CD46 are expressed on the trophectoderm of the blastocyst[7]. Whilst the apparent function of these regulators may be to protect the blastocyst from complement activation within the oviduct and uterus, they have also been shown to contribute to embryotrophic signaling.

Oviductal cells express and secrete complement factors involved in the alternative pathway activation. Factor D and factor B form a C3 convertase and cleave C3 to C3b, leading to a concentration of C3b within the oviductal lumen[35]. Factor I, which participates in the C3b – iC3b conversion, is also secreted by oviductal cells, however, cofactors essential to this conversion are not expressed. The blastocyst itself provides the cofactors for generation of iC3b through Crry expression on the trophectoderm. The generated iC3b has been shown to bind to the trophoblast layer and exert embryotrophic effects, increasing blastocyst size and hatching rate[35,36].

To our knowledge, no *in vivo* studies of complement expression within the inner cell mass exist. However, *in vitro* maintained embryonic stem (ES) cells demonstrate increased susceptibility to complement mediated lysis when compared to terminally differentiated cell types[37]. This increased susceptibility may be a consequence of the sheltered nature of the inner cell mass in the pre-implantation embryo, encased by the protective trophectoderm. Indeed, multiple studies have demonstrated trophectoderm-specific expression of complement regulators on the blastocyst[7,35,36]. The fragile nature of ES cells when exposed to activated complement may also represent a defence mechanism against the formation of teratomas within the mother and embryo, as fetal cells enter the maternal circulation and can survive within the maternal environment for extended time periods[38,39].

Despite the susceptibility of ES cells to complement mediated lysis, complement factors have also been shown to stimulate their proliferation and survival. Embryonic stem cells express the central complement factors C3 and C5 in addition to the two receptors for C5a, C5aR1 and C5aR2[40].

Interestingly, there is no demonstrated ES cell expression of the C3a receptor (C3aR). Akt and Erk1/2 signalling through the C5a-C5aR axis in ES cell culture contributes to proliferation and improves survival[41].

Organogenesis

After implantation the embryo undergoes gastrulation and neurulation laying the foundations for the development of the major organ systems. This period, termed organogenesis, represents the critical peak for potential teratogenic effects[42].

Many components of the complement system are expressed during organogenesis and have been demonstrated across species, including the mouse, *xenopus*, cod and zebrafish[43-45]. The anaphylatoxins, in particular, exhibit unexpected roles in the normal development of the embryo. We recently identified a variety of complement factors expressed during the period of mouse neural tube closure (7.5-10.5dpc) (Chapter 2)[44]. Here, initiators, propagators and regulators of the classical, alternative and lectin pathways were assayed in order to determine which pathways of complement activation were active in the mouse undergoing neurulation. Interestingly, whilst components of all three pathways were present in the whole embryo, all three pathways lacked factors essential for their propagation (Chapter 2). In support of this, a significant portion of the complement system has been localized in the *xenopus* embryo and demonstrates unexpected expression patterns. In particular, *C1qr, C3, C5a* and *C3ar1* localised to the neural structures during neurulation. Other complement factors were also expressed, including terminal components (C6, C8), regulators (CD46, CD55) and propagators (C1s, fH) of complement activation[45].

The neural localization of complement factors during organogenesis is of particular importance as this area has the strongest evidence of physiological function of these molecules outside of their traditional roles. We have previously demonstrated a novel role for C5aR signaling during neural tube closure under conditions of folate deficient stress. In both the human and mouse embryo, C5aR and C5 are expressed by cells of neuroepithelium, and C5aR, in particular, localizes to the apical membrane[44]. Deficiency or pharmacological blockade of C5aR combined with maternal folate deficiency produces a high (~50%) incidence of congenital malformations in the resultant litters[46]. Interestingly, the penetrance of malformations in these litters did not reach 100%, suggesting that C5aR signaling in neurulation is, to some extent, redundant. Additionally, the source of C5a for interaction with C5aR is as yet unknown, however given our previous results demonstrating

incomplete traditional complement pathways at this stage of development, the convertase in this situation is likely to be extrinsic or derived from the C4-independent lectin pathway (Chapter 2).

In *xenopus* and zebrafish development, C3 and C3ar1 are localized to the neural crest and responsible for control of collective migration. Morpholino knockdown of C3aR during zebrafish development caused impaired dispersion of neural crest cells from their normal migratory stream. *In vitro* studies demonstrated a role for C3aR in co-attraction of neural crest during migration, and accordingly neural crest cells have been shown to secrete C3[47]. In addition, both C9 and fH are actively-secreted by migrating neural crest cells, however the functions of these molecules in this environment is, as yet, unknown. Interestingly, the presence of initiating factors of the lectin pathway has also been shown on neural crest cells, and mutations in these factors cause craniofacial defects in human populations. Mutations in MASP1, MASP3 and Collectin kidney-1 have been identified in human populations with craniofacial defects[48]. Accordingly, MASP expression is localised to the brachial arches of zebrafish and mice and, in zebrafish models, morpholino knockdown of MASP1 impairs neural crest cell migration[48].

The apparent importance of the anaphylatoxin receptors in neurulation and neural migration is not translated to homozygous knockout animals, which are born without any gross morphological anomalies. Given that the studies involving acute pharmacological blockade or knockdown demonstrate severe derangement of the normal tissue development, it is pertinent to consider the value of these germline knockouts when investigating the role of complement in the developmental context.

Post-neural tube closure to post-natal period

During the fetal period in humans, the presence of complement factors in the fetal circulation rises in a linear fashion[49,50]. This translates to reduced complement-derived haemolytic activity that is proportional to the complement concentration[50]. It is unknown what percentage of complement activity is attributable to maternal production, however, even in term neonates, complement factor serum concentrations are still significantly below adult levels[49]. In addition, tracing maternal C3 migration into the fetal circulation has previously indicated that there is no transplacental passage of these molecules[51]. Interestingly, there is correlation of fetal and maternal complement activation, as measured by C5a, suggesting that either C5a or the activation factors for complement do exhibit transplacental migration[52].

In the developing organs of the fetus, complement factors demonstrate extra-immune functions. In the developing bones, complement factors of the alternative pathway show a complex and coordinated expression pattern within the growth plate and surrounding regions[53]. C1s, in particular has been highlighted as an initiating factor of cartilage degradation in bone growth, with induction activity of MMP-9[54]. Whilst the presence of the complement anaphylatoxins and their receptors have not specifically been reported in the context of fetal development, they do show specific activity in initiating regeneration post fracture [55,56]. It would be interesting to determine whether the adult model of bone regeneration bears similarities to the induction of fetal bone growth with regards to anaphylatoxin activity.

Complement factors have been shown to play key roles in the developing nervous system (figure 1.4.2). Although reports of complement factor expression in the developing neocortex have been within the literature for the past few decades, few functional characteristics have been ascribed to them[57]. Recently, we showed that C5aR is present on the apical membrane of neural progenitor cells, sharing its cellular location with key drivers of stemness and polarity such as the Par3/Par6/aPKC complex[58]. In this location C5aR signals via aPKC to maintain progenitor cells within the ventricular zone and increase proliferation. In embryonic mice, blockade of C5aR signaling results in behavioural abnormalities in adult life, and reduced neocortical size (Chapter 4). In support of this finding, previously Bénard and colleagues (2008) demonstrated a role for the anaphylatoxins in cerebellar histogenesis post-natally[59]. C5a promotes proliferation of the progenitor cells of the external granular layer, whereas C3a promotes migration to the post-mitotic internal granular layer. Whilst we could not demonstrate a cerebellar anatomical defect resulting from C5aR antagonism from 12.5-14.5dpc, this may be due to a time dependent effect of C5aR on neural progenitor cells. In the neocortex, the peak of murine neurogenesis is within the period we assayed, however formation of the cerebellar layers occurs later in development and extend to the post-natal period in rodents. Interestingly, again C5aR knockout animals demonstrate no gross neurological deficits in brain size nor have any behavioural deficits been reported as yet. However, chapter 6 of this thesis outlines subtle behavioural differences between $C5aR^{-/-}$ / $C3aR^{-/-}$ animals and their wild-type littermates.

The classical pathway of complement activation has been implicated in a novel mechanism of synaptic pruning in the CNS. Complement-mediated synaptic pruning appears to be a phenomenon associated closely with circuits of the visual system postnatally, but not elsewhere in the CNS. Both C1qA and C3 deficient mice, in the post-natal developmental period, exhibit a higher number of

synaptic inputs in the lateral geniculate nucleus[60]. This is due to a failure of synaptic pruning, mediated by the classical pathway, and a subsequent lack of refinement of these afferent signals[61]. This model is an insight into the synapse loss associated with neurodegenerative disease, and outlines a mechanism through which aberrant complement activation may result in cognitive deficits. Additionally, several proteins with phylogenic links to members of the complement cascade have important roles in developmental processes. The C1q-like protein, C1ql1, is a secreted protein that binds to its receptor, Bai. It shares a close evolutionary link to the constituent proteins of C1[62]. C1ql1 is expressed predominantly in brain areas involved in motor function and coordination[63]. Recently, it has been identified as a novel mediator of cerebellar development, specifically it aids in the maintenance of single climbing fiber connections in the cerebellum. It is interesting to note that both C1q and its closely related cousin, C1ql1, exhibit similar roles in managing the synaptic connections of neurons in different areas of the brain. The presence of both shared function and shared homology underlines the pleiotropic nature of complement proteins, especially in their evolutionary history.

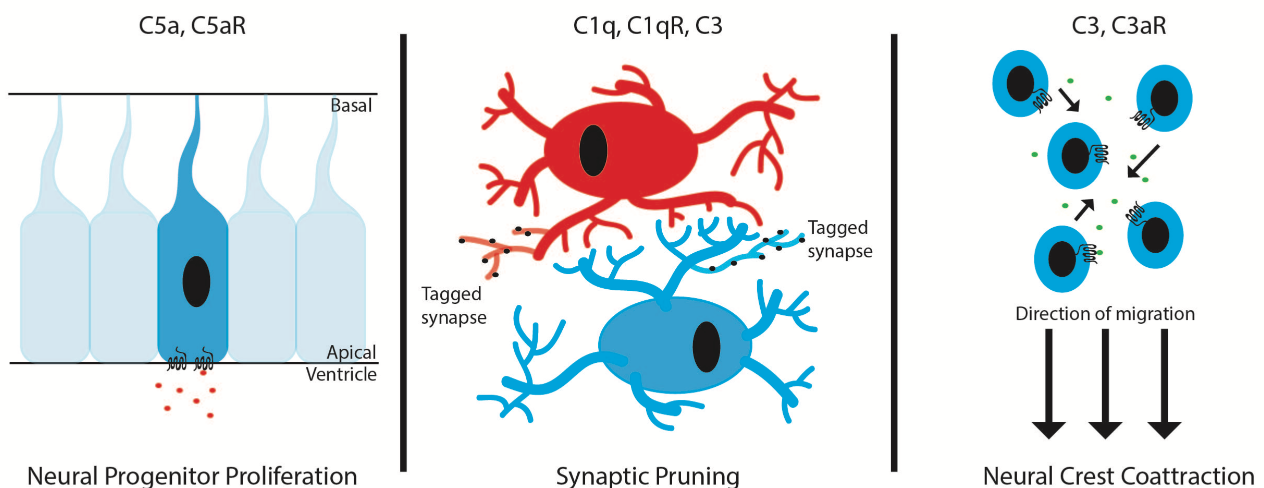

Figure 1.4.2: Novel roles for complement in the developing central nervous system. Left; C5a and C5aR have been demonstrated to promote the proliferation of neural progenitor cells post-infarct and developmentally, which is explored in this thesis. Centre; C1q has been shown to tag redundant synapses for elimination during brain development. Right; C3aR mediates coattraction of neural crest cells during collective migration.

Whilst regulated and directed complement expression is an important component of normal brain development, dysregulation of the inflammatory aspects of complement has been shown to result in adverse developmental outcomes. Models of inflammation-induced preterm birth through the administration of intravaginal LPS demonstrate C5aR mediated fetal brain injury. The induction of inflammation in this model caused significant elevations in C5a concentration in both the amniotic fluid and fetal brain tissue. This elevation in C5a was linked to a reduction in cortical axons and soma as, in this model, C5aR knockout was shown to be protective against LPS-induced injury.

Interestingly, administration of statins *in vivo* and *in vitro* also demonstrated a protective effect against LPS/C5a-induced brain injury, suggesting a statin-mediated interruption of C5aR signaling[64]. The susceptibility of the brain to complement-induced brain injury may also be, in part, explained by the lack of specific complement regulators in the membrane of these cells. Human fetal neurons demonstrate expression of CD59 and CD46, but lack expression of CD55, which regulates complement amplification at the cell surface[65].

Complement and the placenta

Normal pregnancy is associated with systemic maternal complement activation and concentration of complement activation products in the maternal serum positively correlate with gestational age[66]. The presence of the foreign and highly vascular placental tissues in close association with the maternal circulation may go some way to explain this phenomenon. Placental tissue also actively secretes components of the complement cascade and up-regulates the secretions in the presence of pro-inflammatory cytokines, perhaps as a defensive mechanism against potential pathogens[67,68]. Additionally, other novel roles for complement factors are apparent in placental development, such as the secretion of ASP ($C3a_{desArg}$) by placental macrophages correlated to maternal BMI[69]. This has been suggested to alter the fetal insulin homeostasis and result in poor outcomes for births from mothers with high BMI. As such, there has been focus in the available literature on describing the methods of complement regulation by cells of the placenta. However, the complement system, as with the embryo, is not confined solely to mediating inflammation in the context of placental development.

Interestingly, IVF culture causes an decrease in murine placental expression of C3 and C5 at E10.5, in the mouse, and an increase in the terminal factor C9[70]. Complement factors are also increased during the preimplantation period, in the follicular fluid of human IVF cycles[71]. It is interesting to note, in light of this association, that IVF currently is associated with a significantly greater risk of adverse outcomes than spontaneous conception[72]. Additionally, adverse outcomes extend throughout childhood for IVF-conceived infants, with increases in atopic conditions, hospital admissions and systemic disease[73]. However, these outcomes are multifactorial and the sample population is skewed due to the difficulties of conception already present in those self-selecting for IVF treatment. Additionally, there appears to be no gross morphological differences in placentas from IVF-assisted pregnancies and spontaneous conceptions, at least in the first trimester[74]. Regardless, the correlation of increased complement expression in IVF pregnancies raises questions over the level of involvement of dysregulated inflammation in contributing to adverse IVF

pregnancy outcomes, as it does in other placental pathologies explored in this section. It would be interesting to ascertain whether the elevated placental expression of complement factors in IVF pregnancies is a causal or promoting factor in these poor outcomes.

Complement regulators are expressed in the placenta throughout gestation. The expression of CD55, CD59 and CD46 has been found to increase in syncytiotrophoblasts with gestational age[75]. Additionally, there is preferential expression at the fetomaternal interface, as has been shown for CD46, where expression is localised to the embryo-derived trophoblast epithelium on chorionic villi surrounded by the maternal blood spaces[75]. This pattern of expression suggests regulation of the maternal complement system by placental regulators, and indeed in mouse models this hypothesis holds true. Loss of placental *Crry* expression, the analogue of human CD46, causes dysregulated complement activation and C3b deposition on the trophectoderm, leading to embryonic lethality by E16.5[76].

In early placental development, C1q is expressed by extravillous trophoblasts. Action of C1q, through C1qR, in cultured trophoblasts induces Erk1/2 phosphorylation and promotes migration. $C1q^{-/-}$ mice exhibit reduced trophoblast invasion into the decidua, suggesting that the *in vitro* results have biological relevance[77]. It has been suggested that C1q acts to form a molecular bridge between the decidual endothelial cells and migrating trophoblasts in the development of the placental vascular architecture[78]. Absence of C1q results in defective placentation and a predisposition to the development of preeclampsia in mice[79]. Additionally, $C1q^{-/-}$ animals demonstrate reduced fetal weight and increase resorptions, as might be expected given the histological picture of placental dysfunction[77]. Matings between wild type and $C1q^{-/-}$ animal demonstrate that the effect is due to fetal, rather than maternal, loss of C1q[79].

Preeclampsia is characterised histologically by increased deposition of C4b when compared to healthy human controls. Additionally, placental expression of some complement regulators also increase in placentas from preeclamptic pregnancies, notably CD55 and CD59 expression increases, but there is no change in CD46 expression[80]. It is unclear whether the unchanged CD46 in preeclaptic pregnancies contributes to the pathogenesis of preeclampsia by not inhibiting the complement activation. However, this is unlikely given that a recent report demonstrated no increased prevalence of preeclampsia in women with known, function-impairing CD46 mutations[81]. This is interesting given that mice lacking the functional analogue, Crry, exhibit fetal loss by E16.5[76], perhaps suggesting some functional redundancy of complement regulators in human

pregnancy. Mutations in other regulators have been reported in the literature in case-studies and may warrant further investigation as genetic risk factors for development of the disease[82].

At the protein level, complement factors are responsive biomarkers of the inflammation associated with preeclampsia. Early elevation in factor Bb levels in the first trimester is a positive predictor of preeclampsia development[83]. Additionally, active preeclampsia is associated with elevations in C5a, but not C3a, both in the maternal and fetal circulation[52]. Histologically, preeclampsia is associated with MAC deposition on the cytotrophoblasts of the intravillous space. This MAC deposition may be a downstream effect of the disease as, *in vitro,* oxygen deprivation stimulates MAC deposition and the low O_2 environment of preeclamptic placentas could mediate this effect[84]. However, the presence of MAC deposition *in vivo* may further exacerbate the disease process as, in culture, MAC deposition causes an increase trophoblast differentiation[84].

This elevation and activation in complement proteins during preeclampsia may be a cause, rather than result, of the spectrum of symptoms and placental dysfunction. In the DBA/2 x CBA/J model of intrauterine growth restriction (IUGR) and fetal loss, a spectrum of placental dysfunction is apparent that mimics preeclampsia clinically[85]. In this model blocking complement action at several levels, including use of anti-fB, anti-C5, C5aR antagonism and supplemental Crry, results in increased fetal weights and reduced resoptions[86]. It is suggested that the inhibition of the complement system restores the balance of pro- and anti-angiogenic factors in the placenta as complement inhibition was noted to increase free VEGF and decrease the anti-angiogenic factor, sVEGFR-1[86].

This impaired regulation of complement activation also contributes to adverse pregnancy outcomes in other placental inflammatory diseases, and it is not unreasonable to think that the mechanisms remain similar to those outlined in the preeclampsia studies. Antiphospholipid syndrome (APS) is an autoimmune disease that is clinically characterised by the presence of a complement-mediated thrombophilia[87,88]. The syndrome has also been linked to increased risk of developing preeclampsia and other pregnancy complications such as IUGR and fetal loss[89,90]. In mouse models, complement has been shown to be pivotal to the pregnancy complications associated with APS, as inhibition of C3 cleavage, through the use of C3-deficient animals of C3 convertase inhibitors, can prevent the growth restriction and fetal loss[87,91,92].

Active systemic infection, or ascending infection of the female reproductive tract, is also a risk factor for preterm birth and fetal loss during pregnancy. The role of the complement system in

responding to pathogenic insult is well understood, but the complement activation during active infections negatively affects placental health and development. Systemic infection during early placental development in the mouse results in histologically abnormal placentae, with reduced spongiotrophoblast density[93]. Absence of C5aR during this period prevents the loss of spongiotrophoblasts and, in turn, increases fetal survival[93]. In the later stages of pregnancy, systemic inflammation can precipitate pre-term birth, but interestingly, this effect is separate from the mechanisms that precipitate miscarriage, as C5aR knockout animals are not protected[93]. However, the inhibition of C5aR during the inflammation leading to preterm birth can be beneficial in reducing the associated brain injury experienced by the fetus[64]. Systemic inflammation in mouse models of sepsis result in highly elevated C5a concentrations in the amniotic fluid and fetal brain; this aberrant C5a expression results in microstructural cortical abnormalities, and may explain the poor developmental outcomes of children born pre-term[64,94].

As opposed to systemic inflammation, the presence of local vaginal inflammation shows the action of C5aR are directly involved in the cervical ripening leading to preterm birth. Here, C5aR increases the expression of matrix metalloproteinases within the cervical stroma, and leads to the cervical remodeling necessary for delivery[95]. Interestingly, $C5aR^{-/-}$ mice are also resistant to progesterone-inhibitor (RU486)-induced delivery[95], which may be suggestive of some physiological balance between C5aR activation and progesterone in the timing of normal birth.

Conclusion

In conclusion, this section has outlined the evidence for involvement of complement proteins in reproduction, from presence of complement factors on gametes, to the promotion of post-natal brain development. Here complement factors have been described as present in both the embryo and the placenta and, in addition, maternal complement activation can also affect fetal development. The complement system in development functions, in its traditional role, as a barrier to pathogen colonisation, but also has novel and unexpected roles in the histogenesis of the developing embryo. This forms the context of this thesis, which examines specifically the roles of the anaphylatoxin receptors, C5aR and C3aR, on neural progenitor cell populations.

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1.5 C5L2: A controversial receptor of complement anaphylatoxin, C5a

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Published in the **FASEB Journal**

Abstract

C5a is the paramount pro-inflammatory mediator of the complement cascade, and has been previously thought to act only through a single, G protein-coupled, C5a receptor (C5aR, also termed CD88). In 2000, a second C5a receptor, C5L2 (previously known as GPR77), was discovered; yet, despite 12 years of intensive research, its biological, or pathophysiological, function is both enigmatic and controversial. Unlike C5aR, this receptor does not couple to G-proteins, and early studies promoted the hypothesis that C5L2 functions as a decoy receptor. However, recent data have provided other evidence for more complicated and conflicting interactions between C5L2 and other inflammatory mediators. C5L2 has been recently demonstrated to physically interact with both C5aR and β-arrestin to negatively regulate C5aR signalling towards an anti-inflammatory manner, and to reduce pathology, in several disease models *in vivo*. In direct contrast, other groups have demonstrated that C5L2 stimulation caused release of HMGB1 both *in vivo* and *in vitro*, and enhanced pathology in sepsis models, suggesting a clear pro-inflammatory signalling role. These astoundingly contradictory data challenge our precepts and complicate the foundational bases for the possible targeting of C5L2 as a therapeutic option in inflammatory disease: C5L2 may be the great masquerader in complement biology; its function dependent on the cell type, species and disease context. Because of these unusual and unforeseen complexities, we present the current state of knowledge on C5L2 structure, expression and, most controversially, its putative function(s).

Background

The complement system plays a pivotal role in immunity, and is often described as the first line of defence against pathogens. At the apex of the complement zymogen cascade lies C5, a 190kDa precursor to the effector molecules, C5a and C5b. C5b has a well-defined function in initiating the formation of the membrane attack complex, and C5a historically been described as a trigger for the cellular immune response, signalling through its receptor C5aR[1]. Through C5aR, C5a is a potent leukocyte chemoattractant and mediates inflammatory responses, and as such, is involved in many diseases including sepsis, rheumatoid arthritis, asthma, ischemia-reperfusion injuries and inflammatory bowel disease[2]. However, recently a second receptor for C5a has been discovered that does not fit neatly with the perceived functions of C5a, generating controversy in the literature. This second receptor, C5L2, remains a source of intrigue for the complement research community. We now attempt to sift through the reports, mine the frustrations of the frustrated, cut through the mists of ambiguities, and peer into the enlightened darkness of the past, to provide a small beacon of light and hope for the next wave of research into the most recent of many enigmas of complement. Succinctly; what is C5L2, why is it there, what does it do, and why do we care?

Structure

C5L2 was first cloned in 2000 and it has closest homology with C5aR (CD88)[3]. The following year, it was identified that C5L2 is encoded by GPR77 in humans on chromosome 19, q13.33-13.34, located downstream of the gene encoding C5aR, and in 2002, was confirmed that C5L2 binds C5a with high affinity[4]. C5L2 binds to C5a with the same high affinity as C5aR (Kd ~2.5nm), but to its degradation fragment, C5a-desArg, with a much higher affinity (Kd ~12nm) than C5aR (Kd ~660nm). C3a-desArg, also referred to as acylation-stimulating protein (ASP) has been identified to have a direct interaction with C5L2 on adipocytes and macrophages, which may contribute to the inflammatory-adipose phenotype[5]. However, these results are controversial since it has been shown that C5L2 does not bind C3a or ASP/$C3a_{desArg}$ and it is unlikely to be directly related to lipid and carbohydrate metabolism[6]. This viewpoint is currently unresolved, and there are no corroborating studies outside the research group making these claims. How this plays out remains to be seen.

C5L2 is a 337 amino acid, seven transmembrane protein that is grouped in the G-protein family of receptors, together with C5aR. C5L2 shares 38% sequence homology with C5aR, is conserved across mammalian species (figure 1.5.1), however C5L2 exhibits sequence differences in the highly conserved DRY motif located at amino-acid residues 137-139. The DRY motif is an important

region of the third intracellular loop of G-protein coupled receptors (GPCRs), and enables coupling of G-proteins[7]. In C5L2, the DLC sequence of the third intracellular loop appears to be evolutionarily linked to the conserved DRY sequence of other GPCRs. In support of this conclusion, mutating the leucine residue of the DLC sequence to an arginine has been shown to promote intracellular calcium release in C5L2-transfected RBL cells[8]. There is also an apparent lack of an NPXXY motif, which has previously been demonstrated to be essential for phospholipase C and mitogen activated protein kinase activation in formyl peptide receptor signalling, a group of phylogenetically related proteins[9]. In addition C5L2 has a shortened third, typically G-protein binding, intracellular loop (figure 1.5.1). These differences have all contributed to the original prevailing hypothesis that C5L2 is a non-signalling, decoy receptor[2].

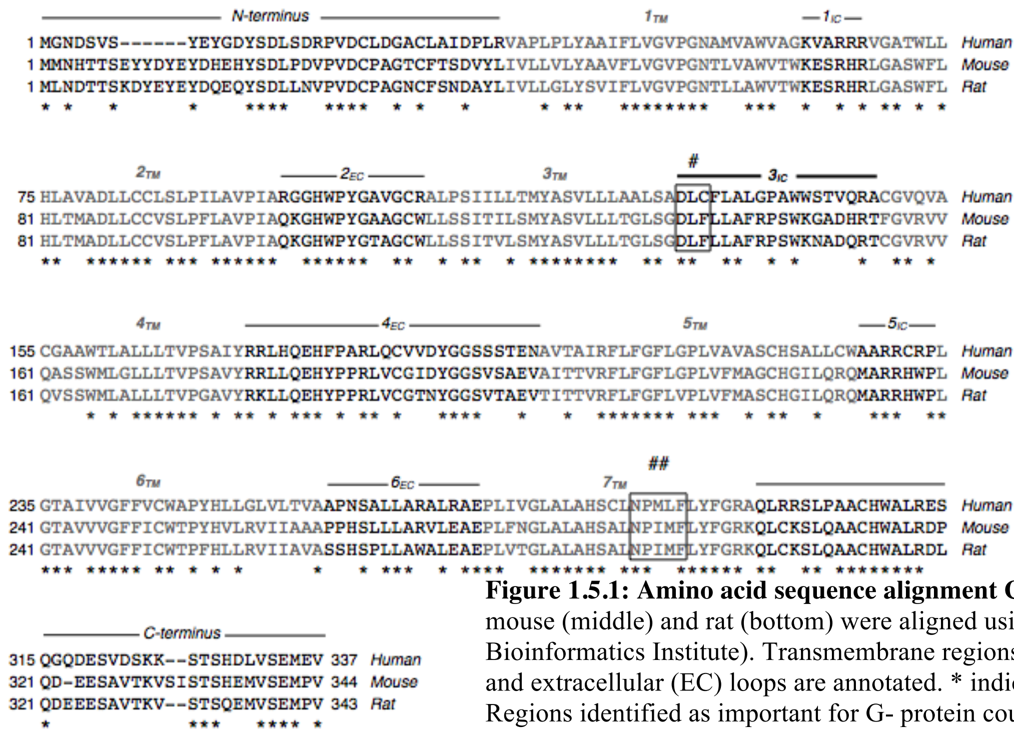

Figure 1.5.1: Amino acid sequence alignment C5L2. Human (top), mouse (middle) and rat (bottom) were aligned using ClustalW2 (European Bioinformatics Institute). Transmembrane regions (TM), intracellular (IC) and extracellular (EC) loops are annotated. * indicates conserved residues. Regions identified as important for G- protein coupling in other GPCRs, but mutated in C5L2, are annotated as # (DRY region) and ## (NPXXY region). Numbers on the left indicate the residue number.

On the extracellular domains, C5L2 has a similar pattern of tyrosine and acidic N-terminal residues to C5aR, which have been shown to be a major feature of extracellular binding of C5a[10]. C5L2 also shares similarities with C5aR in the number of charged and hydrophobic residues in the loops and transmembrane regions, which are involved in interaction with the C-terminus of C5a[11]. Specifically, there is conservation of tyrosine residues 10 and 13 at the N-terminus, which have been shown to be critical for C5a binding by C5aR[2]. However, mutation of these residues in C5L2 diminishes the affinity for $C5a_{desArg}$, but not C5a[10]. Affinity for C5a is reduced in the presence of

mutations to aspartic acid residues 22, 18 and 25, of which D18 was demonstrated to also be of importance in C5a-C5aR coupling[10].

Expression

C5L2 is generally seen to be expressed in similar tissue and cellular locations to C5aR, in agreement with their adjacent location on the genome. Currently, there are no known natural cells expressing just one of the two receptors - for whatever reason(s), they are natively expressed in tandem. The expression of C5L2 has been widely demonstrated on inflammatory cells, including neutrophils, immature dendritic cells, macrophages, lymphocytes and monocytes[3,12]. However, similar to C5aR, it is also expressed on some non-immune cell types such as adipocytes, skin fibroblasts, vascular smooth muscle cells, astrocytes, neurons and cells from tissues such as liver, heart, lung and spleen[3,12-16]. This co-expression of C5L2 and C5aR suggested an functional link between the receptors and seemed to give weight to the 'decoy receptor' hypothesis of C5L2 function – where C5L2 functionality depends on the presence of C5aR. However, recent non-overlapping expression of these receptors has been demonstrated in the diseased nephron[17], indicating differences in the key promotors of expression for C5aR and C5L2.

In relation to the localization of C5L2 within a cell, although C5aR is predominantly expressed on the cell surface expression, in line with the majority of GPCRs; C5L2 appears to be expressed predominantly intracellularly. Using ligand-binding and flow-cytometric analyses, transfected RBL cells have been demonstrated to have lower C5L2 cell surface expression when compared to C5aR[2]. In this model, used extensively for the investigation of C5aR/C5L2 function, C5L2 is abundantly expressed on intracellular vesicles[2]. In relation to primary cells, human neutrophils also display a predominant intracellular localisation under both normal conditions and stimulation with C5a[18]. Indeed, C5L2 was demonstrated, presumably due to its intracellular location, not to take part in the binding of C5a by human neutrophils[18]. Our laboratory has also demonstrated isolated mouse peritoneal macrophages, human THP1 monocytic cells, and isolated human peripheral blood monocytes all show major intracellular expression of C5L2 (figure 1.5.2). This is in direct contrast to the prevailing, and now well-accepted view, for cell-surface expression of C5aR in these same cells (figure 1.5.2). The intracellular localisation of C5L2 is now well established, and could have ramifications for the perceived function of the receptor. However, whether C5L2 remains intracellular between all cell-types and disease states is unknown and could potentially explain some of the disputed functional charecteristics. Indeed, in rat C5L2-transfected HeLa cells, the expression appears to be predominantly cell-surface by confocal microscopy[13], lending weight to this hypothesis.

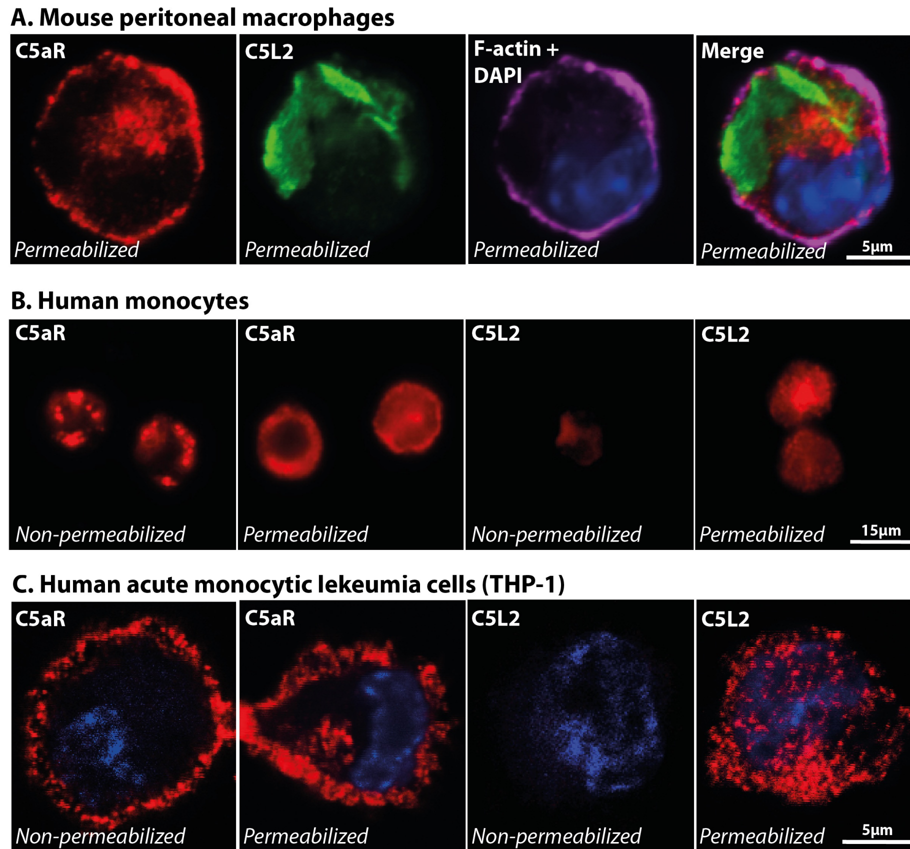

Figure 1.5.2: C5L2 is expressed intracellularly in human and mouse mononuclear cells. Intracellular and surface expression of C5aR and C5L2 were visualised using standard immunofluorescent methods in (A) isolated mouse peritoneal macrophages, (B) human monocytes, and (C) human acute monocytic leukaemia cells (THP-1 cells), adhered to coverslips. Cells in panel A were incubated overnight (4°C) with rabbit anti-mouse C5L2 (1:500 dilution, gifted from P. Monk) and rat anti-mouse C5aR (Clone 10/92, 1:100 dilution, Serotech), with a permeabilisation step (0.25% Triton X-100) prior to primary antibody addition. Cells in panels B and C were incubated with mouse anti-human C5L2 (clone 4C8, 1:500 dilution, gifted by C. Mackay) and mouse anti human C5aR (clone S5/1, 1:500 dilution, Hycult) either with, or without, a permeabilisation step. Cells were then incubated with appropiate fluorescent secondary antibodies (Alexafluor, Invitrogen), and nuclear stained with DAPI (panel A and C), and Alexafluor 688-conjugated phalloidin (F-actin, Invitrogen; panel A). These images demonstrate that whilst C5aR is localised predominantly on the cell surface, C5L2 is predominantly localised intracellularly in all cell types. Panels A and C were imaged at 100x magnification using confocal microscopy. Panel B was imaged at 40x magnification using standard epifluoresence.

The regulation of C5L2 protein expression has been demonstrated to be related to C5a stimulation. It has been shown that C5a decreases C5L2 protein in rat polymorphonuclear cell lysates during sepsis[19]. This control of expression is likely a direct product of the C5a-C5L2 axis, as the presence or absence, of C5aR appears to have little influence on expression of C5L2[19]. In support of this, no significant changes in C5L2 expression have been found between wild type and C5aR-/- mouse neutrophils[20]. Another important regulator of C5L2 expression is the gram-negative bacterial cell coat component, lipopolysaccaride (LPS). LPS is a potent ligand for Toll-like receptor 4 (TLR4). that is of considerable interest to complementologists for its potential signalling interactions with C5aR[21]. In PBMCs, LPS has been demonstrated to increase C5aR-mediated responsiveness to C5a via the downregulation of C5L2 expression[22].

The upregulation of C5L2 expression is regulated by factors such as IFN-γ, noradrenaline, dibutyryl-cAMP insulin, and thiazolidinedione in various cell types[6,15,23,24]. In addition, C5L2 expression also appears to be dependent on the activity of sphingosine kinase 1(Sphk1), an enzyme responsible for generating the signalling lipid, sphingosine-1-phosphate (S1P), which is a key mediator in phagocyte function[25-27]. Mice deficient in Sphk1 have reduced C5L2 expression when compared to wild type mice on the same background, and have no macrophage Erk1/2 phosphorylation in response to C5a. The authors postulated that inflammatory cytokines and C5a activate Sphk1, which can generate S1P during inflammation. S1P then increases expression of C5L2 to negatively regulate inflammation[28]. Oestrogen receptor agonists also increase C5aR mRNA expression, whereas the regulation of C5L2 is dependent on the type of agonist. Oestrodiol increased C5L2 expression, whereas a selective pharmacological agonist of the oestrogen receptor alpha agonist resulted in the downregulation of C5L2 mRNA[29].

Molecular function

The physiological role of C5L2 is still both enigmatic and controversial; three conflicting observations about the function of C5L2 have been presented since its discovery in 2000. Two hypotheses describe an anti-inflammatory role for C5L2 in the immune system[18,30], whereas a third provides evidence for C5L2 acting as a pro-inflammatory entity, triggering release of cytokines such as the damage-associated protein HMGB1[20]. These activities seem mutually exclusive, however, we believe that not only is this not necessarily so, but quite apposite. The evidence for each of the postulated functions is described below and summarized in figure 1.5.3.

Anti-inflammatory role as a recycling decoy receptor

The decoy receptor model for C5L2 was initially described, based on the lack of structural motifs known to participate in G-protein coupling[4,8]. However, Scola and colleagues in 2009 provided the first physical evidence for this model in transfected CHO, HL-60, Hela and RBL cells[30]. The authors noted, C5L2-mediated, clathrin-dependent internalization of C5a and C5a des Arg in differentiated HL-60 and HeLa cells. This effect was not inhibited by a C5aR antagonist, strongly suggesting that ligand degradation occurs after uptake by C5L2. Using human PMNs, C5L2 was confirmed to be the main receptor of C5a-desArg. Their experiments suggested C5L2 functioned by removing active complement fragments from the extracellular environment, preventing C5aR activation[30]. In this model, the binding of C5a to C5L2 induces internalisation of the complex and

endosomal degradation of C5a; C5L2 is then returned to the cell surface. The anti-inflammatory actions of this model depend upon C5L2 sequestering the available C5a (and C5adesArg) to prevent the pro-inflammatory interaction between C5a and C5aR.

Anti-inflammatory role via beta-arrestin pathway

Bamberg and colleagues developed another anti-inflammatory model of C5L2 function, whereby C5L2 complexes with beta arrestin to negatively regulate inflammation. Whilst, antibody blockade of C5L2 induced an increased level of C5a-mediated chemotaxis and ERK1/2 phosphorylation, there was no influence on ligand uptake or C5aR endocytosis in human PMNs. This result contrasts with the previous hypothesis that C5L2 acts as a decoy receptor. Bamberg and colleagues (2010) demonstrated that C5L2 functions independently of calcium mobilisation, and inhibits the C5aR-mediated response to C5a through interactions with β-arrestin[18]. It was proposed that binding of C5a to C5aR activates intracellular C5L2 and triggers phosphorylation of both receptors by G-protein receptor kinases (GRK), facilitating their association with β-arrestin. The C5aR-β-arrestin complex activates ERK1/2, but the C5L2-β-arrestin complex results in ERK1/2 inhibition. The net signal is determined by the summation of the two competing pathways[18].

The evidence for this hypothesis is supported by the intracellular localisation of C5L2. Bamberg and colleagues used human PMNs to demonstrate a contrasting expression pattern for C5aR and C5L2; C5aR is localised to the cell membrane, whereas C5L2 is expressed predominently intracellularly. Indeed, in the transfected RBL and CHO cells used to support the decoy receptor hypothesis, the majority of C5L2 appears peri-nuclear, which remains unexplained in the 'decoy receptor' model[30]. C5aR and C5L2 were also demonstrated to colocalise after administration of C5a. However the investigators used a high dose of C5a (100nM) to induce co-localisation, despite demonstrating that C5L2 negatively regulates C5a induced ERK1/2 phosphorylation at 1nM. This is an important discrepancy to highlight as the investigators rely heavily on co-localisation of C5aR/C5L2/β-arrestin to support their hypothesis. Other studies have also investigated C5L2/β-arrestin co-localisation in different cell types with contrasting results, suggesting that this hypothesis may not be globally applicable[30-32]. However, a recent study has supported this hypothesis through demonstrating green fluorescent protein-labeled beta-arrestin-2 localisation to cytoplasmic vesicles upon stimulation with C5a, demonstrating an EC_{50} for this effect of 2nM. Additionally, these authors demonstrated that the beta-arrestin coupling was independent of any G-protein coupling[33]. Although there have been previous non-supportive studies for this hypothesis, these studies focussed their attention on beta-arrestin-1, rather than beta-arrestin-2[30].

Pro-inflammatory role through intracellular signalling, and release of cytokines (IL-6, TNF- and HMGB1).

In direct contrast to both the 'decoy receptor' and 'beta-arrestin' models of C5L2 function, a pro-inflammatory function for C5L2 has been postulated. It has been noted by several groups that the cytokine expression profile of wild type and C5l2-/- neutrophils differs when exposed to C5a. C5L2 appears to be the trigger in the C5a induced upregulation of IL-6, TNF-α and complement receptor-3 in neutrophils[13]. C5l2-/- neutrophils also demonstrate impaired C3aR/C5aR-induced ERK1/2 and

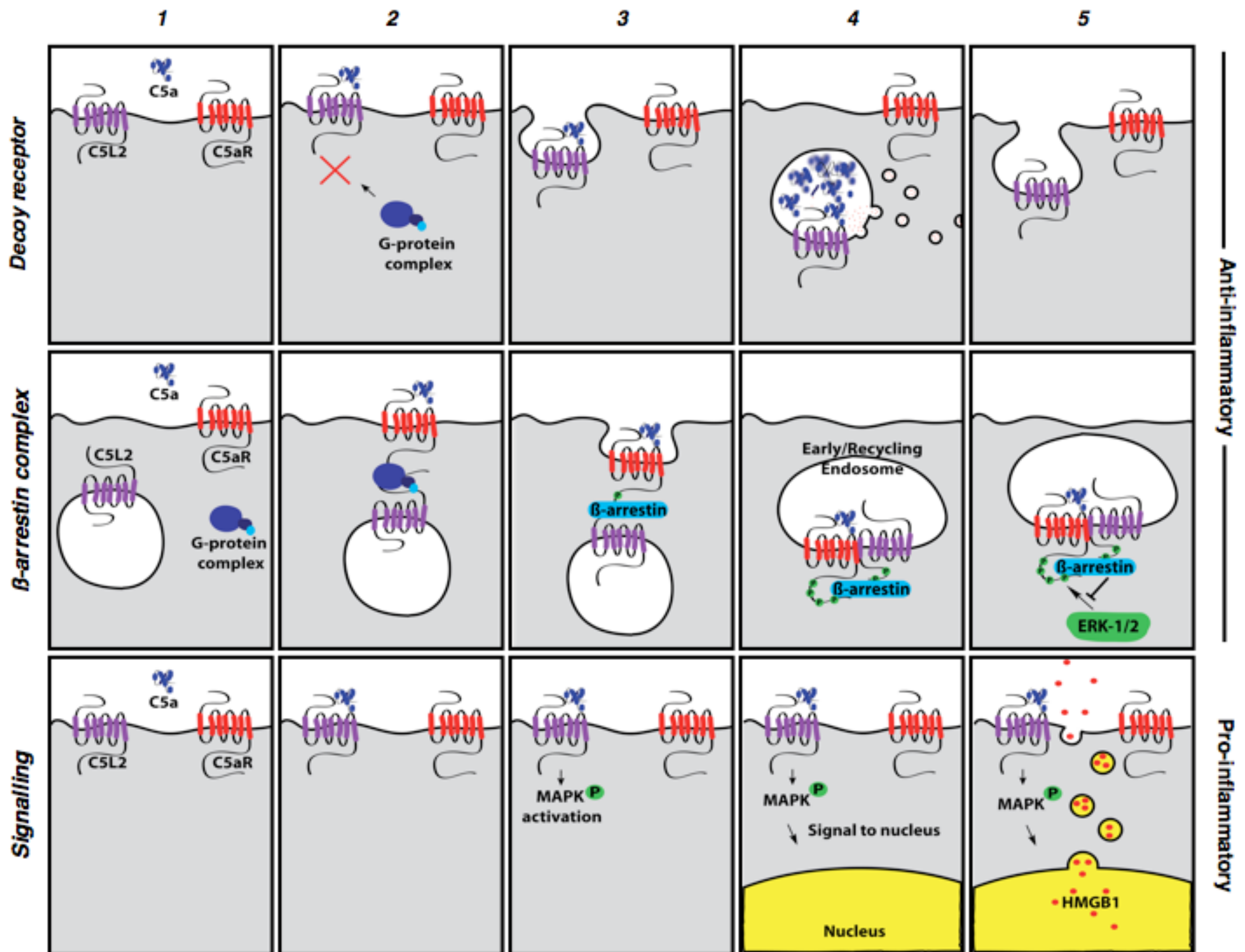

Figure 1.5.3: Three conflicting mechanisms for C5L2 function. Each row represents a separate mechanism of C5L2 function. *Decoy receptor model* (Top row); 1. C5L2 binds C5a, preventing C5a-C5aR interaction. 2. C5L2 cannot couple G-proteins, no second messenger signalling occurs 3. C5L2 internalises upon binding C5a. 4. C5 is degrade in intracellular vesicles. 5. C5L2 returns to the cell surface. *Beta-arrestin complex* (Middle row); 1. C5L2 is located intracellularly, C5a binds C5aR at the cell surface. 2. Upon C5a binding a C5aR/G-protein/C5L2 complex is formed causing phosphorylation of the C-terminus of both receptors. 3. Phosphorylation allows beta- arrestin binding. 4. Complex of receptors and beta-arrestin is internalised in early recycling endosome. 5. Presence of beta-arrestin prevents MAPK pathway signalling through C5aR. *Signalling* (Bottom row); 1. & 2. C5L2 competes with C5aR for C5a binding. 3. Binding of C5a to C5L2 results in MAPK phosphorylation independent of C5aR action. 4. MAPK pathway signals through unknown mechanism to the nucleus. 5. Signalling results in HMGB1 release from nucleus. The top two rows, *Decoy receptor* and *Beta-arrestin*, are hypothesised to be anti-inflammatory in action. Whereas, the bottom row, *Signalling*, is hypothesised to be proinflammatory in action.

AKT activation, suggesting that C5L2 is a positive modulator for both C5a- and C3a-induced responses[12].Perhaps the most compelling evidence of a pro-inflammatory function for C5L2 comes from the caecal-ligation and puncture model of sepsis. C5l2-/- mice had reduced mortality when compared to wild-type littermates, and significantly reduced plasma inflammatory cytokine concentrations[20]. The function of C5L2 in this system was postulated to be as a trigger for the release of cytokines, including transcription factor and pro-inflammatory mediator HMGB1, from circulating leukocytes. Rittirsch and colleagues used inhibitors of MEK1/2, JNK1/2 and p38 to attenuate the release of HMGB1 in peritoneal macrophages[20]. In contrast to the 'beta-arrestin' model of C5L2 activity, these data suggest that the release of HMGB1 via C5L2 signalling is dependent on MAPK activity, an intriguing finding given that C5L2 lacks the traditional intracellular moieties that permit G-protein coupling. In addition, the HMGB1 release in C5L2-/- macrophages was also attenuated when stimulated with LPS, indicating LPS signaling via Toll-like receptor 4 (Trl4) depends on the integrity of C5L2. In addition, deficiency of C5l2 leads to a reduced inflammatory cell infiltration in vivo and C5a-induced downstream signalling in cells. C3a-induced signalling and F-actin formation were reduced in C5l2$^{-/-}$ cells. Also, defective in vivo inflammatory responses and haematopoietic cell regeneration in c5l2$^{-/-}$ mice were reported[12]. Other observations also support a proinflammatory function for C5L2, for instance, C5L2-deficient mice are protected from the development of airway hyperresponsiveness mucus production in two models of experimental allergic asthma[34].

This seemingly proinflammatory role for C5L2 is contradictory to the observations in C5L2 knockout mice, where the biological activity of the C5a/C5aR axis is enhanced both in vivo and in vitro. In studies in non-disease models, the biological role of C5L2 thus appears to be limiting to the pro-inflammatory response to the anaphylatoxin. Other studies demonstrate that up-regulation of C5L2 may be of benefit in inflammatory states driven by C5a, including sepsis, asthma, cystic fibrosis, and chronic obstructive lung disease[35]. Deficiency of C5L2 results in reduced inflammatory cell infiltration, suggesting that C5L2 is critical for optimal C5a-mediated cell infiltration in certain *in vivo* settings. Furthermore, like mice incapable of C3a/complement 3a receptor (C3aR) signalling, C5L2-deficient mice are hypersensitive to LPS-induced septic shock, show reduced ovalbumin (OVA)-induced airway hyper-responsiveness and inflammation, and are mildly delayed in haematopoietic cell regeneration after gamma-irradiation[12].

Therefore, the question remains, how to integrate these seemingly conflicting observations to develop a complete understanding of C5L2. At least in part, some answers may lay in the various models used to investigate the function of this protein (which may indeed be central to the lack of

consensus surrounding the function(s) of C5L2). The models using transfected CHO and RBL cell lines to investigate C5L2 function, although useful for investigating ligand-receptor interactions, come with major caveats with regard to function. For instance, the expression of the putative second messenger for C5L2, beta-arrestin2, may be lower or absent from these cell lines.

C5L2 physiology and pathophysiology

C5a has been implicated in many diseases such as sepsis[36], rheumatoid arthritis[37], asthma and allergy[35,38-40], ischemia-reperfusion injuries[41-43], inflammatory bowel disease[44-46], atherosclerosis[47], and neurodegeneration[16,48-50]. However, many of these studies have focussed on C5aR without specifically investigating the role of C5L2. Modulating the actions of C5L2 may yet prove to be a useful therapeutic tool. However, the function of C5L2 in disease is still a highly debated topic. The function of this receptor, as to whether it attenuates or propagates inflammation, varies between disease state and even between cell types in the same disease.

Sepsis

In mouse models of sepsis blocking C5L2 produced increased levels of pro-inflammatory mediators, IL-6 and TNF-a[13]. Moreover, rat C5L2 was shown to have anti-inflammatory properties as its reduction resulted increased NOS2 and kB gene expression[15]. In addition, a higher level of C5L2 in PMNs from sepsis patients who survived was found in comparison to non-survivors, a finding which appears to cement an anti-inflammatory role for C5L2 in sepsis[19]. However, there is evidence that the organ dysfunction associated with sepsis may be mediated, in part, through C5L2. In rats 24 hrs after CLP there is significant apoptosis of adrenomedullary cells, as assessed by the TUNEL technique. These effects were reversed by dual-blockade of the C5a receptors, C5aR and C5L2, however the authors did not attempt blockade of each receptor independantly[51]. Since blockade of both C5a receptors abolished adrenomedullary apoptosis in vivo, it was suggested that C5aR and C5L2 may be promising pharmacological targets in the clinical setting of sepsis. Although, such targetting of C5L2 may exacerbate the other sequalae of sepsis that C5L2 has been demonstrated to mitigate.

To add to the confusion of the specific role for C5L2 in sepsis, there is the observation that C5L2 knockout mice have increased survival in mid-grade sepsis[20]. This series of experiments demonstrated that C5L2 was required for release of the pro-inflammatory mediator, HMGB1, in the presence of both C5a and LPS. In addition, HMGB1 release from C5aR$^{-/-}$ macrophages was

diminished in the presence of inhibitors to MEK1/2, JNK1/2 and Akt, indicating that C5L2 is involved in intracellular signal transduction in this model. However, in high-grade sepsis only the blockade of signalling from both C5aR and C5L2 improved survival, perhaps suggesting that the severity of the disease affects the functions of C5L2.

Metabolism

C5L2 knockout mice demonstrate insulin resistance and high pro-inflammatory serum cytokines when placed on a high fat/high sucrose diet[52]. There is some evidence that this diabetogenic phenotype is the result of a disrupted interaction between C5L2 and acetylation-stimulating protein (ASP), which is also known as C3a-desArg, the breakdown product of C3a. The ASP/C5L2 interaction may induce adipocyte differentiation and it is suggested that the downregulation of C5L2 in adipocytes contributes to insulin resistance. Interestingly, progesterone has been demonstrated to downregulate C5L2 in both mature adipocytes and preadipocytes, providing and interesting angle for research into gestational diabetes[53]. In these studies C5L2 has been reported to have a high binding affinity for both human and mouse C5L2 (k_d 118 and 31nM, respectively), however this direct interaction has been disputed by other groups that have been unable to demonstrate any interaction between C5L2 and ASP[5,6].

Despite the controversy over the putative interaction between C5L2 and ASP, both proteins have been demonstrated to effect metabolism at the level of the central nervous system. Both C5L2 and C5aR are present in the anterior pituitary gland and C5a was demonstrated to stimulate adrenocorticotropic hormone release, however whether this effect is through C5aR or C5L2 is unclear[54]. In addition, administration of ASP into the third ventricle promotes an anorexogenic state through modulation of neuropeptide Y and pro-opiomelanocortin expression[55].

Asthma

Although C5aR has previously been demonstrated to promote the development of asthma[39], little attention has been given to C5L2. However, Zhang and colleagues (2010) show that C5L2 knockout mice are protected from the development of allergic asthma[34]. This protection was determined to be due to C5L2 expression on dendritic cells promoting the inflammatory T-cell phenotype of asthma. Interestingly, the altered cytokine production of C5L2 dendritic cells was also present *in vitro* when cells were stimulated with house dust mite allergen, suggesting that the role of C5L2 in this model may not be through the direct binding of C5a. Rather, C5L2 may act as a modulator of other pattern recognition receptors to promote the asthma phenotype.

Conclusion and future therapeutic perspectives

The actions of C5L2 in health and disease is not only far from conclusive, the field is in considerable and seemingly paralysed disarray. In contrast to the well-documented, global pro-inflammatory role for C5aR, relentlessly emerging evidence strongly indicates that the situation for C5L2 is much more complex, or at the very least, bewildering. There may be no such thing as a unifying theory for C5L2 function, and its role in various contrived experimental, and clinical situations; indeed, there is no reason, *a priori*, to so suppose. Three separate models of C5L2 receptor function in complement biology currently exist; two anti-inflammatory models and one proinflammatory model. Indeed, the three models of C5L2 function need not be mutually exclusive; all three rely upon evidence gathered from separate species, cell types, and experimental models. The anti-inflammatory models of C5L2 function have been originally based on observations made without immune challenge, whereas the proinflammatory hypothesis has its major genesis in observations of C5L2 function in septic animals. In addition, the original observation regarding the lack of G-protein coupling motifs is not unprecedented, for in recent years other GPCR-classed receptors have been found to signal via pathways other than coupling G-proteins such as β2-adrenergic and AT1a receptors[56]. C5L2 may turn out to be the great masquerader of complement-mediated inflammology, its function dependent on the cell type or disease present.

Based on the multifarious described roles for C5L2, the therapeutic targeting of C5L2 (be it for activation or inhibition), becomes a more difficult endeavour. We have to realise the proper functional role of C5L2 in host immunity and disease pathology before rationally targeting it for future clinical treatment. If C5L2 has an anti-inflammatory role, increasing its expression or activity may be beneficial. However, if it plays a pro-inflammatory role, such as suggested in sepsis, then specific C5L2 inhibitors or antibodies may bring benefits. Perhaps it has all these roles in some situations: perhaps it has some of these roles in all situations.

To date, there is no selective activator of C5L2 available, although recent preliminary findings with our collaborating researchers have identified potential selective C5L2 activating peptides[57], which are currently under further investigation in our laboratories. Polyclonal[20] and monoclonal[18] antibodies have been developed to target mouse and human C5L2 respectively. In addition, the C5a mutant A8Delta71-73, originally reported as an inhibitor of C5aR, is also known to inhibit C5L2[58]. At the pharmacological level, C5L2 is also a difficult receptor to target using classical methods, the intracellular location of C5L2 means that ligand access is diminished. The lack of G-protein

coupling, clearly defined signalling and consequences of activation also make C5L2 therapeutic screening a challenge. In addition, C5L2 may also be functionally dependent on other receptors, meaning that therapeutics developed from transfected cell lines may not translate well into the clinical scenario.

Quo vadis. The puzzle with C5L2 is not that it cannot be effectively studied, but rather that it can be, and has been, for more than a decade, with the full armamentarium of the tools of modern biology, at every level of investigation- molecular, cellular, tissue and whole animal. The puzzle is, that it is still such a puzzle. Unlike the classical receptor for C5a, C5aR, which is both historically and universally acknowledged to be of a proinflammatory bent, the specious nature of C5L2 has engendered confusion within the community of adepts. This quagmire leads us to propose the following scenario: it will remain the proverbial riddle for the proverbial near future, but the reasons for this are neither complex, nor controversial. There have been many reports now, from many reputable complement laboratories, on C5L2: it can be reasonably said that there are diverse views and that these are sometimes seemingly contradictory, but this is not so unusual, nor so unexpected. But, is this normally the case for a single discrete receptor, especially in light of its hugely-studied cognate primary receptor, the latter for which there is little disagreement as to its fundamental *raison d'être*? No, it is not; C5L2 seems different in this way. Pharmacologically, single receptors have clearly restricted, and somewhat ideologically comforting functions, albeit with varying degrees of cellular expressions or locales: C5L2 is apparently breaking free from this ideology. How can one receptor do obverse things in diverse cells or diverse systems? Because it can- or, because it must?

Perhaps we can offer a conciliatory viewpoint for C5L2. It is a strange receptor, in that its behaviour seems so different when studied by different groups in different systems at different times. But, perhaps, this is indeed the reality, and our simple minds seek a simple explanation, which is simply not yet available. Or, perhaps it is the *Janus* receptor, the ultimate masquerader at the complement ball, there to confuse and taunt us to a new level of understanding. Certainly, it is quite unlikely to have evolved for this reason, so the challenge for us is now clear. We need to clean up this mess-possibly of our own making, and resulting from our limitations- and resolve whether the situation is more complex than we allow, or whether we are merely asking the wrong questions. Neither situation carries with it a mutual exclusivity, but nor does it offer a clear set of experiments to solve our dilemma. Rarely has the function of a receptor been so troublesome, and it is especially galling is that it is happening now, when we have so many technically sophisticated

investigative tools to employ in our quest to comprehend. This is the both our conundrum and our dilemma. Complement is never easy, and C5L2 has made it less easy.

The situation is truly more complicated than we are used to, for we have been led astray by the boss receptor for C5a, with its relatively simplistic and easily comprehensible functions, so that we feel dismayed and confused that there is no similarly easy nor shining path for clarifying the proper place of this infuriating mole receptor. We are used to receptors (like C5aR) having stupefyingly clear roles in multiple systems, and we are disappointed if new receptors like C5L2 do not fall into our little boxes, because it is all so new and we are not mentally prepared for the new. Is this because it is a rogue mutated GPCR that is no longer coupled to G proteins for signal transduction? Has this then skewed its function over evolutionary time in all the various cells and species so that we now are all at sea in our primitive attempts to unravel its place in complement biology? Perhaps so, or even, probably, more so. Uncomfortably, we do not know, for we have been caught unawares, and we have now not the mentation, or perhaps force-of-will, to proceed clearly apace in any given direction. We need a new and fresh perspective; one that does not easily gather at cosy international meetings.

The case now seems to us to be this. C5L2 may indeed have many different roles and functions in many different systems, cells, tissues, organs and/or species. This is an uncomfortable place to inhabit, for this will make it difficult to do both predictable or predictive research, and to reach an enabling consensus about C5L2 in the foreseeable future. So rarely is the challenge so clear, or so great, by a new ligand-binding site for C5a (dare we yet call it a receptor without clear agreement as to its signalling properties?). However, we seem to agree that all the signs are that C5L2 is an important player in C5a biology, and the portents are that agents modulating C5L2 receptors may well have therapeutic carry: we just can't say what right now these agents are-or may be, nor what utility they may have. Agonists, maybe; antagonists, perhaps- these are the challenges, both proximal and distal: they are multifarious and guidance will neither come soon, nor easily, but we believe the area to be both intrinsically interesting and worthy of pursuit, and it could well reap clinical dividends in the not-so-soon future. The place of C5L2 in complement pathophysiology is yet to be unravelled from its tangled skein, but it certainly seems a worthy and challenging target at which to aim, if we have a renewed force of clear and determined intent, to not be defeated by this arriviste.

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1.6 Is the complement activation product C3a a pro-inflammatory molecule? Re-evaluating the evidence and the myth

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Published in **The Journal of Immunology**

Abstract

The complement activation product, C3a, is often described as a pro-inflammatory mediator, alongside its downstream cousin, C5a. However, this description may be misleading and too simplistic to encapsulate the true functions of this so-called anaphylatoxin, in health and disease. Recent, and emerging studies have shown that C3a has several anti-inflammatory facets *in vivo*. For example, in the acute inflammatory response, C3a appears to act in direct opposition to C5a, though via different receptors. C3a is a functional or physiological antagonist of C5a, in these circumstances, where C3a prevents the accumulation of neutrophils in inflamed tissues by independently regulating bone-marrow mobilization of inflammatory cells. This acute, protective, and opposing activity of C3a to C5a is also illustrated in models of endotoxic shock and bacteremia. These studies at the whole animal level have demonstrated a more complicated and multifaceted picture of C3a biology than has previously been seen *in vitro*. Here, we briefly reinvestigate the discovery and original classification of C3a as a pro-inflammatory mediator; this has inadvertently entrenched a simplistic view, and in our opinion, wrongly so, of the roles of this molecule. We then highlight the emerging studies demonstrating anti-inflammatory effects for C3a in the immune response, with a particular focus on the *in vivo* environment in acute disease models. It is our hope this review will illuminate these apparently contradictory roles for C3a in responding to immune insult, and challenge the general dogma surrounding C3a, which, historically, is ubiquitously described as a pro-inflammatory mediator.

Introduction

The complement cascade is a key component of the immune system, and is essential in providing a balanced response to injury and infection. Complement can be activated through multiple initiating pathways, but all routes will lead to the cleavage of the central complement components C3 and C5 to generate the small bioactive fragments C3a and C5a[1]. Both C3a and C5a are frequently and consistently described in the literature as 'pro-inflammatory mediators', despite a limited number of reports of true pro-inflammatory activity for C3a *in vivo*. Furthermore, many reports in fact demonstrate an anti-inflammatory role for this major complement cleavage product in infection and disease. To add to the confusion, the only *in vivo* C3a receptor (C3aR) 'antagonist' (SB290157), which is frequently used in research reports, has been demonstrated to act as a partial agonist[2], thus clouding interpretations of results in disease models where the agent has been used [3]. We wish to use this review to draw much-needed attention to the potential anti-inflammatory roles of C3a, so as to refocus the discussion on its revised position in the immune response, allowing new strategies for therapeutic targeting of C3a.

Whilst the modern literature surrounding C3a and C5a frequently describe divergent functions for these peptides, their discovery and characterisation may explain why they are so often portrayed together as pro-inflammatory mediators. Through the work of Hans Buchner, Jules Bordet and Paul Ehrlich in the late 19th century it was shown that serum contained both a heat-labile and a heat-stable bacteriocidal element. The heat stable element, in immunised animals, was later demonstrated to be antibody, whereas the heat labile element was termed complement by Ehrlich, as it 'complemented' the antibody response. The anaphylatoxins were discovered through incubation of serum with antigen-antibody complexes, thus activating the classical pathway, and named for their histamine-releasing ability[4]. Although unconfirmed at the time, the origin of the anaphylatoxin activity was suspected to be complement due to its heat-labile nature[5]. This anaphylatoxin activity was finally ascribed to complement split products with the identification of multiple serum components of complement and the purification of C3a and C5a in the late 1960's. Initial experiments demonstrated that the release of the elusive anaphylatoxin was dependent on the presence of the initiating factors of the complement cascade and that the anaphylatoxin was released at, or shortly after, the involvement of C3[6]. With purification, both C3a and C5a were shown to induce smooth muscle contraction through release of histamine in a mutually redundant manner, both having similar 'activity' at the guinea pig ileum[7,8]. This led to the adoption of the term 'complement anaphylatoxin' for these peptides, although this label has been challenged recently[9] as it does not encompass their widespread functions.

C3a Generation, Structure and Signalling

Cleavage of C3 results in the formation of two split products, C3a and C3b. C3a is generated at the convergence of all known complement activation pathways and its sibling at the point of cleavage, C3b, continues the propagation of the complement cascade[10]. Classically, cleavage of C3 results from the action of one of the C3 convertases, which are multi-protein complexes formed from the upstream cleavage events of complement activation. Additionally, C3 can be cleaved by multiple serine proteases present at sites of inflammation, most notably blood proteases such as thrombin, and immune cell-derived proteases such as cathepsin [11-13].

Structurally, C3a is a 77-amino acid peptide consisting of three helical regions and a series of non-regular residues at the C-terminal responsible for binding at C3aR[14,15]. Short portions of the C-terminus have been demonstrated to be necessary both for agonist activity at C3aR, and intrinsic antimicrobial activity [15-19]. The activity at C3aR of the terminal residues in isolation is far lower than that of the native molecule and there is evidence that these flexible C-terminal residues are stabilized in the conformation necessary to bind C3aR by the upstream alpha helix [20]. This C-terminal region of C3a has hence been a nidus for drug design research, both in terms of therapies capable of modulating C3aR activity and those that take advantage of the intrinsic antimicrobial abilities of the molecule [16,21-23].

After its generation, C3a is cleaved quickly at the C-terminal arginine to form C3a-desArg. This molecule has no detectable activity at C3aR [24], but has been shown by some groups to bind the second receptor for C5a, C5aR2 (C5L2) [25,26]. The interactions with C5aR2 been shown to have effects on metabolism and in this sphere C3adesArg is also homologous to acetylation-stimulating protein (ASP). There is now much interest in the ASP-C5aR2 interaction, which appears to regulate metabolism at both the central nervous system (CNS) and adipose tissue levels[27,28].

This review focuses on the interaction of C3a with its canonical receptor C3aR. However it should be noted that there are additional factors emerging in the literature that also intersect with the C3a-axis (detailed below and summarized in Figure 1.6.1). C3aR is a classical 7-transmembrane, G-protein coupled receptor, which shares close homology to the receptors for C5a, C5aR1 and C5aR2[29]. C3aR binds C3a through a proposed 'two-site' binding model, consisting of functionally different recognition and activation sites[30]. This is, in essence, very similar to the general model reported for C5a acting at C5aR1, which may be expected, owing to the shared evolutionary origins of both proteins[31]. However, unlike C5aR1, the N-terminus of C3aR is not charged and is functionally insignificant with regards to C3a binding[32]. At the C-terminus, C3aR couples to

heterotrimeric G-proteins and has demonstrated a promiscuity of G-protein interaction dependent on cell type[33-36]. Intriguingly, the internalization and recycling of C3aR, which is dependent on GRK mediated receptor phosphorylation, is inhibited by the presence of C5a in human granulocytes[37,38]. Studies in this area were perhaps the first reports of functional antagonism between C3a and C5a at the molecular level. Until recently, C3a was thought to be the only ligand for C3aR, however the neuropeptide TLQP-21, a cleavage fragment of the VGL pro-peptide, has been shown to bind and activate murine C3aR through conformational change of the ligand upon receptor binding[39,40].

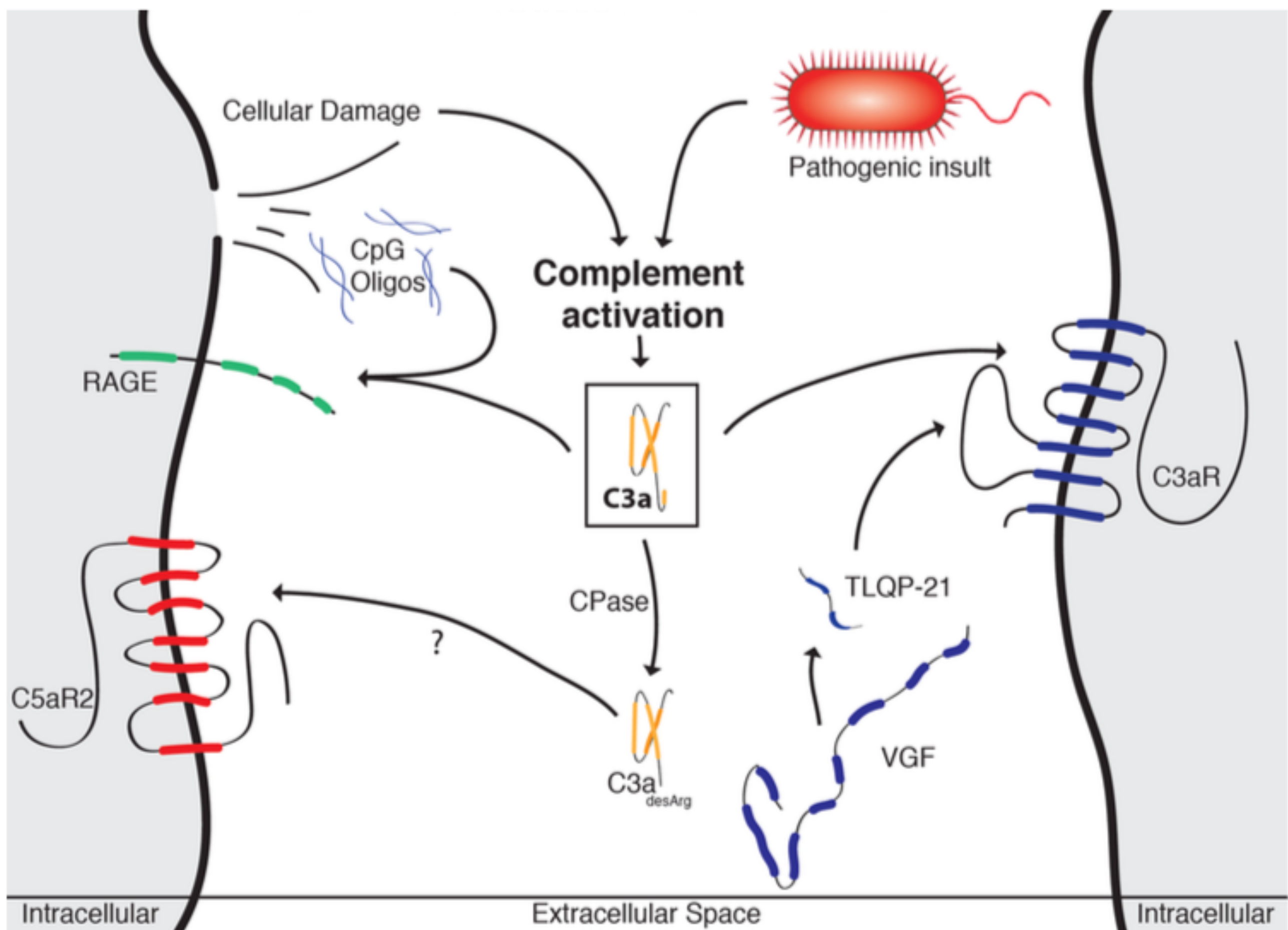

Figure 1.6.1: Diagrammatic representation of receptor-ligand interactions surrounding C3a. C3a, generated from complement pathway activation interacts with its canonical receptor, C3aR, and the receptor for advanced glycosylation end-products (RAGE). Functional interaction with RAGE is dependent on formation of a C3a-CpG oligonucleotide (oligo) complex. The VGF cleavage product, TLQP-21, is also a ligand of C3aR. The terminal arginine of C3a is cleaved by serum carboxypeptidases (CPase) to form $C3a_{desArg}$, which is homologous to acetylation-stimulating protein (ASP). $C3a_{desArg}$ may bind and signal through one of the receptors for C5a, C5aR2.

Of further note, the actions of C3a need not be mediated solely through its canonical receptor, C3aR. For example, there is some evidence of a second, unidentified, receptor, at least in the CNS. Using a transgenic mouse expressing a recombinant C3a peptide under the control of the GFAP promoter, Boos and colleagues demonstrated that elevated CNS C3a worsened outcomes in experimental autoimmune encephalitis (EAE)[41]. Accordingly, in animals that lacked C3aR the disease outcomes were improved. However, *C3ar1*$^{-/-}$ animals with ectopic C3a expression

demonstrated significantly better clinical outcomes than *C3ar1*$^{-/-}$ animals alone, suggesting that the actions of C3a in CNS disease may not be confined solely to its interaction with C3aR. This finding was corroborated in a second paper demonstrating a protective effect for CNS C3a expression in LPS-induced endotoxic shock, that was not diminished in the absence of C3aR[42].

Similarly, C3a has also been shown to bind to the receptor for advanced glycosylation end-products (RAGE) with a high affinity, but this interaction is more complex than a simple ligand-receptor interaction[43,44]. RAGE is a promiscuous receptor that primarily recognizes a plethora of molecules associated with cellular damage[45]. Ruan and colleagues (2010) demonstrated that C3a is able to form a complex with CpG oligonucleotides to enhance IFN-α release from mononuclear leukocytes. Intriguingly, there is as yet no demonstrable effect of a C3a-RAGE interaction alone, despite strong and specific binding between the two[43].

C3a Activity on Immune Cells

C3aR is expressed by all leukocytes of myeloid (and several non-myeloid) lineages, however the functional characteristics of the receptor, and the response to C3a, are dependent on cell type. In the past few decades the literature concerning C3a and its actions at the cellular level has demonstrated a 'bipolar' role for this molecule in different immune cell types. For example, despite a canonical *pro-inflammatory* histamine degranulation response for C3a on mast cells[46], our laboratory has recently shown a potent *anti-inflammatory* response for C3a on neutrophils, by attenuating their mobilization into the circulation following injury[47,48]. Uninhibited mobilization can lead to an increase in neutrophil numbers at the site of injury, which can significantly worsen disease progression. Figure 2 summarizes the opposing actions of C3a on different immune cell populations, which are detailed further below.

The reports on the function of C3a on neutrophils are complicated in the literature, owing to the difficulty in the past of obtaining pure neutrophil cell isolates. Indeed, many of the earliest reports indicating that C3a induced neutrophil activation, were later refuted due to contamination with non-neutrophil granulocytes[49]. What is now clear, is that despite high expression of functional cell-surface C3aR on neutrophils, C3a does not chemo-attract, or stimulate degranulation of neutrophils, despite inducing downstream signalling through Erk1/2 and Akt [49,50]. Interestingly, the signaling produced by C3a stimulation of neutrophils was shown to be dependent on the presence of C5aR2, suggesting that C5aR2 may contribute to C3a signaling through interactions with C3aR[50]. Any chemotactic or degranulating activity previously ascribed to C3a action on neutrophils was suggested to be a byproduct of eosinophil or basophil contamination of the cultures, leading to the

release of secondary factors causing the observed effect[49]. However, our recent studies indicate that at the level of the bone marrow, C3a prevents migration of neutrophils into the circulation by acting in direct opposition to neutrophil mobilizing factors such as granulocyte colony stimulating factor (G-CSF), in a manner reminiscent of stromal derived factor-1 (SDF-1)[47].

C3a and C3adesArg can also induce signalling in human monocytes and monocyte-derived macrophages, however, this interaction, with TLR-4 co-stimulation, induces the production of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6 and PGE_2 [34, 51-56]. This suggests that in the chronic phase of inflammation where monocyte/macrophage responses become more predominant over neutrophils, C3a may indeed act as a classical pro-inflammatory mediator; this view is supported by evidence that *C3ar1$^{-/-}$* animals have modest pathology reductions in chronic disease models such as rheumatoid arthritis[57]. Interestingly, by contrast to the effects of C3a on purified monocytes[56], *in vitro* investigations into the effects of C3a on LPS-induced cytokine release by peripheral blood mononuclear cells (PBMCs; a pooled mixture of monocytes and lymphocytes) demonstrate differences in action depending on the type of the cell. C3a augmented the proinflammatory cytokine production of adherent cells, but suppressed that of non-adherent cells[52]. Depending on the adhesive state of cells, C3a and C3adesArg suppressed TNF-α, IL-1β and IL-6 synthesis in lipopolysaccharide (LPS)-primed non-adherent peripheral blood mononuclear cells (PBMCs) and B lymphocytes, and enhanced their synthesis in adherent cell systems[52,53,58].

Interestingly, despite a marked lack of degranulation effect of C3a on neutrophilic granulocytes, in other granulocytes, C3a induced a degranulation response. For example, in human eosinophils, C3a induces calcium mobilisation, oxidative burst and degranulation[59,60], and in mast cells, C3a and $C3a_{desArg}$ can induce histamine secretion in a dose-related fashion[61,62 63]. This eosinophil and mast cell-specificity has highlighted C3aR as a drug-target candidate for allergic conditions. Such inferences have been strengthened through observations that *C3ar1$^{-/-}$* animals exhibit reduced responses in atopic diseases such as allergic asthma and allergic dermatitis[64], again suggesting a pro-inflammatory role for C3a in these situations.

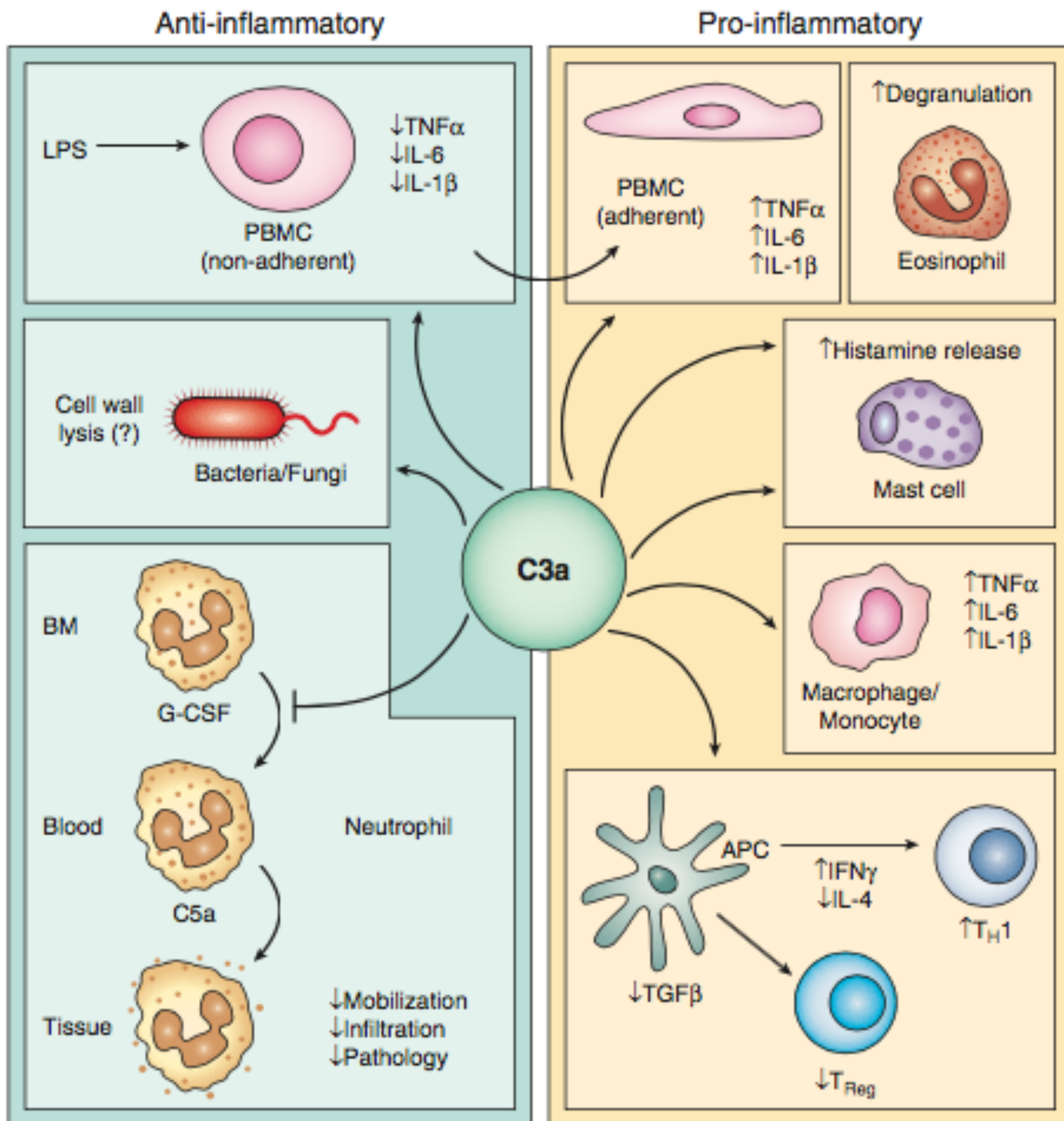

Figure 1.6.2: Functions of C3a on the cell types of the immune system. Right panel - C3a acts in a pro-inflammatory manner to: increase cytokine release from lipopolysaccharide(LPS)-primed, adherent peripheral blood mononuclear cells (PBMCs); induce degranulation in eosinophils and mast cells; increase inflammatory mediator production in macrophages/monocytes; modulate the T-cell response through the suppression of regulatory T cells (T_{reg}) and induction of a T_H1 polarised reponse. Left panel – C3a acts in an anti-inflammatory manner to: decrease cytokine release from LPS-primed, non-adherent PBMCs; Induce direct antimicrobial actions on pathogens; inhibit the mobilization of neutrophils from the bone marrow reservoir.

Administration of C3a has also been demonstrated to augment the T-cell response, promote T-cell proliferation and prolong the inflammatory response through suppressing regulatory T-cells (T_{reg}) production[65-67]. Additionally, it has recently been discovered that T-cells generate intracellular C3a that contributes to survival[68]. In support of this, T-cell populations are reduced in *C3ar1*$^{-/-}$ mice in several models of disease[65,67,69]. Whilst broad T-cell expression of C3aR remains controversial[70], T-cell receptor stimulation upregulates C3aR mRNA expression in isolated T-cell populations and the presence of C3aR appears to promote a T_h1 response, as measured by the defining cytokine, IFN_{γ}[66].

This is supported by observations, *in vivo,* of increased IFN_{γ} production in mice lacking the complement-suppressive factor, *Daf*[71]. C3aR-signalling also acts in concert with C5aR1-signalling to suppress the production of TGF-β1 from dendritic cells, reducing the stimulus for differentiation to T_{reg} cells[67]. The induced T_{reg} cells heavily suppress the proinflammatory $CD4^{+}$ T-cell function, leading to abrogation of the inflammatory process. In addition, C3aR signaling in the tissue antigen-presenting cells suppresses IL-4 production, inhibiting a $T_{H}2$ polarised response[72]. However this effect on T-cell biology is not entirely APC-mediated, as altered T_{reg} responses also occur with adoptive transfer of *C5ar1*$^{-/-}$/*C3ar1*$^{-/-}$ T cells into a wild type animal[67]. *C5ar1*$^{-/-}$/*C3ar1*$^{-/-}$ T_{reg} cells also demonstrate prolonged survival and enhanced function, suggesting that C3aR/C5aR1 stimulus of T cells is also important in regulating inflammatory responses[69].

C3a in Disease

(i) Ischemia-reperfusion injury

Gut ischemia-reperfusion (IR) injury, through transient occlusion of the mesenteric arterial tree, causes an increase in circulating neutrophils upon reperfusion that are critical to the generation and promulgation of tissue injury[73]. Tissue injury also directly activates the complement system leading to the generation of the anaphylatoxins, C5a and C3a[46]. The role for C5a in the progression of this pathology is both very well understood and unambiguous; C5a functions at the level of injury to cause extravasation and degranulation of the circulating neutrophils[74-76]. By direct contrast, in a model of gut IR we have demonstrated that the role for C3a in this acute pathology is overwhelmingly anti-inflammatory[47]. Thus, the historical model for C3a as being a “weaker” form of C5a as a pro-inflammatory mediator is no longer uncomplicated.

C3a levels in both blood and intestinal tissues increase after a period of ischemia, likely from activation of the complement system in response to damage-associated molecular patterns[47,77]. One function of this C3a release, through its interactions with C3aR, is to attenuate the neutrophilia associated with injury by confining unmobilized neutrophils to the bone marrow reservoir. Mice lacking C3aR have a significantly greater increase in circulating neutrophils post-IR than their wild type counterparts[47]. This correlated with increased neutrophil migration into ischemic tissue and worsened histopathological outcomes. The effect of the C3a-C3aR axis in this acute injury also appears to be restricted solely to an effect on neutrophils, as circulating levels of other leukocytes remained similar to wild type animals. Additionally, the wild-type phenotypic response to IR could be fully restored through wild-type bone marrow transfer to *C3ar1*$^{-/-}$ animals, indicating that the anti-inflammatory actions of C3a take place at the level of the bone marrow rather than the site of

injury[47]. Finally, we showed a direct requirement for C3aR to restrict G-CSF mediated neutrophil mobilisation from the bone-marrow, in the absence of tissue injury[47].

As neutrophils are the major contributing cell type responsible for propagation of the acute inflammatory response, this emerging evidence of anti-inflammatory activity for C3a through the retention of neutrophils in the bone marrow forms the crux of the thesis for this review: that C3a should not be grouped with C5a as a purely pro-inflammatory anaphylatoxin. Indeed, several other *in vivo* studies in the literature demonstrate a more complex role for C3a in disease models, particularly in the acute setting.

(ii) Sepsis

In sepsis, it has previously been noted that elevated C3a and C5a correlated with fatal outcomes in hospital patients, but determining cause and effect in these studies is not possible[78,79]. The significant difficulty with research into the effects of the complement anaphylatoxins is that their genesis also amplifies the terminal elements of the complement cascade. In the past, without the use of knockout mice or specific antagonists, it has been difficult to tease these aspects apart.

C3 deficient animals have been utilised for investigation of complement-mediated pathogen clearance during sepsis, and it has been demonstrated, perhaps unsurprisingly, that the lack of C3 reduces survival rates and increases pathogen load in experimental models[80]. It might be expected that the increased pathogen load due to the loss of the terminal elements of the complement system, (namely C3b opsonisation, C5a-induced activation of leukocytes and membrane attack complex lysis of pathogens) explains the premature death of C3 deficient animals. It remains to be seen whether the scale of the direct antimicrobial actions of C3a are of sufficient magnitude to contribute towards this effect[17]. However, this concept seems to be supported by demonstration of a protective effect for administration of exogenous C3 in septic animals[81].

Attributing the effects of C3 deficiency to an attenuation of complement action is complicated by the fact that the presence of C3 is not required for the formation of the terminal elements of the complement cascade, as C5 can be cleaved by serum proteases[13,82]. These proteases are upregulated in $C3^{-/-}$ animals, leading to pathophysiologically significant generation of C5a and MAC[83]. In keeping with this, the differences demonstrated in bacterial load between C3-deficient and C3-supplemented animals do not appear as dramatic as the survival curves suggest [81]. It is difficult to appreciate in this study whether the bacteriocidal or the C3a-mediated actions of C3 supplementation contributed to improved sepsis survival, but the initial report using a *C3ar1* knockout mouse included an investigation into sepsis survival, following lipopolysaccharide (LPS)

challenge, concluded that C3aR signaling contributed to improved survival[84]. Additionally, this study also demonstrated elevated circulating levels of the pro-inflammatory cytokine IL-1β in the *C3ar1*$^{-/-}$ animals. Further studies by the same group used heat-inactivated *Escherichia coli* administration as a model of gram-negative bacteremic sepsis. This also demonstrated a protective effect for C3aR, as *C3ar1*$^{-/-}$ animals had significantly elevated mortality rates when compared to their wild-type counterparts[85]. *C5aR1*$^{-/-}$ animals, conversely, were protected from the bacteraemia, again demonstrating the divergent roles of the two anaphylatoxins[85].

Although these authors did not include measurements of circulating neutrophils in the LPS- or *E. coli*-challenged animals, the recent studies surrounding the actions of C3a in inflammatory disease suggest that a reactive neutrophilia may have been the cause of the reduced survival in the *C3ar1*$^{-/-}$ animals. In support of this, the authors note that the elevated IL-1β previously attributed to high *C3ar1*$^{-/-}$ mortality in the LPS model was unlikely to be the cause of death in bacteremia and instead suggested that the role of C3a is to regulate the inflammatory response generated through C5a signaling[85,86].

(iii) Renal disease

Several studies have outlined the pathogenic role complement plays in the development of autoimmune lupus nephritis; indeed, blockade of C5a signaling can attenuate disease progression in mouse models [87]. High concentrations of C3aR and C3 are present in the glomeruli, correlating with disease severity[88], seemingly suggesting a pathophysiological role for the C3a-C3aR axis in this disease, and, perhaps, a therapeutic target for treatment of lupus nephritis. It has previously been demonstrated that infusion of the C3aR antagonist, SB290157, in the MRL/*lpr* mouse model of lupus nephritis resulted in increased survival and reduced renal inflammation[89]. However, the same mouse model backcrossed with C3aR knockout mice indicates that the loss of C3aR accelerates renal injury and increases mortality[90]. This contradictory result was attributed to the ill-defined pharmacological activity of the widely used C3aR antagonist, SB290157, which has been reported to have agonist activity upon binding C3aR[2]. However, although the pharmacology of SB290157 may be controversial, the conclusion drawn by the authors may now be reinterpreted to take into account the acute ‘anti-neutrophil’ effects of C3aR discovered recently[47]. It is uncontroversial that, at least at the level of the nephron, C3aR is involved in the pathogenesis of renal disease, including the induction of metaplasia in nephron epithelial cells central to the disease[91,92]. These actions appear synergistic to the role of C5aR1 at the tissue level, but may be redundant in the presence of

elevated C5a concentrations, which is much more potent than C3a as a pro-inflammatory mediator at the receptor level.

Contrary to reports of gut IR, there is a slight improvement in disease outcomes for *C3ar1*$^{-/-}$ animals subjected to renal IR injury[91]. In this study the authors demonstrated that C3a administered directly to renal tubule epithelial cells increased the production of chemotactic factors directing leukocytes to the site of injury. Interestingly, a decrease in global leukocytes was demonstrated in the diseased *C3ar1*$^{-/-}$ kidney isolates, as indicated by CD45^{+} flow cytometry. However, there was no difference from wild type mice with regards to granulocyte infiltration, suggesting the dynamics of this model may be more complex than presented. Quantification of circulating granulocytes was not recorded in this model, but it is tempting to hypothesise that the *C3ar1*$^{-/-}$ animals exhibited an injury-induced neutrophilia, as has previously been reported[47], which is tempered by the decreased cytokine production at the tissue level, as noted by these authors. This hypothesis could also explain the lack of a difference in granulocyte tissue infiltration observed.

Finally, preliminary work on pyelonephritis also suggests that C3a reduces neutrophil infiltration and tissue damage. Intriguingly, *C3ar1*$^{-/-}$ animals demonstrated a higher bacterial load within the infected kidney, despite a greater neutrophil response[93]. This highlights the intricate and subtle balancing act performed by the complement system in the acute response to injury.

Conclusion

The terminology surrounding the effects of C3a in the literature warrants revisitation. C3a is frequently and consistently described as pro-inflammatory in nature in the literature, and grouped conceptually with its cousin, C5a, as a like-minded anaphylatoxin. The reality is rather more subtle and nuanced. It is true that C3a does indeed have several pro-inflammatory facets, but here we have highlighted numerous studies from a wide variety of independent laboratories that consistently point to significant anti-inflammatory functions for C3a, in direct functional opposition to C5a. It should also be noted here that different mediators that arise from the same metabolic pathway, as do C3a and C5a, are often physiological antagonists *in vivo*. A good example are the prostanoids, where individual mediators (eg PGE_2 versus $PGF_{2\alpha}$, or Thromboxane A_2 versus PGI_2), are well recognized to act on their cognate receptor, and, with opposing effects, are therefore physiological antagonists of each other. We should therefore not be surprised by the concept that C3a and C5a may also function as physiological antagonists of each other. In fact, C3a and C5a have directly opposing effects of blood pressure mediated entirely through prostanoid activity[94,95]. Collectively, the studies presented in this review contravene the current general dogma, and suggest a change to

the nomenclature, or at least a wide-ranging recognition of the unforeseen complexity of the pathophysiological roles of C3a. At the very least, there should be earnest attention and debate, to leaven our understanding of what is clearly a complex and multifaceted issue.

The response of C3a to injury is a dichotomy. In the acute setting, C3a prevents mobilization of neutrophils, limits their accumulation into tissues, and therefore reduces the inflammatory response at the tissue level. In certain chronic disease models however, such as asthma and rheumatoid arthritis, C3a clearly demonstrates certain pro-inflammatory actions and contributes to disease progression[57,64,96]. The difference in response of inflamed tissues to C3a, between the acute and chronic phases of inflammation, may well be due to the differing cell types involved (eg. neutrophils vs monocyte/macrophages; see Figure 1.6.2). However, the different facets of C3a in disease are not mutually exclusive, but instead depend upon the balance between the pro- and anti-inflammatory effects of the molecule (Figure 1.6.3). Even in models of disease that demonstrate a

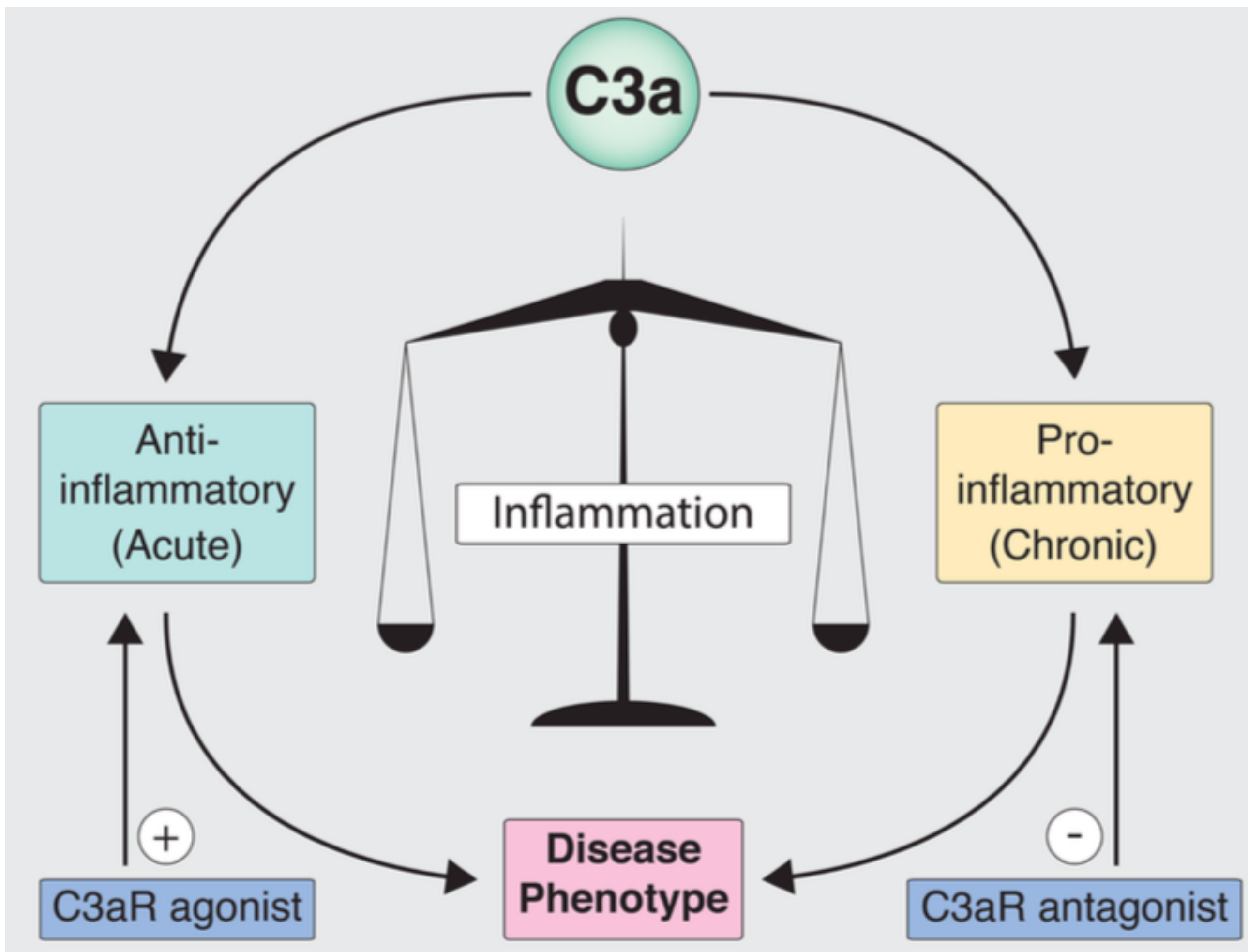

Figure 1.6.3: The balance of C3a actions determines the disease phenotype. C3a induces both pro- and anti-inflammatory effects that contribute to the disease phenotype. Anti-inflammatory effects of C3a dominate the acute phase of inflammation and targeting of C3aR with an agonist at this stage may result in beneficial effects on pathological outcomes. In contrast, the pro-inflammatory aspects of the molecule dominate the chronic phase of inflammation and diseases characterized by their chronicity, such as rheumatoid arthritis and asthma, may benefit from treatment with a C3aR antagonist.

worse outcome with disruption of the C3a-C3aR axis there are also measurable benefits, such as reduced tissue pro-inflammatory cytokines in the gut IR model[47]. It is the balance of these actions in disease that determines the ultimate outcome (Figure 1.6.3).

This dual role of C3a in inflammation also poses significant opportunities for the development of therapeutics. It may be possible to harness the anti-inflammatory activity of C3a in the acute inflammatory phase through the use of C3aR agonists, of which there are emerging several promising candidates[97,98]. We recently demonstrated the validity of this approach in the intestinal I/R model[47]. Equally, the pro-inflammatory aspects of C3a in chronic disease may warrant the targeting of C3aR using a “clean” and selective antagonist to reduce, for example, the cytokine-mediated pro-inflammatory response. Future rational design of therapeutics targeting the C3aR should take these dual roles of this receptor in the inflammatory response into account.

In this review we aimed to revisit the historical and recent literature on the pathophysiological role of C3a in complement-mediated inflammation. In our view, several major conceptual errors have been casually promulgated in the last 30 years over C3a. These first appeared with the earliest experiments comparing C5a- and C3a-induced degranulation of mast cells *in vitro*. Pharmacological activity should not be confused with potency or efficacy. That C3a and C5a both cause efficacious mast cell degranulation *in vitro*, but at widely differing potencies (varying by at least an order of magnitude)[99], does not make them both potent pro-inflammatory mediators. This is where the myth began, aided by the imprecision of terminology carried forth by succeeding researchers. It is time for a rethink, and a careful, and more nuanced consideration, of the roles of C3a in inflammation, which seem far more catholic than its more famous and celebrated cousin, C5a. We suggest that future authors recognize this detail through the description of C3a as an *inflammatory modulator*, rather than a purely pro-inflammatory peptide.

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1.7 This Thesis

Thesis Statement

This PhD project focuses on the physiological role played by the anaphylatoxin receptors in the development of the brain. This research expands the understanding of neural progenitor physiology in the hope that a greater understanding of the physiological processes underlying development may be applied to the discovery and prescription of novel therapeutics to treat developmental and degenerative diseases of the CNS.

Hypothesis

It is hypothesised that the role of the anaphylatoxin receptors on the neural progenitors of the embryonic brain is likely to be that of a modulator of progenitor function.

Aims

The present thesis aimed to demonstrate the role of the anaphylatoxin receptors on neural progenitor biology through the following aims:

1. *To investigate the localisation and expression of C5aR and C3aR during brain development.*
2. *To determine the functional role of C5aR and C3aR on neural progenitors during brain development.*
3. *To elucidate the second messenger signalling involved after C5aR activation on neural progenitor cells.*

Structure of this Thesis

This thesis projected comprises five experimental chapters linked by the theme of the complement system in neural development. In chapter 2 the presence and location of complement factors is demonstrated within the neurulating embryo. In chapter 3, an attempt at creating a transgenic mouse capable of lineage tracing *C5ar1*-expressing cells is described, with the aim that the mouse be used for developmental studies. Chapter 4 comprises the majority of the work associated with this thesis and represents an investigation into the role of C5aR on neural progenitor cells of the embryonic telencephalon. Chapter 5 describes similar work with a shifted focus to C3aR1. Chapter 6 explores adult behavioural abnormalities attributable to genetic absence of the complement anaphylatoxin receptors. Finally, Chapter 7 is a summary of the experimental chapters and provides insights on the future directions of the field.

Chapter 2

Expression of Innate Immune Molecules During Embryonic Development

2.1 Background

The following chapter is comprised of a manuscript submitted to *Developmental and Comparative Immunology* describing the presence of numerous innate immune molecules throughout the period of neurulation in the mouse embryo. The significance of this manuscript lies in the fact that we were unable to demonstrate the existence of all components of the three canonical pathways at this period of embryogenesis. This observation then begs the question of individual function for each of these moieties, independent of their place within the complement cascade.

Coordinated Expression of Innate Immune Molecules During Mouse Neurulation

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Published in **Molecular Immunology**

2.2 Abstract

The innate immune system is the first line of defence against pathogens and infection. Recently, it has become apparent that many innate immune factors have roles outside of immunity and there is growing evidence that these factors play important functional roles during the development of a range of model organisms. Several studies have documented developmental expression of individual factors of the toll-like receptor and complement systems, and we recently demonstrated a key role for complement C5a receptor (C5aR1) signalling in neural tube closure in mice. Despite these emerging studies, a comprehensive expression analysis of these molecules in embryonic development is lacking. In the current study, we therefore examined the expression of key innate immune factors in the early development period of neurulation (7.5-10.5dpc) in mice. We found that complement factor genes were differentially expressed during this period of murine development. Interestingly, the expression patterns we identified preclude activation of the classical and alternative pathways and formation of the membrane attack complex. Additionally, several other classes of innate immune molecules were expressed during the period of neurulation, including toll-like receptors (TLR-2, -3, -4 and -9), receptor for advanced glycation end-products (RAGE), and their signalling adapters (TRAF-4, TRAF-6, TAK-1 and MyD88). Taken together, this study highlights a number of innate immune factors as potential novel players in early embryonic development.

2.3 Introduction

The first discovery of function of a protein often has the consequence of branding that factor with a label that is difficult to remove. This consequently may impart subtle and unintended bias on subsequent experimental endeavours. This is true for many innate immune factors; for example, the misguided labelling of complement factor C3a as a pro-inflammatory mediator (Coulthard and Woodruff, 2015). However, there are many examples of individual proteins possessing multiple, mutually-exclusive and context-dependent functions. Such bifurcation of function is particularly evident in the developmental context.

There are multiple recent reports of early developmental expression of innate immune factors (Carmona-Fontaine et al., 2011; Denny et al., 2013a; Hawksworth et al., 2014). In the adult, the hepatocytes of the liver are responsible for the production of many of the circulating factors of the innate immune system. In the developing mouse embryo, the liver bud forms around 9.5dpc (Bort et al., 2006) and contains hepatoblasts, capable of differentiating to cholangiocytes and hepatocytes from 13.5 dpc onwards (Kung et al., 2010). The early hepatoblasts of the liver bud have been demonstrated to produce several circulating serum proteins, such as albumin and alpha-fetoprotein (Germain et al., 1988), however their ability to form the complement factors produced by the adult liver has not, to our knowledge, been assayed. Cells of the myeloid lineage likely to express the receptors of the innate immune system first appear in the yolk sac of the developing embryo, before migration into the aortic region and liver bud (Palis et al., 1999). These progenitor cells reach the primitive circulatory system around 8.5dpc and the liver around 9.5dpc (Palis et al., 1999).

Recently, our group demonstrated that the complement C5a receptor (C5aR1) is expressed by neuroepithelial cells during human and mouse neurulation, and is critical for proper mammalian embryonic development under conditions of folate-deficiency (Denny et al., 2013a). C5a is the ligand for C5aR1, and is produced as a cleavage product of complement factor C5 (Klos et al., 2013). In the immune system, C5a can be generated through four separate initiation pathways: classical, alternative, lectin and extrinsic protease, each with its own unique propagating zymogens (Manthey et al., 2009; Woodruff et al., 2014). Additionally, numerous regulators modulate the generation of C5a and the action of other cleavage by-products such as C5b, which goes on to form the terminal membrane attack complex (MAC) (Woodruff et al., 2011). Given that C5a may be generated through multiple pathways, and that complement generation and deposition is highly regulated in an immune context, there remains an unanswered question: how C5a may be generated

and modulated in the context of mammalian embryonic development, given the importance of its canonical receptor in neurulation (Denny et al., 2013b).

Aside from the complement system, the innate immune system also comprises factors such as toll-like receptors (TLR), formyl peptide receptors (FPR), chemokines, and cytokines. This diverse collection of molecules acts to immediately recognise, opsonise and neutralise pathogens and damaged cells either directly or through the recruitment of leukocytes. In addition, these factors stimulate the activity of the adaptive immune system to respond to the pathogenic insult. Interestingly, whilst many of these factors have well-established roles in innate immunity, many of them were either first discovered, or have recently been discovered, to have non-immune functions (Hashimoto et al., 1988; Hori et al., 1995; Lathia et al., 2008; Okun et al., 2010).

Toll-like receptors were initially discovered for roles in dorso-ventral patterning of developing drosophila larva (Hashimoto et al., 1988) and were only later demonstrated to be part of a larger family with roles in pathogen recognition. In mice, this family extends to 13 members, each recognising molecular patterns associated with tissue damage or pathogen invasion (Arumugam et al., 2009). Conversely, the complement system has long been investigated as an important player in innate immunity, and only recently ascribed non-immune roles (Carmona-Fontaine et al., 2011; Denny et al., 2013a; Haynes et al., 2013; Kimura et al., 2003; McLin et al., 2008; Rahpeymai et al., 2006; Rooryck et al., 2011).

McLin and colleagues demonstrated that a number of complement factors are expressed during the early development of xenopus (McLin et al., 2008). This has piqued interest in what early developmental processes complement may be facilitating. Subsequently, complement C3a and its cognate receptor C3aR have been demonstrated to direct cranial neural crest cell chemotaxis (Carmona-Fontaine et al., 2011), and the complement lectin pathway components, collectin 11 (collec11) and MASP1, have also emerged as important participants in craniofacial development (Rooryck et al., 2011).

Given the pleiotropic nature of many innate immune molecules, it is not clear if an early, non-immune developmental role is unique to complement or whether expression of innate immune factors during development is a broader phenomenon. This study, therefore, sought to investigate which innate immune factors were expressed in early stages of embryonic development, specifically during the period of neural tube formation and closure (7.5 – 10.5dpc).

2.4 Methods

Tissue Collection

Animal breeding and tissue collection were performed in accordance with guidelines from the National Health and Medical Research Council of Australia and was approved by the University of Queensland Animal Ethics Committee. Pregnant C57BL/6J dams at 7.5 to 10.5 days post conception (dpc) were obtained from University of Queensland Biological Resources for use in this study. The day of vaginal plug discovery was designated 0.5 dpc. Mice were sacrificed by cervical dislocation and embryos were removed from the uterus and extra-embryonic membranes and transferred into a 4% formaldehyde/PBS solution freshly prepared from paraformaldehyde, or snap-frozen in liquid nitrogen.

RT-PCR

Total RNA was obtained from aggregated litters at 7.5, 8.5, 9.5 and 10.5dpc using RNeasy kit (Qiagen, The Netherlands) according to the manufacturer's instructions. RNA was DNase-treated (Turbo-DNase, Life Technologies, USA) and 1 µg of total RNA was reverse transcribed (Tetro RT, Bioline, UK). Specific primers for each gene were designed using the NHMC primer design tool. The primers and corresponding annealing temperatures used are detailed in table 2.4.1.

Table 2.4.1; Primer sequence, amplicon length and PCR conditions used in this study.

Name	Forward	Reverse	Product	Temp
C1qb	5'-CAGAACACCAGGATTCCATACA-3'	5'-AAACCTAGAAGCAGCAGTAACA-3'	102bp	60°
C2	5'-CTCATCCGCGTTTACTCCAT-3'	5'-TGTTCTGTTCGATGCTCAGG-3'	872bp	58°
C4	5'-ACCCCCTAAATAACCTGG-3'	5'-CCTCATGTATCCTTTTTGGA-3'	320bp	58°
Cfb	5'-ACAGAGACCATCGAAGGAGC-3'	5'-GGTTCTATTCCAGCCTTCAGG-3'	426bp	59°
Cfd	5'-ACTGCATGGATGGAGTGACG-3'	5'-TTCGATCCACATCCGGTAGG-3'	554bp	58°
Mbl-2	5'-CCCTGCCTGCAGTGACACCA-3'	5'-AGCACCCAGTTTCTCAGGGCT-3'	443bp	58°
Masp1	5'-ACTTTCCGGTCAGATTTCTCCA-3'	5'-TAGCCACCGATGTAGTTGTGA-3'	143bp	60°
C3	5'-CCAGCTCCCCATTAGCTCTG-3'	5'-GCACTTGCCTCTTTAGGAAGTC-3'	159bp	58°
C5	5'-GCTGCTAAGTACAAACATAGTGTGCC-3'	5'-GGACAGGTTTATGGGGGCTTTCT-3'	184bp	58°
C9	5'-TCTCCCCAGCAGAACACTCT-3'	5'-TGCTTTGTTTCCTGAAGGCT-3'	911bp	58°
AI182371	5'-CCGGGATTGCCTCTTTTCCT-3'	5'-TCTGGGCGTTACAAGTCGTC-3'	79bp	58°
C3ar1	5'-CCCCCAGCCTCTTCTTTATC-3'	5'-AGCCTAAGGCCCTTCTCTTG-3'	732bp	56°
C5ar2	5'-AACCACACCACCAGCGAGTATTATG-3'	5'-AGCCCTCTTGCCTACACCGGC-3'	1040bp	56°
C1qr	5'-ATCGCTTGATGCTGATGCGATGC-3'	5'-ATGCTGAGTCGATGCTGGATGCT-3'	869bp	58°
Cd11b	5'-CCATGACCTTCCAAGAGAATGC-3'	5'-ACCGGCTTGTGCTGTAGTC-3'	147bp	58°
Crry	5'-TCCTTGTGAGATACCCCCAG-3'	5'-TTCTCATTTCACGTTGCTGC-3'	858bp	58°
Cd55	5'-CAAGTACAGGAACCCCCTCA-3'	5'-CTGTGGCGATTCTGCTTACA-3'	729bp	58°
Cd59a	5'-TGTGAAGCCTGTGGAAACTG-3'	5'-TTTTGAGCGTGTCAGAGTGG-3'	895bp	58°
Cfh	5'-CATGGGTCAGTTTCTTGCATTAG-3'	5'-CTTCAGCACTCTACACTTCTCC-3'	90bp	58°
Properdin	5'-TTCACCCAGTATGAGGAGTCC-3'	5'-GCTGACCATTGTGGAGACCT-3'	149bp	58°
Tnfα	5'-TGTCCCTTTCACTCACTGGC-3'	5'-CATCTTTTGGGGGAGTGCCT-3'	117bp	60°
Mcp-1	5'-AGCACCAGCCAATCTCACT-3'	5'-TCTGGACCCATTCCTTCTTG-3'	301bp	60°
Tlr2	5'-GGGTGTGTGATGGCCGCTCC-3'	5'-TCGCCGAGCCCATTGAGGGT-3'	781bp	58°

Name	Forward	Reverse	Product	Temp
C1qb	5’-CAGAACACCAGGATTCCATACA-3’	5’-AAACCTAGAAGCAGCAGTAACA-3’	102bp	60°
C2	5’-CTCATCCGCGTTTACTCCAT-3’	5’-TGTTCTGTTCGATGCTCAGG-3’	872bp	58°
C4	5’-ACCCCCTAAATAACCTGG-3’	5’-CCTCATGTATCCTTTTTGGA-3’	320bp	58°
Cfb	5’-ACAGAGACCATCGAAGGAGC-3’	5’-GGTTCTATTCCAGCCTTCAGG-3’	426bp	59°
Cfd	5’-ACTGCATGGATGGAGTGACG-3’	5’-TTCGATCCACATCCGGTAGG-3’	554bp	58°
Mbl-2	5’-CCCTGCCTGCAGTGACACCA-3’	5’-AGCACCCAGTTTCTCAGGGCT-3’	443bp	58°
Masp1	5’-ACTTTCCGGTCAGATTTCTCCA-3’	5’-TAGCCACCGATGTAGTTGTGA-3’	143bp	60°
C3	5’-CCAGCTCCCCATTAGCTCTG-3’	5’-GCACTTGCCTCTTTAGGAAGTC-3’	159bp	58°
C5	5’-GCTGCTAAGTACAAACATAGTGTGCC-3’	5’-GGACAGGTTTATGGGGGCTTTCT-3’	184bp	58°
C9	5’-TCTCCCCAGCAGAACACTCT-3’	5’-TGCTTTGTTTCCTGAAGGCT-3’	911bp	58°
AI182371	5’-CCGGGATTGCCTCTTTTCCT-3’	5’-TCTGGGCGTTACAAGTCGTC-3’	79bp	58°
C3ar1	5’-CCCCCAGCCTCTTCTTTATC-3’	5’-AGCCTAAGGCCCTTCTCTTG-3’	732bp	56°
C5ar2	5’-AACCACACCACCAGCGAGTATTATG-3’	5’-AGCCCTCTTGCCTACACCGGC-3’	1040bp	56°
Tlr3	5’-GAAACGCGCAAACCCCGGTG-3’	5’-AGGACGCCTGCTTCAAAGTCCC-3’	756bp	58°
Tlr4	5’-CTGAGCAGCCGCTCTGGCATC-3’	5’-GCCAGGCTATCTGTGAGCGTGT-3’	763bp	58°
Tlr9	5’-CCGCAAGACTCTATTTGTGCTGG-3’	5’-TGTCCCTAGTCAGGGCTGTACTCAG-3’	260bp	58°
Fpr1	5’-CATTTGGTTGGTTCATGTGCAA-3’	5’-AATACAGCGGTCCAGTGCAAT-3’	101bp	60°
Fpr2	5’-TGGGCTCAGAACCACCGCAC-3’	5’-GTGCCCAAAAGGGCCACAAGC-3’	401bp	60°
Fpr3	5’-TGGGCTCAGAACCACCGCAC-3’	5’-CACATCCCACAGCCCCCTCCT-3’	724bp	60°
Rage	5’-CAGCGTGGCTCGAATCCTCCC-3’	5’-GCCACAGGATAGCCCCGACG-3’	869bp	58°
Traf4	5’-CAAGTGTGGGGCCCGCATGA-3’	5’-GAGGTCCGGCCCCCAATCCA-3’	978bp	60°
Traf6	5’-ACGTGGGCGAGCTCAAACGG-3’	5’-CATGGACGCTACACCCCCGC-3’	601bp	60°
MyD88	5’-CAGGCGAGCGTACTGGACGG-3’	5’-GCAAGGCGGGTCCAGAACCA-3’	914bp	60°
Tak1	5’-GGAGCTCCGGCAGTTGTCACG-3’	5’-GCCCGTTACCTGCAGTCGCC-3’	992bp	60°
Actb	5’-GTGGGCCGCCCTAGGCACCAG-3’	5’-CTCTTTGATGTCACGCACGATTTC-3’	540bp	60°

Section In situ hybridisation

Section *in situ* hybridisation was performed as per Simmons *et al.* (Simmons et al., 2007). Briefly, cRNA digoxigenin-labelled probes were generated through ligation of PCR products into a pGEM-Teasy vector (Promega, USA) and transcription with SP6 and T7 RNA polymerase (Promega). Resulting probes were diluted 1:2000 in hybridisation buffer and hybridised overnight on 16 µm 9.5dpc embryo sections at 65°C. Following washes with 50% formamide and RNase treatment to remove excess probe, sections were blocked and exposed to anti-digoxigenin-AP antibody (Roche, Switzerland) at 1:2500 dilution overnight at 4°C. For color development, embryos were incubated in 40 mg/ml NBT and 20 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Roche, Switzerland). Slides were re-fixed in 4% paraformaldehyde, mounted with DEPEX (Sigma-Aldrich, Missouri) and imaged using a digital slide scanner (Aperio, AT2).

Whole mount in situ Hybridisation

In situ hybridization was performed as per Christiansen *et al.* (Christiansen et al., 1995). Briefly, embryos at 9.5 dpc were fixed overnight at 4 ℃ in 4% paraformaldehyde/0.01M PBS, and taken through a dehydration and rehydration series with methanol. Embryos were permeabilized using 10 mg/ml proteinase K and incubated at 65 ℃ overnight with 0.5 mg cRNA probe. After removing

excess probe, embryos were blocked with 10% goat serum/2% BSA in TBS. Alkaline phosphatase–conjugated anti-digoxigenin IgG (Roche), preadsorbed against embryo antigens, was added in the pre-blocking solution and incubated overnight at 4 ℃. For color development, embryos were incubated in 40 mg/ml NBT and 20 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Roche). Embryos were taken through an ethanol dehydration series to remove excess color, re-fixed in 4% paraformaldehyde overnight, and imaged using a stereomicroscope (Leica, M205 FA stereomicroscope).

2.5 Results and Discussion

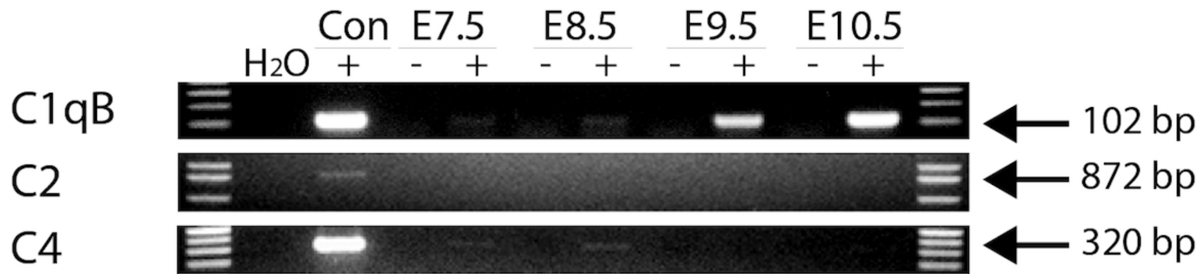
In the present study, we demonstrate that complement factors normally produced for circulation by adult hepatocytes were also expressed in the murine embryo during the period of neurulation. This period coincides with the early stages of liver development and the movement of myeloid progenitors into the embryo proper. Indeed, in this study, we demonstrate the clear presence of the myeloid cell marker CD11b at 9.5 and 10.5dpc, whereas it was beyond detection at 7.5dpc. This coincides with the migration of myeloid progenitors into the embryo. We have also demonstrated the production of a number of circulating complement factors preceding mature hepatocyte development, suggesting that alternative cell types are responsible for complement factor production in the early embryonic period.

3.1 Expression of factors essential for complement activation

To determine whether the canonical complement pathways are active during the period of neurulation in the mouse embryo, we assayed key factors essential for their propagation. The classical pathway was investigated with the expression of C1qB, complement C2 (C2) and complement C4 (C4). Complement C2 was not detectable by RT-PCR throughout the period of neurulation in mouse embryos 7.5-10.5dpc. Very little complement C4 was detectable at 7.5 and 8.5dpc, and was beyond detection at 9.5 and 10.5dpc. In contrast, C1qB, part of the initiating factor of the classical pathway (but also a factor with complement-independent roles), was only expressed at E9.5 and E10.5 (figure 2.5.1). This suggests that the combination of factors usually required to initiate the classical pathway, are either not expressed at all (C2), or are not expressed co-ordinately (C4 and C1qB), during the same period. Thus, it is unlikely the classical pathway is used for complement activation during the period of neurulation.

Activation of the alternative pathway is characterised by cleavage of complement factor B (CfB) into the fragments Ba and Bb, which leads to the formation of a C3 convertase. This cleavage of CfB occurs through factor D (CfD), an obligatory and rate-limiting serine protease that catalyses the formation of the alternative pathway C3 convertase (Klos et al., 2009). While CfB could be detected during the period of neurulation, CfD could not (figure 2.5.1), suggesting that the C3 convertase cannot be assembled via the alternative pathway at this stage in embryonic development.

Classical Pathway



Alternative Pathway

Lectin Pathway

Figure 2.5.1. Complement activation pathway gene expression is restricted during the period of neurulation. RT-PCR analysis was conducted on embryos ranging from E7.5 to E10.5. '-' represents a negative reverse transcriptase control. 'Con' represents a positive control tissue relevant for the gene of interest. Arrows indicate expected band and size. C1qB, complement factor 1q, subunit B; C2, complement factor 2; C4, complement factor 4; CfB, complement factor B; CfD, complement factor D; MBL-2, mannose-binding lectin 2; MASP1, mannose-binding lectin serine protease 1; ActB, beta actin.

Finally, we assessed expression of lectin pathway components mannose-binding lectin-2 (MBL-2) and mannose-associated serine protease 1 (MASP1). While MBL-2 was most apparent at 7.5 and 8.5dpc, MASP1 was clearly expressed at all stages (7.5-10.5dpc; figure 2.5.1), unlike any of the other pathway components assayed. Importantly, the lectin pathway has been shown to be able to act independently of C4/C2 (a C4/C2-bypass pathway) (Selander et al., 2006), and so despite the limited and absent expression of C4 and C2 in the embryo, this complement activation pathway could still be functional. Expression of MASP was also demonstrated by *in situ* hybridisation at

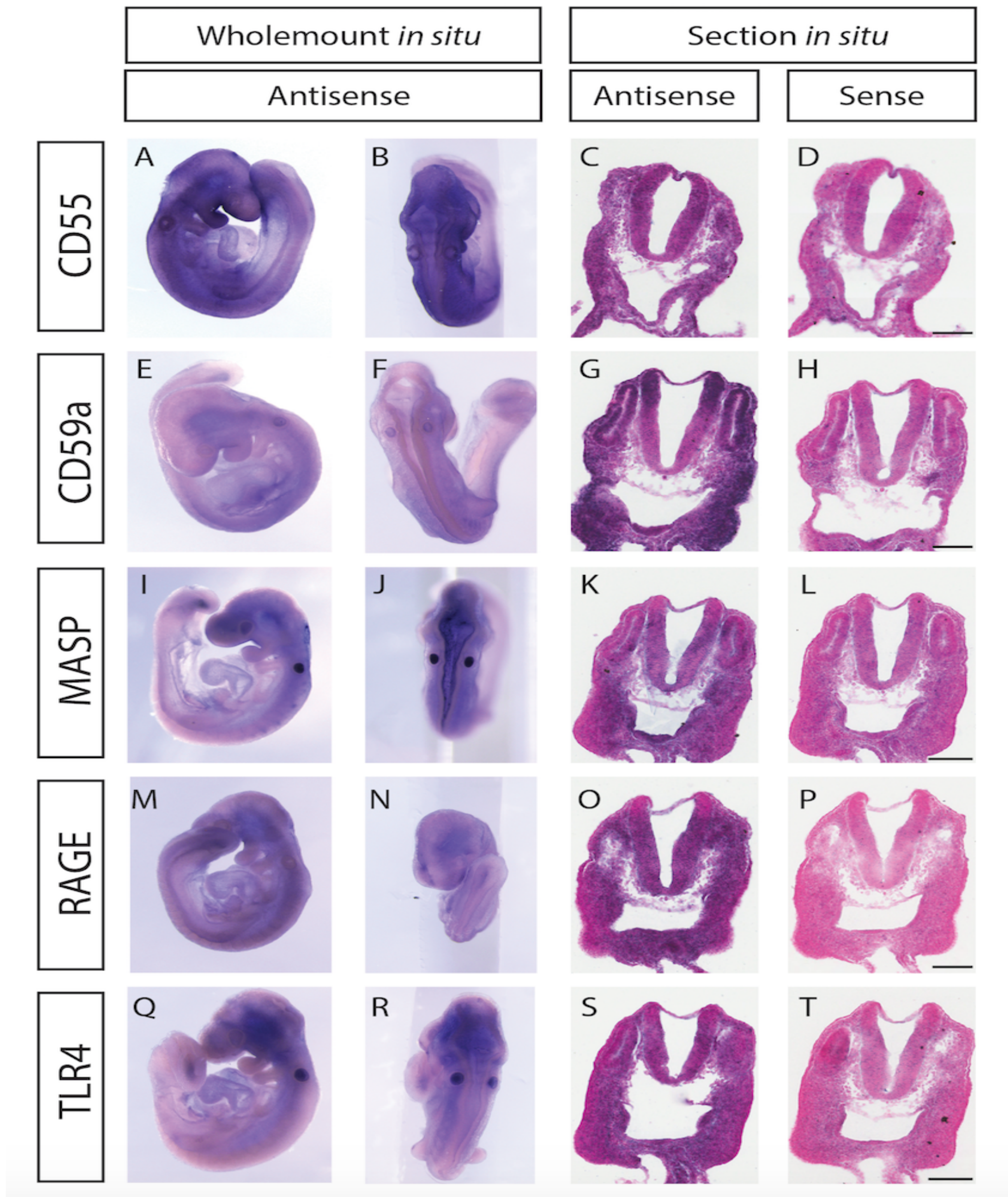


Figure 2.5.2. In situ hybridisation reveals restricted expression patterns of innate immune factors in the developing mouse embryo. Wholemount and section *in situ* hybridisation was performed on E9.5 embryos for the complement regulators CD55 (A-D) and CD59a (E-H), the lectin pathway component MASP1 (I-L), and innate immune receptors RAGE (M-P) and TLR4 (Q-T). All five genes were expressed in the neuroepithelium with CD59a expression enriched in the dorsal part of the neural tube, whereas MASP1 and RAGE appeared more prominent in ventral portions of the neural tube. The sense (negative control) probe is shown in D, H, L, P, T. Section *in situs* were counterstained with nuclear fast red. Scale bar = 100 um.

9.5dpc, and was prominent in the mesoderm and ventral neural tube. The dorsal neuroectoderm and ectodermal tissues demonstrated no expression (figure 2.5.2).

3.2 Expression of central complement factors and regulators

To investigate the expression of the broader complement system, a number of factors, receptors and regulators were screened for expression during mouse neurulation. The central anaphylatoxin precursors, C3 and C5, were both expressed throughout the period of 7.5-10.5dpc (figure 2.5.3). Additionally, we evaluated the expression of the gene with designation AI182371, which is located upstream of murine C5 on chromosome 2, and annotated as *haemolytic complement-like*. AI182371 was also expressed throughout the period of neurulation (figure 2.5.3). By contrast to the central complement factors C3 and C5, the terminal complement component C9 was absent (figure 2.5.3), suggesting that formation of the MAC is not possible in the early embryo, and not the central aim of complement activation at this stage of embryogenesis. Both C3 and C5 cleavage fragments, C3a and C5a, have already been demonstrated to have extra-immune functions in developmental processes such as neural crest cell migration (Carmona-Fontaine et al., 2011) and proliferation of neural progenitors in rat cerebellum (Bénard et al., 2004).

Other receptors that mediate the effects of complement factors were evaluated for expression in mouse embryos. C1qR expression was detectable throughout the period of 7.5-10.5dpc, whereas the expression of CD11b, a component of complement receptor 3 (CR3), was only evident towards the end of neurulation (9.5-10.5dpc) (figure 2.5.3). We have previously demonstrated the presence and a function for C5aR1 during this period of development (Denny et al., 2013a), however here we now show that the related complement peptide receptors C3aR and C5aR2 (C5L2) are also expressed throughout neurulation (figure 2.5.3). The expression of these receptors, in coordination with their respective ligands (C1q, C3, C5), suggests they may transduce signals from generated complement components during this period of embryogenesis.

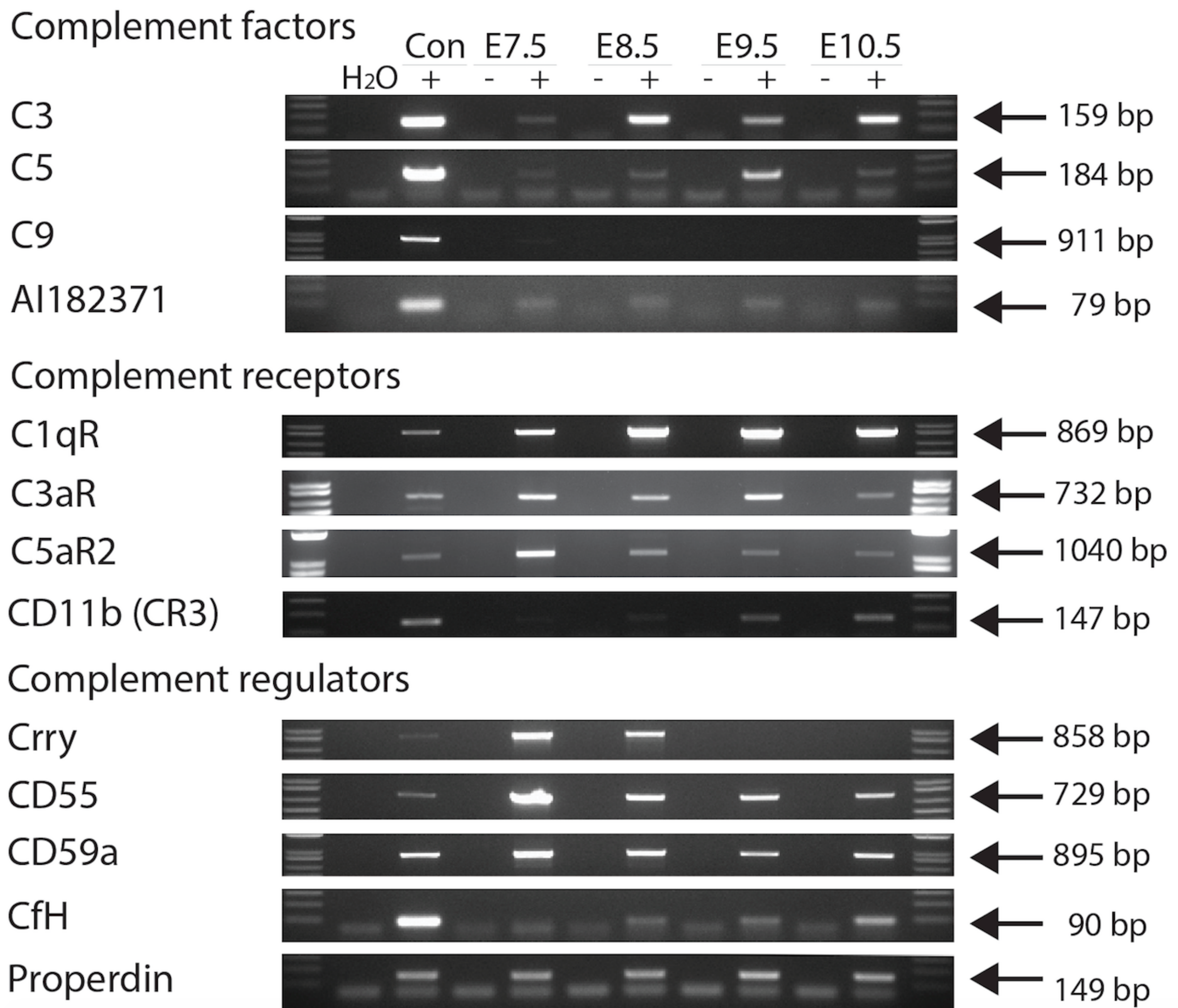


Figure 2.5.3. Complement factors and regulators are expressed during the period of neurulation. RT-PCR analysis was conducted on embryos ranging from E7.5 to E10.5. '-' represents a negative reverse transcriptase control. 'Con' represents a positive control tissue relevant for the gene of interest. Arrows indicate expected band and size. C3, complement factor 3; C5, complement factor 5; C9, complement factor 9; AI182371, haemolytic complement-like factor; C1qR, complement factor C1q receptor; C3aR, complement factor 3a receptor; C5aR2, complement factor 5a receptor 2; CD11b, cluster of differentiation molecule 11B (integrin alpha M); Crry, complement receptor 1-related gene/protein y; CD55, cluster of differentiation molecule 55 (decay-accelerating factor, DAF); CD59a, cluster of differentiation molecule 59a (membrane attack complex inhibition factor); CfH, complement factor H; Properdin (factor P).

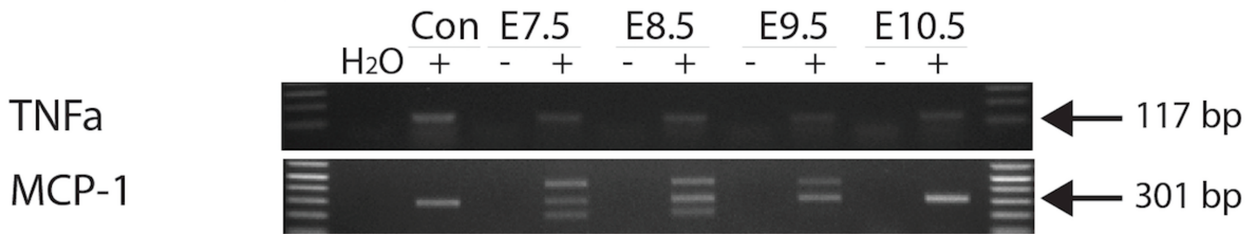
Finally, the complement system is under tight regulation by a range of factors that either enhance or dampen convertase formation or activity. CD55, CD59a, complement factor H (CfH) and properdin were all expressed throughout the period of neurulation (figure 2.5.3). Complement receptor-1 related gene/protein Y (Crry), which functions to inhibit the formation of the C3 convertase, was only detectable at 7.5 and 8.5dpc. Furthermore, both CD55 (a C3/C5 convertase inhibitor) and CD59a (a terminal MAC inhibitor) had a broad expression pattern in embryos at 9.5dpc, as shown by *in situ* hybridisation (figure 2.5.2). Interestingly, in the neural tube, while CD55 appeared to be

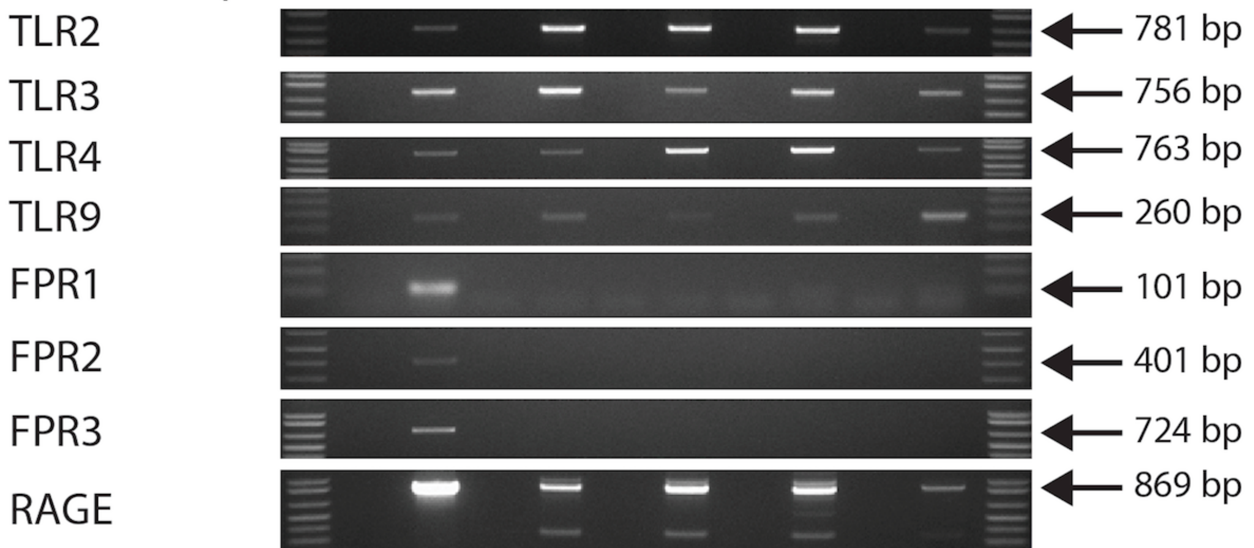
evenly expressed throughout, CD59a appeared to be enriched in the dorsal half of the neural tube (figure 2.5.2). Given the absence of C9 at these stages of embryogenesis, this suggests CD59a may play a role in development that is unrelated to its role in regulation of the complement terminal complex.

Collectively, these data suggest that many of the components of the complement system, including activators, factors, receptors and regulators, are expressed throughout the period of neurulation in the developing mouse. However, not all factors necessary for the propagation of the complement pathways of innate immunity (classical, lectin, alternative) are present at this period of development (figure 2.5.5). We therefore postulate that a MASP-1-mediated pathway, or activation by extrinsic proteases, may mediate the formation of C3a and C5a, in these early stages of development.

3.3 Expression of other innate immune system factors

Our studies also highlight the presence of other innate immune factors during the period of neurulation in the mouse. TLR-2, -3, -4 and -9 were all found to be present throughout this period (figure 2.5.4). Expression of TLR4 was demonstrated by *in situ* hybridisation at 9.5dpc and showed a broad expression pattern, particularly concentrated in the cephalic regions (figure 2.5.2). These results are consistent with previously demonstrated roles in development for these receptors, however, to our knowledge this is the earliest demonstrated mammalian expression within the available literature. Expression of TLR-2, -3, -4 and -9 has previously been established in the embryonic brain for 13.5dpc onwards (Kaul et al., 2012). Functionally, TLR-2 and TLR-3 signalling has been shown to inhibit neurogenesis at later stages of embryonic development (Lathia et al., 2008; Okun et al., 2010). A functional role for TLR-4 and -9 in mouse embryogenesis has yet to be elucidated. TNFα was detected by RT-PCR from 7.5-10.5dpc (figure 2.5.4). Expression of TNFα has previously been demonstrated in 10dpc embryos (Chie et al., 1994). However, little is known of the function of this molecule during embryogenesis. TNFα knockout mice have no reported developmental abnormalities and do not differ in embryonic weight or litter size when compared with their wild-type cousins (Pasparakis et al., 1996). Whilst TNFα has a demonstrated role in miscarriage, its presence during normal development in embryonic tissues suggests an as yet unknown role for this factor (figure 2.5.4).





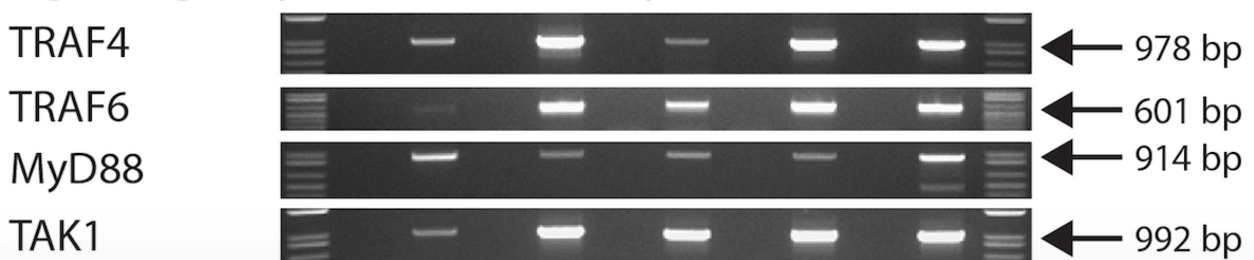


Figure 2.5.4. Expression of innate immune factors and signalling adaptors during the period of neurulation. RT-PCR analysis was conducted on embryos ranging from E7.5 to E10.5. '-' represents a negative reverse transcriptase control. 'Con' represents a positive control tissue relevant for the gene of interest. Arrows indicate expected band and size. TNFa, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; TLR, toll-like receptor; FPR, N-formyl peptide receptor; RAGE, receptor for advanced glycation endproducts; TRAF, TNF receptor associated factor; MyD88, myeloid differentiation primary response gene 88; TAK1, transforming growth factor-beta-activated

To our knowledge, this is the earliest demonstrated expression of MCP-1/CCL-2 during embryogenesis, detectable at 7.5-10.5dpc (figure 2.5.4). MCP-1 has previously been assayed at the blastocyst stage of development, and was found to be undetectable, however the blastocyst was found to exhibit control over MCP-1 production in endometrial cultures (Caballero-Campo et al., 2002). Interestingly, polymorphisms of the *CCL*-2 gene resulting in lower MCP-1 serum

concentrations have been linked to increased risk of neural tube defects in human populations (Lu et al., 2008). However, previous experiments in our laboratory have been unable to demonstrate an increased risk of neural tube defects in MCP-1 deficient mice even in the presence of maternal folate-deficiency as an environmental stressor (*unpublished data*). Interestingly, multiple bands were detectable with these primers at all ages except 10.5dpc. As these extra bands were not present in the liver or 10.5dpc cDNA, they may represent other isoforms of MCP-1 that are restricted in terms of their temporal expression during embryonic development.

The formyl-peptide receptor family, like TLRs, responds to endogenous damage associated molecular patterns, and functions in the immune response to bacterial infection (Le et al., 2002). Although FPR expression has been demonstrated in human umbilical vein endothelial cells, no reports of FPR expression in development exist (Lee et al., 2008). In the present study, we assayed the three known members of the formyl peptide receptor family for expression during the period of mouse neurulation by RT-PCR. Interestingly, despite abundant expression of complement factors and toll-like receptors, we found no evidence of expression for FPR-1, -2 or -3 from 7.5-10.5dpc in the mouse. The absence of these receptors, and the presence of other immune factors, suggests an orchestrated pattern of immune molecule expression, as opposed to embryonic expression of the adult immune system. Interestingly, the FPR genes and complement receptor C5aR1 are grouped into the same sub-family of G-protein-coupled receptors, based on sequence homology. That C5aR1 is expressed during neurulation and the FPRs are not, demonstrates specificity in embryonic expression of C5aR1 rather than widespread expression of innate immune factors in general.

RAGE has previously been noted to be expressed in the later stages of rodent development, and has a functional role in neurogenesis in this period (Fang et al., 2012; Hori et al., 1995). Additionally, RAGE is also present in the early placenta and blastocyst stages of development (Haucke et al., 2014; Konishi et al., 2004). The present study has demonstrated through RT-PCR that RAGE is also expressed throughout the period of neurulation in mouse development (figure 2.5.4). Additionally, *in situ* hybridisation analysis revealed expression in a range of tissues at 9.5dpc, particularly in the mesoderm and ventral neural tube. No expression was evident in the dorsal neural tube (figure 2.5.2).

In the immune context, many of the innate immune receptors we have shown to be expressed in the early embryo, require a number of intracellular second messenger signalling molecules. Thus, we finally investigated the transcripts of four common adaptor proteins during neurulation (TRAF-4, TRAF-6, TAK-1, MyD88) and found all to be expressed throughout this developmental period

(figure 2.5.3). This suggests that many of these innate immune receptors may in fact utilise these same second messengers for signalling during embryonic development, as they do in their immune context.

This study raises the major outstanding question of function for these molecules at this point in development; what does expression of these factors mean in a biological context? In terms of the complement components, we have provided evidence that the generation of the anaphylatoxins C3a and C5a may be via restricted pathways, lectin and extrinsic protease, during neurulation, as there are significant factors required for canonical pathway propagation that are lacking (figure 2.5.5). This may be due to the anaphylatoxins merely acting as signalling cues in development, rather than having to respond to a wide variety of stimuli and insults, as in the context of mounting an immune response. However, this study does not preclude the transfer of maternal circulating complement components into the embryonic tissue, which may rescue potential deficiencies in canonical complement activation. However, this scenario raises the further question of why some, but not all, complement pathway components are produced by the embryo, and specifically why there is an absence of factors central to canonical complement activation and propagation. Importantly, complement expression is evident in tissues other than the liver, which supplies much of the circulating complement factors in the adult. These data, coupled with emerging reports demonstrating novel roles for a diaspora of innate immune factors in development (Carmona-Fontaine et al., 2011; Denny et al., 2013a; Hawksworth et al., 2014), suggest that we look again at the function of innate immune factors during neurulation.

Figure 2.5.5. Diagrammatic representation of active complement pathways during mouse neurulation. The three canonical complement activation pathways (classical, alternative, lectin) are depicted. Green boxes indicate factors that are expressed throughout the period of neurulation, orange boxes indicate variable expression and red boxes indicate factors that were not detected during this period. Black arrows demonstrate theoretically intact complement pathways, whereas grey arrows demonstrate pathways that are limited by the non-expression of complement factors. Importantly, only the C2/4-independent lectin pathway and C3/5 cleavage by intrinsic proteases are theoretically functional at this stage of development. Formation of the membrane attack complex is limited by a lack of detectable C9 expression.

2.6 Conclusions

This study highlights the expression of innate immune molecules throughout the period of mouse neural tube closure. The expression of these factors appears before the development of the murine immune system and suggests alternative functions within this period of development, although maternal supplementation of the absent factors cannot be excluded. Whilst the functions of these molecules at this juncture remains to be determined, and the corollary of human expression confirmed, their presence could potentially inform the use of anti-inflammatory therapeutics targeted towards these factors in pregnancy. We have previously shown that inhibition of complement C5a activity throughout this embryonic period induced severe neurulation-related defects when combined with a folate-deficient diet (Denny et al., 2013a), and it is not inconceivable that the milieu of innate immune molecules identified within this study could have roles of similar importance. Given that the developmental role of C5aR1 was only uncovered under conditions of folate deficiency, it would be of interest in future studies to re-examine the knockout or pharmacological inhibition of other immune factors under similar types of stressors in order to evaluate whether novel gene-environment interactions additionally exist for these factors.

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Chapter 3

Development of a Transgenic Mouse for the Purpose of Lineage Tracing C5aR-Expressing Neural Progenitors

3.1 Introduction

A desirable tool in developmental biology is one that allows for the marking of specific cells and their progeny in order to discern the effects of stimuli on the fate of cells. To this end, this PhD project aimed to develop a transgenic mouse model that allowed for lineage tracing of C5aR positive cells, specifically for demonstrating the effect of C5aR modulation during neurogenesis.

The Structure of murine C5ar1

The genetic structure of *C5ar1* has been reviewed in chapter 1.1, however a brief summary is contained here. *C5ar1* is comprised of two exons and a 10kb intron. The initial exon contains a 131bp 5' UTR and the first codon of the coding sequence (ATG). Exon 2 contains the remainder of the C5aR sequence and a relatively large (~1.5kb) 3'UTR. The promotor region of *C5ar1* is ill-defined, but significant regulatory sequences are present within the 500bp prior to the transcriptional start point; these include C/EB, AP-4 and NFκB-binding site sequences[1]. Importantly, the importance of each regulatory motif differs between cell type, and suggests a complex, multifaceted expression pattern[1].

Methods of creating a transgenic mouse

Two broad methods exist for inserting a transgene into an the genome of an organism. The first involves random recombination (RR) of the transgene into the genome and the second uses targeted homologous recombination (HR) to modify existing genes. There are both advantages and drawbacks to each method.

The initial attraction to the HR method is due to the targeted approach, the ability to predict and test where the transgene will insert into the genome has the advantage of reducing the risk of adverse outcomes for the mouse line. The RR method however, runs the risk of inserting within essential portions of the genome that may be involved in any aspect of the resulting organisms physiology. This has the potential to distort future experimental results that subsequently become attributed to the expression of the transgene rather than its unfortunate insertion in the genome. Consequently, the researcher must be aware of this limitation when designing experiments with this type of transgenic animal.

The advantage of the HR method in terms of targeted insertion also comes with the caveat that there will invariably be loss or alteration of endogenous gene expression. A previous attempt at creating a

C5ar1-IRES-GFP transgenic mouse by utilising the HR method was frustrated by the aberrant expression of C5aR in the transgenic lines. In this mouse the researchers had attempted to control GFP expression from the C5aR promoter without compromising endogenous C5aR expression, however they discovered that the alteration of 3' genomic sequences resulted in an intracellular expression of C5aR protein, rather than the natural cell surface expression[2]. The alteration of endogenous gene expression is not a desirable characteristic of a mouse designed to study the physiological or pathophysiological role of the gene product and, in this instance, the RR method has the advantage.

Additionally, the HR method, by virtue of its targeted nature, is limited to two copies of the transgene existing in the genome of the transgenic animal. The RR method, by contrast, has the potential for the insertion of hundreds of transgene copies into the genome, this may provide for greater sensitivity in the case of low-expression genes.

Initial attempts at achieving a lineage tracing mouse

In order to achieve this aim this project employed the modified BAC method of inserting the desired transgene. This method is useful as it allows for expression of the transgene from the *C5ar1* promoter without compromising expression of endogenous C5aR. The transgene comprised an oestrogen-inducible cre recombinase and enhanced green fluorescent protein separated by an internal ribosome entry site (CreErt2-IRES-EGFP). In addition, a floxed kanamycin/neomycin resistance cassette was included to allow for *in vitro* selection of successful recombination. The insertion site for the transgene was the beginning of the second exon of C5aR immediately after the termination of the single 10kb intron. This site was chosen as earlier experiments using 15kb of the 5' sequence immediately before the first exon inserted into the linear pJazz vector failed to yield any demonstrable expression when transfected into known C5aR-expressing cell lines (*data not shown*). From this initial attempt, we hypothesised that the 10kb intron in the *C5ar1* sequence may contain elements essential for the efficient transcription of C5aR and therefore decided upon the BAC method to retain more of the peripheral motifs surrounding the *C5ar1* gene.

3.2 Methods

Plasmids

The plasmids used in this chapter are presented in table 3.2.1. BAC clone RP24-556B7 was obtained from the Children's Hospital of Oakland Research Institute (CHORI), containing the genomic sequence for *C5ar1* and surrounding regions of chromosome 7. pSC101-BAD-gam-tet was a kind gift from Dr David Simmons, and is commercially available as part of the Red/ET recombination system (GeneBridges). The transgene was generated through standard cloning techniques to incorporate elements of pIRES-EGFP, pErt2CreErt2 and pML3 in order to generate a sequence of an oestrogen-inducible cre recombinase (Ert2CreErt2), internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) under the control of a CMV promotor, with a distal floxed kanamycin/neomycin resistance cassette (FLP-kan/neo-FLP) (plasmid 7, table 3.2.1).

Table 3.2.1: Description of plasmids utilised in generation of the transgene

#	Plasmid Name	Source	Description	Antibiotic Resistance
1	BAC – clone RP24-556B7	BACPAC (CHORI, California, USA)	Bacterial artificial chromosome of the region of mouse chromosome 7 containing *C5ar1*	Chloramphenicol
2	pSC101-BAD-gam-tet	Dr David Simmons	Commercially available plasmid expressing proteins that mediate recombination. Temperature sensitive *ori* allows for propagation at 30°C	Tetracycline
3	pIRES-EGFP	Dr David Simmons	Expression of EGFP under control of CMV promotor. EGFP is proceeded by an multiple coloning site/internal ribosome entry sequence allowing for addition of a coexpressed protein	Ampicillin
4	pErt2CreErt2	Dr David Simmons	Expression of tamoxifen-inducible cre recombinase under the control of CMV promotor	Ampicillin
5	pML3	Dr David Simmons	Contains FLP-kan/neo-FLP to aid in transgene selection through antibiotic resistance	Ampicillin/Kanamycin
6	pErt2CreErt2-IRES-EGFP	Generated from plasmids 3 & 4	Ert2CreErt2 sequence inserted within the MCS of pIRES-EGFP	Ampicillin
7	pML3-Ert2CreErt2-IRES-EGFP	Generated from plasmids 5 & 6	Ert2CreErt2-IRES-EGFP sequence inserted proximal to FLP-kan/neo-FLP sequence to provide template for transgene amplification	Ampicillin/Kanamycin

HeLa cell culture and transfections

HeLa cells expressing a cre-responsive DsRed-nuc reporter gene were cultured at 37°C/5% CO_2 in Dulbecco's modified eagle medium (DMEM, Sigma) supplemented with 10% fetal calf serum and 100U/mL penicillin / 100μg/mL streptomycin. For transfection, cells were grown to 70% confluency in 24 well plates and transfected using the Lipofectamine 3000 lipid-mediated transfection system (Life Technologies) according to manufacturer's instructions. Cells were examined for the presence of fluorescence 24-48 hours post-transfection under fluorescent microscopy. To determine the functionality of plasmids containing an oestrogen-inducible cre recombinase, cultures were supplemented with 1μM 4-OH-tamoxifen and observed for the development of nucleus-localised red fluorescence.

Generation of transgene through PCR

The transgene was amplified from pML3-Ert2CreErt2-IRES-EGFP using primers directed to the start sequence of the Cre recombinase and just distal to the second FLP of the kan/neo resistance cassette. The forward primer was supplemented at the 5' end with a 50bp homology sequence corresponding to the intronic sequence just proximal to the beginning of the second exon of *C5ar1*. The reverse primer was similarly supplemented at the 5' end with a 50bp homology sequence complementary to the initial 50bp of exon 2. Primer sequences are listed as primer set 1 in table 3.2.2. Amplification was achieved over 35 cycles with Phusion high-fidelity taq using an annealing temperature of 63°C and an extended extension time of 5 minutes to account for the amplicon length of 5kb. The resulting product was isolated using 1% agarose gel electrophoresis and a Zymoclean gel extraction kit (Zymo research). Concentration was assessed using a nanodrop spectrophotometer (Thermo Scientific).

Recombination

BAC-containing *E. coli* were grown inoculated into LB media containing 25μg/mL chloramphericol (Sigma) and grown to logarithmic growth phase (2.5hrs, 37°C, 225rpm). Cells were centrifuged and washed twice before resuspension in 50μL ice-cold dH_2O. The resuspension was then complemented with 50ng pSC101-BAD-gam-tet plasmid and incubated on ice for 5 minutes before transfer to 1mm electroporation cuvettes. The cell-plasmid suspension was electroporated using the GenePulser system (Biorad) with settings as follows; 1800V, 25μF, 200Ω. This method and settings was also used for all subsequent bacterial electroporations. Post-electroporation, 950μL SOC media (NEB) was added and the cell suspension incubated at 30°C, 225rpm for 70 minutes. The culture was then plated onto pre-warmed LB plates containing

25µg/mL chloramphenicol/15µg/mL tetracycline for growth overnight at 30°C. Lower growth temperatures after transformation with pSC101-BAD-gam-tet allows for plasmid replication through the temperature sensitive *ori* site. Viable colonies were expanded in 3mL cultures overnight at 30°C and used to inoculate 1.4mL LB broth cultures for growth at 30°C. After two hours cultures were supplemented with 10% arabinose solution and incubated for a further hour at 37°C to induce expression of the mediator proteins required for recombination from the pSC101-BAD-gam-tet plasmid. Cultures were then collected and

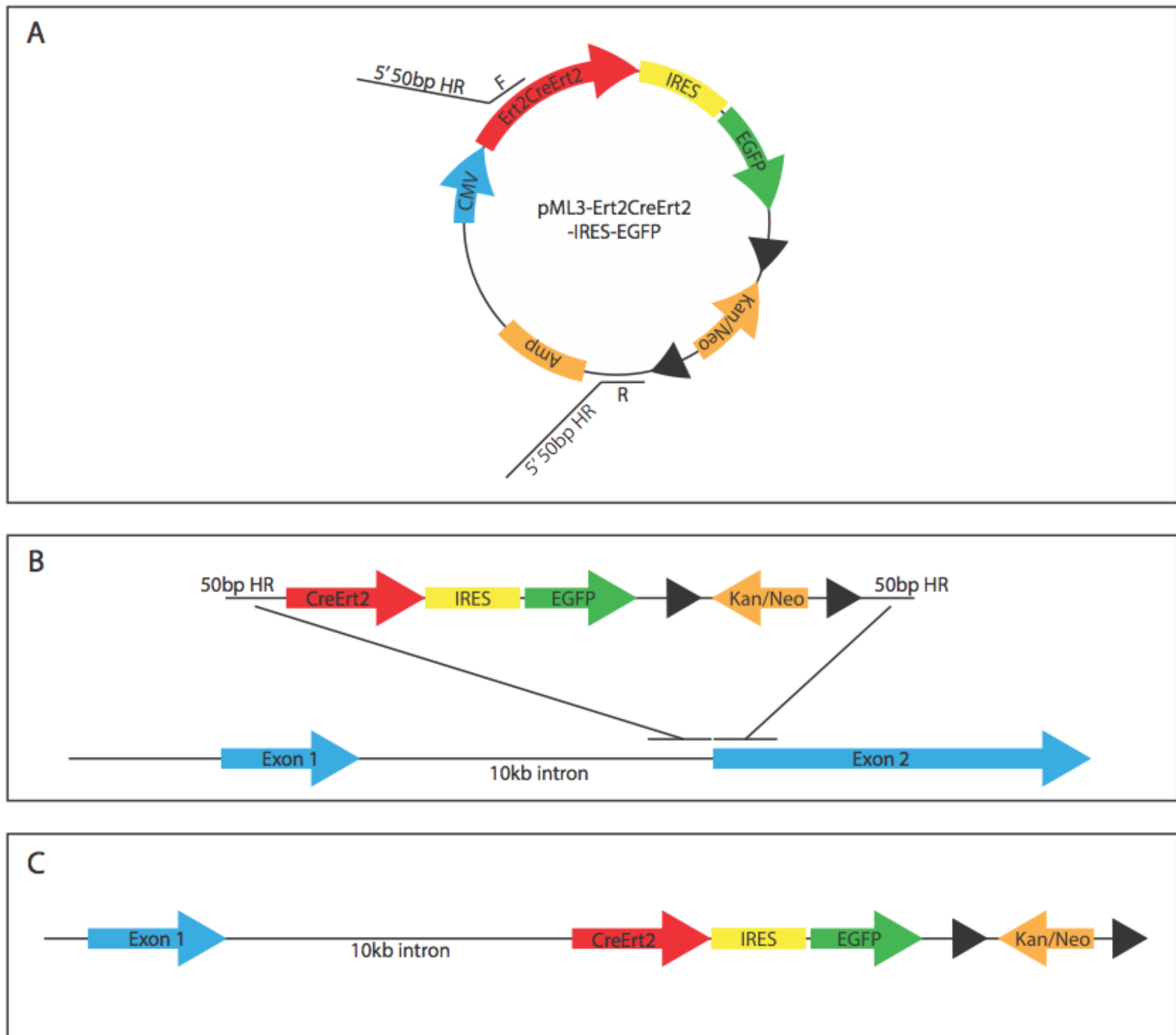

Figure 3.2.1: **Diagram demonstrating the recombination method used in creating the transgene.** (A) Template plasmid indicating sites used for primer amplification of transgene, primers contained 5' 50bp homology regions to the insertion site in the BAC. (B) *C5ar1* genomic sequence (below) and transgene (above) indicating the site of insertion at the start point of exon 2. (C) Final sequence of recombineered BAC at the *C5ar1* locus.

electroporated with 50ng of the transgene product, as previously described, before plating for overnight growth on LB agar containing 25µg/mL chloramphenicol/50µg/mL kanamycin to select for bacterial cells that had successfully recombined the transgene into the BAC (figure 3.2.1). Resulting colonies were screened for correct recombination by PCR using primers directed against

the transgene and known surrounding *C5ar1* sequence (table 3.2.2; primer set 3). Correct recombination was confirmed through sequencing of the PCR products (table 3.2.2; primer 4) at the Australian Genome Research Facility (AGRF).

Pronuclear injection

The recombineered BAC was prepared for pronuclear injection by linearisation and dialysis with microinjection solution (10mM Tris HCl, 0.1mM EDTA, 100mM NaCl, 30µM spermine, 70µM spermidine, pH 7.5) across a 0.05µm membrane (Millipore). The resulting solution was diluted in microinjection solution to a concentration of 1ng/µL and delivered to the Transgenic Animal Service of Queensland (TASQ) to perform the pronuclear injections. Four viable mouse lines resulted from pronuclear injection of the recombineered BAC and were screened for presence and function of the transgene.

Tissue collection and tamoxifen treatment

To assess for the genomic presence of the transgene within the mouse lines, tail-tips were digested in DNA digestion buffer (50mM Tris-HCl, 100mM EDTA, 100mM NaCl, 1% SDS, pH 8.0) supplemented with 0.5mg/mL proteinase K (Sigma) overnight at 55°C. PCR utilising transgene specific primers, aligned with the cre-recombinase sequence (table 3.2.2; primer set 2), was used to assess for the presence or absence of the transgene within the crude lysates. Mice in all colonies were bred to produce heterozygous or wild-type progeny through the mating of a wild-type C57BL6 male to heterozygous females.

For *in situ hybridisation*, tissues were fixed via intracardiac perfusion of DEPC treated 4% paraformaldehyde (PFA), collected and sectioned at 16µm using a cryostat. For RNA or protein isolation, mice were sacrificed via cervical dislocation and tissue samples snap-frozen in liquid nitrogen.

For tamoxifen treatment to induce cre-mediated recombination, heterozygous female mice were bred to males of the cre-reporter line, B6;129S6-Gt(ROSA)26Sor$^{tm(CAG\text{-}tdTomato)Hze}$/J, a kind gift from the A/Prof Linda Richards (Queensland Brain Institute). Offsping were genotyped for the presence of both the cre transgene and the tdTomato-cre responsive allele. Eight week-old mice positive for Cre/tdTomato or tdTomato alone were administered 75mg/kg tamoxifen (Sigma Aldrich, USA) dissolved in corn oil, or corn oil alone as a vehicle control, by intraperitoneal injection over a five day period. Mice were perfused by intracardiac injection of 4% PFA seven days after the last

tamoxifen administration. Brain, kidney, liver and spleen tissue samples were isolated into 4% PFA for histological analysis.

RT-PCR

RNA was isolated from snap-frozen tissues using an RNeasy column purification kit (QIAGEN) and DNase treated with TurboDNase (Ambion) to remove contaminating genomic DNA. RNA was reverse transcribed to cDNA with tetro reverse transcriptase (Bioline) for use in RT-PCR analysis. Presence of transgene transcript was assessed in mice determined heterozygous for the transgene through genotyping and wild-type littermates were utilised as a negative control. PCR for cre recombinase was performed as outlined in table 3.2.2 (primer set 2) and PCR products were analysed using 1% agarose gel electrophoresis.

Table 3.2.2; A detailed listing of primer sets used in this chapter.

#	Name	Forward Sequence	Reverse Sequence	Amp. length	Anneal. temp.
1	Transgene	CTGGGAATGTGTCTACCAACTCACA CAATCTACCTGTTTGATTTGCTTAGG CT GGA GAC ATG AGA GCT GCC	GTTCCATAGTGATCATAGTTGATTTC AAAGCTGCTGTTATCTATGGGGTCCT TTCGATTGACGGTCGAGGAAGTT	5kb	62°C
2	Cre	CGTACTGACGGTGGGAGAAT	TGCATGATCTCCGGTATTGA	500bp	60°C
3	BAC screen	AGAGGAGGTCGGCCACCG	CGGGATGGGCGGAGTTAG	624bp	60°C
4	BAC Seq.	AGTAGATGGACCACCATGAT		N/A	60°C
5	C5ar1 RT-PCR	GCTGGTTACCACAGAACCCAGG	CGGTACACGAAGGATGGAATGG	559bp	63°C
6	C5ar1 qPCR	GGGATGTTGCAGCCCTTATCA	CGCCAGATTCAGAAACCAGATG	131bp	60°C
7	Cre qPCR	CGTACTGACGGTGGGAGAAT	CCCGGCAAAACAGGTAGTTA	166bp	60°C
8	r18s qPCR	GATCCATTGGAGGGCAAGTCT	CCAAGATCCAACTACGAGCTT	103bp	60°C

LPS injections and qPCR analysis

Heterozygous males from the transgenic colony were subjected to intraperitoneal injection of 1mg/kg lipopolysaccharide in 100µL dH_2O, in order to promote a systemic inflammatory response or 100µL dH_2O as a sham control. Mice were sacrificed after 24 hours by cervical dislocation and tissues snap-frozen for RNA analysis. RNA was isolated as previously described and resulting cDNA used to determine relatively transcript levels of *C5ar1* (table 3.2.2; primer set 6) and cre recombinase (table 3.2.2; primer set 7). Transcript levels were determined by qPCR with SYBR

green mastermix (Life Technologies) according to manufacturer's instructions. Relative transcript levels were normalised against levels of *r18s* (table 3.2.2; primer set 8) as an internal control.

In situ hybridisation

In situ hybridisation was performed using sense and antisense digoxigenin (DIG)-labelled cRNA probes specific for cre recombinase. Probes were generated through ligation of isolated cre PCR product (table 3.2.2; primer set 2) into the pGEM-Teasy vector system (Promega) and subsequent linearization and transcription using T7 and SP6 RNA polymerase in the presence of DIG RNA labeling mix (Roche). Tissues were pretreated with proteinase K before incubation with sense or antisense probes overnight at 65°C. The presence of hybridized cRNA was detected after washing through the use of an alkaline phosphatase conjugated antibody (Roche) specific for the DIG epitope. Slides were incubated with a BCIP/NBT alkaline phosphatase substrate solution (Roche) and monitored for colour development. Tissues were counterstained using nuclear fast red and mounted for imaging with an AT2 digital slide scanner (Aperio).

Western Blot

Proteins for western blot analysis were extracted from snap-frozen tissues through lysis in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche). Protein concentration was determined by BCA assay (Thermo scientific) and 20μg protein was loaded onto 10% acrylamide gel for electrophoresis. Separated protein samples were then blotted onto activated PDVF membrane and probed using anti-cre recombinase antibody (CST, USA) at 1:1000 dilution overnight. Following incubation with an isotype specific IRDye conjugate secondary antibody (LiCor) at 1:10,000 dilution, blots were washed and visualised on an Odyssey infrared imaging system (LiCor).

Immunofluorescence

Tissues from mice subjected to intraperitoneal of tamoxifen or corn oil solutions were prepared in 12μm sections on a cryostat. Nuclear localisation was achieved with DAPI and sections were imaged using fluorescent microscopy for the presence of tdTomato fluorescence as a marker of cre nuclear translocation. Additionally, sections were probed for the presence of C5aR using a rat anti-mouse C5aR antibody (Serotech, clone 10/92, 1:250). Sections were blocked for one hour at room temperature in blocking buffer (10% goat serum, 0.5% Triton X-100 in PBS) before overnight incubation at 4°C with primary antibody or blocking buffer alone. Alexafluor 488 goat anti-rat IgG antibody (Life Technologies, 1:1000) was used to probe for bound primary and incubated overnight at 4°C, nuclear staining with DAPI and mounting.

3.3 Results

Transfection of HeLa cells with transgene constructs

Prior to the recombination of the BAC, the construct containing the transgene was tested under the control of a CMV promotor within the pML3-Ert2CreErt2-IRES-EGFP plasmid. The preceding plasmid, lacking the floxed-kan/neo cassette was also tested as a positive control. After 48 hours HeLa cultures in both the pErt2CreErt2-IRES-EGFP and pML3-Ert2CreErt2-IRES-EGFP groups demonstrated detectable green fluorescence whereas none was detectable in the lipofectamine-only control (Non-transfected; figure 3.3.1). Addition of 1µM 4-hydroxy-tamoxifen to the cultures successfully transfected with the test plasmids caused expression of nuclear localised DsRed. Importantly, the DsRed expression occurred only within cells positive for EGFP expression, and was not evident without the addition of 4-hydroxy-tamoxifen to the cultures.

Recombination results

Recombination of the transgene into the BAC was tested by PCR amplification across the insertion site and sequencing of the PCR product. Sequencing results demonstrated satisfactory insertion of the transgene at the beginning of exon 2 of *C5ar1* (data not shown). Pronuclear injection of linearised BAC at AGRF resulted in the development of four genotypically-positive lines on a C57BL6/J background. Genotyping PCR demonstrated good distinction between heterozygous and wt/wt animals through the presence or absence of a 500bp band amplified by primer directed against the cre sequence (figure 3.3.2:A). Two lines were initially culled as no PCR gene product was detected by RT-PCR. An additional line had recombination into the locus for *Rbfox1*, an RNA splicing modulator, as determined by inverse PCR and sequencing (data not shown). This line exhibited epileptic seizures, as has previously been reported in *Rbfox1* knockout animals[3]. Therefore, the one remaining line was used for all following experiments.

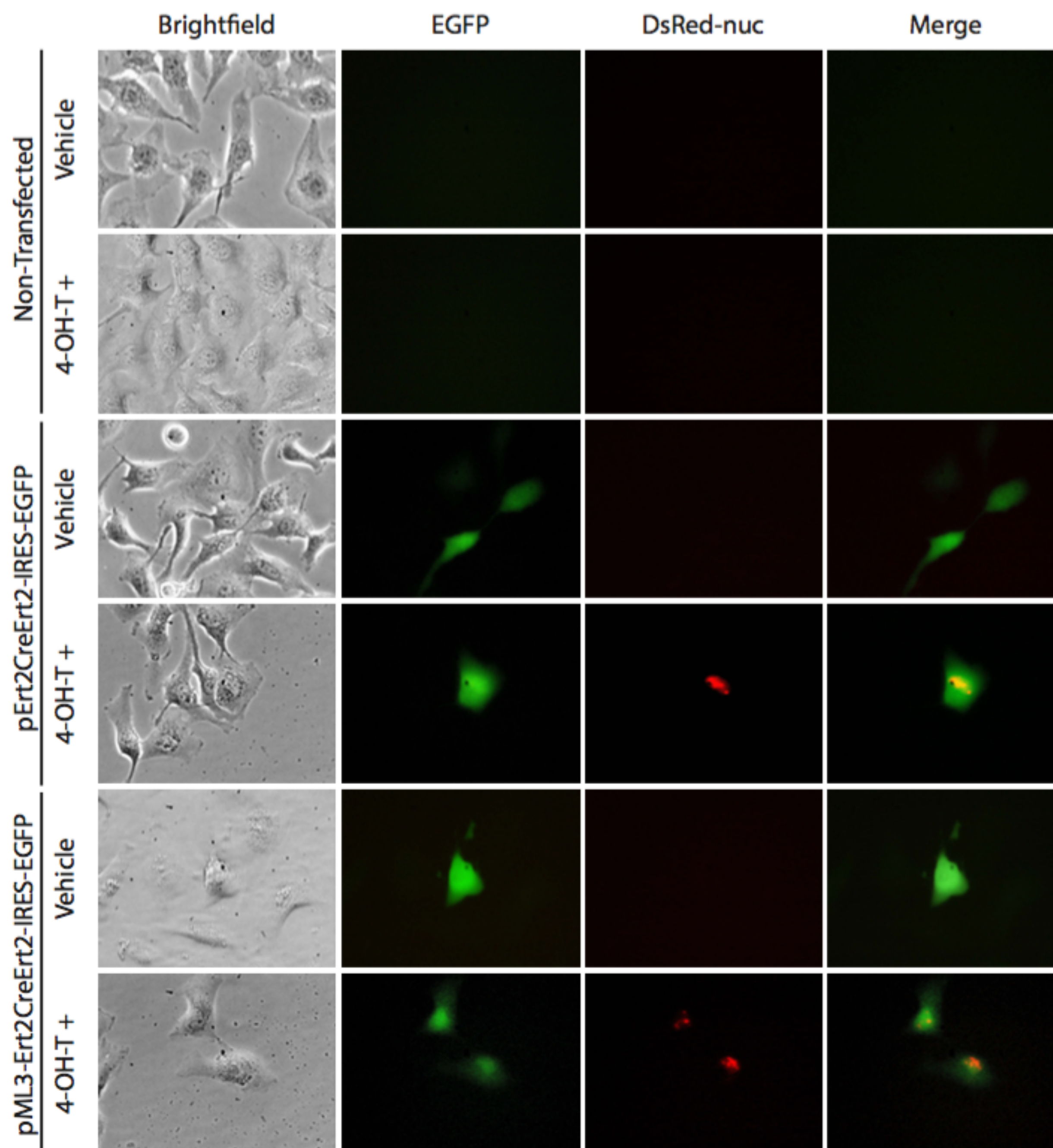

Figure 3.3.1; **Transfection of HeLa-DsRednuc cre-reporter cells with transgene constructs demonstrates effective transgene function.** Cells transfected with no plasmid (top rows), pErt2CreErt2-IRES-EGFP (middle rows) or pML3-Ert2CreErt2-IRES-EGFP (bottom rows) and treated with or without 4-hydroxytamoxifen (4-OH-T) and imaged after 48 hours in culture. Cre containing plasmids demonstrate enhanced green fluorescent protein (EGFP) expression and treatment with tamoxifen stimulates expression of nuclear localised DsRed. Brightfield images are shown to demonstrate cell location.

Testing for presence of transgenic gene product

RT-PCR demonstrated the presence of RNA transcript from the transgene within brains of cre/0 animals, whereas tissue from wt/wt animals produced no band within this region (figure 3.3.2:B). Protein expression of the CreErt2 fusion protein was also shown at 74kDa within the liver, brain and spleen of Cre/0 animals and was absent within wt/wt littermate lysates (figure 3.3.2:C).

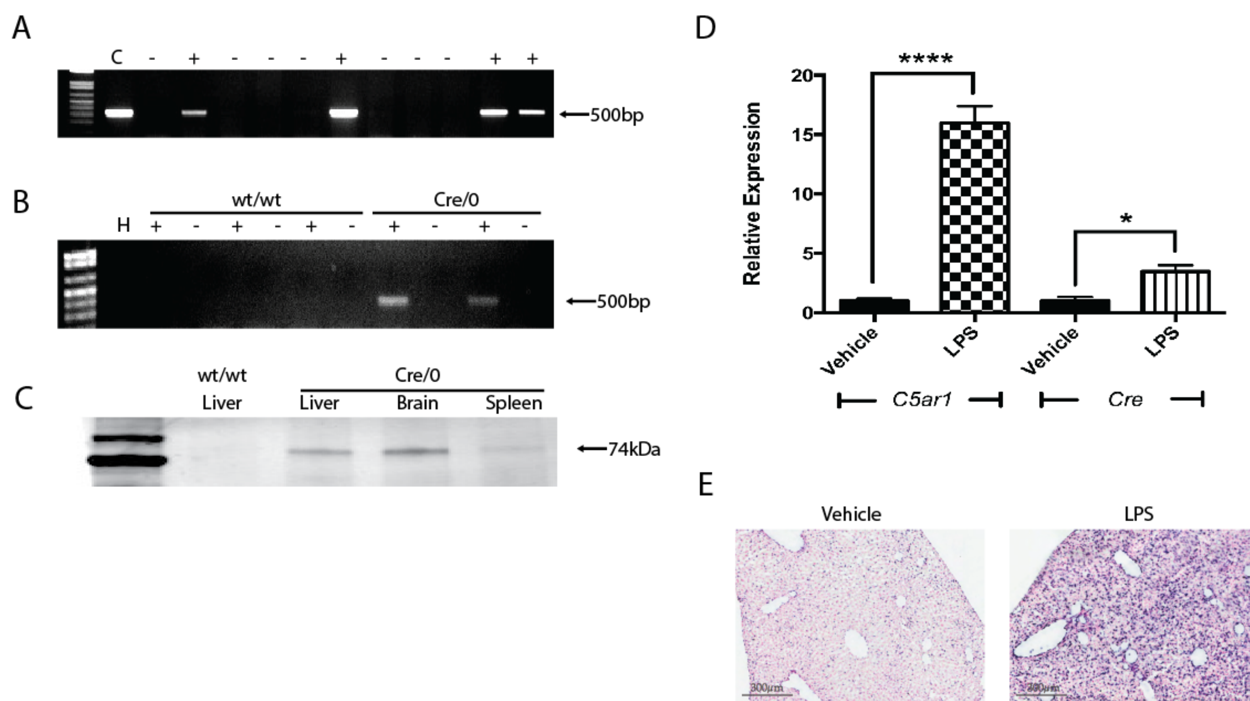

Figure 3.3.2: **Transgene is expressed in a manner similar to C5aR1**. A. Representative genotyping PCR from wt/wt x Cre/0 litter. "C" indicates control PCR with pML3-Ert2CreErt2-IRES-EGFP plasmid as template. 500bp band indicates a Cre/0 genotype, whereas absence of a band indicates a wt/wt genotype. B. RT-PCR demonstrating presence of cre transcript in cre/0 cDNA. "H" indicates dH_2O negative control, "-" indicates negative reverse transcriptase controls for gDNA contamination. C. Western blot for the presence of CreErt2 fusion protein at 74kDa. CreErt2 is present in liver, brain and spleen from Cre/0 animals and absent from wt/wt liver. D. Bar graph demonstrating the change in liver *C5ar1* and *creErt2* transcript expression by qPCR 24 hours post LPS or vehicle administration to Cre/0 mice. * indicates $p<0.05$, **** indicates $p<0.0001$. E. *In situ* hybridisation analysis of *CreErt2* transcript expression and localisation in livers of Cre/0 from experiment described in D.

Testing responsiveness to C5ar1 promotor

To determine whether RNA expression of the transgene was subject to the *C5ar1* promotor Cre/0 and wt/wt littermates were subject to intraperitoneal injection of 1mg/kg LPS. LPS produced an significant upregulation in liver *C5ar1* expression, as has previously been reported[4]. Addtionally, *Cre* transgene expression was also significantly upregulated in LPS treated mice, but did not demonstrate the same degree of change as *C5ar1* (figure 3.3.3:D). *In situ* hybridisation for *cre* in livers isolated from LPS and vehicle treated Cre/0 mice also shows an broadened and elevated level of transcript expression (figure 3.3.2:E).

Investigating transcript localization in the brain

As the aim of this thesis is to demonstrate a function of C5aR in neural progenitor cell physiology, the localisation and expression of transgene product in the adult brain was a priority. *In situ* hybridisation showed transgene expression strongest within cortical layers IV and VI (figure 3.3.3:C), the medial habenular nuclei (figure 3.3.3:D,E), the dentate gyrus and CA1 region of the hippocampus (figure 3.3.3:F,G) and the subventricular zone of the lateral ventricles (figure 3.3.3:H,I). Antisense probe on Cre/0 sections was controlled for through the use of a sense probe on Cre/0 sections (figure 3.3.3:B,C,E,G,I) and antisense probe on wt/wt sections (data not shown). Both sense and wt/wt sections demonstrated no significant staining.

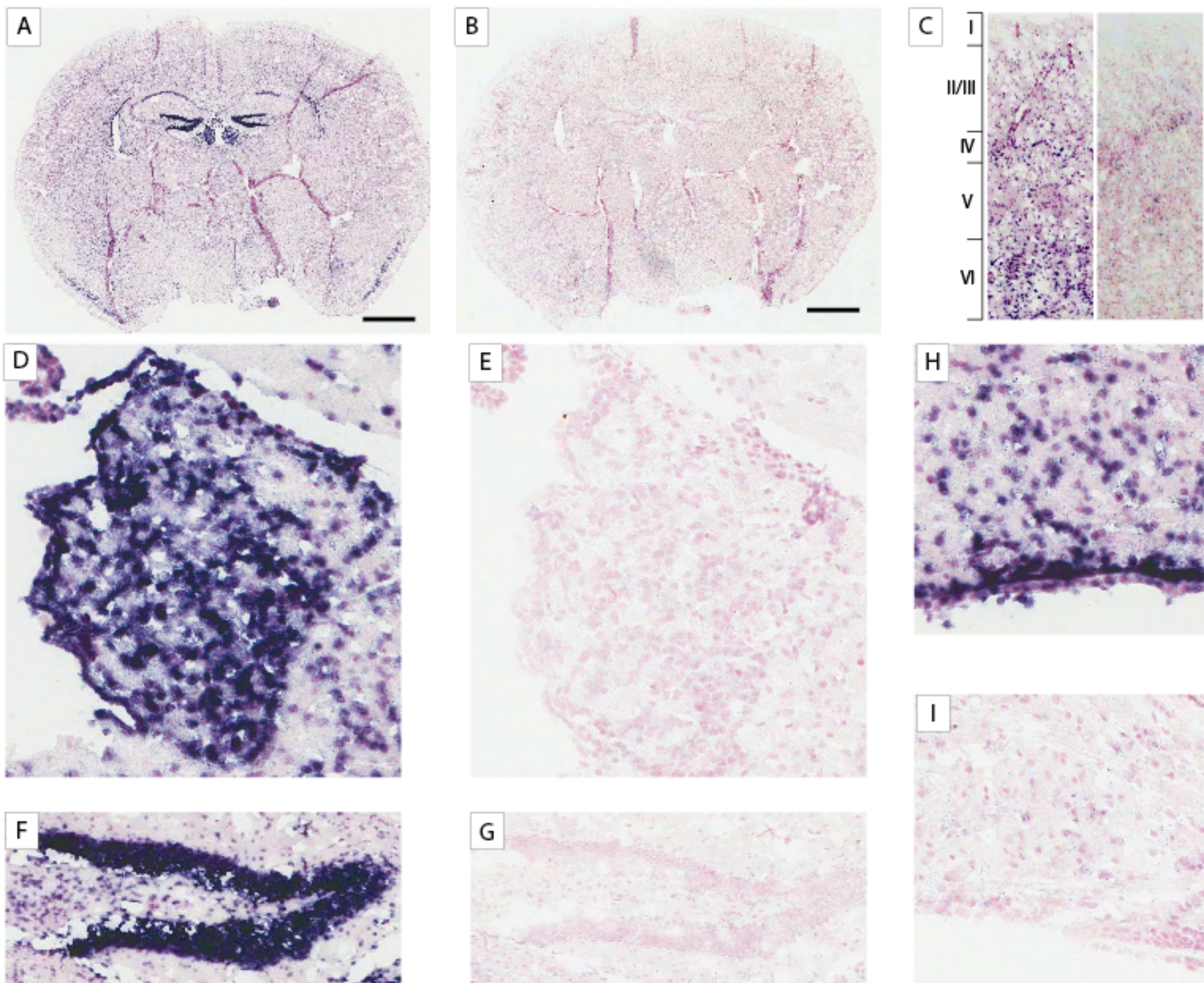

Figure 3.3.3: ***In situ* hybridisation analysis of *CreErt2* expression in the brains of Cre/0 animals.** Images represent whole brain (A, antisense; B, sense), cortical layers (C, antisense, left; sense, right), median habenular nuclei (D, antisense; E, sense), dentate gyrus of the hippocampus (F, antisense; G, sense) and subventricular zone of the lateral ventricle (H, antisense; I, sense). Approximate location of cortical layers in C is denoted by roman numerals. Scale bar in images A and B indicates 1mm.

Investigating transgene functionality

Transgene functionality was investigated through the use of tamoxifen or vehicle intraperitoneal injection over a period of five days administered to C5aR-CreErt2-IRES-EGFP crossed with a tdTomato Cre reporter mouse, or reporter mouse alone. Sections of brain, kidney, liver and spleen did not demonstrate tdTomato or EGFP fluorescence in all samples, despite being all being positive for C5aR immunostaining (brain and kidney shown; figure 3.3.4). The medullary region of the kidney, and choroid plexus is displayed. C5aR positive cells in these areas likely represent capillary endothelial-cells.

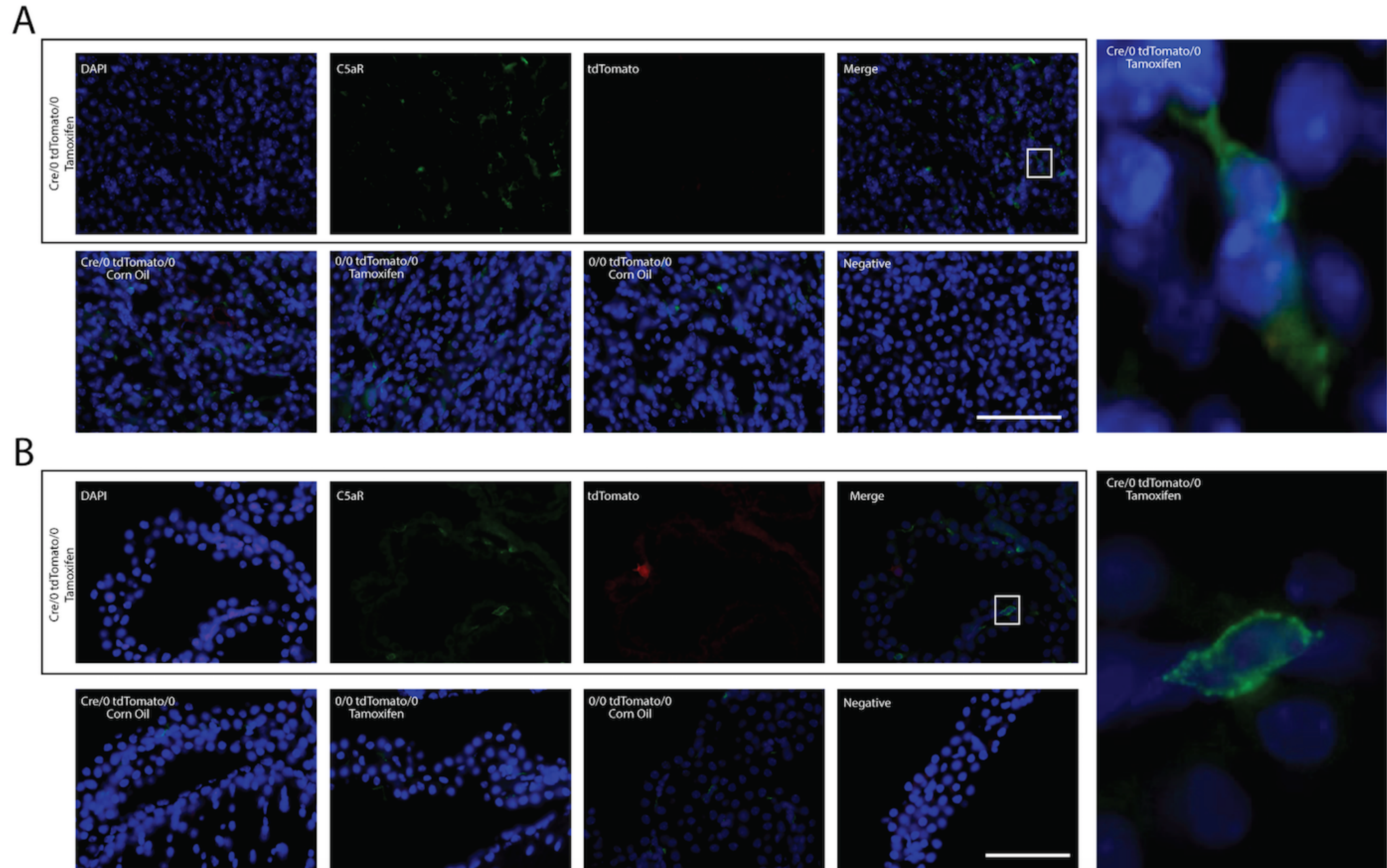

Figure 3.3.4; Immunofluorescent analysis of cre functionalty. Mice heterozygous for C5aR-CreErt2-IRES-EGFP (Cre) crossed with homozygous tdTomato cre reporter line, B6;129S6-Gt(ROSA)26Sor$^{tm(CAG-tdTomato)Hze}$/J (tdTomato), generated Cre/0 tdTomato/0 double positive mice and 0/0 tdTomato single transgenics. Both genotypes were administered 75mg/kg/day tamoxifen or vehicle control (corn oil) for five days. A) Representative kidney sections at the medulla co-labelled for C5aR, large right panel is enlarged area demonstrated by the white box in merge panel. B) Representative sections of the choroid plexus co-labelled for C5aR, large right panel is enlarged area demonstrated by the white box in merge panel. White scale bars in negative control images indicate distance of 100µm.

3.4 Discussion

There is a need for genetic models to investigate *C5ar1* physiology. Currently, there is only the C5aR knockout mouse available to explore the role of C5aR in pathology and physiology. Whilst this model suffices for elucidating the functions of C5aR in acute models of inflammatory disease, it falls short when investigating its functions over short time frames of development. This chapter aimed to create a transgenic mouse that could function as a way of lineage tracing C5aR-expressing progenitor cells for use in understanding the function of C5aR in development. Unfortunately, this chapter failed to address its major aims as the expressed cre-recombinase in this mouse was non-functional.

The transgenic mouse, created in this chapter, demonstrated expression of the transgene that was responsive to LPS stimulation, in a similar manner to C5aR. Additionally, mRNA expression within the brain and confined to anatomical regions already reported in the literature for *C5ar1 in situ* hybridisation[5]. Protein expression at the correct molecular weight was shown by western blot for the CreErt2 fusion protein, probed for by Cre-recombinase antibody. However, no functionality of the CreErt2 protein could be shown by tamoxifen treatment after crossing to a cre-reporter line, despite expected functioning of the transgene *in vitro*. The EGFP, which should have been present in cells currently expressing C5aR via IRES-mediated expression, was also not evident in the isolated tissues.

Interestingly, the *in situ* hybridisation results for cre transcript within the brain correlate well with previous *in situ* hybridisation results for *C5ar1*[5]. *In situ* hybridisation demonstrated strong cre-expression within the subventricular zone surrounding the lateral ventricles, the medial habenular nuclei, the piriform cortex and the CA1 region/dentate gyrus of the hippocampus. In addition, cortical staining was strongest within layers IV and VI. However, this result is unable to be replicated using immunofluorescence methods to demonstrate protein expression within these regions. The antibody used in these experiments (clone 10/92, Serotech) has previously been shown to specifically bind to murine C5aR in transfected cells[6], however experiments in our laboratory have demonstrated additional binding to an unknown intracellular protein on knockout tissue (John Lee, *personal communication*). These previous results encouraged the aim of this chapter, as a healthy skepticism of the results derived from antibody experiments emerged; the transgenic animal proposed here would have also allowed for real-time, indirect monitoring of C5aR expression via EGFP.

The *C5ar1* gene in the mouse is ill-characterised and it is presently unclear how much of the surrounding sequence (both 3', 5' and intronic) contributes to endogenous expression. Previous attempts at using the *C5ar1* promotor for expression of transgenes have also yielded unexpected results. A recent report demonstrated that disruption of the 3'UTR through insertion of a IRES-EGFP sequence prevented C5aR1 cell surface expression and resulted in intracellular accumulation of the receptor[2]. In the present study the results suggest that there is both expression and translation of the transgene under control of the C5aR promotor, as evidenced by the *in situ* hybridisation and western blot results. It is most likely that the protein produced is non-functional, perhaps due to a chance mutation during production.

It is disappointing that this transgenic mouse is unable to be used for the intended purpose of developmental cell-linage tracing. However, several other mouse models that may be suitable to this purpose are currently being developed, or have recently been reported. These include a mCherry-tagged C5aR1 mouse being engineered under the direction of Prof. Rick Finnel's laboratory at the University of Texas (*Personal Communication*), for use in intracellular trafficking experiments. This mouse is an exciting prospect as it is an inducible *C5ar1*-knockout, which reports successful Cre-recombination by expression of EGFP. However, there have been issues with the targeting of this vector into embryonic stem cells, which has slowed progress on this model (Trent Woodruff, *personal communication*). Due to the apparent importance of the 3'UTR of *C5ar1*, another, unconventional, attempt was made to generate a *C5ar1* reporter line. The approach of adding the EGFP-IRES sequence at the 5' end of exon 2 has resulted in the successful creation of a GFP-reporter mouse that retains cell surface expression of C5aR1. This mouse has the added benefit of allowing conditional knockout of C5aR1, with LoxP sequences flanking the second exon[7]. These new mouse models under development limit the future use of the model presented in this chapter. Together, these mouse models represent a significant step-forward in the investigation of C5aR1 physiology and allowing for temporally-dependent knockout of the receptor is especially important in the developmental context. In particular, this will allow for the unmasking of any compensatory mechanisms to chronic loss of the receptor and further delineate its role in directing cell fate. Future use of these models will allow for more thorough interrogation of the role of C5aR1 on neural progenitor population in a similar way to the aims of the transgenic mouse presented in this chapter.

In conclusion, the major aim of this chapter, to create a transgenic mouse capable of lineage-tracing *C5ar1* expressing cell during development, was not met. Despite detected mRNA and protein

expression, the transgene was experimentally shown to be non-functional. However, there are several other mouse models, currently available or under construction, that may meet the needs of investigating the role of C5aR1 from a developmental perspective.

3.5 References

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Chapter 4

Investigating C5aR in Neural Progenitor Cell Physiology

4.1 Background

The present chapter comprises the bulk of the work associated with this thesis and is an investigation into the physiological role of C5aR1 in embryonic neural progenitor cells. I have presented it in format of a draft journal article submission in order to acknowledge the assistance I received from co-authors during the undertaking of this work. Additionally, we plan to submit this work for publication with the inclusion of further experiments to enhance the paper. Unfortunately, the inclusion of these experiments are beyond the temporal scope of this thesis submission but include; characterisation of NE-4C cell polarity changes with C5a treatment, viral transduction of C5aR1 into the embryonic telencephalon to track cell fate, and histological analysis of the brain samples used for the behavioural/*ex vivo* MRI sections. Despite the absence of this work, this chapter provides tantalising evidence of the importance of C5aR1 function in directing neural progenitor proliferation and polarity.

C5aR1 Signalling Promotes Polarity and Proliferation of Embryonic Neural Stem Cells

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In preparation for submission

4.2 Introduction

The complement proteins and their associated receptors have a long association with the innate immune system; this function of the complement cascade as the first line of defence against pathogenic insult is undisputed. As a result of the activation of the complement cascade, complement factor 5a (C5a) is generated as one of two cleavage products derived from complement factor 5. The other cleavage product, C5b, is the initiating factor of the membrane attack complex. Until recently it was assumed that the cleavage of C5a could only occur as the end product of one of the three complement pathways that are initiated by pathogenic surfaces or antibody complexes. However, recently it has been demonstrated that C5a can be generated from C5 directly through the actions of a wide variety of serine proteases. This 'extrinsic' pathway of C5a generation means that C5a can be generated locally, in a controlled manner, without the need for the systemic zymogenic amplification that occurs in response to pathogenic insult. This extrinsic generation of complement split products has leant credence to numerous studies that have demonstrated surprising expression and function of the complement anaphylatoxin receptors outside of their traditional innate immune roles[1-3].

C5 is reportedly expressed in the neural plate and cephalic regions during *Xenopus* development and in the wound epithelium of regenerating newt limbs[2,4]. Our laboratory has demonstrated similar expression during neural tube closure in the mouse for both C5 and C5aR1[5]. In this study we demonstrated that the combination of C5aR1 blockade *in utero* and maternal folate deficiency caused severe and frequent neural tube defects. However, we also demonstrated neuroepithelial apical localisation of C5aR1 that persisted following the formation of the neural tube in untreated embryos, suggesting a broader, physiological role for the receptor in progenitor populations.

The zone of neurogenesis, surrounding the ventricles of the embyonic brain is a pseudostratified layer. Progenitor cell nuclei are positioned between the apical and basal surfaces depending on their stage of the cell cycle. All progenitor cells within this zone maintain an attachment to the apical surface and the inheritance of this attachment point upon division appears to correlate with continued stemness[6]. The apical attachment is a congregation point for several proteins involved in promoting the proliferation and polarity of neural progenitors. Principal amongst these is the PAR3/PAR6/aPKC complex, which is expressed in all epithelial cells and functions to maintain apical polarity[7]. Localisation of the PAR3/PAR6/aPKC complex to the apical membrane is essential for self renewal of neural progenitors[8,9]. The signalling protein aPKC (PKCζ), in particular, has been demonstrated to increase proliferation and symmetrical division of progenitor cells,

maintaining stemness[10-12], and is involved as a second messenger downstream of the growth factor receptors FGFR and EGFR[13]. Signalling through aPKC in this manner results in activation of the MAPK pathway, leading to an increased proliferation of progenitor cells[13]. The pivot toward neurogenesis in the neural stem cell population has been demonstrated to be controlled by cell cycle length[14,15]. Shortening of G_1 phase by artificial expression of cyclin D_1 maintains pluripotency of neural progenitor cells and prevents differentiation[14]. Rapid cell cycle progression is also maintained by endogenous mitogens, such as EGF or FGF, acting through their respective receptors; it may be hypothesised that loss of the machinery needed for rapid cell cycle progression from the apical membrane is the cause of neuronal differentiation through a slowed cell cycle.

The known importance of the apical membrane attachment to neural progenitor cell fate makes the apical localisation of C5aR1, in the context of neurodevelopmental abnormalities, of great interest[5]. Therefore, the aim of this current study was to determine the normal physiological role of C5aR1 during embryonic development.

4.3 Methods

Tissue Collection and Processing

All animal experiments in this study were performed with prior approval from the animal ethics committee of the University of Queensland. Animal housing and time-mating of mice was provided, with thanks, from University of Queensand Biological Resource. Tissues used in this study were collected after sacrificing the mouse by cervical dislocation. Tissues preserved for RNA/protein analysis were snap frozen in liquid nitrogen and stored at -80°C until extraction. Protein was extracted using modified RIPA buffer prepared in house. Tissues used for histological analysis were incubated at 4°C overnight in freshly prepared 4% paraformaldehyde. Tissues were prepared for cyrosection by sequential passaging through sucrose solutions (10%, 20%, 30%), removed into OCT for freezing and sectioned at 12μm, unless otherwise stated.

Embryonic cerebrospinal fluid (CSF) was obtained from 13.5dpc embryos using a pulled glass pipette attached to a vacuum. Pooled CSF from three litters was used in analysis of C5a concentration through ELISA. CSF was treated with EDTA (5mM final concentration) to prevent coagulation and extrinsic complement activation, and stored at -80°C until analysis.

Reagents

Mouse recombinant C5a was obtained from Sigma Aldrich and reconstituted in 0.25% BSA in PBS. PMX53 was synthesized in house, stored lipophilised and reconstituted in water before use.

RT-PCR and qPCR

RNA was extracted using RNeasy plus spin columns (QIAGEN, The Netherlands) and treated for gDNA contamination using Turbo DNase (Life Technologies, USA). All RNA was additionally checked for gDNA contamination by PCR anaylsis. Primer sequences and PCR conditions can be found in table 4.3.1. qPCR performed using SYBR green PCR mastermix (Ambion, USA) and machine settings according to the manufacturer's instructions. The ΔΔCt method to assess fold-change of gene expression was employed and all data points within an individual sample were referenced back to *18s* expression levels.

Table 4.3.1; Table of primer sequences, amplicon length and annealing temperature used in this study.

Name	*Forward Sequence (5'-3')*	*Reverse Sequence (5'-3')*	*Amplicon*	*Temp*
C5aR1	*GACCATCTCCCAAGTGTCGT*	*CACCACACCCAGGTCTTCTT*	*566bp*	*58°C*
C5aR1 qPCR	*GGGATGTTGCAGCCCTTATCA*	*CGCCAGATTCAGAAACCAGATG*	*131bp*	*60°C*
ActB	*GTGGGCCGCCCTAGGCACCAG*	*CTCTTTGATGTCACGCACGATTTC*	*540bp*	*58°C*
Cyclin B1	*CTTGCAGTGAGTGACGTAGAC*	*CCAGTTGTCGGAGATAAGCATAG*	*94bp*	*60°C*
Cyclin D1	*GCGTACCCTGACACCAATCTC*	*ACTTGAAGTAAGATACGGAGGGC*	*94bp*	*60°C*
Cyclin E1	*GTGGCTCCGACCTTTCAGTC*	*CACAGTCTTGTCAATCTTGGCA*	*101bp*	*60°C*
Cyclin E2	*ATGTCAAGACGCAGCCGTTTA*	*GCTGATTCCTCCAGACAGTACA*	*198bp*	*60°C*
r18s	*CCCTCCAATGGATCCTCGTT*	*TCGAGGCCCTGTAATTGGAA*	*61bp*	*60°C*

Western blot

Protein samples (20µg) were subjected to electrophoresis at 100V on a 10% polyacrylamide gel until good separation was achieved. Primary antibodies directed against C5aR1 (1:500, HBT clone 10/92), phospho-Erk (1:1000, CST #9106), total-Erk (1:1000, CST #9102) and beta-tubulin (1:2000, Sigma Aldrich clone TUB2.1) were diluted in 0.5x odyssey blocking buffer (LiCor, Germany) and incubated with the membrane rocking overnight at 4°C. Incubation with specific Licor odyssey secondary antibodies was carried out according to the manufacturer's instructions. Blots were imaged using the Licor odyssey system and software. Optical densitometry values were derived from analysis of the image in ImageJ (NIH, USA).

Immunofluorescence

Tissues or cells were permeablised and blocked using 0.1% Triton X-100/4% goat serum in PBS for one hour. In the case of live staining for NE-4C cultures, primary antibody was added to unfixed cells on ice for 30 minutes prior to fixation. Primary antibodies C5aR1 (HBT clone 10/92, 1:200), Pax6 (R&D #MAB1260, 1:500), Phophohistone H_3 (CST #9706, 1:1000), doublecortin (CST #4604, 1:500), Sox2 (CST #3728, 1:500), acetylated alpha-tubulin (Sigma Aldrich #T7451, 1:500), Zo-1 (Life Technologies, #402300, 1:500) or isotype control antibodies were incubated overnight at 4°C. Appropriate alexafluor secondary antibodies (Invitrogen, USA, 1:1000) were incubated with the samples for 2 hours at room temperature before counterstaining (1µg/mL DAPI, 5 min) and mounting.

C5a ELISA

Maternal and embryonic brain sample concentrations were determined by BCA assay (ThermoScientific, USA). Aliquots of each sample were measured in technical triplicate for C5a concentration by enzyme-linked immunosorbent assay (R&D systems, USA) according to manufacturer's instructions. C5a concentrations were normalised to protein concentration (ng/mg, brain samples) or volume (ng/mL, CSF).

Neurosphere culture

Telencephalon from litters of 14.5dpc C57BL6/J mice were isolated and mechanically dissociated. Cells were maintained in DMEM/F12 media supplemented with 1x B27 supplement, L-glutamine 10ng/mL bFGF, 10ng/mL EGF and penicillin/streptomycin. To assess the effect of C5aR1 modulation on neurosphere growth 10^3 cells at passage 3 were seeded into each well of a 96 well plate in the presence or absence of 10nM C5a or 1μM PMX53. Wells were imaged after one week in culture and the number and diameter of neurospheres assessed. For monolayer culture, neurospheres were split into plates coated with PEI. Before treatment with 10nM C5a cells were deprived of growth factors (bFGF/EGF) for 6 hours. For qPCR analysis cells were harvested 12 hours post-treatment for RNA qPCR analysis.

NE-4C culture

NE-4C cells were acquired from ATCC and expanded in MEM (Sigma Aldrich) supplemented with 10% FCS (Lonza, Switzerland), L-glutamine and Non-essential amino acids (Life Technologies, USA). For migration assay cells were plated into poly-L-lysine coated 24-well plates and grown to confluency. A scratch within the monolayer was achieved using a p200 pipette tip and each well was imaged. PKCζ inhibitor GF109203X (Sigma Aldrich 2μM final concentration) or vehicle was added 30 minutes before addition of 10nM C5a or vehicle (0.25% BSA). Wells were imaged for cell migration at 18 hours and the decrease in scratch diameter was estimated using the distance between polylines plugin for ImageJ (NIH, USA).

For transwell culture, NE-4C cells were plated on poly-L-lysine coated 0.2μm transwell membranes in a 24-well plate. Cells were maintained in media as described above and treatment (C5a or vehicle) was added to the upper compartment 12 hours before fixation for immunofluorescence.

In utero injections

Time mated dams at E13.5 were anaesthetised under 1% isofluorane for surgery. 1uL of 100nM C5a, 10μM PMX53 or respective vehicle control was injected into the ventricular system of the

embryos. Abdominal inscisions were closed with sutures and dams were administered 0.1mg/kg buprenorphine for analgesia post-surgery. Dams were sacrificed and tissues collected at 24 hours post-surgery for tissue analysis.

For analysis of proliferation post-drug administration the embryonic telencephalon was sectioned coronally and sections at the level of the preoptic area were used for histological analysis. M-phase cells, as determined by phosphohistone H_3 staining, were counted using ImageJ at the apical surface of the telencephalic ventricular zone. Phosphohistone H_3 positive cells per 100μm was calculate for each individual embryo and differences between treatment groups analysed by students T-test.

Treatment of animals for behavioural experiments and MRI

Twelve time-mated dams were acquired from UQBR and housed under standard conditions under the care of animal house staff. Mice were administered 1mg/kg PMX53 or sterile water vehicle control in a 100μL volume via intraperitoneal injection over three days (12.5dpc – 14.5dpc). Dams were allowed to litter in individual cages. Gestational age at birth was defined as the number of days after discovery of the vaginal plug (0.5dpc). Litter number, weight, crown-rump length and cranial width were taken at birth. In addition, pup weight was tracked over the first five weeks of life to determine if any differences existed in growth parameters.

At eight weeks of age male and female mice from the litters were randomly selected for participation in behavioural experiments (n = 8 per group). After behavioural experiments mice were anaethetised with zylazine/xoletil cocktail and perfused with PBS followed by 4% PFA via an intracardiac cannula situated in the left ventricle. Whole heads were incubated in 4% PFA for a further 3 days before washes with PBS and careful removal of the brain. Brains were stored in fresh PBS until MRI analysis.

Grip strength

Mice were assessed for motor weakness using the grip strength test. Briefly, mice gripped a bar attached to a force transducer. The experimenter gently pulled backwards on the base of the tail until the mouse dislodged from the bar. The maximum force recorded over three trials was designated as the grip strength. Both forelimb and hindlimb grip strength was assessed.

Balance Beam

Mice were assessed for higher motor coordination using the balance beam test. The apparatus consisted of a 70cm (length) x 3mm (width) beam suspended 1m above a surface. The beam was

held in a room with bright overhead lights kept at a constant output of 150 lumens, a covered platform was set at the end of the beam. Mice were trained, through four training attempts, to move towards the covered platform through the use of a training beam of 80mm width. After training, mice were exposed to the test apparatus. Time taken to cross beam and foot fall errors were recorded. A footfall error was deemed to have occurred if the paw of the animal moved from a position on the beam and crossed a threshold 10mm beneath the beam.

Open Field Test

The open field test utilised 50 x 50cm infrared photobeam tracking arenas (Med associates, USA) to measure activity in a novel environment. Mice were placed in the center of the arena and, after a 30s initiation period, movement in the x, y and z planes was tracked for the following 30 minutes. Arenas were cleaned with 70% ethanol and allowed to dry between experiments. Thigmotaxis over the initially 5 minutes was used as a measure of anxiety in the new environment and was assessed as beam breaks within the centre (25 x 25cm) square of the arena. Other measurements calculated included average velocity, distance travelled, time immobile and rearing activity (z beam breaks).

Y-maze

The Y-maze consisted of a Y shaped maze of opaque white plastic with three identical arms set at 120° angles. The arms were consisted of a home arm, of plain design and two exploratory arms where the walls were decorated with different repetitive geometric patterns. For the exploratory task, one exploratory arm was blocked from the maze by use of a plastic divider. A subject was placed in the home arm and allowed to explore the home arm and remaining exploratory arm for 5 minutes. The subject was then re-introduced to the maze after a 30 minute period with the arm divider removed, allowing for entry into the second, novel, exploratory arm. The movement of the mouse around the maze was tracked with EthoVision video tracking software (Noldus, The Netherlands). Frequency of entry into the novel arm was used as a measure of short term memory.

MRI analysis of brain regions

Brains stored for MRI analysis were washed extensively in PBS, followed by 48h incubation in gadolinium contrast agent (0.2% Magnevist, Bayer Healthcare Pharmaceuticals, in PBS). Brains were imaged on 16.4T small animal vertical wide bore NMR spectrometer (Bruker BioSpin) at the Centre for Advanced Imaging, University of Queensland. Brains were immersed in fomblin oil (Solvay Solexis, Italy) inside a glass test tube of 10mm diameter and fitted inside a quadrature birdcage coil (M2M imaging Inc., USA). T_1 weighted images and three dimensional neurite orientation and density diffusion images (NODDI) were obtained within a total scan time of 18

hours as previously described[16]. Briefly, NODDI datasets were composed of three B_0 and sixty optimised direction diffusion-weighted images obtained from Camino software package. Three separated *b* values were used to generated the NODDI dataset, 1000, 4000 and 8000s/mm^2, δ/Δ = 1.1/22, 2.3/7 and 5.6/23ms respectively. The three b value shells were used for generation of the NODDI data (ficvf, odi) and the b4000 shell used for generation of the diffusion parameters (FA, RD, AD, MD).

Volumetric analysis of the obtained T_1 images was achieved using Advanced Normalisation Tools (ANTs) software. Briefly, all T_1 images were warped to produce a common template image. Warp fields containing Jacobian values for the individual images were subjected to a modified T-test using the *randomise* function of FSL (Oxford centre for functional MRI of the brain software library, Oxford, UK) in order to determine significantly different Jacobian value voxels between the vehicle and PMX53 treated groups. Inverse warp fields were applied to anatomical area mapping of the common template to generate volumetric values for regions of sample brains. T_1 images and generated anatomical masks were visualised and refined in ITKsnap software (University of Pennsylvania, USA). Differences in brain regions volume were tested for using Student's T-test.
NODDI data was obtained using Matlab (MathWorks, USA) and the NODDI Matlab toolbox (UCL, UK). NODDI was used to obtain estimates of intracellular volume fraction (ficvf) and neurite orientation and dispersion index (odi). Voxel values for ficvf and odi range from 0 – 1, with the scale being correlated to estimated degree of intracellular volume and neurite dispersion, respectively. Images were registered using the FSL linear registration tool in order to compare anatomically similar voxels between samples. Comparison of each of the diffusion parameters was achieved using the *randomise* function of FSL to generate a probability map of differences between vehicle- and PMX53-treated samples. Probabilty maps were thresholded to significance ($p \leq 0.05$) and displayed on a generated template image.

4.4 Results

C5aR1 is expressed in neural progenitor cells and localised in vivo to the apical ventricular zone.

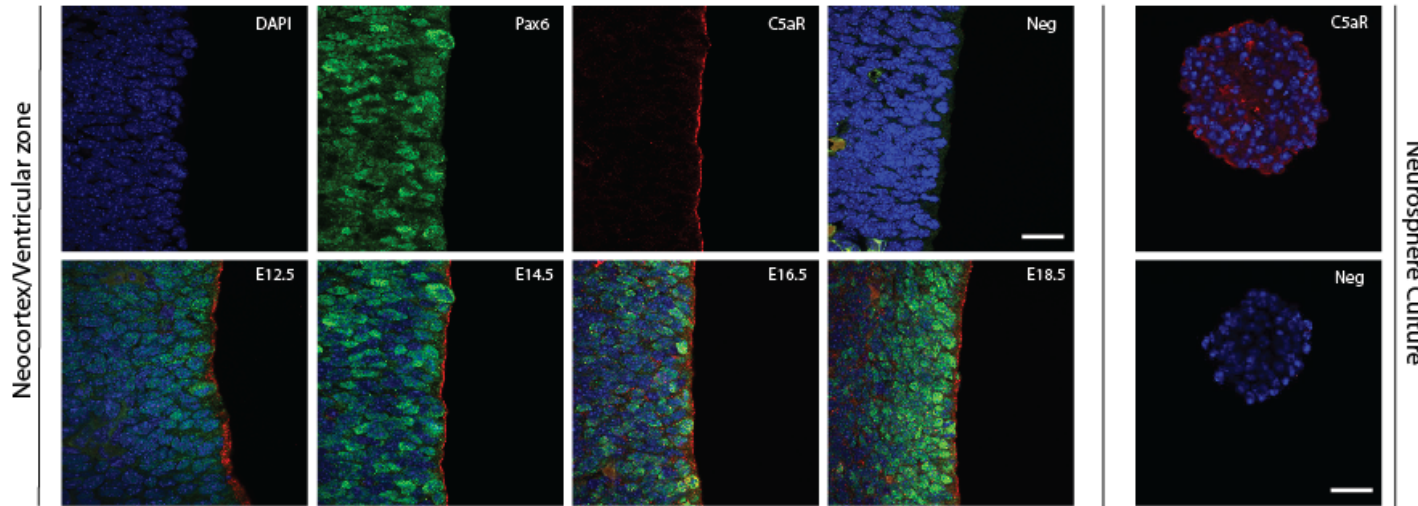
RT-PCR analysis of whole brain RNA extracts of embryos 12.5-18.5dpc demonstrated *C5ar1* expression throughout the period of embryonic neurogenesis (Figure 4.4.1C). In addition, neurospheres derived from the telecephalon of E14.5 mice and the immortalised neural progenitor cell line, NE-4C also demonstrated *C5ar1* expression by RT-PCR analysis (figure 4.4.1C). Protein expression of C5aR1 was demonstrated in embryonic brain, neurospheres and NE4C cell lines (representative blot for NE4C culture; figure 4.4.1E) by Western blot at the correct molecular weight (50kDa). *In vivo,* C5aR1 was shown to localise to the apical surface of the ventricular zone (figure 4.4.1A). The progenitor cells of the ventricular zone were immunostained with a Pax6 antibody and nuclei were counterstained with DAPI. *In vitro,* C5aR1 cell surface expression was evident on some, but not all, NE-4C cells in culture when unpermeabilised cells were stained (figure 4.4.1D). Membrane permeabilisation through detergent treatment of cells demonstrated significant intracellular expression of C5aR1 (*data not shown*).

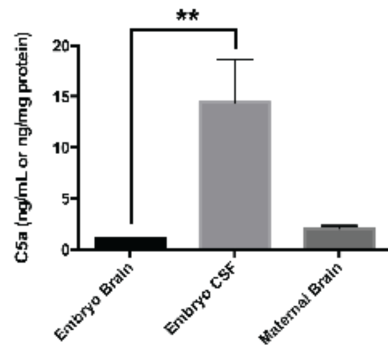
Expression of C5aR reduces as neural progenitor cells differentiate

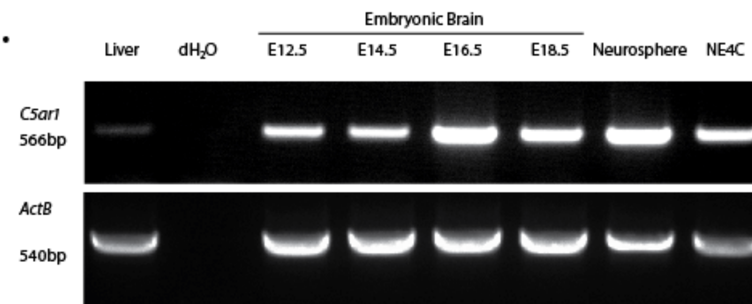
NE4C cells differentiated in the presence of retinoic acid to neurons over 12 days were collected at defined morphological stages[17]. C5aR1 mRNA expression exhibits a significant reduction in abundance between stage II and IV of differentiation, corresponding to the beginning of neuronal outmigration in the cultures. Interestingly, the abundance of C5aR1 protein as measured by semi-quantitative Western blot analysis remains static until reducing at stage VI, the beginning of gliogenesis in culture (figure 4.4.1F).

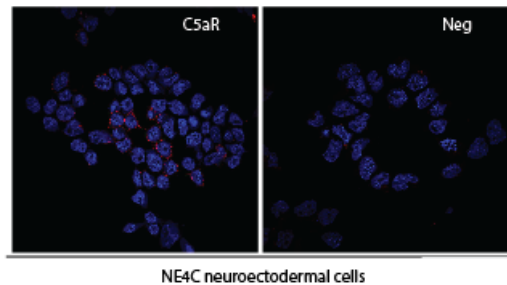
C5a is present in the embryonic CSF at E13.5

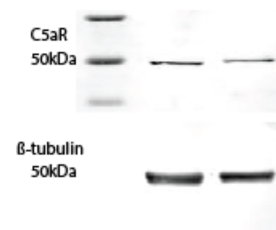
Given the localisation of C5aR1 at the apical ventricular zone *in vivo* the presence of C5a was assayed in the embryonic CSF (figure 4.4.1B). Embryonic and maternal brain tissues were used as reference points for the assay. Isolated embryonic CSF demonstrated significantly higher C5a concentrations at 13.5dpc than the embryonic telecepalon (14.45 ± 4.189 ng/mL vs. 1.054 ± 0.017 ng/mg). There was no significant difference found between embryonic brain and maternal brain tissues for C5a concentration (figure 4.4.1B).











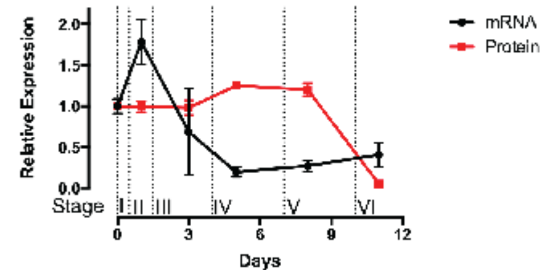


Figure 4.4.1; Localisation of C5aR1 and ligands. A) C5aR1 (red) is expressed in the developing neocortex at the apical surface of the ventricular zone from 12.5-18.5dpc. Apical progenitors are labeled with Pax6 (green), nuclei are labeled with DAPI (blue). Upper panels show individual images from 14.5dpc brain. Lower panels demonstrate merged images from 12.5-18.5dpc. Additionally, C5aR localises to the apical surface of neurospheres in culture (right panels). Scale bar = 50µm. B) Embryonic CSF contains C5a at significantly greater concentrations than embryonic or maternal brain tissue. C) RT-PCR demonstrates C5aR1 expression in embryonic brain tissue, culture primary neural progenitors (neurosphere) and immortalized neuroepithelial line (NE-4C). D) Non-polarised NE-4C cells grown in monolayer exhibit inconsistent cell surface expression of C5aR1 (red) as determined by live-staining. Blue indicates DAPI nuclear stain E) C5aR is detected within NE-4C cultures by western blot at the predicted molecular weight. F) Expression of C5aR1 mRNA and protein decreases with differentiation of NE-4C cells. Stages indicate morphologically distinct progression of NE-4C differentiation. I – Non-induced; II – Initial changes; III – Aggregate formation; IV – Neuronal outmigration; V – Neuronal maturation; VI – Onset of gliogenesis. Relative expression is normalised to stage I.

C5a causes MAPK signaling in neurosphere culture through a PKCζ dependent pathway

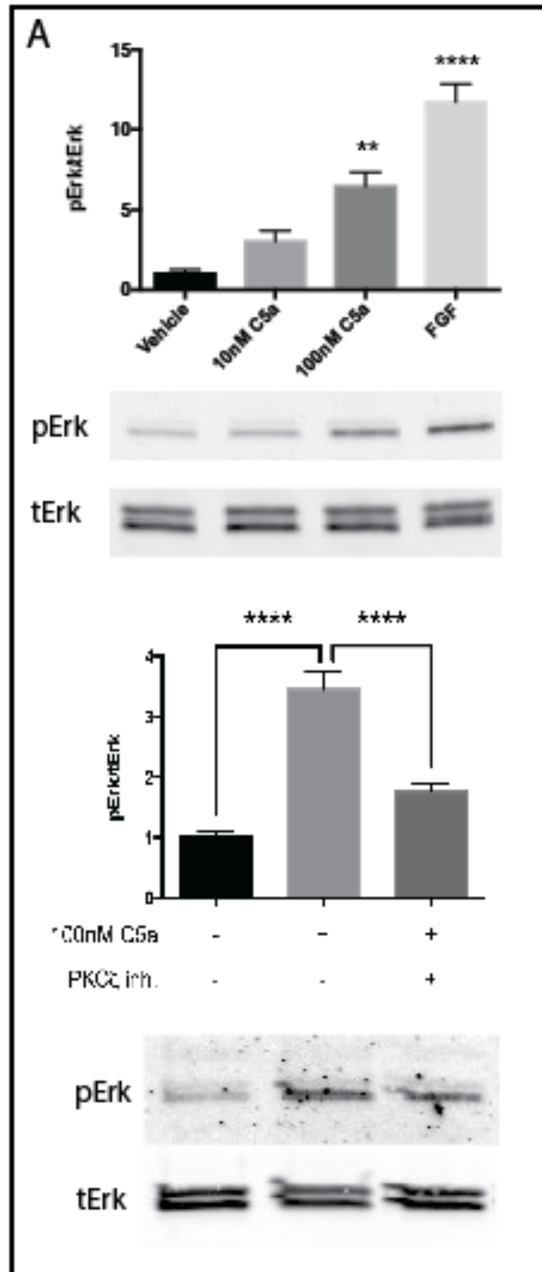
Western blot analysis of neurosphere cultures treated with 100nM C5a demonstrated a significant increase in phosphorylated p42/44 (Erk) over those treated with vehicle (figure 4.4.2A). Treatment with 10nM C5a demonstrated a trend towards increased p42/44 phosphorylation, although this was not statistically significant. bFGF was used as a positive control for this assay. The increase in p42/44 phosphorylation was inhibited by the use of a specific PKCζ pseudosubstrate, GF109203X (figure 4.4.2A), indicating a signaling role for PKCζ in the propogation of extracellular signals through C5aR.

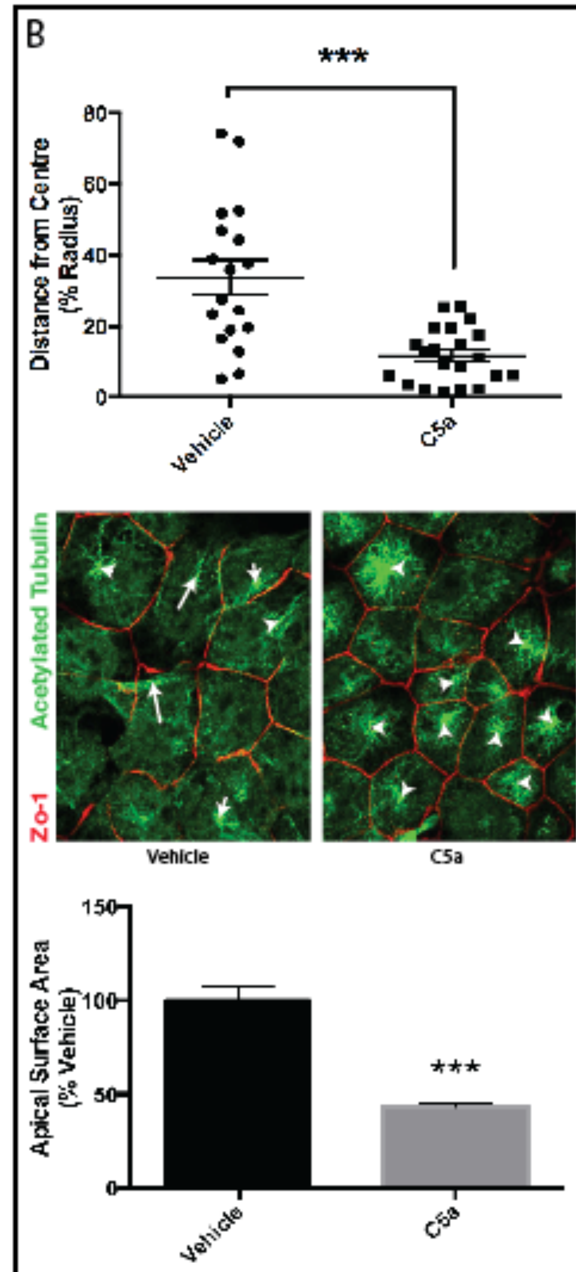
C5aR1 signalling in NE4C culture causes increased cell polarity

NE4C cells grown to confluency on transwell supports demonstrate secondary characteristics of increased polarity. Treatment with C5a for 6 hours caused a close association of the microtubule organising centre (MTOC) with the calculated centre point of the apical membrane (figure 4.4.2B). In addition, the apical surface area of C5a-treated cells was markedly reduced when compared to the vehicle treated group, suggesting active constriction of this area in response to C5a.

C5a increases NE4C migration in scratch assays

To assess the downstream actions of C5aR1 on second messengers within neural progenitor cells we employed the NE-4C line *in vitro*. Migration assays demonstrated a significant increase ($p=<0.01$, *one-way ANOVA*) in migration after 12 hours upon administration of C5a, an effect that could be blocked through the use of the PKCζ inhibitor GF109203X ($p=<0.01$, *one-way ANOVA*) (figure 4.4.2C). There were no significant differences in migration between the vehicle wells and those with either PKC inhibitor alone, or PKC inhibitor + C5a. We were unable to demonstrate Erk or Akt phosphorylation by Western blot, however this may be due to only a fraction of these cells expressing membrane bound C5aR1 at any one time (figure 4.4.1D).





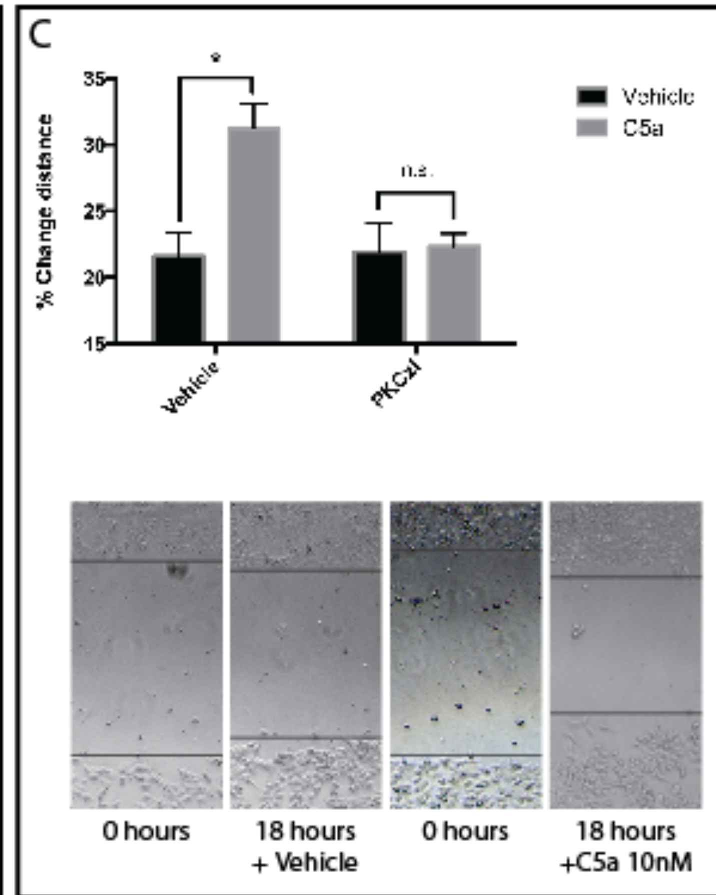


Figure 4.4.2; Signalling through C5aR1 is PKCζ dependent. A) Treatment of neurospheres with C5a causes a dose-dependent increase in phospho-p42/44 (erk), which is abrogated through pre-treatment with PKCζ inhibitor. B) NE-4C cells grown on a transwell membrane respond to 10nM C5a treatment through constriction of the apical surface area (area within ZO-1 stain, red) and centralisation of the MTOC, marked by acetylated alpha-tubulin stain (green). White arrows indicate distance between apical centre point and MTOC. C) C5a induces migration of NE-4C cells in scratch assay and can be inhibited through pretreatment with PKCζ inhibitor. Representative images with approximate cell boundaries are displayed. * = p≤0.05, ** = p≤0.01, *** = p≤0.001.

C5a increases the number and diameter of neurospheres in culture

Repassaging and growth for 7 days of neurospheres in media containing C5a resulted in neurospheres of greater number and diameter than those repassaged in media containing vehicle (figure 4.4.3A&B). However, administration of PMX53 did not alter either diameter or number of neurospheres, suggesting that there is negligible endogenous generation of C5a by neurospheres *in vitro*. Culture of neural progenitors over 3 days in the presence of C5a caused an increase in proliferation as measured by nucleic acid indirect fluorescence (figure 4.4.3C). Administration of C5a to neural progenitor culture also results in an upregulation of cyclin genes, significant upregulation of cyclin B1 (G_2-M transition) and D1 (G_1-S transition) were noted (Diagram, figure 4.4.3D).

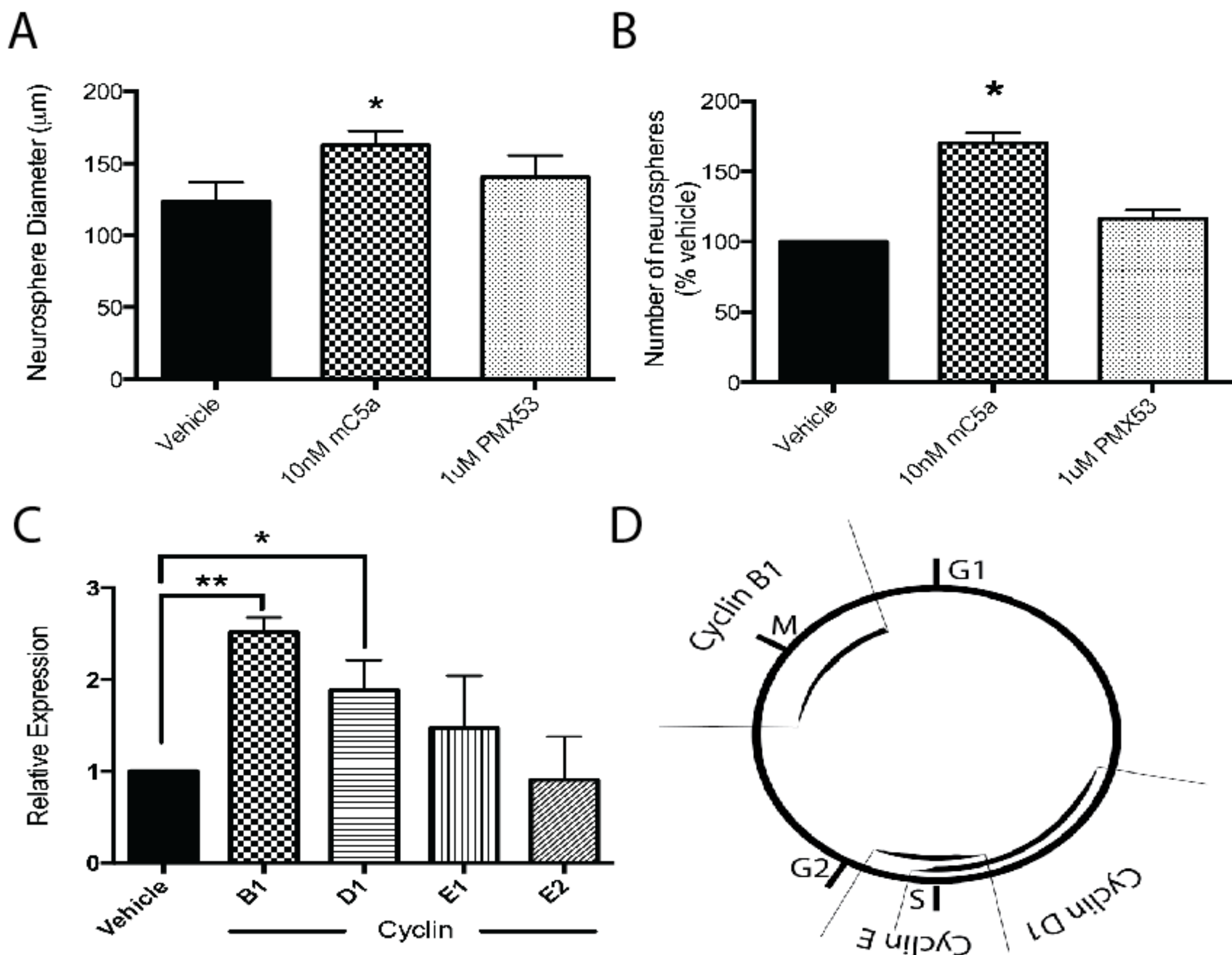
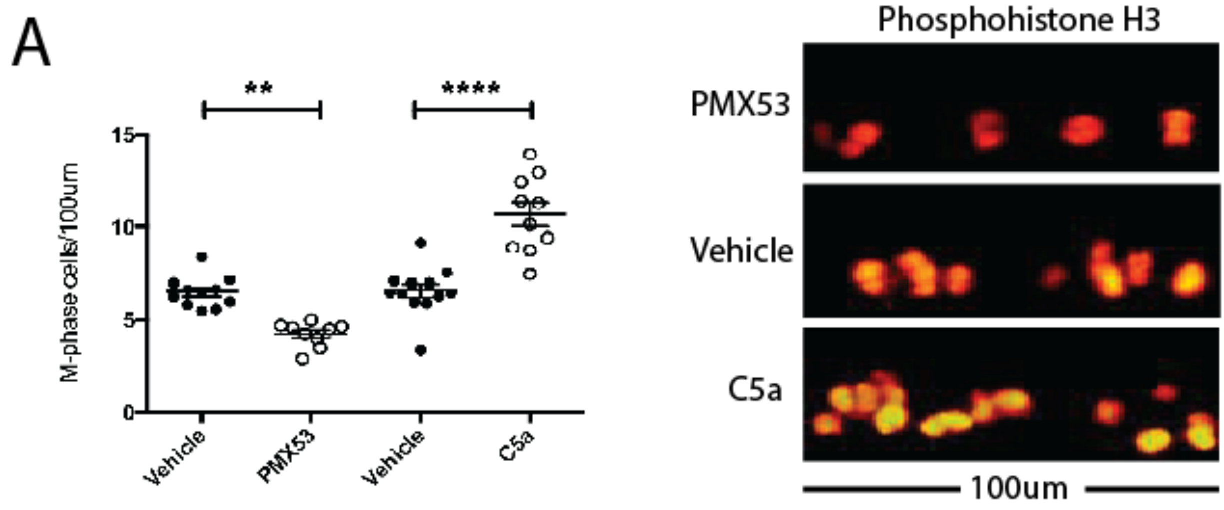
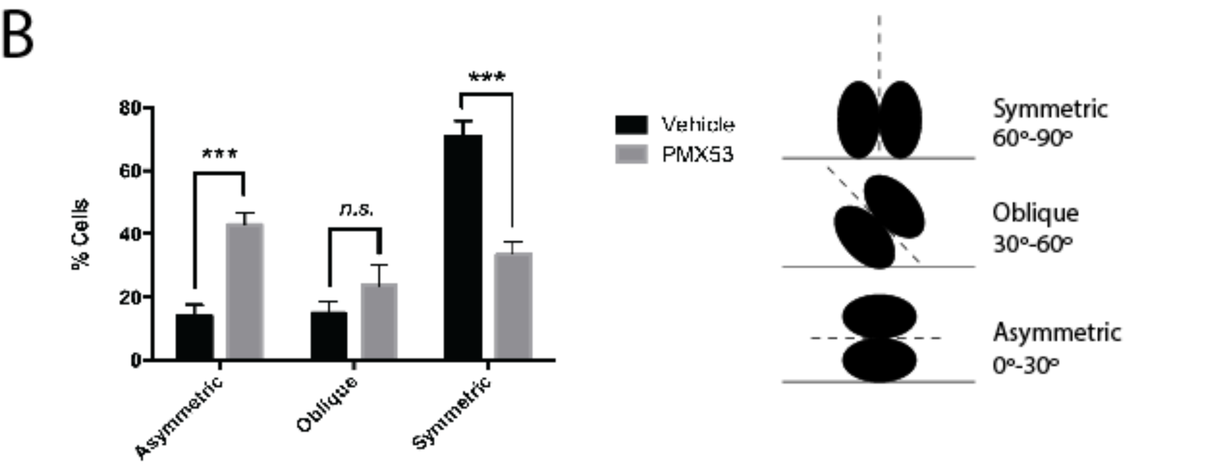


Figure 4.4.3; C5a treatment increases proliferation of progenitors *in vitro*. A) Neurosphere diameter is significantly greater in cultures containing 10nM C5a. The presence of a C5aR1 antagonist, PMX53, did not change neurosphere diameter. B) Neurosphere number in culture is significantly increase in the presence of 10nM C5a, PMX53-treatment did not change number. C) There is upregulation of cyclins B1 and D1 in neurosphere culture after 12 hours in the presence of 10nM C5a. D) Diagramatic representation of cyclin expression throughout the cell cycle. *p≤0.05, **p≤0.01.





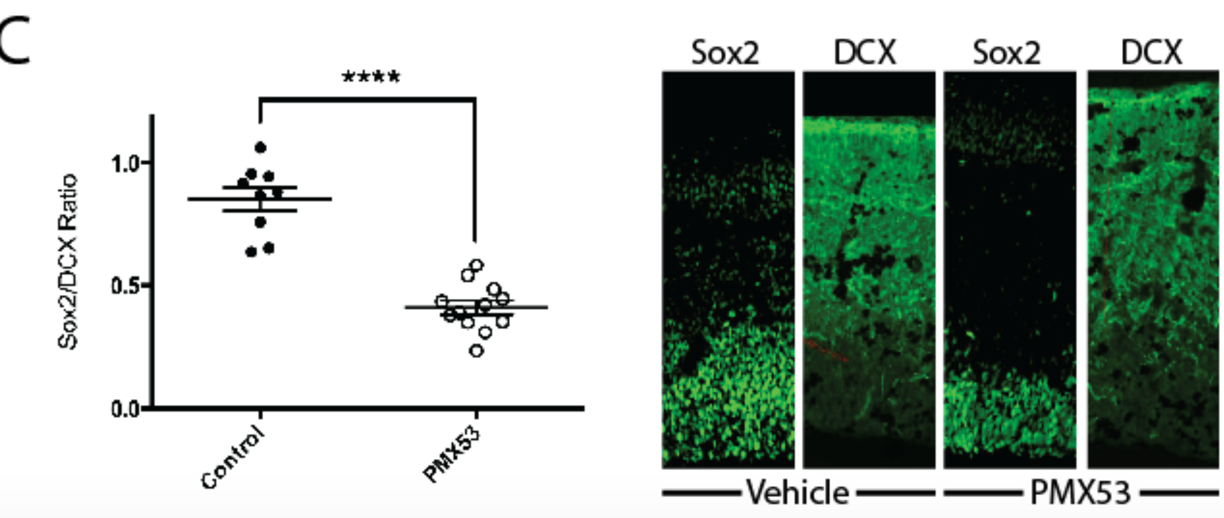


Figure 4.4.4; C5aR1 signalling results in increased proliferative divisions *in vivo*. A) *In utero* intraventricular injection at 13.5dpc of 1µL 100nM C5a results in an increased number of M-phase cells per 100µm in the telecephalic ventricular zone after 24 hours. Conversely, injection of 10µM PMX53 reduces the number of M-phase cells. Representative images of M-phase cells marked by phosphohistone H_3 staining are displayed. B) *In utero* treatment with PMX53 increases the number of asymmetric divisions at the expense of symmetric divisions. Cleavage planes of apical ventricular zone cells were catagorised as symmetric (60-90°), oblique (30-60°) or asymmetric (0-30°) according to the diagram on the right. C) Systemic treatment of dams from 12.5-14.5dpc with 1mg/kg/day PMX53 resulted in a reduced ratio of mitotic (Sox2) to post-mitotic (Dcx) layer thickness in 15.5dpc embryos. Representative images are displayed at 100µm sections of telencephalon. **p≤0.01, ***p≤0.001, ****p≤0.0001.

C5aR1 signalling increases the proliferation of neural progenitor cells of the embryonic ventricular zone.

C5aR1 was stimulated or inhibited in ventricular zone neural progenitors by *in utero* injection of C5a or C5aR1 antagonist, PMX53, into the embryonic ventricular system at 13.5dpc. 24 hours post-injection embryos administered C5a demonstrated a significant increase in the number of apical progenitors in M-phase of the cell cycle as indicated by phosphohistone H_3 staining. Blockade of C5aR1 signalling by injection of PMX53 resulted in a decrease in the number of M-phase apical progenitors after 24 hours when compared to vehicle injected littermates (figure 4.4.4A). In addition, analysis of the cleavage plane of actively dividing cells in these samples demonstrated a significant shift from symmetric to asymmetric division with PMX53 treatment (figure 4.4.4B).

Blockade of C5aR1 from 12.5-14.5dpc resulted in behavioural abnormalities in the adult mouse

To determine whether this apparent cell cycle inhibition resulted in an increase in neurogenesis we administered PMX53 over an extended period to pregnant dams and use markers of mitotic and post-mitotic cells to evaluate gross changes in cell populations. Daily intraperitoneal administration of PMX53 to the dam over the period of 12.5-14.5dpc, to globally block C5aR1 signalling resulted in a reduction in the size of the ventricular zone in the embryo. The reduction in progenitor cells in the ventricular zone, as measured by Sox2 staining, was accompanied by an increase in the thickness of the maturing cortex demonstrated by the post-mitotic maturing neuron marker doublecortin (DCX). Pharmacological blockade of C5aR in these animals resulted in a significantly decreased Sox2/DCX ratio (figure 4.4.4C).

Mice subjected to PMX53 dosing *in utero* demonstrated behavioural abnormalities in adulthood (experimental timeline; figure 4.4.5A). Litters showed normal fetal number (figure 4.4.5B), weight at birth (figure 4.4.5C) and growth postnatally (figure 4.4.5E), however there was a slight, but significant, reduction in crown-rump length of the PMX53 treated litters. On behavioural testing the adult mice showed no significant difference in peripheral nervous system motor scores, as measured by grip strength (figure 4.4.5F). However, on balance beam testing of motor coordination, PMX53-treated animals demonstrated significant differences from their vehicle treated counterparts. PMX53-treated males also showed and increase in time-taken to cross the beam and footfall errors for both hindlimb and forelimb (figure 4.4.5G-H). PMX53-treated females did not

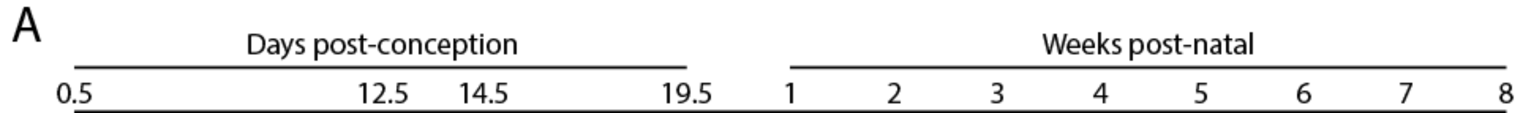


Figure 4.4.5; PMX53-treatment during development causes behavioural abnormalities in the adult. A) Diagrammatic representation of time-line for experiment. Animals were administered 1mg/kg/day PMX53 or vehicle control 12.5-14.5dpc. Animals were tracked from birth and underwent behavioural testing between 6 and 8 weeks postnatal. Animals were then culled for *ex vivo* MRI analysis. B) Fetal number per litter (n≥5). C) Birth weight (n≥30). D) Crown-rump length E) Weight gain over 33 days post-natally F) Grip strength. F, forelimb. H, hindlimb. G & H) Balance beam, time to cross(G), footfall errors (H) F, forelimb. H, hindlimb. I) Open field test, distance moved in centre area over 5 minutes. J) Y-maze, frequency of entry to novel arm. K) Forced swim test, time spent immobile. n≥8 per group (F-K). n.s, not significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$.

exhibit a difference in time to cross and only showed a significant difference in the number of footfall errors of the forelimb (figure 4.4.5G-H). This difference between gender likely represents the weight difference between males and females at this age, accentuating any errors made in motor coordination. On open field test, PMX53 animals of both genders demonstrated a decrease in distance travelled in the centre areas of the cage (figure 4.4.5I). This increase in thigmotaxis is often interpreted as a relative measure of anxiety in the novel environment. The Y-maze was used to assess short-term memory, and frequency of entry into the novel arm was reduced in both genders of the PMX53-treated animals. Finally, PMX53-treated animals also demonstrated a significantly decreased time spent immobile in the forced swim test, used to assess depressive symptoms in laboratory mice.

Blockade of C5aR1 from 12.5-14.5dpc resulted microstructural differences on MRI analysis

Ex vivo MRI analysis of the brains of male PMX53-treated animals from the behavioural experiments demonstrated microstructural differences when compared to their wild-type counterparts. In the volumetric analysis, warp fields of the Jacobian values required to shape each sample to a collective template were compared. Jacobian values are inversely correlated to the amount of stretch required to fit an area to the template image, and therefore are suggestive of volumetrically different areas between the sample groups. Vehicle treated animals showed significantly ($p < 0.05$) increased clusters of Jacobian values throughout the cortex and thalamus (volumetric red/yellow figure 4.4.6) indicating that more stretch was required to fit the PMX53 images to the template in these areas. However, analysis of whole brain regions of the T_1 weighted images failed to show any significant volume differences (table 4.4.1). Interestingly, in multiple brain regions the data for the PMX53 treated animals showed wider variation in values than the vehicle treated group (table 4.4.1), suggesting a perturbation of normal development.

Table 4.4.1; Anatomical area MRI volumes derived from T_1 weighed images.

	Vehicle treatment			PMX53 treatment			
Area	**Volume (mm^3)**	**Std Error**	**n**	**Volume (mm^3)**	**Std Error**	**n**	**p value**
Amygdala	10.24	0.1425	7	10.18	0.2929	9	0.8764
Caudate/Putamen	23.25	0.4326	7	22.85	0.5933	9	0.6175
CC and External Capsule	8.213	0.102	7	7.891	0.331	9	0.4203
Central Grey Matter	4.476	0.05	7	4.339	0.07	9	0.1762
Cerebellum	46.96	0.3563	7	42.83	1.764	9	0.0624
Fimbria	2.007	0.028	7	1.928	0.05576	9	0.2702
Globus Pallidus	2.485	0.055	7	2.368	0.046	9	0.1209
Hippocampus	24.71	0.287	7	24.31	0.5846	9	0.5832
Hypothalamus	11.61	0.162	7	11.17	0.284	9	0.234
Neocortex	125.5	1.84	7	121.5	3.45	9	0.3668
Olfactory Bulbs	17.88	0.2216	6	16.53	0.7673	9	0.1858
Thalamus	25.19	0.095	7	24.69	0.732	8	0.5444

Microstructural analysis demonstrated multiple regions of difference in the FA values between PMX53- and vehicle-treated animals. FA values were higher in the white matter tracts, olfactory bulbs, hippocampus and frontal cortex of PMX53 treated animals (figure 4.4.6). This is suggestive of increased myelination, increased axonal density or a reduction in crossing fibres in these areas[18]. Directional diffusion (RA, AD, MD) demonstrated no significant difference between treatments (figure 4.4.6). NODDI parameters were compromised by shadowing due to brain movement during scanning, which is likely the cause of the unilateral appearance of significant voxels. Ficvf values showed a significant increase in the vehicle treated brain in the posterior neocortex. Specific areas demonstrating increased ficvf include; visual/auditory cortex, primary somatosensory cortex, posterior parietal association cortex and CA1 pyramidal layer of the hippocampus. Significantly increase odi values were detected in PMX53 samples in the amygdala, CA1/dentate gyrus regions of the hippocampus, external and internal capsules, and the visual/somatosensory regions of the neocortex (figure 4.4.6).

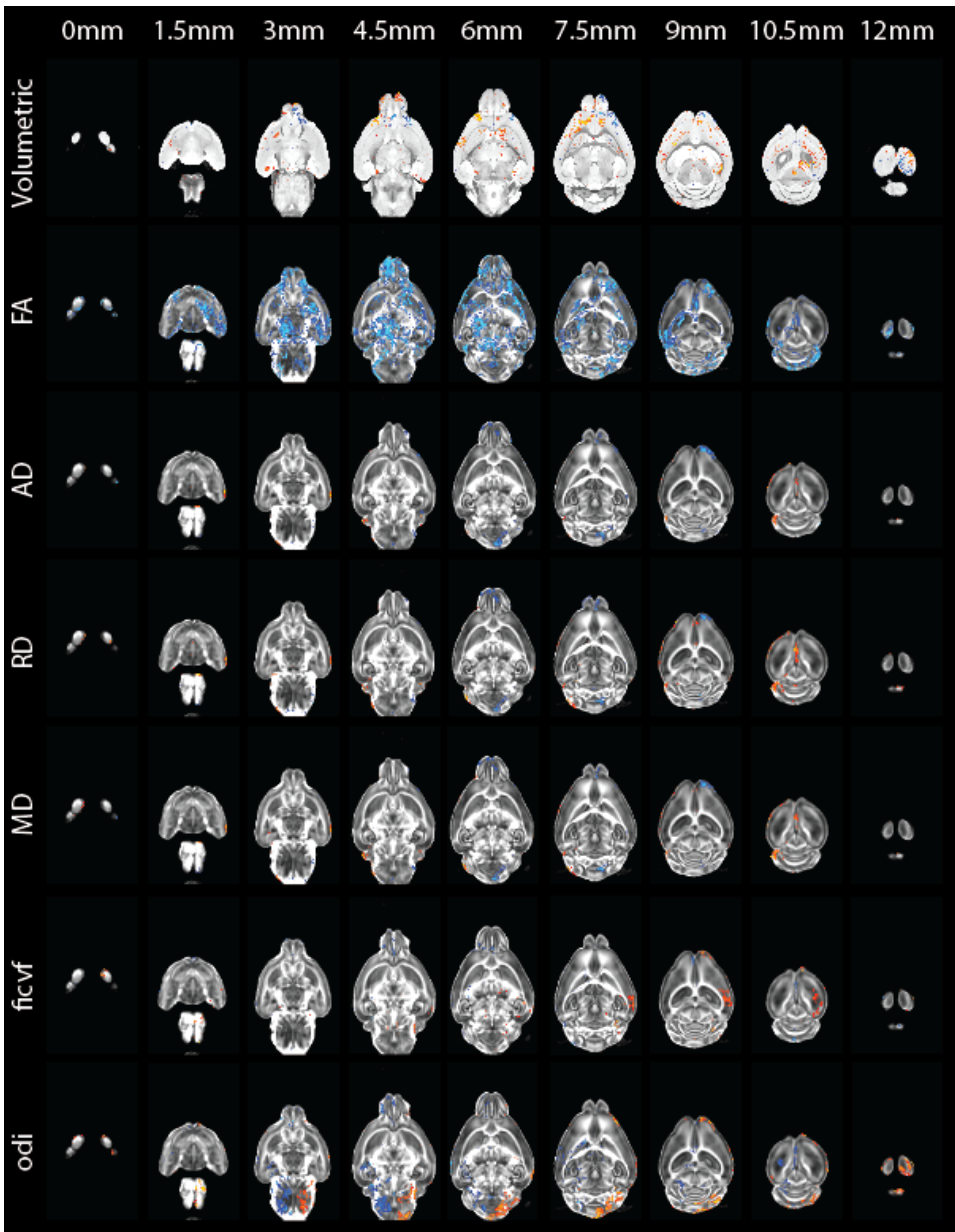


Figure 4.4.6; MRI analysis of adults brains subjected to vehicle- or PMX53-treatment (1mg/kg/day 12.5-14.5dpc) *in utero*. Sagittal sections of template images are masked with significant areas of difference between groups. Red masking represents areas for which voxels within the vehicle-treatment voxel signal is significantly greater than PMX53-treatment. Conversely, blue masking represents voxels where PMX53>Vehicle. Significance threshold was set at $p \leq 0.05$. FA, fractional anisotropy; AD, axial diffusivity; RD, radial diffusivity; MD, mean diffusivity; ficvf, intracellular volume fraction; odi, neurite orientation and dispersion index.

4.5 Discussion

This study follows on from the novel demonstration that the complement anaphylatoxin receptor, C5aR1, is present during embryonic neurogenesis on neural progenitor cells[5]. Proteins of the complement system have previously been shown to be present during embryogenesis and play novel roles in regeneration[1,2,4]. However, here we have shown for the first time that C5aR1 functions *in vivo* to retain neural progenitors within the ventricular zone, likely through PKCζ activation.

C5aR1 was found to localise to the apical membrane of ventricular zone progenitor cells throughout embryonic development (12.5dpc – 18.5dpc). This localisation is intriguing given the importance of the apical membrane attachment to the fate of the progenitor cell[6]. The apical membrane of ventricular zone progenitors is crucial for the polarisation of the cell and loss of this apical attachment during mitosis is a catalyst for differentiation toward a post-mitotic state. The apical membrane attachment acts as an anchor for determinants of polarity such as the Par3/Par6/aPKC complex and stem cell marker, prominin-1[6]. Additionally, in neurosphere culture, expression of C5aR1 also localised to the apical (outer) surface of the neurosphere, indicating a predilection of the receptor to the apical membrane in this cell type. Interestingly, in NE-4C monolayer culture, live staining revealed that C5aR1 was not readily expressed on the cell surface, despite significant mRNA and protein expression. Given that we also demonstrated a reaction of these cells to C5a when polarized, either by scratch assay or transwell culture, it is tempting to hypothesise that the polarity of the cell dictates C5aR1 cell surface expression. NE-4C cells also demonstrated a >5-fold down-regulation of C5aR1 expression, both protein and mRNA, with the onset of differentiation, and loss of polarisation.

Given the apical localisation of C5aR1, it was suspected C5a concentrations within the embryonic CSF would be elevated when compared to the surrounding brain tissue as it directly borders the site of C5aR1 expression. Indeed, this was found to be the case and significantly differed from C5a concentrations in both embryonic and maternal brain tissue. Little is known about the concentrations of C5a present in normal adult CSF, but studies in humans suggest that the concentrations found in the present chapter are equivalent to those in survivable pneumococcal meningitis, and greater than those found in patients with multiple sclerosis[19,20]. This suggests that the C5a concentrations in mouse embryonic CSF, found here in the order of 1nM, are biologically relevant.

In vitro, we have demonstrated that C5aR1 signals via PKCζ to induce p42/44 phosphorylation in neural progenitors. This association with PKCζ fits with the subcellular localisation of C5aR1 *in vivo* at the apical membrane of neural progenitor cells[21]. Here PKCζ acts upstream of p42/44 to promote proliferation and maintenance of cell polarity[21,22]. Functionally, blockade of PKCζ inhibits p42/44 phosphorylation in neurospheres and the migrational effect of C5a in a NE-4C scratch assay. Additionally, in NE-4C cells grown to confluency on a transwell membrane, C5a treatment appears to increase characteristics of polarisation, as evidenced by the centralised MTOC and constricted apical membrane. However, this observation warrants further investigation, specifically z-stack images incorporating staining for apical membrane markers. However, taken together with the localisation and signalling data, it is likely that C5aR1 promotes polarity through PKCζ in these progenitor cells.

In neural progenitor cells, polarity and the cell cycle are intrinsically linked[6,14]. In neurosphere culture, C5a treatment increased neurosphere diameter and neurosphere number over the period of a week in culture. This suggests that C5a promotes the proliferation and survival of neural progenitor cells and is supported by the observation that 12 hours of C5a treatment increases cyclin B1 and D1 expression in neurosphere culture. *In vivo*, we have demonstrated that intraventricular injection of C5a to embryos *in utero* results in an increased number of apical progenitors in M-phase. Conversely, blockade of C5aR1 signalling results in a decreased number of apical progenitors in M-phase. Combined with our findings of C5a in embryonic CSF, this supports an endogenous physiological role for C5a-C5aR1 signalling in neurogenesis. Systemic treatment of dams with the C5aR1 antagonist, PMX53, over the period of 3 days (12.5-14.5dpc) also demonstrated a reduction in the ratio of mitotic/post-mitotic cells in the embryonic telencephalon. This finding may be explained by analysis of the cleavage plane of apical progenitors post-injection of PMX53, which shows an increase in the number of asymmetric, neurogenic division at the expense of symmetric, proliferative divisions. Again, these observations support a role for C5aR1 in the maintenance of polarity in the apical progenitor population.

Whilst the cellular functions of C5aR1 in this chapter have focused on the apical progenitors of the embryonic telencephalon, its actions on neural progenitors may have wider implications. There have been previous reports of C5aR1 stimulating progenitor proliferation in post-natal cerebellar development and post-ischaemic stroke[23-25]. The behavioural and MRI analysis in the present chapter targeted the effect of C5aR in the period of telencephalic neurogenesis by administering systemic PMX53 to the dams throughout the period of 12.5-14.5dpc[6]. However, the results from these experiments demonstrate a broad pattern of impaired neural development, rather than an effect

concentrated within the neocortex. Behaviourally, mice treated with PMX53 displayed impaired central motor control (suggestive of cerebellar anomalies), impaired memory (suggestive of hippocampal involvement) and increased anxiety (a marker of limbic system function). Additionally, the forced swim test, typically a measure of depressive symptoms as recorded by time spent immobile, showed that PMX53-treated animals spent less time immobile than their wild-type counterparts. This test is contentious in the literature as, although it is responsive to a test of human anti-depressants, its use in the elicitation of neurobiological causes of depression is less concrete[26,27]. Regardless, the behavioural data presented in this chapter are suggestive of broad neurological differences as a result of acute C5aR1 antagonism *in utero*.

Structurally, C5aR1 blockade *in utero* did not result in any significantly altered volumes of anatomically distinct brain areas. However, whilst brain region volume was tightly clustered in the vehicle-treated group, animals subjected to PMX53 treatment demonstrated a wider range of brain region volumes. Additionally, Jacobian analysis of volume suggests small clusters of decreased volume within the neocortex of the PMX53 treated animals, that volumetric analysis of whole regions may not be sensitive enough to detect. Interestingly, previous studies in our laboratory that treated folate-deficient dams systemically with PMX53 demonstrated a 50% penetrance of neural tube defects, with the remainder of the litters being morphologically normal[5]. It may be that the treatment dose of 1mg/kg/day intraperitoneally is towards the lower end of efficacy. This dose is frequently used for studies of immune-mediated disease, however in these models distribution of the drug is not impeded by the feto-maternal interface and additional pharmacokinetics of the embryo[28,29]. In future studies, it would be valuable to analyse the pharmacokinetics of PMX53 in this model.

Despite a lack of volumetric differences between vehicle- and PMX53-treatment groups, there is tangible evidence of microstructural differences on MRI. PMX53-treated animals showed a significant, anatomically broad increase in FA values when compared to vehicle-treated controls. The FA value represents the confinement of water diffusion within a given voxel, with high values indicating restriction of diffusion to one axis, and low values indicating free diffusion, or crossing of equally restricted fibres[30]. The FA value is not easily correlated to pathology, as multiple studies have indicated that deviation from the normal FA value is seen in several neural pathologies. For instance, lower FA values are present, predictably, in demyelination disorders, such as multiple sclerosis[31]. However higher FA values do not necessarily indicate better neuronal function, and are evident in cases of alcoholism, meningitis and traumatic brain injury[32-34]. No differences were evident in the rate of axial or radial diffusion between the two treatment groups.

NODDI imaging allows for estimation of the compartmentalisation of water within the brain tissue and gives outputs of estimated intracellular volume fraction (ficvf), extracellular volume fraction (iso) and neurite orientation and dispersion (odi)[16]. The NODDI analysis demonstrated differences between the vehicle and treated groups for ficvf, where increased intracellular volume was found vehicle-treated brains in the posterior neocortex and is suggestive of a greater cell density in these areas. Additionally, odi analysis showed an increased neurite orientation and dispersion index throughout the amygdala, hippocampus and posterior neocortex of PMX53 treated animals. Increased odi typically denotes a less organised or immature microstructure, and is suggestive of impaired brain development within the PMX53 treated group. Collectively, the MRI data suggest a lower cell density and reduced branching of the neurons within the PMX53 treated brains. These MRI results, whilst promising, need to be further evaluated through detailed histological analysis to truly interpret their meaning.

C5aR1 knockout mice have not been reported to have any apparent gross neuronal or neurological deficits. This is further discussed in chapter 6, where knockout animals are taken through a series of behavioural tests. However, given the marked differences, both at the molecular and functional level, with the pharmacological modulation of C5aR1 signalling during embryonic brain development shown in the present chapter it is tempting to hypothesise that C5aR1 deficiency from conception onwards promotes early compensatory mechanisms within the embryo. C5aR1 is present and functional at the earliest points of development on human embryonic stem cells[35], an observation that suggests compensatory mechanisms for function must be developed at least at the blastocyst stage. Addtionally, folate-deficient stress of the C5aR1 knockout embryo leads to impaired brain development, indicating that C5aR1 signalling is not entirely redundant despite the presence of any compensatory mechanisms for loss of function[5]. Currently, the pharmacological approaches to modulating C5aR1 signalling in the embryo had the caveat of global inhibition of C5aR1. However, there is a C5aR1 conditional knockout under construction (Trent Woodruff, *personal communication*) that may help elucidate the receptors true role in the development of the brain, without the question of compensation present in the full knockout.

In conclusion, the present study has demonstrated a novel signalling pathway and function for C5aR1 on neural progenitor cells within the mouse embryo. With regards to clinical implications, this discovery should serve as a warning to the development of C5aR1 antagonists as a treatment of pregnancy-related inflammatory disease. In addition, in combination with recent work that demonstrates a two-phase role for C5aR1 in neural injury (promoting inflammation followed by

repair), it is becoming clear that use of C5aR1 modulators in these pathologies should be temporally regulated to avoid potential unintended inhibition of tissue regeneration[25,36].

4.6 References

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Chapter 5

C3aR promotes differentiation of neural progenitor cells

5.1 Introduction

This thesis has previously discussed, in chapter 4, the role of C5aR in regulating neural progenitor cell fate. C5a is often grouped with its upstream cousin, C3a, as a complement anaphylatoxin. While this nonclamenture has recently been challenged[1], there is no doubt that these molecules share evolutionary and, some, functional similarities[2]. This chapter aims to investigate the role of the canonical receptor for C3a, C3aR in neural progenitor proliferation.

An extensive background on C3aR and its ligand, C3a, can be found in appendix A and chapter 1.6, respectively. These chapters summarise the literature in general surrounding C3a, C3aR and their interactions. This introduction briefly recaps the pertinent points for the understanding of this study.

C3aR in the CNS

C3aR is expressed within the adult CNS on both neurons and glia and has been implicated in the response to CNS disease[3]. The expression pattern of neuronal C3aR in adult brains appears restricted to the granular layers of the neocortex, the hippocampal neurons and Purkinje cells of the cerebellum[3,4]. Expression on astrocytes and microglia within the non-diseased brain is low and restricted to subgroups of cells, but significantly upregulated in disease states[4-6].

Importantly, the expression of C3aR in neural stem cells within the adult brain has also been reported. In culture, neural progenitor cells derived from adult brains show altered Erk1/2 response to SDF-1α in the presence of C3a[7] and increase migration[8]. *In vivo*, interruption of C3aR signalling results in reduced basal neurogenesis[9]. These data demonstrate that modulating the C3a-C3aR axis has the potential to aid in neurogenic recovery post-cerebral ischaemia. However, in the disease state, the experimental conditions may affect the conclusions drawn over whether stimulation or inhibition of C3aR would be beneficial[10]. Seemingly contradictory reports of infarct volume size following MCA stroke have been reported in *C3*$^{-/-}$ mouse models; infarct size was increased in permenant MCA occlusion of *C3*$^{-/-}$ animals when compared to wild types, but relatively reduced in a transient model[9,11]. Additionally, *C3*$^{-/-}$ animals have been reported to exhibit reduced neurogenesis in response to cerebral ischaemia, whereas treatment with C3aR antagonist, SB290157, increases neurogenesis and functional outcomes post-injury[9,12,13]. The relative balance between the inflammatory and regenerative aspects of the C3a-C3aR axis specific to each experimental model may go some way to explain these perplexing results[1].

Intriguingly, C3a may not be the sole perpetrator of C3aR-mediated actions within the CNS. It was recently discovered that the cleavage product of neuropeptide VGF, TLQP-21, is a ligand for

C3aR[14]. This peptide has been demonstrated to exert effects on metabolism and energy storage, and perhaps provides and route of C3aR activation devoid of immune implications[15].

C3aR in development and regeneration

The C3a-C3aR axis has been reported to have functional roles in both adult tissue regeneration and the developing embryo. There is strong evidence of a role for C3aR in the developing brain. Cerebellar development in rodents continues post-natally and there is proliferation and differentiation present within the granular layers[8]. Administration of C3aR agonists during the postnatal period initiated differentiation of progenitor cells within the cerebellum resulting in marked histological differences between C3aRA- and vehicle-treated animals[8]. In non-mammalians, *Xenopus laevis* embryos express both *C3* and *C3ar1* during development. *C3* appears as markedly restricted expression to the neural groove and follows the path of neural crest migration during the period of neural tube closure[16]. C3aR stimulation acts to mediate co-attraction between neural crest cells within the migratory streams, and abrogation of this signal leads to disordered migration within *Xenopus* embryos[17].

Additionally, the C3a-C3aR axis has been reported to have functional roles in the regeneration of both embryonic and adult tissues. In the chick retina post-retinectomy *in ovo*, exogenous C3a acts through STAT-3 signalling to induce retinal regeneration from the stem cell niche[18]. Similarly, after toxic injury to the liver, $C3^{-/-}$ mice demonstrate reduced proliferation and tissue repair when compared to wild type counterparts. The increased proliferation in $C3^{+/+}$ animals was shown to be mediated entirely through the actions of C3a, as exogenous C3a could restore the phenotype of $C3^{-/-}$ mice[19]. C3a also acts on Kupffer cells, post-hepatectomy, to induce the production of factors necessary for hepatocyte survival and regeneration[20]. In support of this, $C3^{-/-}$ animals demonstrate increase liver necrosis and reduced proliferation post-surgery[20]. In newt limb and lens regeneration *C3* is expressed at the mRNA level in the wound ectoderm and suggested to signal to the blastema due to the observation of elevated C3 split products in that region [21,22].

Interesting work with cardiac stem cells demonstrates that C3a is able to promote the migration and proliferation of this population. However, its role here and putative functions post-infarct, are a double-edged sword. Whilst the promotion of regenerative potential is promising, C3a also acts to guide these cells through a myofibroblast differentiation pathway, increasing the likelihood of scar formation over functional tissue repair[23].

A New Approach to C3aR in Embryonic Neurogenesis

In light of the perspectives offered by the literature on C3aR in development and regeneration, this chapter investigates a role for C3aR on embryonic neural progenitor cells. This aspect of C3aR function has recently come to the fore on multiple types of progenitor cells, in addition to reports of a functional response to C3a on both adult and postnatal neural progenitor cells[7-9]. Here we ask whether C3aR contributes to the normal physiological development of the CNS, and whether it alters neural progenitor physiology in culture.

5.2 Methods

Neurosphere Culture

Neurospheres were cultured as previously described (Chapter 4). Briefly, the neocortex of E14.5 embryos was dissected using sterile technique in Hanks balanced salt solution (HBSS) (Sigma Aldrich, USA). Trypsin-EDTA solution (Sigma Aldrich, USA) supplemented with 2 kU Bovine pancreas DNase (Sigma Aldrich, USA) was used to dissociate the tissue and a single cell suspension was achieved by passing the dissociate through a 40µm cell filter (Biologix, USA). Trypsin was removed from the culture through 2 wash/centrifugation cycles with 1x HBSS. Cells were cultured to form neurospheres for one week in DMEM/F12 (Sigma Aldrich, USA), 1x B27 supplement (Life Technologies, USA), 1x Penicillin/Streptomycin (Sigma Aldrich, USA), 20ng/mL FGFb (Millipore, Germany) and 20ng/mL EGF (Millipore, Germany) at 37°C, 5% CO_2. Passaging of neurospheres was achieved as described above, using Trypsin-EDTA/DNase solution after centrifugation.

Immunofluoresence

For immunofluorescence analysis of C3aR expression, wild type C57BL6/J time-mated mice were sacrificed at E14.5. Embryonic brains were dissected into 4% PFA and fixed overnight at 4ºC before the generation of coronal cryostat sections at 12µm. Matched sections of the mid-neocortical region were used in this study. After washing with PBS, sections were blocked in 5% goat serum/0.1% Triton X-100 and incubated with primary antibody (1:250 Chicken anti-mouse C3aR BMA Biomedical, Switzerland; 1:300 Rabbit anti-mouse CD133, AbCam, UK) overnight at 4ºC. Sections were washed and incubated with a complementary secondary antibody (1:1000, Alexafluor secondaries, Life Technologies, USA) for 2 hours at room temperature. Nuclei were stained with 1:50,000 dilution of 4',6-diamidino-2-phenylindole (DAPI) before mounting using Prolong gold (Life Technologies, USA). Sections were imaged using an Olympus BX51 confocal microscopy system.

PCR

Neurospheres were collected for RNA isolations by centrifugation from normal culture conditions. LPS-stimulated, BV-2 control cells, a mouse microglial cell line, were collected as a positive PCR control. RNA was extracted using a RNeasy kit (QIAGEN, The Netherlands) according to the manufacturer's instructions. Post-extractions RNA was treated with DNase-I (New England Biolabs, USA) to removed genomic DNA contamination and 1µg RNA reverse transcribed to

cDNA using a Tecto reverse transcription kit with random hexamer priming (Bioline, UK) according to the manufacturer's instructions. Primers for *C3ar1* (F – CCCCCAGCCTCTTCTTTATC; R – AGCCTAAGGCCCTTCTCTTG, 60°C annealing temperature, 732bp expected product), *C3* (F – ATGCTGATGCTGGATGCTAGGCTGA; R – TAGGCTGATCGGATGCTGAGCTAGCT, 60°C annealing temperature, 567bp expected product) and *ActB* (F – GTGGGCCGCCCTAGGCACCAG; R – CTCTTTGATGTCACGCACGATTTC, 540bp expected product) were used to amplify transcript using MyTaq Redmix (Bioline, UK), 60°C annealing temperature, 35 cycles. PCR products were visualized under UV light post agarose gel electrophoresis and staining in 0.5μg/mL ethidium bromide solution.

In utero delivery of drug

Time mated C57BL6/J mice at E13.5 were obtained from University of Queensland Biological Resources (UQBR). E13.5 embryos were subjected to surgical delivery of C3aR modulators as previously described (Chapter 4). Briefly, anaesthesia of dams was achieved using 1.5% isofluorane in 2L/min O_2. The uterus was exposed in the abdominal cavity through an incision following the linea alba. 100nM EP141 (Wuxi Peptides, China) in dH_2O, 10μM SB290157 (Calbiochem, USA) dissolved in dH_2O/0.05% DMSO/0.95% ethanol and appropriate vehicle controls were supplemented with DNA loading buffer (New England Biolabs, USA). Within a litter embryos were designated to receive a treatment or vehicle control and approximately 1μL of solution was delivered to the embryonic ventricle through microinjection using a pulled glass pipette. Post surgery the dams were provided 0.1mg/kg subcutaneous burprenorphine analgesia and subcutaneous fluid resuscitation with ~2mL Ringer's solution. 24 hours post surgery dams were sacrificed by cervical dislocation and embryonic brains isolated to 4% paraformaldehyde for fixation.

*Determining M-phase cell density at E14.**5***

M-phase cell density in the embryonic ventricle was assessed as previously described (Chapter 24 ref). Briefly, sections were incubated with anti-phosphohistone H_3 antibody (#9706 1:1000 CST, USA) overnight at 4ºC, washed and subjected to secondary antibody incubation with alexafluor anti-mouse 555 (A-21422 Molecular Probes, USA) at room temperature for two hours. Sections were imaged at 20x magnification on an upright BX51 fluorescent microscope (Olympus, Japan). Phosphohistone H_3 positive cells were counted and displayed as mean positive cells per 100μm. Significance was determined by ANOVA with Dunnett's post-test using Prism 6 software (Graphpad, USA). Final n's per group for this analysis were; EP141-treated - 6, vehicle control – 6; and SB290157-treated - 7, vehicle control - 7 embryos.

BrdU assay

Neurospheres were taken to single cell suspension as described above and plated 1000/cells per well in 200µL media, 96-well cell culture plate. After three days of culture BrdU was added to culture media to a final concentration of 3µg/mL and wells were treated with either DMEM/F12 (vehicle control), 10nM EP141, 100nM EP67 (Wuxi Peptides, China) or 1µM EP54 (Wuxi Peptides, China), 3 wells per group. Cells were incubated at 37°C/5% CO_2 f r 48 hours before centrifugation and lysis. Proliferation in neurospheres within the 48 hour time period was assessed using the BrdU cell proliferation kit (EMD Millipore, Germany) according to manufacturer's instructions. Cell lysates were probed for the presence of BrdU through the use of an anti-BrdU antibody and horse-radish peroxidase conjugated complementary secondary antibody. BrdU incorporation was quantified colourmetrically using a 3',3',5,5'-tetramethylbenzidine substrate and spectrophotometric analysis at 650nm.

qPCR for Tis21

Mature neurospheres were washed in HBSS and plated in DMEM/F12 media +/- 100nM EP67. Spheres were incubated at 37°C/5% CO_2 for 12 hours before centrifugation and lysis for RNA extraction. RNA extraction and cDNA synthesis was performed as described for RT-PCR. Primers for *Tis21* (F – ATCAGTTGTCCTGAGCTCCGTCTG, R – TGGAAGCAACCTCGTCACAAACC) and *18s* (F – CCCTCCAATGGATCCTCGTT, R – TCGAGGCCCTGTAATTGGAA) were used at 70nM concentration with 1x Platinum SYBR green qPCR mix (Applied Biosystems, USA). qPCR was performed on a OneStep system (Life Technologies, USA) at 60°C annealing temperature and *18s* Ct values were used to normalize the *Tis21* data. All data was normalized to the average control value. Data was analysed in Prism 6.0 (Graphpad, USA) using a student's T-test.

5.3 Results

C3aR is expressed on neural progenitor cells in vivo

In vivo, C3aR is localised to the apical surface of the ventricular zone in embryonic brains, from the point of neural tube closure to birth (figure 5.3.1). The expression pattern for C3aR bears a striking resemblance to the expression pattern of C5aR1, previously described in chapter 4. In contrast to C5aR1, C3aR expression is more prominent on distinct cells within the subventricular zone and cortical plate, which may represent newly-born migrating neuroblasts.

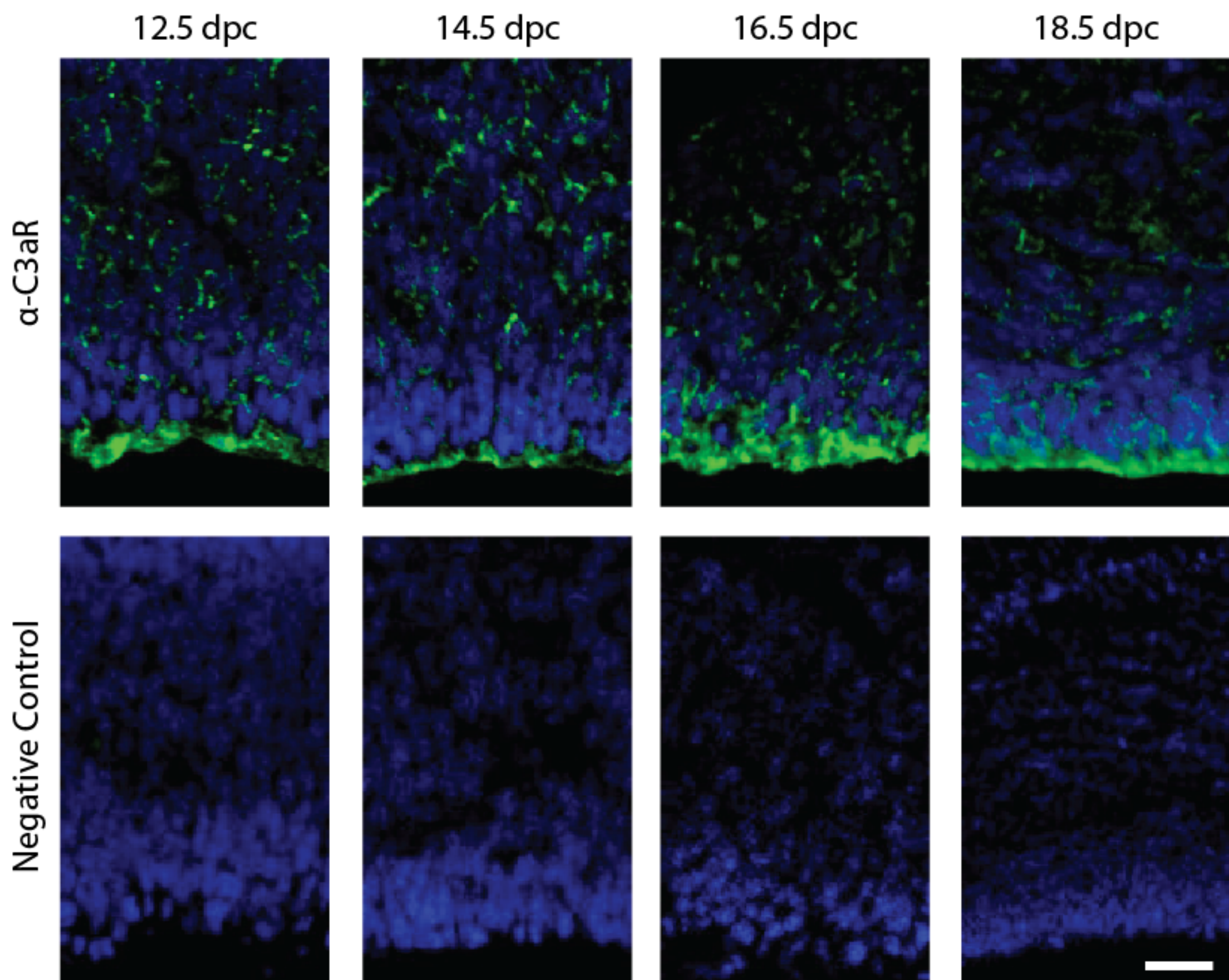

Figure 5.3.1 C3aR is expressed by neural progenitor cells throughout the period of embryonic neurogenesis. Immunofluorescence demonstrating C3aR expression (green) concentrated on the apical surface of the ventricular zone in the embryonic neocortex. Upper panel images from left to right indicate representative images from samples 12.5-18.5dpc. Lower images demonstrate matched secondary-only controls. Ventricular surface is the inferior portion of the image. White scale bar represents 20μm.

C3aR is expressed on neurospheres in vitro

In order to utilise neurosphere culture as a model system for the investigation of C3aR in neural progenitor physiology, the expression of C3aR was demonstrated and compared to the *in vivo* results. RT-PCR demonstrated the expression C3aR in RNA extracts from neurosphere cultures seven days after passaging. Interestingly, the precursor for the C3a, C3, was also detected in neurosphere culture.

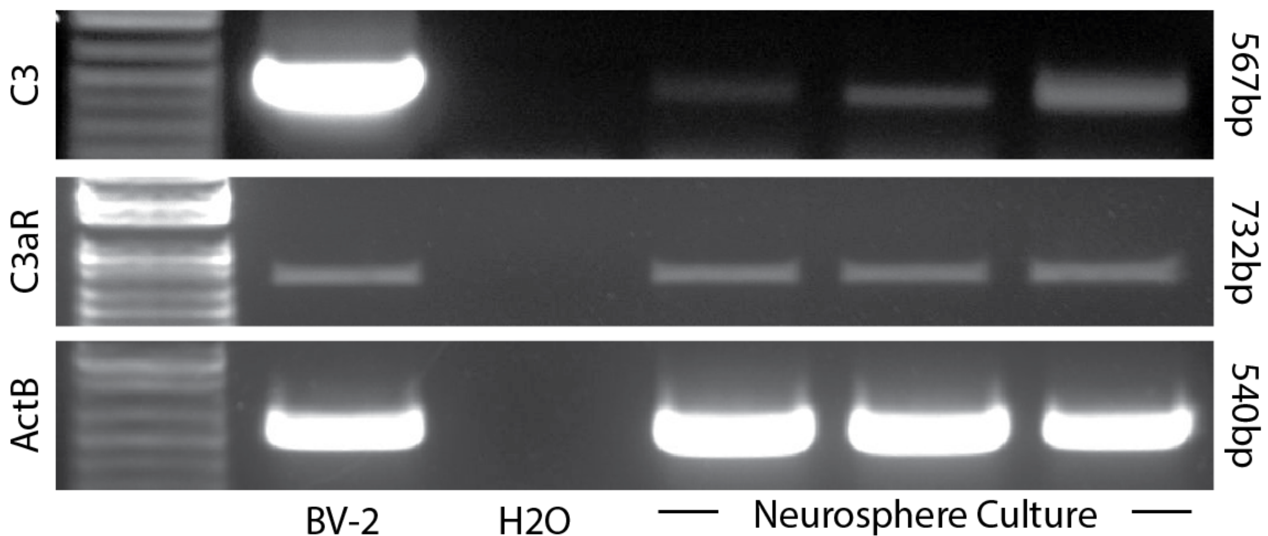

Figure 5.3.2 C3aR is expressed by neurospheres. Neurospheres seven days in culture after passage one were used for RNA extraction and analysed for the presence of C3 (top panel) and C3aR (middle panel). Activated microglial cells line (BV-2) was used as a positive control, and water (H2O) as a negative control. Beta-actin expression (bottom panel) controlled for gDNA contamination through the use of primers spanning an intron (540bp product RNA; 1060bp product gDNA).

Neurospheres were collected for immunofluorescence analysis in tandem with RT-PCR samples. Neurospheres were sectioned at 12μm in order to determine localisation of C3aR within the culture model. C3aR was localised in neurospheres to the apical surface, represented within culture by the outer portion of neurospheres (figure 5.3.3). Here, C3aR demonstrated co-localisation to apical surface – and progenitor cell marker – prominin-1 (CD133). This pattern of expression in culture, mimics the observations *in vivo* (figure 5.3.1).

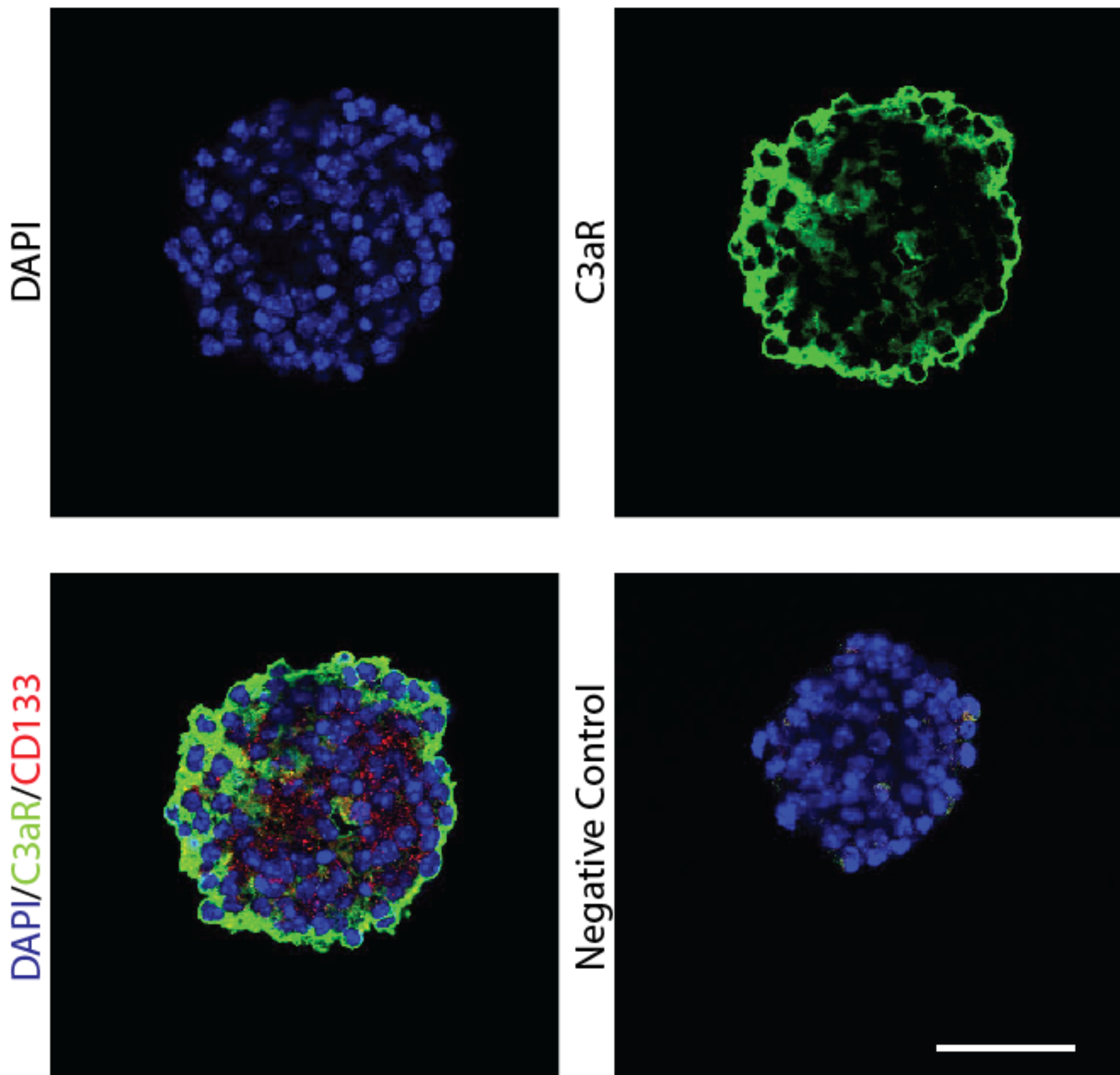

Figure 5.3.3 C3aR is expressed on the apical surface of neurospheres. Neurospheres seven days in culture after passage one were isolated and sectioned for immunofluorescence analysis. C3aR (top right) localises to the apical (outer) surface of neurospheres, with little to no staining evident within the core. Nucleus is visualised with DAPI staining (top left). Merge image (bottom left) includes neural progenitor marker prominin-1 (CD133). Negative, secondary-only control image shown (bottom right) with scale bar representing 30μm.

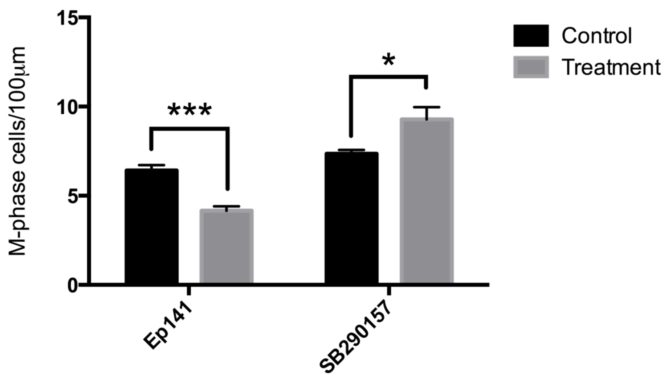

Figure 5.3.4 C3aR signaling contributes to decreased apical ventricular zone proliferation. In utero ventricular injection of C3aR agonist EP141 (1µL, 100nM) at E13.5 results in a significant decrease in M-phase cells per 100µm at the apical ventricular zone when assayed at E14.5, in comparison to vehicle-injected littermates. Administration of C3aR anatagonist, SB290157 (1µL, 10µM) results in a significant increase in ventricular zone M-phase cells. Columns represent mean +/- SEM,* $p < 0.05$, *** $p < 0.01$. $n \geq 8$ per group

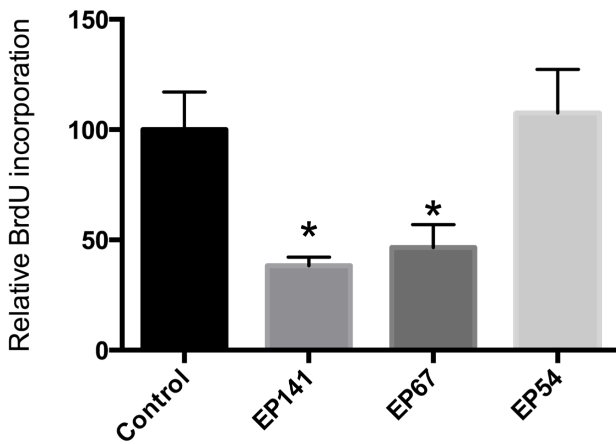

Figure 5.3.5 C3aR signaling contributes to decreased BrdU incorporation in neurosphere culture. Neurospheres derived from E14.5 embryos and treated with C3aR agonists EP141 (10nM), or EP67 (100nM) demonstrated significantly decreased BrdU incorporation over 48 hours when compared to vehicle control. In contrast, treatment with dual C3aR/C5aR agonist, EP54 (1µM), showed no significant difference when compared to vehicle treated neurospheres. Columns represent mean +/- SEM,* $p < 0.05$ $n = 3$ per group

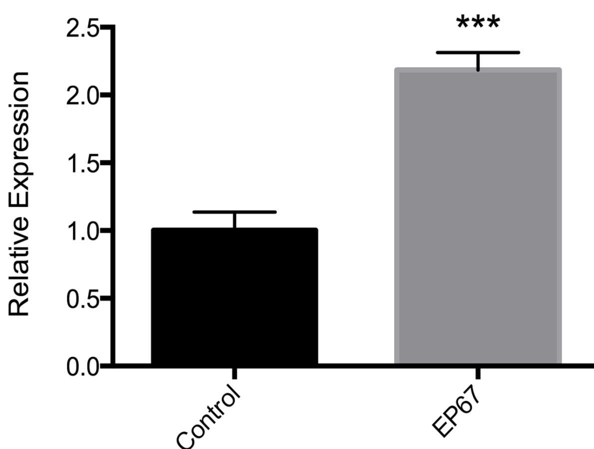

Figure 5.3.6 C3aR signaling increases *Tis21* transcript in neurosphere culture. Neurospheres derived from E14.5 embryos were treated with C3aR agonist EP67 (100nM) for 12 hours and assayed using qPCR for *Tis21* transcript. Columns represent mean +/- SEM, *** $p < 0.01$ $n \geq 4$ per group.

C3aR signalling results in decreased neural progenitor proliferation

In utero injection of C3aR agonist EP141 (1μL, 100nM) into the embryonic ventricles of E13.5 C57BL/6J mice caused a decrease in M-phase cells within the ventricular zone at E14.5, as measured by phosphohistone H_3 immunofluorescence analysis (Fig 5.3.4). By contrast, the use of C3aR antagonist, SB290157 (1μL, 10μM), caused an increase in proliferating cells in this region (Fig 5.2). Use of C3a agonists in neurosphere culture demonstrated this effect was not limited to the *in vivo* environment, as treatment with the C3aR agonists EP141 (10nM) and EP67 (100nM) caused a decrease in proliferation of neurospheres, as measured by BrdU incorporation over 48 hours. Intriguingly, treatment with the dual C5aR/C3aR agonist EP54 (1μM) showed no difference in BrdU incorporation when compared to control cultures (Fig 5.3.5). Neurospheres were then assayed after 12 hours of EP67 treatment for relative expression of *Tis21,* a marker of differentiation in neural progenitors. C3aR stimulation through EP67 treatment in differentiation conditions resulted in a significant increase in *Tis21* mRNA expression (Figure 5.3.6).

5.4 Discussion

This study demonstrates a role for C3aR in neural progenitor physiology that has not previously been ascribed to such an early stage of development. The results suggest that C3aR plays an essential and non-redundant function in embryonic brain development.

Here we show, for the first time, that C3aR is expressed by embryonic neural stem cells during normal development. Previously, C3aR has been demonstrated to be expressed by adult neural progenitors in culture and to function in the neurogenic response to ischaemia[9]. In the embryo, expression of C3aR is restricted to the apical surface of the ventricular zone *in vivo* and the apical surface of neurospheres in culture (Figures 5.3.1, 5.3.3). This expression pattern is similar to other fate determining proteins in neural progenitors, such as CD133, Par3 and aPKC[24,25].

Expression of the receptor at the apical surface also put it in direct contact with embryonic cerebrospinal fluid. In adults, CSF has been demonstrated to contain C3a in physiologically active concentrations (~100-300 ng/mL), with the concentration increasing exponentially in the presence of CNS disease or trauma[26-28]. The results of the present study beg the question of whether the overall effect of increased C3a in the CSF is detrimental to functional outcomes. There is positive correlation of C3a concentration to severity of disease, but little is known about the relative contributions of CSF-derived C3a to CNS pathology[29,30]. It is likely that C3a may have both physiologically beneficial and pathologically detrimental actions in this situation, as has previously been highlighted in the setting of the immune response[1]. To our knowledge, there has been no study investigating the presence or concentration of C3a in embryonic CSF.

Treatment of embryos *in utero* through injection of C3aR modulatory compounds into the lateral ventricles demonstrated a role for C3aR in modulating proliferation of neural progenitors. At 24 hours after injection, treatment with C3aR agonist, EP141, caused a significant decrease in M-phase cells at the ventricular surface (figure 5.3.4). Conversely, treatment with the C3aR antagonist, SB290157, resulted in increased proliferation as measured by M-phase cells (figure 5.3.2). This was supported by *in vitro* results demonstrating decreased BrdU uptake in primary neural progenitors treated with the C3aR agonists EP141 and EP67. Interestingly, treatment with the dual agonist of C3aR and C5aR, EP54, resulted in no significant difference in BrdU uptake when compared to the vehicle treated group (figure 5.3.5). Given our previous results demonstrating a role for C5aR in promoting neural progenitor proliferation (chapter 4), it is interesting to consider whether there is physiological antagonism between C3aR and C5aR in neural progenitors, resulting in no overall change from control when treating with the dual agonist. Future experiments using EP54 combined

with a C5aR1 antagonist, or use of a future commercially available rC3a, could address this problem.

Previously, C3aR has been implicated in the migration of post-natal neural progenitor cells *in vivo,* and the regulation of SDF-1 mediated differentiation *in vitro*[7,8]. In light of these previous results we assayed EP67-treated cultures for *Tis21* expression, an early marker of neural progenitor differentiation. EP67 treated cultures demonstrated double the *Tis21* expression when compared to vehicle treated controls (figure 5.3.6), indicating that the reduction in neural progenitor proliferation seen in the BrdU assay (figure 5.3.5) may be attributed to an increase in terminal differentiation in these cultures.

Interestingly, there have been no reports of gross functional defects in *C3ar1*$^{-/-}$ animals, which may be surprising given the important role we have highlighted here in neurogenesis. However, we show in chapter 6 of this thesis that there are subtle defects in cognitive processes reliant on neurogenesis in *C3ar1*$^{-/-}$ mice, indeed this is the first reported investigation of cognition in these knockouts. However, developmentally, the knockout animals appear grossly normal, and not in keeping with the magnitude of effect demonstrated in this study. This may be an example of redundancy developed during development in the knockout that is not appreciable with acute pharmacological modulation of the receptor.

In summary, this chapter has provided evidence for the localisation and function of C3aR on embryonic neural progenitor cell as a driver for differentiation. This is in keeping with studies of C3aR function in adult neural progenitors[9], and suggests conserved mechanisms of progenitor fate determination between developmental and regenerative processes. Future studies in this area should address the intracellular mechanisms behind this action and the effects of modulating C3aR signalling throughout development. Utilisation of the methods employed in chapter 4, with a focus on C3aR, would strengthen this research and add gravitas to these preliminary results. In addition, given the apparent physiological antagonism between C3aR and C5aR (chapter 4), and the results presented here using a dual-agonist, it would be interesting to know at what point this antagonism occurs.

5.5 References

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Chapter 6

C3aR and C5aR1 deficient mice exhibit behavioural deficits in adulthood

6.1 Introduction

This thesis has previously demonstrated the role of C3aR and C5aR1 in neural progenitor physiology during embryonic development. Given the previous results of altered neurogenesis with pharmacological blockade, we aimed to determine whether *C3ar1* and *C5ar1* knockout animals exhibited similar deficits in neural development. There have been no reports of gross developmental abnormalities associated with the knockout animals, particularly no reports associated with abnormalities in neuronal function. Our laboratory has previously demonstrated a dramatic increase in neural tube defects in *C5ar1*$^{-/-}$ animals under conditions of folate stress[1] and similar results have been obtained under the same conditions in *C3ar1*$^{-/-}$ animals (Angela Jeanes, *personal communication*). However, these defects in neurulation are not apparent in folate-sufficient mice, suggesting that any physiological roles that the anaphylatoxin receptors take during this stage of development can be compensated for under normal developmental conditions.

A short experimental report has previously analysed the rate of basal neurogenesis at several areas in the adult cerebrum between C5aR1$^{-/-}$ mice and their wild type littermates and demonstrated no observable difference[2]. This study also demonstrated that the expression of C3a and C5a-like molecules under the control of GFAP promoter also resulted in no changes in basal neurogenesis. However, in the anaphylatoxin expression experiments, the authors were unable to detect any C3a or C5a expression by immunohistochemistry, which raises some doubts about the validity of the model for examining the effects of anaphylatoxins on basal neurogenesis. Additionally, this result contrasts with reports in pathological and developmental scenarios where complement anaphylatoxins have been shown to be actively involved in normal development and the response of neural progenitor cells to injury[3-5].

This thesis has previously summarised the evidence in the literature supporting a role for the anaphylatoxins in neural development (chapter 1.4). Briefly, the initial reports of function on progenitor cells in the developing CNS came from studies into post-natal cerebellar development. Final organisation of the cerebellar layers occurs postnatally in rodents and corresponds to a transient increase in *C5ar1* and *C3ar1* cerebellar expression. The movement of progenitor cells from the external granular layer to maturation in the internal granular layer is promoted by subdural injections of C3a, whilst C5a stimulates proliferation[3]. *In vitro*, C3a stimulated increased migration of isolated granule layer progenitor cells and this effect could be inhibited through use of a C3aR

antagonist. Taken together, these results suggest a mutually antagonistic role for C3aR and C5aR1, with C3aR stimulating migration and differentiation in the internal granule layer and C5aR1 encouraging proliferation of the progenitor population.

This view of the roles of C3aR and C5aR1 in neurogenesis is supported by studies into cerebral ischaemia. The presence of progenitor cells, in this model, is reduced in both $C3^{-/-}$ and $C3ar1^{-/-}$ animals[5], suggesting a similar role for C3aR in this area of neurogenesis as the cerebellar observations. This also demonstrates that the altered neurogenesis is a result of the C3a-C3aR axis, rather than other, or unknown, ligands for C3aR[6]. This study also demonstrated C5aR expression on neural progenitor cells of the adult brain, although the function of this receptor is not investigated[5].

Whilst these studies provide solid evidence of anaphylatoxin directed migration and proliferation of neural progenitors, there have been no reported studies into the effect of knockout of anaphylatoxin receptors on neurological function in the adult mouse. Behavioural studies have been employed on complement knockout mice for which behaviour is a key marker of disease recovery, such as in spinal cord injury or neurodegenerative disease[7-9]. However, to our knowledge, no study has specifically investigated behavioural differences between naïve complement knockouts and their wild type counterparts. Thus, this chapter aimed to elucidate this.

It is not particularly surprising that the previous chapters have demonstrated a role for C3aR and C5aR1 signalling in neural development, and no behavioural abnormalities have been reported in the knockout animals. The perturbation of embryonic neurogenesis has previously been shown to result in subtle behavioural phenotypes, rather than gross morphological defects. In 17dpc rats (15dpc equivalent in mice), injection of a transient, neuronal-specific, antimitotic compound caused deficits in special memory that were associated with histological abnormalities in the pre-frontal cortex[10]. Alterations in developmental neurogenesis at this age also affect the electrophysiological properties of individual neurons and result in a schizophrenia-like phenotype behaviourally[11]. These studies lend credence to the investigation of behavioural abnormalities in complement knockout mice that appear grossly normal, but may have subtle underlying neuronal deficits. Thus, here we examine neuronal function in $C3ar1^{-/-}$ and $C5ar1^{-/-}$ mice using a range of behavioural tests in order to assess locomotor function, affect, learning and memory.

6.2 Methods

Tissue collection

C5aR1$^{-/-}$, C3aR$^{-/-}$ and C5aR1$^{+/+}$, C3aR$^{+/+}$ (wild type) mice were generated through mating heterozygous knockout animals to generate C5aR1$^{-/-}$ and C5aR1$^{+/+}$ (and C3aR$^{-/-}$ and C3aR$^{+/+}$) littermates. The presence of a vaginal plug was deemed E0.5 and dams were sacrificed at E14.5 or E16.5. The embryonic dates of E14.5 and E16.5 were chosen as a direct comparison to previous experiments using complement modulatory drugs (chapter 4). Embryonic brains were dissected into 4% PFA and fixed overnight at 4ºC before the generation of coronal cryostat sections at 12µm. Embryonic tail tissue was collected for genotyping embryos. Matched sections of the mid-neocortical region were used in this study.

Determining M-phase cell density at E14.5

M-phase cell density in the embryonic ventricle was assessed as previously described (chapter 4). Briefly, sections were incubated with anti-phosphohistone H_3 antibody (#9706 1:1000 CST, USA) overnight at 4ºC, washed and subjected to secondary antibody incubation with alexafluor anti-mouse 555 (A-21422 Molecular Probes, USA) at room temperature for two hours. Sections were imaged at 20x magnification on an upright BX51 fluorescent microscope (Olympus, Japan). Phosphohistone H_3 positive cells were counted and displayed as mean positive cells per 100µm. Significance was determined by ANOVA with Dunnett's post-test using Prism 6 software (Graphpad, USA). Final n's per group for this analysis were; wild-type 12, C3aR$^{-/-}$ 17 and C5aR1$^{-/-}$ 21 embryos.

Calculating Sox2/Dcx ratio at E14.5

Sox2 to doublecortin (Dcx)-positive ratios for knockout and wild type littermates were determined as previously described (chapter 4). Briefly, serial sections were incubated with either rabbit anti-mouse Sox2 (#3728 1:400 CST, USA) or rabbit anti-mouse Dcx (#4604 1:300 CST, USA) overnight at 4ºC, washed and subjected to secondary antibody incubation with alexafluor anti-rabbit 488 (A-11088 Molecular Probes, USA) at room temperature for two hours. Sections were imaged at 20x magnification on an upright BX51 fluorescent microscope (Olympus, Japan). Thickness of Sox2- and Dcx-positive layers of the neocortex was calculated using ImageJ (National Institutes of

Health, USA) and displayed as a ratio of Dcx to Sox2 thickness. Statistical analysis and graphing was performed using Prism 6 software (Graphpad, USA), significance was determined by ANOVA with Dunnett's post-test. Final n's per group for this analysis were; wild-type 9, C3aR$^{-/-}$ 7 and C5aR1$^{-/-}$ 6 embryos.

Animals used for behavioural experiments

C5aR1$^{-/-}$ (n = 8), C3aR$^{-/-}$ (n = 8) and wild type (n = 7) male mice at six months of age were used for the following behavioural experiments. Wild type mice were sourced from the heterozygous matings in the knockout colonies to reduce inter-litter variation. Mice were transferred to the behavioural testing facility housing at TetraQ, within the University of Queensland, one week before testing to habituate to a new environment. Mice had previously been housed under SPF conditions within the University of Queensland Biological Resources facilities.

Open field protocol

Activity was tracked in a 50 x 50 x 40cm arena using Ethovision software (Noldus, The Netherlands) for 30 minute periods over five days. Lighting and temperature conditions in the test room were controlled to match the housing conditions of the mice. Mice were placed in the centre of the arena and, after 30s habituation, the behavioural parameters of distance moved, average velocity, distance from centre, time immobile and mean turn angle were scored by the software. Faecal boli were manually counted on conclusion of each test. The arena was cleaned and dried with a 70% ethanol solution between tests.

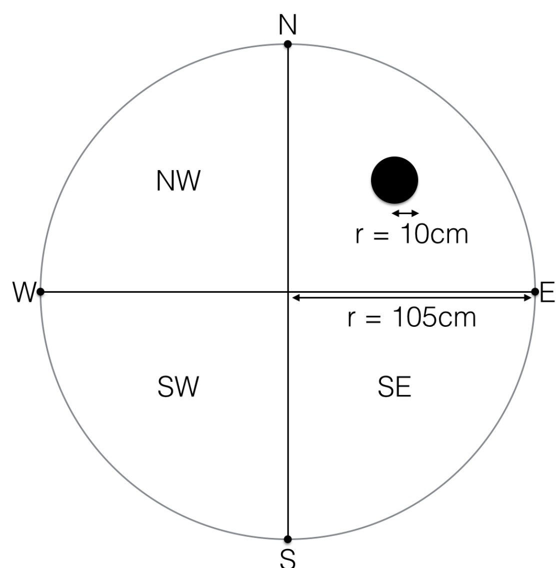

Figure 6.2.1: Layout of the Morris Water Maze. The water maze (r = 105cm) was divided through the North-South and East-West axes into four quadrants labelled NW, SW, SE and NE (unlabelled). The platform was placed within the NE quadrant 1cm below the water surface.

Morris water maze protocol

Mice were assessed for learning and memory using the Morris water maze protocol. A 105cm radius

tank with sides of 51cm was filled with ambient temperature water supplemented with opacifier 631 (Morton SA, France). The arena was divided into four segments through the North-South and East-West axis, a platform of 5cm radius was placed 1cm beneath the water surface in the centre of the North-East quadrant (figure 6.2.1). Distal visual cues, temperature and ambient light were constant throughout the training and test periods. Mice were released into the area, facing the arena wall, at one of the North, South, East and West points of the maze. The sequence of release was randomised throughout the five days of the training protocol for each of the four trials per day. Each animal was tracked in the arena using Ethovision software (Noldus, The Netherlands) for a period of five minutes or until the platform was reached. Parameters assessed were distance moved, mean velocity, latency to enter platform quadrant, percentage time in platform quadrant, time to reach platform and mean distance to platform. After five days of training, mice were subjected to a test of memory by removing the platform from the arena. Mice were released from the South release point for the trial day, and tracked for 5 minutes within the arena. Parameters assessed in the test were distance moved, mean velocity, latency to enter platform quadrant, mean distance to platform zone and frequency of entry to platform zone.

Statistical Interpretation

Data from the open field test and Morris water maze was analysed using Prism 6 software (Graphpad, USA). Two-way analysis of variance was employed to determine effects of trial day and genotype followed by Sidak's post-hoc test to determine intra-trial day differences and overall changes between genotypes. Significance was set at $p \leq 0.05$.

6.3 Results

Anaphylatoxin knockout animals have altered neural progenitor proliferation rates, but no changes in mitotic cell populations

At E14.5, C3aR$^{-/-}$ animals demonstrated reduced numbers of M-phase cells per 100µm than wild type animals (p<0.05). In contrast, M-phase cells per 100µm in C5aR1$^{-/-}$ mice were increased (p<0.001)(Figure 6.3.1A). This apparent difference in proliferative rates at E14.5 does not translate into differences in the mitotic/post-mitotic cell ratio at E15.5 (Figure 6.2B).

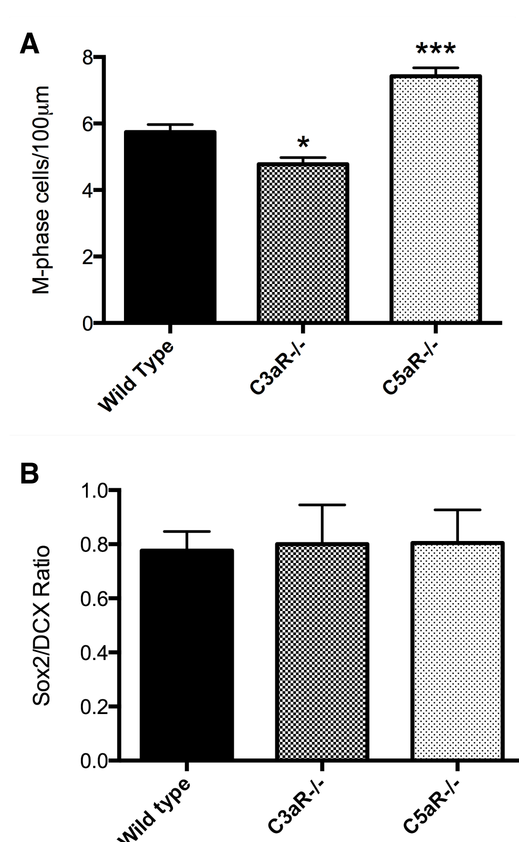

Figure 6.3.1: Developmental analysis of anaphylatoxin receptor knockout mice. (A) Bar graph demonstrating phosphohistone H_3 positive cells per 100µm at the ventricular surface of E14.5 mouse embryonic brains. **(B)** Bar graph displaying the ratio of mitotic (Sox2-positive) to post-mitotic (doublecortin-positive) cells in the embryonic neocortex at E15.5. Bars represent mean ± standard error. *p < 0.05. ***p < 0.001. p-values determined by one-way ANOVA with Dunnett's post-test.

C3aR$^{-/-}$ animals display reduced anxiety-related behaviours in the open field test

Open-field testing demonstrated differences between the wild-type and anaphylatoxin receptor knockout mice in multiple parameters. There was a significant time-dependent decrease in total distance travelled in all groups (figure 6.3.2A), with no significant difference demonstrated overall between wild-type and knockout mice (table 6.3.1). There was a trend towards increased distance travelled in the C3aR$^{-/-}$ animals (p=0.0644), which only deviated significantly from the wild type animals on day 4 of the protocol. In contrast, C5aR1$^{-/-}$ animals demonstrated no difference when

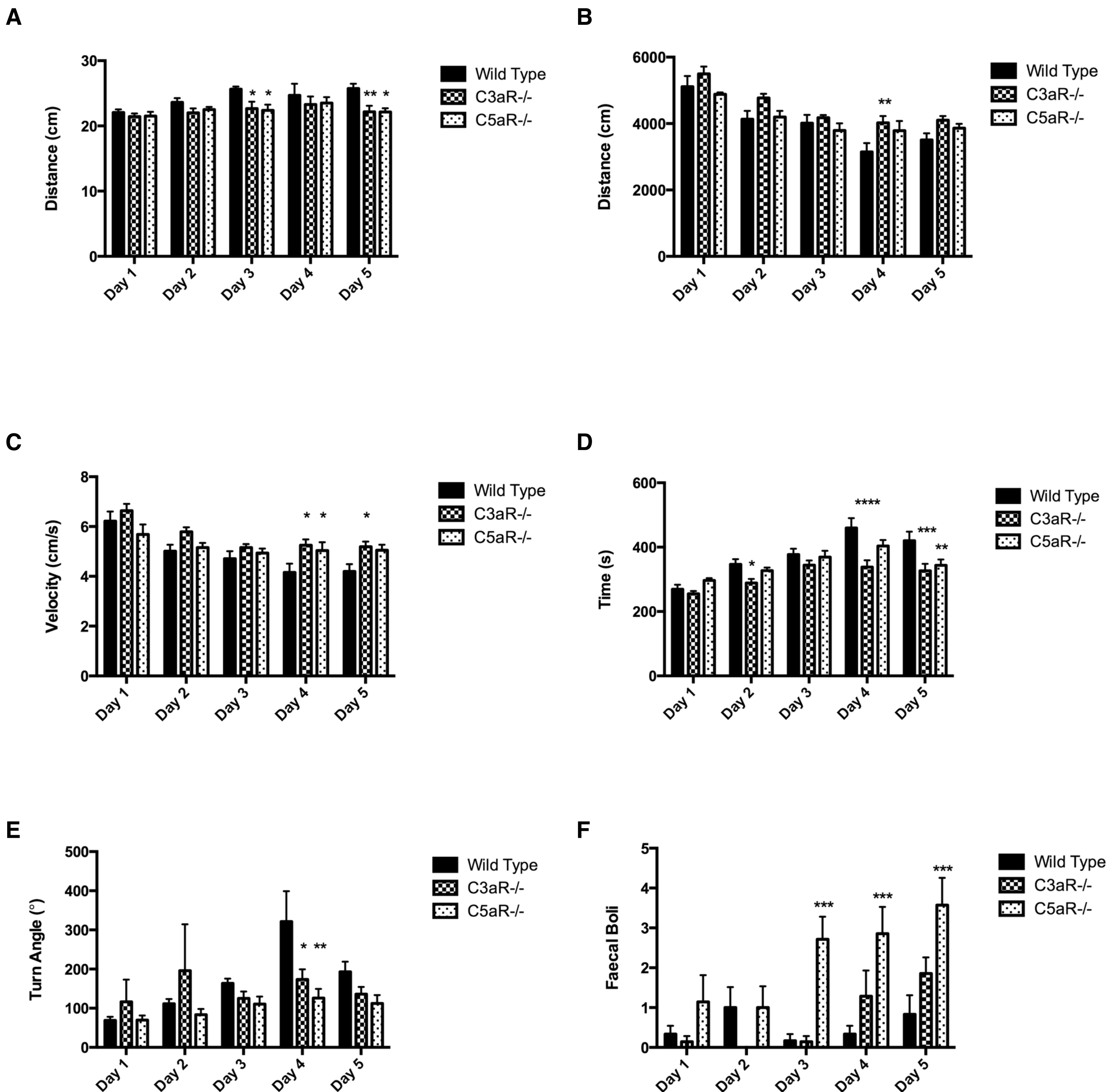

Figure 6.3.2: Open field analysis of anaphylatoxin receptor knockout mice. Bar graphs representing change in open field parameters over the five trial days. **(A)** Mean distance from centre of field **(B)** Total distance travelled **(C)** Average velocity **(D)** Time spent immobile **(E)** Mean turn angle **(F)** Faecal boli in field. Bars represent mean ± standard error. *p < 0.05. ***p < 0.001. p-values determined by two-way ANOVA with Dunnett's post-test.

compared to wild-type mice in this parameter. Anxiety, is generally held to be represented in the open field test by an increase in thigmotaxis, immobility and risk-assessing behaviour (turn angle). Thigmotaxis was measured indirectly from this dataset through calculation of the mean distance to the centre of the arena throughout each trial. In this parameter, both C3aR$^{-/-}$ and C5aR1$^{-/-}$ animals demonstrated a significant reduction in distance to centre on days 3 and 5 of the protocol (figure

6.3.2A), however overall there was no significant effect (table 6.3.1). The time spent immobile was significantly positively correlated to the trial day (table 6.3.1). Overall, C3aR$^{-/-}$ animals spent a decreased proportion of time in the arena immobile (table 6.3.1), an effect that was most pronounced towards the final days of the open field test period (figure 6.3.2D). Although the time spent immobile for C5aR1$^{-/-}$ animals did not differ significantly from wild type mice overall, there was significant deviation from the wild type animals at trial day 5 (figure 6.3.2D). Risk assessing behaviour was analysed using mean turn angle as a measure of the directional changes in the arena. Mean turn angle was positively correlated with trial days and both C3aR$^{-/-}$ and C5aR1$^{-/-}$ animals demonstrated a significant reduction in turn angle when compared to wild type mice (table 6.3.1). Finally, faecal boli is a disputed measure of anxiety in open field testing, as there is debate on whether this is a true measure of anxiety in mice[12]. In the present study, there was a significant increase in faecal boli over time (table 6.1), and C5aR1$^{-/-}$ animals produced significantly more faecal boli than than their wild type counterparts (figure 6.3.2F).

Table 6.3.1: Two-way ANOVA summary data for open field test.

		Two-way ANOVA effect				Overall difference vs Wild Type			
Parameters	**Indication**	**Time**		**Genotype**		**C3aR$^{-/-}$**		**C5aR1$^{-/-}$**	
Distance from Centre	Anxiety/Exploration	↑	***	-	ns	-	ns	-	ns
Distance Travelled	Exploration	↓	****	-	ns	↑	*	-	ns
Mean Velocity	Locomotor activity	↓	****	-	ns	↑	*	-	ns
Time Immobile	Anxiety/Exploration	↑	****	↓	*	↓	**	-	ns
Turn Angle	Risk Assessment	↑	****	↓	*	↓	**	↓	*
Faecal Boli	Anxiety/Emotionality	↑	***	↑	***	-	ns	↑	**

C3aR$^{-/-}$ and C5aR1$^{-/-}$ mice demonstrate deficiencies in memory, but not learning, in the Morris water maze protocol

Learning in the anaphylatoxin receptor knockout animals was assessed over five trial days of the Morris water maze. Parameters assessed during the training period are displayed in figure 6.3.3. Both C5aR1$^{-/-}$ and C3aR$^{-/-}$ animals demonstrated no overall difference when compared to wild type in distance moved (figure 6.3.3A), average velocity (figure 6.3.3B), latency to enter platform quadrant (figure 6.3.3C), percentage of time in platform quadrant (figure 6.3.3D), time to platform (figure 6.3.3E) and mean distance to platform (figure 6.3.3F). Both knockout groups demonstrated a significant reduction in latency to enter platform quadrant from the wild type animals on day one of the protocol, however this trend was no carried forward over the five-day trial period (figure 6.3.3C). On the test day, both knockout groups showed no significant difference when compared to wild type in distance moved (figure 6.3.4A), average velocity (figure 6.3.4B), mean distance to platform (figure 6.3.4C) and latency to enter platform quadrant (figure 6.3.4D). However, the frequency of entries into the platform zone was significantly reduced in both C3aR$^{-/-}$ and C5aR1$^{-/-}$ animals, indicating a deficit in spatial memory (Fig 6.3.4E).

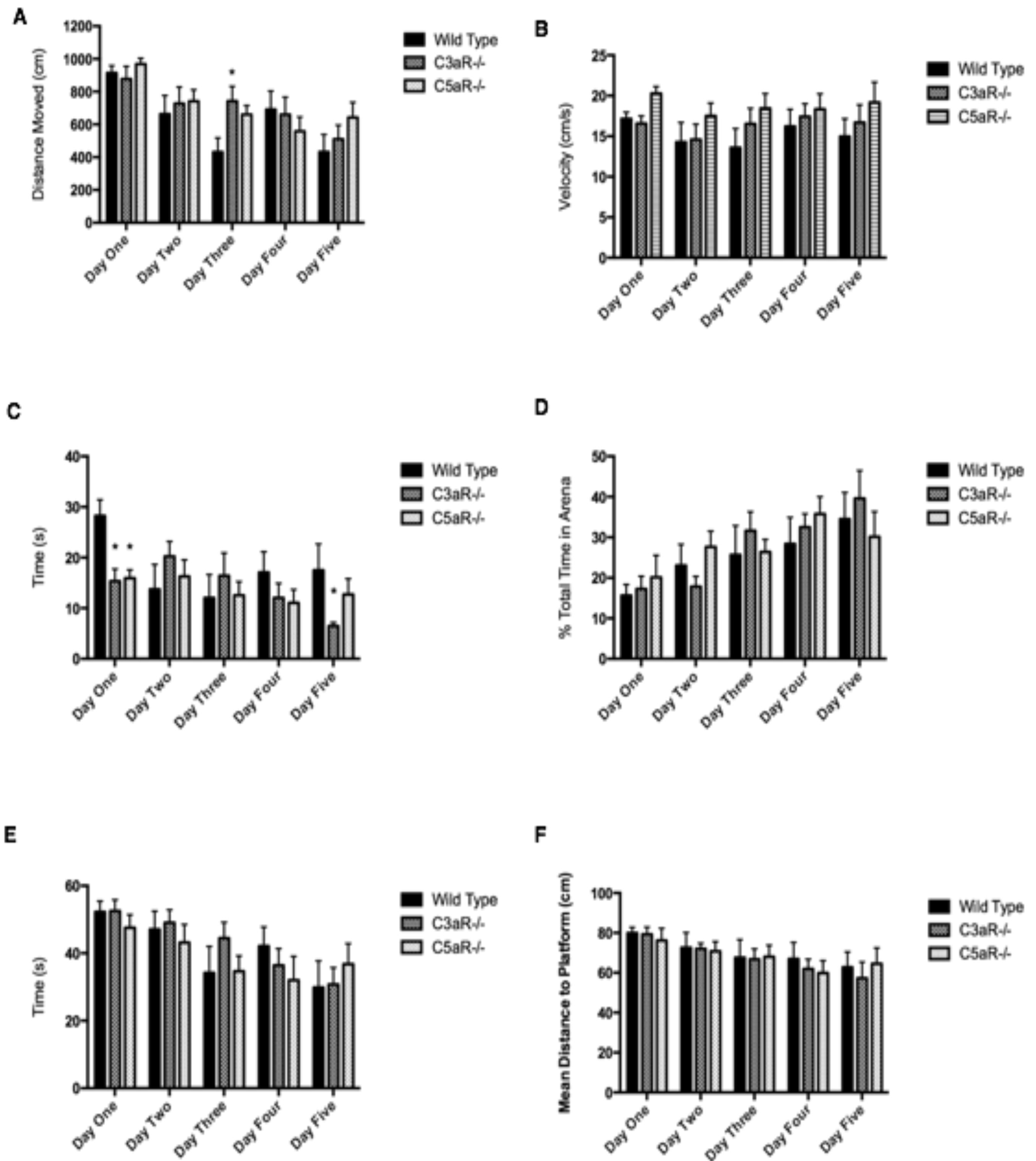

Figure 6.3.3: Water maze trial analysis of anaphylatoxin receptor knockout mice. Bar graphs representing change in water maze parameters over the five trial days (x-axis). **(A)** Total distance moved. **(B)** Average velocity. **(C)** Latency to enter platform quadrant. **(D)** Percentage of time spent in platform quadrant **(E)** Time to reach platform **(F)** Mean distance to platform. Bars represent mean ± standard error. *p≤0.05. ***p≤0.001. p values determined by two-way ANOVA with Dunnett's post-test.

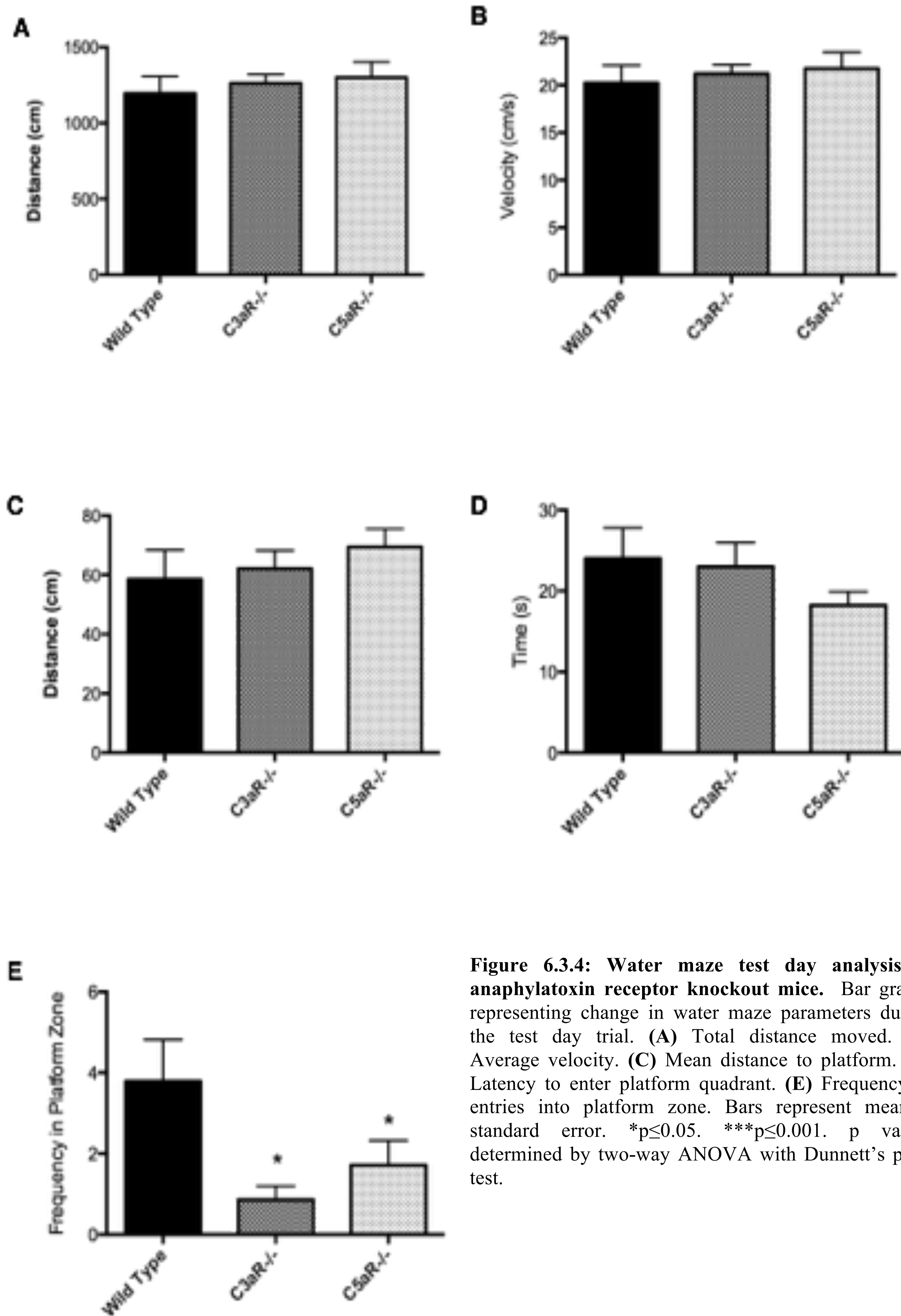

Figure 6.3.4: Water maze test day analysis of anaphylatoxin receptor knockout mice. Bar graphs representing change in water maze parameters during the test day trial. **(A)** Total distance moved. **(B)** Average velocity. **(C)** Mean distance to platform. **(D)** Latency to enter platform quadrant. **(E)** Frequency of entries into platform zone. Bars represent mean ± standard error. *$p \leq 0.05$. ***$p \leq 0.001$. p values determined by two-way ANOVA with Dunnett's post-test.

6.4 Discussion

To our knowledge, although anaphylatoxin receptor knockout mice have been present in the scientific literature for the greater part of two decades, no behavioural screen has been performed to assess for any overt neurological differences[13,14].

Immunofluorescence analysis

Immunofluorescent analysis of M-phase cells at the apical surface of the neocortex in *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ animals yielded some unexpected results. Previously, in this thesis we have demonstrated C5aR1 activity to promote proliferation of neural progenitor cells, whereas C3aR stimulation may promote differentiation, and therefore a reduced population. The results in the current study run contrary to those results previously obtained by pharmacological methods, *C3ar1*$^{-/-}$ animals demonstrated a decreased number of M-phase cells, whereas *C5ar1*$^{-/-}$ animals showed a significant increase. These results did not translate into any observable difference between the gross size of mitotic and post-mitotic populations at E14.5, however cell density or layer composition in the neocortex was not assessed. Therefore this analysis does not rule out the presence of subtle histological differences between the groups.

However, the seemingly contradictory results between using pharmacological and genetic means of suppressing complement receptor signaling may be attributable to the chronicity of treatment, and perhaps the activation of adaptive mechanisms for promoting normal neurogenesis in the absence of these receptors. Previously we have demonstrated the presence of C5aR1 on embryonic stem cells and its function as a signaling mechanism for cell survival and growth in this environment [15]. It is conceivable that, given the early expression of this molecule, adaptive mechanisms to cope with the loss of input may be active early on to direct normal embryogenesis. Indeed, the application of folate-deficiency as a stressor in *C5ar1*$^{-/-}$ embryogenesis leads to gross morphological defects that are not present in the wild type counterparts[1]. This suggests that the role of C5aR1 may only be redundant under normal developmental circumstances, and not under any maternal or embryonic physiological stressor.

Open field test

Given the previously demonstrated importance of C3aR and C5aR1 in neurogenesis, open-field testing was conducted to analyse the behaviour in a novel environment and the effect of learned environment on the *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ animals. Interestingly, previous groups have demonstrated no difference in adult basal neurogenesis between *C5ar1*$^{-/-}$ animals and wild type littermates, using

a pulse-chase BrdU protocol[2]. However, this study did not subject the mice to an enriched-environment, which may actively stimulate neurogenesis in these animals. Additionally, in the open-field test, a balance exists within the individual mouse between the compulsion to explore and the fear of dangers in a new environment. Ultimately, this balance will shift in favour of exploration with increasing time spent in the environment, with the perceived likelihood of danger from predation decreasing. This habituation to the new environment requires the formation of memories specific to the situation, as a form of spatial learning.

No differences existed between *C3ar1*$^{-/-}$, *C5ar1*$^{-/-}$ and wild type mice in the initial exposure to the activity cage, with results demonstrating similar levels of exploratory- and anxiety-related activity in all groups (figure 6.3.4). However, *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ animals demonstrated impaired habituation to the activity cage environment, which may be associated with impaired learning or memory of the novel environment. Wild type mice demonstrated habituation to the novel environment through a progressive increase in time immobile, increase in exploration as measured by turn angle, decrease in average velocity and decrease in distance travelled (figure 6.3.2). Both *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ animals demonstrated some significant differences in these parameters at days four and five of the protocol, not demonstrating the same magnitude of change in behavioural parameters as their wild type counterparts.

Morris water maze

Interestingly, in the Morris water maze protocol, *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ animals did not differ, in terms of habituation, from the wild type. However, habituation to the activity cage environment involves a milieu of subtle behavioural facets that may not be tested in the MWM, the balance between anxiety and exploration and the memory of the learned environment all contribute to behavior in the activity cage[12]. In the MWM environment, the driving force behind movement is the survival instinct, as time progresses mice learn where the hidden platform can be found using the visual cues in the room. This compulsion for movement in the MWM may explain the discrepancy between the results seen with regards to habituation.

Over the 5 days of trials, *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ did not differ significantly from the wild type group, suggesting that spatial learning is not impaired in these animals. However, the test day showed that memory of the platform's position was not as accurate in both the *C3ar1*$^{-/-}$ and *C5ar1*$^{-/-}$ groups. The knockout groups demonstrated only half the passes into the platform area when compared to the wild type group (figure 6.3.4E). Interestingly, the mean distance to platform (figure 6.3.4C) and latency to enter platform quadrant (figure 6.3.4D) did not demonstrated any difference from the

wild type group, suggesting that any spatial memory deficit present in the knockout groups is subtle.

Conclusion

The foundation of learning and memory in the adult is based on an active process of neurogenesis. Given that we have previously demonstrated a role for C3aR and C5aR1 in the regulation of embryonic neurogenesis we aimed in the present study to identify any functional deficits in adult knockout mice through the perturbation of their embryonic neurogenesis, or impairment of learning and memory as adults. Here we have shown that there are differences in mitoses at E13.5 that are opposed to the pharmacological investigations. Additionally, adult anaphylatoxin knockout animals demonstrate subtle behavioural deficits in memory. However, additional experiments are required before these can be conclusively linked to any deficits in neurogenic potential. There are promising mouse models in production (detailed in chapter 3) that will further research in this area. In particular, the use of a conditional C5aR1-knockout animal during development would allow for further understanding as to whether these behavioural abnormalities are of a developmental or adult neurogenesis origin. As it stands, the use of absolute knockout animals, such as in this chapter, is unable to provide this distinction. In addition, this chapter only assessed the effect of anaphylatoxin receptor knockout on male mice, therefore, whilst this data may be reasonably extrapolated to female animals, it is unclear whether sex alters the role of the anaphylatoxin receptors in the mouse brain.

6.5 References

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Chapter 7

Discussion and Concluding Remarks

7.1 Discussion

This thesis investigated the function of the anaphylatoxin receptors of the complement system on neural progenitor cells during mouse embryogenesis.

The aims of this thesis were to:

1. *Investigate the localisation and expression of C5aR and C3aR during brain development.*
2. *Determine the functional role of C5aR and C3aR on neural progenitors during brain development.*
3. *Elucidate the second messenger signalling involved after C5aR activation on neural progenitor cells.*

Summary of the aims and findings

This thesis demonstrated expression of C5aR and C3aR on the apical membrane compartment of apical neural progenitor cells *in vivo*. This localisation was maintained in 3-dimensional, polarised neurosphere culture, but lost in monolayer culture, suggesting an importance for the development of cell-polarity before membrane expression of the anaphylatoxins in this cell type. With regards to aim 2, this thesis demonstrated opposing roles for the anaphylatoxin receptors within the same cell type. Whilst chapter 4 covers the role of C5aR as a driver of neural progenitor proliferation, the evidence provided in chapter 5 indicates that C3aR, conversely, is a driver of differentiation. This is in keeping with previously reported observations demonstrating that both receptors direct the balance of proliferation and differentiation in the post-natal rat cerebellum[1,2]. Aim 3 was addressed within chapter 4 and showed that C5aR stimulation, in neural progenitor cells, initiates Erk1/2 signalling through a PKCζ-dependent mechanism (summarised in figure 7.1.1). This correlates well with the localisation of the receptor *in vivo*, as PKCζ has previously been shown to be apically located within this cell type.

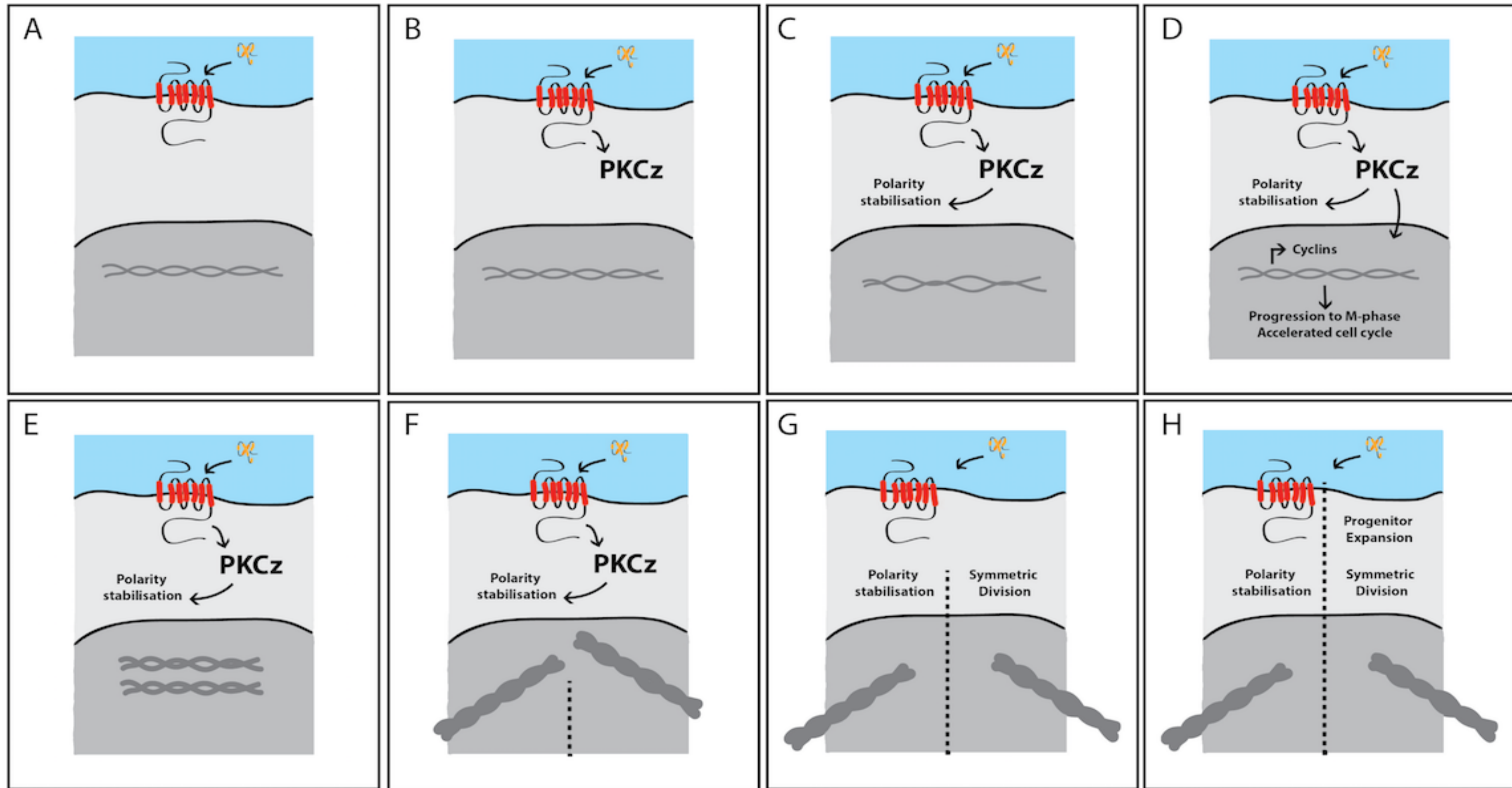

Figure 7.1.1: Diagrammatic representation of the suggested signalling cascade for C5aR1 demonstrated in this thesis. From A-H: C5aR is present on the apical membrane of neural progenitor cells within the ventricular zone. Interaction with C5a leads to PKCζ activation. Actions of PKCζ, through MAPK signalling, leads to a short G_1 phase and faster progression through the cell cycle. Additionally, PKCζ acts to stabilise polarity increasing the likelihood of symmetric division. Symmetric division of progenitor cells results in expansion of the progenitor pool at the expense of neurogenesis.

In addition to the major aims, this thesis also demonstrated the presence of complement and other innate immune factors in early neural development (chapter 1). Significantly, there was absence of a number of propagating factors of the canonical pathways of the complement cascade, and absence of C9 of the membrane attack complex. Given the fragmented and toothless nature of this pattern of expression, it is not unreasonable to consider alternate roles for complement components expressed at this stage of development, outside of innate immunity. Finally, a preliminary investigation into the behaviour of anaphylatoxin knockout animals was included (chapter 6). Despite the evidence garnered in chapters 3 & 4, demonstrating a role for the anaphylatoxins in directing proliferation in the developing brain, the receptor knockout animals are not embryonically lethal, nor have they been reported to have any neurological deficits. Chapter 6 demonstrated some subtle behavioural differences between the knockout mice and wild-type littermates. However, although slight numerical differences were demonstrated within M-phase apical progenitors during development, these were opposite to the effect of acute pharmacological treatment and the mitotic population pool did not differ from wild-type littermates. These data suggest that the effects of the anaphylatoxin receptors in brain development are somewhat redundant and their chronic, genetic loss may be compensated for adequately.

Wider implications of innate immune molecule expression within the developing embryo

The demonstration of widespread expression of both complement- and other innate immune factors within the early embryo should provide an impetus for further research into the roles of these factors in embryogenesis. This thesis has demonstrated a role for C5aR in embryonic neurogenesis and shown that disruption of signalling can result in impaired neurological function in adulthood (chapter 4). There is increasing evidence of important roles for other components of the complement system in neurodevelopmental processes including, C1q/C3b in synaptic pruning[3], C3aR in neural crest migration[4] and C3aR/C5aR in cerebellar developement[2]. In addition, other innate immune factors, such as the toll-like receptors, regulate the balance of proliferation and differentiation in the neural progenitor population[5,6].

These observations of immune molecules in development present problems, clinically, for the treatment of inflammatory diseases of pregnancy. Complement factors in particular have been highlighted as potential targets for the placental inflammation associated with pre-eclampsia[7,8]. In addition, dysregulated complement activation has also been shown to causes poor pregnancy outcomes, such as intrauterine growth restriction and miscarriage[9-11]. This thesis, and similar research, suggests that in treating these diseases, by targeting factors of innate immunity, the

clinician gambles with the risk of altering processes of embryogenesis. It is difficult to track outcomes of anti-inflammatory treatment during pregnancy in human populations as it is unknown whether poor fetal outcomes are a result of treatment, or disease, or both. A balance must be achieved for both the health of the mother and fetus, and at present it is unclear where this lies.

Another consideration lies in the fact that a large proportion of embryonic neural development, related to the stages examined within this thesis, occurs within the first trimester, often when the mother is unaware she is pregnant. Thus, this cautions generically against the use of anti-complement therapies in females of childbearing age, where the therapeutics may conceivably enter the developing embryonic brain.

Linking damage and repair

The canonical immune role of the complement system is well defined and delineated. Activation occurs via pathogen recognition or cellular damage, and numerous damage associated serine proteases (e.g. thrombin) are also able to cleave the central complement components[12]. However, there has been an increasing focus within complement research in the past decade on the extra-immune roles of complement and, increasingly, on the idea that innate immunity and developmental or regenerative processes are intimately linked[13,14].

It has previously been noted that there is an inverse correlation, within organisms, between the maturity of the adaptive immune system and the capacity for regeneration[15]. In animals with poor adaptive immunity there is capacity for regeneration of whole limbs or body regions. The hypothesis is that an organism could deal with damage via two distinct mechanisms, proactively detecting and avoiding damage through adaptive immunity or regenerating tissues lost to inevitable damage. A striking example of this inverse relationship is the hydra. This animal is undoubtedly phylogenically ancient, possessing only two germ layers and no mobile adaptive immunity. The hydra has the capacity to regenerate into two individuals when bisected through regeneration of the foot and head regions within the two halves. Interestingly, within the regenerating region the hydra expresses a host of recognisable genes of the innate immune system as characterized by RNA sequencing[16]. Hydra analogues of complement factors, including C1q and MASP, are up-regulated at the site of regeneration[16].

This phenomenon remains in more complex animals. After surgical removal of the limb or eye lens the newt displays a capacity for regeneration of these tissues. Again, complement factors are

expressed within these regions, long after the initial insult. C3 and C5 are expressed in the regenerating lens vesicle and limb blastema, although there is no detectable expression within the mature tissues[17,18]. In both the newt and hydra models, the presence of complement factors at the site of regeneration is observational and no function has been ascribed experimentally. It could be argued that their expression in these regions functions as protection for the compromised regenerating tissue from pathogen colonisation. However, this hypothesis does not adequately explain the persistence of expression after the formation of an epithelial barrier.

Higher order vertebrates provide greater experimental clues as to the biological roles of these molecules in regeneration. Although in these phyla regenerative capacity is restricted, certain organs, such as the liver and the brain, show capacity for regenerative repair. Liver regeneration in mice post-hepatectomy is reliant on the presence of C3 and C5, signalling through C3aR and C5aR. Genetic absence of these factors results in reduced proliferation and arrest of hepatocytes in G_1 phase in the regenerating liver[19,20]. These effects were able to be rescued through the addition of their split product, C3a and C5a[19]. This suggests that the important signalling events in this instance are through the anaphylatoxin receptors, rather than through formation of terminal elements of the complement cascade. Similar results are found in bone remodelling, where $C3^{-/-}$ and $C5^{-/-}$ animals demonstrate reduced callus formation and reduced bone growth post-osteotomy[21]. Knockout animals also exhibit reduced bone strength that may represent impaired remodelling from physiological fractures, as there are no noted bone deformities that might indicate impaired osteogenesis. Recently, a specific and potent role for C3a in inducing retinal regeneration in the embryonic chick also adds weight to the argument for the importance of the anaphylatoxins in regenerative processes[22]. In this context these studies reflect the conclusions of this thesis, that abrogation of anaphylatoxin signalling in the acute setting elicits defined roles not evident during linked developmental processes in the knockout animal.

The studies of ischaemic stroke and neuronal injury illustrate the functional linkage between the roles of the anaphylatoxin receptors in inflammation and regeneration; it has been described in this area as a ‘double-edged sword’[23-25]. Initially, blockade of C5aR1 was demonstrated to be beneficial with regards to outcomes in neuronal injury, in models with a short time to assessment of outcomes[26]. C5aR1 and C3aR were then demonstrated to be present on basally migrating neural progenitors of the mouse, and in the context of middle cerebral artery occlusion (MCAO), $C3^{-/-}$ animals demonstrated reduced neurogenesis up to three weeks after the ischaemic event[23]. The models focussing on initial outcomes have the drawback of only capturing the inflammatory aspect of complement functions, a restriction based on the ethical considerations in stroke research. However, it also realised that the anaphylatoxin receptors exhibit a biphasic, opposing function with

regards to neurological outcome[27]. Antagonism of these receptors, whilst beneficial in the inflammatory phase, may be detrimental as the tissue progresses towards repair and regeneration[27].

Interestingly, previous research demonstrates that there is no deficit of basal neurogenesis in C5aR1$^{-/-}$ adult animals[28]. This is almost contrary to the present thesis, which shows a crucial role for endogenous C5a-C5aR1 signalling, through use of the antagonist, PMX53, in normal CNS development. However, this thesis also demonstrated no histological differences between C5aR1$^{-/-}$ brains and those of wild type littermates. There are caveats to both the pharmacological and knockout models that must be considered in the interpretation of results. Pharmacological targeting of C5aR1 has the benefit of being able to be administered acutely, to target a distinct time point in development. However, this benefit is tempered by dose-dependent effects, pharmacokinetics, and, possible off-target effects on other aspects of cell biology. The genetic knockout models provide complete absence of the protein, but raise the spectre of upregulated compensatory mechanisms for developmental processes. Using knockout animals for C3 and C5 also further complicates the interpretation. It is often unclear, and difficult to experimentally determine, whether observed differences result from interruption of direct receptor-ligand interactions (i.e. C3a-C3aR, C5a-C5aR1/C5aR2) or the opsonin (i.e. C3b) or terminal elements (i.e. C5b-9) of the complement cascade. Additionally, in the case of C3$^{-/-}$ mice, results are further compromised as complement pathway compensation through extrinsic protease C5 cleavage has been demonstrated to occur in the absence of C3[29]. This quagmire of interpretation may be navigatable with the emergence of inducible knockouts in this area of research, which will allow for the specific and time-dependent abrogation of receptor signalling, without the potential for affecting other aspects of embrogenesis[30].

Physiological antagonism of the anaphylatoxin receptors

This thesis demonstrated an apparent physiological antagonism for the anaphylatoxin receptors in neural progenitor cell physiology; C5aR promotes proliferation, whilst C3aR promotes differentiation. This concept is not new in complement biology, however it is often overlooked as both receptors are described as 'pro-inflammatory mediators'. We have recently attempted to dissuade the use of this term by researchers through highlighting the anti-inflammatory facets of C3a, in contrast to the largely pro-inflammatory nature of C5a (chapter 1.7)[31].

For instance, in models of ischaemia-reperfusion (IR) injury knockout animals for C3aR and C5aR show opposing responses to induced injury. C5aR$^{-/-}$ mice demonstrate reduced pathological scores

and increased survival in models of sepsis and gut IR, which results from a reduction in neutrophil activation and migration into the affected tissues[32,33]. In contrast, $C3aR^{-/-}$ exhibit worsened outcomes to IR injury, suggesting that C3aR signalling is protective in these models[34]. The mechanism behind this apparent opposing activity is the role that C3aR plays in keeping the reserve neutrophil pool within the bone marrow; the knockout animal demonstrate a persistent neutrophilia that leads to worsened outcomes in disease[34].

However, the range of antagonistic activities extends far deeper than the respective roles of the receptors in the immune system. Previous research has shown distinct and opposing haemodynamic responses to the anaphylatoxins in the rat. Infusion of C5a causes a transient hypotension, whereas infusion of C3a induces hypertension[35]. In both cases, these effects can be prevented through pretreatment with indomethacin, indicating a prostanoid-mediated response[35,36]. This demonstrates distinct and physiologically opposing signalling effects for C3a and C5a which contrasts with the persistent class grouping of these molecules in the literature[31].

In the developing brain, this phenomena is perhaps best illustrated by the opposing roles of C3aR and C5aR in the post-natal rat cerebellum. Both receptors are expressed in the progenitor-rich external granule layer of the cerebellum and expression is reduced with cerebellar maturation[1]. This observation is similar to that shown for C5aR in this thesis, where expression reduces with differentiation of NE-4C cells (chapter 4). Both receptors have effects on the fate of cerebellar progenitor cells. Sub-dural administration of a C5aR agonist results in an expansion of the external granule layer and an increase in BrdU-positive cells within this region[2]. By contrast, administration of a C3a agonist results in expansion of the internal granule layer at the expense of the progenitor population. It is hypothesised that this is due to effects of C3a in mediating differentiation and migration of the progenitor cells[2]. The results of this thesis, in treatment of telencephalic neurosphere cultures with C3aR modulators (chapter 5), and that of previous research demonstrating reduced neuroblast migration in $C3^{-/-}$ animals, suggests that this role for C3aR may extend across all types of neural progenitor cell[23].

7.2 Concluding Remarks

The studies presented in this thesis add to the understanding of embryonic neural development and support recent, exciting findings into the new aspects of complement biology. It is becoming clear that the complement system is not solely confined to roles in innate immunity, but rather links the twin aspects of damage control and regeneration[15]. In this context, the appearance of complement factors in developmental processes is less surprising. As ever though, there is always more to do.

There is currently a deficit in technology available to study complement in developmental processes. This thesis has demonstrated that the current conventional knockout animals may not portray a truly representative depiction of neural development. Clearly, they respond very differently compared with direct pharmacological modulation of the anaphylatoxin receptors, perhaps due to compensatory mechanisms. The proliferation of new genome editing technologies, such as the recently introduced CRISPR system, and the production of complement receptor inducible knockout mice[30] provide exciting new avenues through which to expand this research.

The plethora of innate immune molecules identified in the early embryo in this thesis, and the key roles for the anaphylatoxin receptors in directing neurogenesis should caution against rapid clinical uptake of anti-complement therapeutics in pregnancy. Currently, anti-complement therapeutics are valuable targets in the treatment of inflammatory diseases of pregnancy (e.g. preeclampsia) and inflammatory diseases of the mother. Their specific and targeted effect, when compared to other available treatments such as corticosteroids, makes them an attractive avenue for drug design. However, given the results presented in this thesis, and the previous demonstration of C5aR1 also on the neuroepithelium of human embryos[37], it would be wise to proceed cautiously in the introduction of anti-complement therapeutics until the role of complement in developmental processes is more fully understood.

This thesis has identified a key new player in embryonic neural development in C5aR1, which add to the milieu of factors currently identified in the literature. It is still unknown how this fits in with other key players occupying the same niche of the apical neural progenitor cell; whether C5aR1 signalling acts mainly in isolation, or is part of the greater orchestra of factors that mould a cells fate. However, this thesis has provided a basis for the exploration of these important questions.

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Appendix A

The Classical and Novel Roles for Complement Factor 3a Receptor (C3aR) in Health and Disease

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In preparation for submission as a review article

Abstract

The complement cleavage product C3a exerts its biological effects through the 7-transmembrane G-protein coupled receptor, C3aR. Until recently, research on the infamous cousin of C3aR, C5aR, has taken precedence in anaphylatoxin literature. This may have stemmed from the traditional interpretation of the complement cascade as beginning with an immune challenge and ending with the cleavage of C5, to form C5a and the membrane attack complex (C5b-9). In contrast to C5aR, which has well-established pro-inflammatory functions in a number of diseases, the function of C3aR is more complex. It has now been demonstrated to exhibit multiple biological effects, which are dependent on the cell type and disease model. In recent years, novel advances have also more definitively characterised the role of C3aR expression on cells that previously had an unknown, or only loosely defined, role for C3aR, particularly neutrophilic granulocytes and certain lymphocyte subsets. The multiple functions of C3aR complicate the targeting of this receptor as a therapy in inflammatory disorders. Indeed both C3aR antagonists and agonists could be viable therapies depending on the disease context. In addition, the lack of research on the receptor has left a dearth in pharmacological modulators, although this is a burgeoning area of interest. Here we present the current state of knowledge on C3aR and the possibilities for therapeutics targeting inflammatory diseases.

Introduction

Complement factor 3 is the convergence point of the complement activation pathways and is cleaved to form the anaphylatoxin, C3a. C3a is a 77-amino acid polypeptide, with carboxyl-terminal residues largely responsible for activating its corresponding receptor, C3aR. Acting via C3aR, C3a has traditionally been regarded as a highly potent effector of inflammation and plays several crucial roles in immune responses and inflammatory processes. Unlike its infamous cousin, C5a, resting concentrations of C3a are high due to high circulation concentrations of C3 (~1.2mg/ml in humans), and the continual degradation of C3 through the alternative tickover pathway[1]. However, elevation of serum C3a concentrations is still an early marker of infections or inflammatory disease. C3a is also readily degraded by serum carboxypeptidase-mediated removal of the C-terminal Arginine to form C3a-desArg, which is unable to bind to C3aR[2]. Although the C3a-C3aR axis was originally associated with the onset of acute inflammatory reactions leading to complications such as acute graft rejection[3], local tissue injury, and multiorgan failure[4], further investigations have revealed more multifaceted roles for this signalling axis. The actions of C3aR, to promote, moderate or attenuate inflammation, depend on the disease and cell type. In addition, C3aR has now been demonstrated to be involved in processes outside of the boundaries of innate immunity, such as organogenesis[5], tissue regeneration[6,7], and neural development[8,9].

Expression, structure and signaling

C3aR was initially shown to be expressed on leukocytes of myeloid lineage, such as neutrophils, basophils, eosinophis, mast cells, and monocyte/macrophages[10]. However, it is now well-accepted that C3aR is expressed on cells as diverse as endothelial cells, smooth muscle cells, and cells of brain origin, astrocytes, microglia and neurons[8,11-13]. Recently, C3aR expression has also been demonstrated in mesenchymal, neural and haematopoietic stem cells[8,14,15]. The expression in stem cell populations may suggest a role in recruitment for the repair phase of inflammation. Although C3aR is widely expressed there are dramatic differences in both the control of expression and associated second messenger coupling between cell types.

The C3aR gene (*C3AR1*) has the chromosomal location 12p13.31 in humans and 6F1 in mice, the major animal model for C3aR investigation. In both species, the gene consists of two exons separated by a large intron. Exon 1 contains no coding sequence, but is the site of the regulatory sequences that determine expression. Both species exhibit a dense, conserved region 70 to 35 bases upstream of the transcriptional start site, that are critical to cell-type specific regulation[16]. This region contains a GATA, Ets-like and AP1 site, but the relative contribution of these sites to gene

transcription has been demonstrated to be dependent on the cell-type. For instance, transcriptional control in myeloid derived cells, which are reliant on the functionality of both the ETS and AP-1 elements, differs to that reported in astrocytes, where there is heavy reliance on the AP-1 site, but deletion of the ETS-like site does not reduce transcription significantly[17,18]. Additionally, although transcription from the AP-1 site in macrophages was initiated by c-Jun binding, there was no indication of this interaction occurring in primary astrocytes or the Ast2.1 cell-line[18].

At the protein level, C3aR is a classical 7-transmembrane, G-protein coupled receptor, which shares close homology to the receptors for C5a, C5aR and C5L2[19]. C3aR binds its only known ligand, C3a, using a proposed 'two-site' binding model, consisting of functionally different recognition and activation sites[20]. This is similar to the model reported for C5aR, which may be expected due to the shared evolutionary origins of both proteins[21]. However, unlike C5aR, the N-terminus of C3aR is not charged and is functionally insignificant with regards to C3a binding[22]. C3aR also differs in structure through the presence of a large second extracellular loop of 172 amino acids[23]. The size this extracellular loop is largest in mammals, with cloning of Rainbow trout and Xenopus C3aR demonstrating smaller loops that are not as remarkable, in terms of size, as the mammalian form[21]. The loop is of interest because the residues that are important for C3a binding appear to be well conserved, raising questions over why the mammalian version is so exaggerated[21,24,25]. It is unlikely that this evolutionarily-sequential extension of extracellular loop 2 is without benefits, and it is hypothesised that the loop may contribute to the binding of other, as yet unidentified, ligands[24].

At the C-terminus, C3aR couples to heterotrimeric G-proteins. C3aR has demonstrated a promiscuity of G-protein interaction dependent on cell type, for instance, in leukocytes, C3aR couples to the pertussis toxin (PT)-sensitive Giα and to the PT-insensitive Gα16, whereas the receptor couples to Gα12/13 in endothelial cells. This alternating coupling of G-proteins affects the types of second messenger signalling resulting from the C3a/C3aR interaction. Generally, the stimulation of C3aR results in the activation of MAPK pathways, activation of protein kinase C (PKC) and mobilisation of intracellular calcium. Although C3aR exerts traditional pro-inflammatory roles via p38/PKC in cells such as astrocytes, mast cells and endothelial cells[12,26,27], intriguingly, the more novel functions now assigned to C3aR are not consistently mediated in this manner. For instance, in neural precursors C3aR-activation functions as an inhibitor of MAPK phosphorylation, demonstrating the pleiotropic functional nature of C3aR[28].

C3a-induced desensitisation of C3aR involves phosphorylation of the receptor by GPCR kinases (GRK) in a rapid dose-dependent and reversible fashion[29]. This desensitisation is augmented by receptor internalisation in response to C3a. Internalisation of the C3a receptor has been demonstrated in granulocytes, to be dependent on the activity of PKC, and is insensitive to pertussis toxin inhibition of C3aR signalling[30].

Additionally, C5a reduces C3a-stimulated C3aR internalisation, an observation that adds weight to the view of the C3aR/C5aR system as analogous to yin and yang, rather than a purely pro-inflammatory duo[30]. In addition to GRK, recently, β-arrestin proteins have been shown to play a novel role on C3aR desensitisation, internalisation, degranulation, NF-$_k$B activation and chemokine production in human mast cells[31]. β-arrestin proteins can also regulate the activity of C3aR by inhibiting G-protein-dependent ERK1/2 phosphorylation[32]. Interestingly, this MAPK signalling inhibition by β-arrestins is one of the postulated downstream pathways of the C5a-C5L2 axis[33]. C5L2 has already been demonstrated to regulate C5aR signalling in this manner, although C5L2 regulation of C3aR signalling remains, as yet, uninvestigated[34].

The Function of C3aR on Circulating Leukocytes

The role of C3aR in the immune system is complicated by seemingly contradictory reports. C3aR has opposing roles at the local and systemic level, meaning that broad statements about the general function of this receptor in immunity present a superficial and oversimplified view. At the site of inflammation, C3aR has several pro-inflammatory properties overlapping with C5aR, however C3a is much less potent than C5a in this regard and also has anti-inflammatory facets[20]. *In vitro* investigations into the effects of C3a on LPS-induced cytokine release by PBMCs demonstrate of difference of action depending on the state of the cell. C3a augments the proinflammatory cytokine production of adherent cells, but suppresses that of non-adherent cells[35]. Depending on the adhesive state of cells, C3a and C3adesArg suppress TNF-α, IL-1β and IL-6 synthesis in lipopolysaccharide (LPS)-primed non-adherent peripheral blood mononuclear cells (PBMCs) and B lymphocytes, and enhance their synthesis in adherent cell systems[35-37]. There is a certain elegance in this hypothesis when applied to the in vivo environment - C3a, a split product of one of the most abundant blood proteins, enhances the inflammatory response in the local environment of the insult, but inhibits a systemic cytokine storm by suppressing production from non-adherent cells.

Myeloid Lineage

C3aR is expressed by all leukocytes of a myeloid lineage, however the functional characteristics of the receptor are dependent on cell type. In human eosinophils, C3a induces calcium mobilisation,

oxidative burst and degranulation[38,39]. In mast cells, C3a and $C3a_{desArg}$ can induce histamine secretion in a dose related fashion[40-42]. Both C3a and C3adesArg can also increase intracellular calcium levels in human monocytes, and induce production of IL-1β, TNF-α, IL-6 and PGE_2 [12,35,36,43,44,45]. This eosinophil and mast cell-specificity has highlighted C3aR as a drug-target candidate for allergic conditions. Such inferences have been strengthened through observations of $C3aR^{-/-}$ animals that exhibit reduced responses in atopic diseases such as allergic asthma and allergic dermatitis[46]. Although the C3a/C3aR axis has been demonstrated to cause chemotaxis of eosinophils and basophils, interestingly, and in stark contrast to C5a/C5aR activation, neutrophils do not undergo chemotaxis, granular release or oxidative burst in the presence of C3a[47]. Some studies have previously reported a functional role for C3aR on neutrophils[38], however these were observations of indirect mechanisms. For instance, C3a has been shown to cause chemotaxis of neutrophils in cultures that contain >5% eosinophils, and eosinophils release a soluble factor in reponse to C3a that causes neutrophil chemotaxis[47]. However, we have demonstrated a unique, and direct, role for C3aR in regulating the mobilisation of neutrophils from the bone marrow.

Emerging role in neutrophil/HSC mobilisation

An unprecedented but critical role in hematopoiesis and stem cell trafficking has emerged in the last decade. It is well established that the α-chemokine stromal-cell-derived factor 1 (SDF-1/CXCL12), expressed by osteoblasts, bone marrow stromal cells and vascular endothelial cells, plays a central role in haematopoeitic stem/progenitor cell (HSPC) trafficking, and has become the textbook example of HPSC migration[48-51]. In 2003, it was demonstrated that C3 is a physiologic constituent of the bone marrow (BM) milieu following its secretion by BM stromal cells[14]. Less than a year later, it was reported that G-CSF-induced mobilisation is associated with complement activation and the local cleavage of native C3 into fluid-phase C3a, which is degraded into C3a-des-arg and C3b/iC3b; the latter is deposited on viable BM cells of G-CSF-treated animals[52]. Human BM-derived and mobilised peripheral blood $CD34^+$ HSPCs, as well as in $CD34^+$ cell-expanded lineages (including myeloblasts, megakaryoblasts, and erythroblasts) analysed by RT-PCR and FACS have been reported to express C3aR, and that administration of C3a to these cells stimulates intracellular Ca^{2+} flux in mobilised peripheral blood $CD34^+$ and lineage-expanded hematopoietic cells[14].

Investigations of this functional significance discovered that although administration of C3a alone did not affect the proliferation, survival or chemotactic response of human and murine $CD34^+$ HSPCs and $CD34^+$ lineage-committed clonogenic hematopoietic progenitors, it greatly enhanced their chemotactic response to a low-dose (10ng/ml) SDF-1 gradient[14,53]. Similarly, using reconstituted basement membrane Matrigel to measure the invasive potential of human BM-derived

HSPCs and $CD34^+$ peripheral blood cells, C3a alone did not increase trans-Matrigel migration, but it significantly augmented SDF-1-dependent migration[14]. Human $CD34^+$ cells stimulated by C3a also secreted 2.6 fold more MMP-9 into the conditioned media than non-stimulated cells, indicating C3a facilitates basement membrane degradation[14]. Finally, C3a was shown to promote HSPC homing by enhancing the SDF-1-dependent adhesion of VLA-4 on human CD34+ cells to VCAM-1, suggesting cross talk between C3a and the SDF-1-CXCR4 signalling axis may increase the propensity of HSPCs to attach to the vascular endothelium or BM niches[14].

The role of C3a in HSPC trafficking has been further defined using in vivo studies. In lethally irradiated mice, transplanted $Sca\text{-}1^+$ ($Ly6A/E^+$) BM HSPCs that were primed before transplantation with C3a demonstrated significantly enhanced bone marrow engraftment and accelerated hematopoietic recovery compared with animals that received transplants of $Sca\text{-}1^+$ cells primed with C3a-des-Arg or non-primed cells[14]. Although $C3aR^{-/-}$ mice are haematologically normal under steady state conditions, they display significantly increased G-CSF-induced mobilization of circulating mononuclear cells (MNCs) and colony-forming unit-granulocyte macrophage progenitors (CFU-GM) in their peripheral blood compared to wild-type mice[52]. We have independently confirmed that $C3aR^{-/-}$ mice exhibit accelerated G-CSF-induced mobilisation of granulocytic populations into the circulation[54]. BM chimeric mice have confirmed that C3aR deficiency on transplanted and not resident cells are responsible for the increased sensitivity to G-CSF-induced mobilisation observed in $C3aR^{-/-}$ mice[52]. Similarly, transplantation of $C3aR^{-/-}$ HPSCs into lethally irradiated mice results in delayed recovery of platelets and leukocytes and decreased number of donor-derived granulocyte-macrophage progenitors detectable in the BM[55]. Further, antagonism of C3aR with SB290157 significantly accelerated G-CSF-induced mobilization of MNCs and CFU-GM progenitors in WT, but not $C3aR^{-/-}$ mice[52]. Again this particular agonist also has off-target agonist activity[56]. HSPCs from $C3aR^{-/-}$ mice exposed to C3a secrete less MMP-9 and show impaired adhesion to stromal cells. C3a may therefore directly modulate HSPC homing by augmenting C3aR-mediated secretion of MMP-9 and cell adhesion[55]. On a molecular level, C3a could potentiate HPSC responses to SDF-1 gradients by increasing the incorporation of CXCR4 into membrane lipid rafts, facilitating its assembly with its downstream signaling molecules[57].

Interestingly, evidence suggests that the C3a-enhanced responsiveness of HSPCs to SDF-1 is also mediated in a C3aR-independent manner. First, and somewhat contradictory to previous findings, Honczarenko et al. (2005) found that freshly isolated human BM $CD34^+$ and B lineage cells do not express C3aR as assessed by flow cytometry. It was also reported that C3a enhances SDF-1-induced chemotaxis of BM progenitors from $C3aR^{-/-}$ mice similar to BM cells from WT mice[53].

Further, C3a increased the binding affinity of SDF-1 to human $CXCR4^{+}C3aR^{-}$ REH pro-B cells, which could suggest a direct interaction between C3a and SDF-1[53]. Collectively this data indicates C3 is cleaved in mobilised BM into C3a and C3b, and that C3a is a positive regulator of early and more differentiated HSPC retention in the BM. This is via increasing responses dependent on the SDF-1-CXCR4 axis in a C3aR-dependent and independent manner, thus preventing uncontrolled release of HSPCs into peripheral blood during mobilisation.

Lymphoid Lineage

The function of C3aR on cells of the lymphoid lineage has been the subject of some debate as the presence of the receptor on T- and B-cells has yet to be conclusively proven using the gold standard techniques of Western blot, RT-PCR or immunohistochemisty. The C3a-C3aR axis certainly has a role in modulating the response of lymphocytes to a pathogenic insult via the actions of an intermediate cell-types[58-60]. Nevertheless, several studies exist in excellent journals demonstrating functional roles for C3aR within the lymphoid lineage itself. The mystery surrounding C3aR expression in lymphoid cells could perhaps be partially explained by only a subset of cells possessing the receptor. For instance, in one of the first publications investigating a lymphoid C3aR, Werfel and colleagues (2000) demonstrated absence of C3aR on T-cells in both normal human subjects and those with moderate inflammatory disease. However, C3aR-expressing T-lymphocytes were demonstrated in some patients with severe inflammatory skin diseases and C3aR expression could be induced by administration of type I interferons[61]. Administration of C3a has been demonstrated to augment the T-cell response, promote T-cell proliferation and prolong the inflammatory response through suppressing regulatory T-cells (T_{reg}) production[62-64]. In support of this, T-cell populations are reduced in $C3aR^{-/-}$ mice in several models of disease[62,64,65]. T-cell receptor stimulation upregulates C3aR mRNA expression in isolated T-cell populations and the presence of C3aR in these populations appears to promote a T_h1 response, as measured by the defining cytokine, IFN_γ[63]. This is supported by observations, *in vivo,* of increased IFN_γ production in mice lacking the complement suppressive factor, *Daf*[66]. C3aR-signalling also acts in concert with C5aR-signalling to suppress the production of TGF-β1 from dendritic cells, reducing the stimulus for differentiation to T_{reg} cells[64]. The induced T_{reg} cells heavily suppress the proinflammatory $CD4^{+}$ T-cell function, leading to abrogation of the inflammatory process. In addition, C3aR signalling in the tissue antigen presenting cells suppresses IL-4 production, inhibiting a T_H2 polarised response[67]. However this effect on T-cell biology is not entirely APC-mediated, altered T_{reg} responses also occur with adoptive transfer of $C5aR^{-/-}/C3aR^{-/-}$ T cells into a wild type animal[64]. $C5aR^{-/-}/C3aR^{-/-}$ T_{reg} cells also demonstrate prolonged survival and enhanced function, suggesting that C3aR/C5aR stimulus of T cells is also important in regulating inflammatory responses[65].

There is also some interesting preliminary evidence that there may be some direct effect of C3aR signaling on cytotoxic $CD8^+$ T cells. In an experimental model of allograft rejection, which is mediated by $CD4^+$ priming of cytotoxic T cells, $CD4^+$ T-cell-depleted mice have only a weak $CD8^+$ response to heart transplants. Removing *Daf*, and causing local complement dysregulation, removed the protection of $CD4^+$ T cell depletion after heart transplant. The authors postulate that there is a direct activation effect of C3a/C5a on $CD8^+$ T cells, however this has not been confirmed by pharmacological blockade of any complement receptor[68].

C3aR in Pathology

The heterogeneity of C3aR function at the cellular level creates a more complex picture when observing its role in the natural history of a disease. Unlike C5aR, C3aR cannot merely be labelled broadly as a pro-inflammatory entity as its function as a suppressor of neutrophil migration from the bone marrow serves to systemically dampen the inflammatory response. In addition, the more novel roles for the receptor, indicating functions in the regenerative phase post-insult, can further modulate clinical outcomes. As a consequence, when considering the overall contribution of C3aR to pathology, it is best to consider each disease state separately.

Atopic Disease

Atopic dermatitis is one instance where C3aR signalling results in a more desirable clinical outcome. This is linked to the function of C3aR on dendritic and, perhaps, T-cells, to promote a T_H1 response, rather than the T_H2 response associated with atopic disease[63,64,67]. However, dendritic cells still respond to C3aR signalling in a proinflammatory manner by increasing antigen uptake, T-cell recruitment and TNFa production[69,70]. As a result $C3aR^{-/-}$ animals do not differ significantly from wild type littermates in terms of clinical signs of skin inflammation during the sensitisation phase of allergic contact dermatitis[71] but longer term knockout animals demonstrate an serum immunoglobulin profile characteristic of atopy[67].

In allergic asthma this picture is reversed. C3aR is strongly upregulated on bronchial epithelial and smooth muscle cells in mouse models of allergic asthma, and the level of expression in humans in positively correlated with severity of disease[72,73]. This correlation is humans is supported by mouse models demonstrating a protective effect in allergic asthma in C3aR knockout animals[74]. This protection has been demonstrated to be derived from a reduced T_H17 cell population in knockout animals, due to absent C3aR-induced tachykinin release [74,75].

Lupus Nephritis

In autoimmune lupus nephritis, there are high concentrations of C3aR and C3 present in the glomeruli, correlating with disease severity[76]. This seems to suggest a pathophysiological role for the receptor in this disease, and, perhaps, a therapeutic target for treatment of lupus nephritis. Indeed, it has previously been demonstrated that infusion of the C3aR antagonist, SB290157, in the MRL/*lpr* mouse model of lupus nephritis resulted in increased survival and reduced renal inflammation[77]. However, the same mouse model backcrossed with C3aR knockout mice indicates that the loss of C3aR accelerates renal injury and increases mortality[78]. This contradictory result was attributed to the ill-defined pharmacological activity of SB290157, which has been reported to have agonist activity upon binding C3aR[56]. However, although the pharmacology of SB290157 may be controversial, the conclusion drawn by the authors may be overly simplistic, and perhaps not take into account the systemic effects of C3aR discovered recently. It is incontroversial that at the level of the nephron, C3aR is involved in the pathogenesis of renal disease, including the induction of metaplasia in nephron epithelial cells[79] but C3aR$^{-/-}$ mice exhibit neutrophilia and neutrocytosis in response to acute pathology[54]. Collectively, this data may suggest that locally, C3aR activation potentiates pathological outcomes, whereas systemically C3aR activation may attenuate inflammation.

Sepsis

Despite the prolific volume of research examining C5aR in sepsis, the role of C3aR in this disease remains perplexingly understudied. C3 deficient animals have been utilised for investigation of complement-mediated pathogen clearance during sepsis, and it has been demonstrated, perhaps unsurprisingly, that the lack of C3 reduces survival rates and increases pathogen load in experimental models[80]. One might expect that the increased pathogen load due to the loss of the terminal elements of the complement system, namely C3b opsonisation, C5a-induced activation of leukocytes and membrane attack complex lysis of pathogens, explains the premature death of C3 deficient animals. Indeed this is supported by demonstration of a protective effect for administration of exogenous C3 in septic animals[81]. However, the initial report of a *C3ar1* knockout mouse included an investigation into survival in sepsis and, interestingly, concluded that the loss of C3aR contributed to impaired survival[82]. Extra complement pathway, compensation via the upregulation of serum proteases[83,84]. Conversely, elevated C3a is associated with fatal outcomes in sepsis, however this may be a byproduct of uninhibited complement activation rather than a contributor to mortality[1,85].

Preeclampsia

It is still unclear whether the duality of C3aR, in terms of preventing systemic but promoting local inflammation, holds true for all disease states in which C3aR has been implicated. For instance, in preeclampsia the complement system has a well-defined role in placental inflammation[86], and C3 deposition on placental tissue is one of the hallmarks of the disease[87]. Complement activation in preeclampsia causes angiogenic dysregulation through the upregulation and release of sVEGFR-1, although this is a C5a, rather than C3a, mediated phenomenon[87]. Interestingly, in placentas from preeclamptic patients, the expression of C3aR is reduced, whereas C5aR levels are uneffected[88]. Whether this is a cause or effect of preeclampsia is unclear, but the presence of lower concentrations of the receptor in local areas of disease runs contrary to the model that C3aR functions as a proinflammatory agent at this level.

Pneumonia

Additionally, in a model of pneumonia C3aR$^{-/-}$ mice exhibited more efficient killing of *Pseudomonas aeruginosa* than their wild-type counterparts[89]. This increased efficiency was also accompanied by a reduction in the adverse effects of local inflammation, such as vascular leakage and a significant reduction in leukocyte infiltration. Interestingly, this is in contrast to what we have seen in models of intestinal ischaemia-reperfusion injury using C3aR knockout mice, and what would be expected from an animal with exaggerated neutrophil mobilisation in response to injury[54]. The authors also demonstrated an increased antibody response to *P. aeruginosa* in the C3aR$^{-/-}$ animals, adding weight to early reports that C3aR is a suppresor of humoral immunity, although whether C3aR is expressed on B-cells is, as yet, controversial[10,37]. However, the authors claim the increased antibody titre in C3aR$^{-/-}$ mice could not solely account for the enhanced killing of *P. aeruginosa*, leaving a paradoxical situation of a reduced inflammatory response, but also reduced bacterial viability in C3aR$^{-/-}$ animals.

CNS infection

In order to investigate the role of C3a in acute inflammation of the brain, [90] studied the pathogenesis of LPS-induced shock using crosses of C3aR$^{-/-}$ and C3a/GFAP-Tg mice, which overexpress C3a under the control of the GFAP promoter and thus generate biologically active C3a in the CNS without requiring prior complement activation. Interestingly, C3a/GFAP mice were significantly more resistant to endotoxin-induced lethality than WT and C3aR$^{-/-}$ mice and, curiously, C3a/GFAP x C3aR$^{-/-}$ mice were also significantly protected[90]. This suggests that not only can C3a function in vivo as an anti-inflammatory mediator, but that it exerts this effect via an alternative, non-canonical C3aR, possibly C5L2 or Receptor for Advanced Glycation End-Products[91].

C3aR is however directly associated with pathology in other neuroinfectious disorders. Reactive astrocytes, amoeboid microglia and ramified microglia stain strongly and consistently with C3aR in sections of human brain with bacterial meningitis, much greater than in normal human brain sections[11]. This suggests excessive C3aR activity may contribute to progression of this disease. A similar observation was reported in the mouse model of CNS-affecting systemic lupus erythematosus (SLE) (MRL/lpr mice); C3aR is upregulated in the brains of MRL/lpr mice relative to MRL/+ controls[92]. Interestingly, treatment of the experimental lupus brain with a non-specific C3aR antagonist reduced neuronal apoptosis, gliosis, expression of the pro-inflammatory mediators TNFα, IFNγ and iNOS, and modulates neuronal excitability by decreasing AMPAGluR1 expression[92]. Combined with the observation that systemic C3a levels correlate with disease severity in human SLE patients[93], this data indicates that therapeutic antagonism of C3aR may be a novel therapeutic strategy for attenuating human CNS SLE. Although these findings appear somewhat paradoxical compared to the ameliorative effects of C3a in the LPS-induced shock model described above, it is important to note that they are dependent on activation of C3aR, whereas the neuroprotection observed in with C3a in LPS-induced shock was mediated independently of C3aR. Taken together, this data suggests that deregulated complement and excessive C3aR activation results in exacerbation of CNS infectious pathology.

Stroke

Similar detrimental roles for C3aR have been assigned for acute neuroinflammatory conditions resulting from injury. Human patients that sustained an ischaemic stroke less than one month prior to plasma collection have elevated plasma C3a compared to control patients[94]. Although this is only correlative, the animal model of stroke, middle cerebral artery occlusion (MCAO), has provided valuable insights into the functional significance of elevated C3a levels. C3aR mRNA and protein levels in the murine ischaemic cortex are significantly increased on macrophage-like cells and astrocytes by one day post-injury[95-97]. This upregulation was partly attributed to infiltration of leukocytes to the ischaemic core and penumbra, and suggests that C3a may promote inflammatory responses in the ischaemic brain specifically via modulation of infiltrating and glial cell responses. In line with this, *in vitro* there is no effect of hypoxia-re-oxygenation on human NT2-N neuronal C3aR expression[98], even though these cells constitutively express C3aR.

Given these observations, investigations in the last decade have evaluated the effects of modulating C3aR *in vivo*. In transient (60 min) but not permanent MCAO, pharmacological antagonism of C3aR resulted in smaller stroke volumes, less ICAM-1 protein on endothelial cells and less C3aR-positive granulocytes compared to mice treated with vehicle only[99]. These data suggest that

blocking the binding of C3a to C3aR modulates acute inflammatory events following cerebral ischemic-reperfusion injury by limiting the recruitment of neutrophils to the ischemic zone[99]. It is likely that the tissue damage produced with permanent MCAO was too copious for therapeutic effects of C3aR inhibition to be observed. It should be taken into account, however, when interpreting these findings that the antagonist used in this study, SB290157, reportedly has off-target effects, including neutropaenia *in vivo*[100], and full agonist activity in various of cell systems *in vitro*[56,101]. A study by Mocco et al. (2006)[102], which employed mice genetically deficient in C3 or C5, may clarify this situation. Only $C3^{-/-}$ mice displayed improved outcomes compared to WT mice, shown by an average of 34% reduction in infarct volumes, improved neurological deficit scores, and decreased granulocyte infiltration and oxidative stress. It was thus concluded that the improvements observed in $C3^{-/-}$ mice must be due to absence of C3a or C3b activity, and not downstream complement components such as the membrane attack complex (MAC). The authors utilised a C3aR anatagonist in order to distinguish which C3 cleavage product was detrimental to recovery. It was found that C3aR blockade resulted in commensurate neurological improvement and stroke volume reduction compared to the $C3^{-/-}$ mice, indicating that the worsened outcomes in the WT cohort was due to C3a-mediated mechanisms[102]. The use of $C3aR^{-/-}$ mice may more definitively confirm this role in ischemic stroke.

Although only accounting for 15% of all strokes[103], haemorrhagic strokes result in a disproportionate degree of morbidity[104]. Interestingly, it has been observed that in plasma[105] and cerebrospinal fluid (CSF)[106] levels of C3a were shown to correlate with functional outcome after subarachnoid aneurysm rupture, suggesting that inflammatory processes involving C3a may contribute to neurologic dysfunction. C3a/C3aR has therefore been investigated as a putative therapeutic target in this pathology. Treatment of mice with a C3aR antagonist prior to intracerebral haemorrhage (ICH) induction has been shown to improve neurologic function, brain water content, and granulocyte infiltration relative to vehicle-treated animals when assessed at 72 hours post-trauma[107]. It would be of interest to determine if these effects would be as substantial if this antagonist were administered subsequent to ICH, as this would be more clinically applicable. A study by Garrett et al. (2009)[104] suggests that C3aR inhibition post-intracerebral hemorrhage may have therapeutic potential. Although the authors did not evaluate the effects of a C3a antagonist alone, they found that combined C3aR antagonist with a C5aR antagonist improved functional outcomes and reduced inflammation and oedema to a greater extent than that observed with a C5aR antagonist alone, suggesting C3aR and C5aR function synergistically to modulate the infalmmatory response. The authors concluded that simultaneous blockade of the C3a and C5a receptors represents a promising neuroprotective strategy in haemorrhagic stroke[104].

Traumatic Brain injury

There is a paucity of literature on the role of C3aR in traumatic brain injury resulting from mechanical trauma, but it would appear to be a good candidate as a therapeutic target here as well. Our recent work has however determined an ameliorative role for C3aR in the acute-to-chronic stages after experimental contusive spinal cord injury (SCI). Unlike cerebral injury, C3aR$^{-/-}$ mice subjected to SCI perform worse than WT mice in functional, anatomical and histological outcome measures, and this is associated with augmented granulocytic inflammatory infiltrate. This indicates that C3a/C3aR plays a key role in acute CNS injury, but its therapeutic potential must be tailored to specific pathological contexts in order to optimally harness its therapeutic potential.

Neurodegenerative Disease

There is also a growing body of work examining the role of C3a in chronic neurodegenerative diseases. The following section will discuss the known literature regarding C3a/C3aR activation in Alzheimer's disease (AD), Huntington's disease, multiple sclerosis, and cuprizone-induced demyelination.

In regard to AD, plasma concentrations of C3a partly explain the variance in whole brain volume in AD patients, suggesting plasma C3a may represent a predictor of *in vivo* AD[108]. Another study examined the CSF of patients with Alzheimer's disease (AD) and vascular dementia (VD) in order to determine candidate protein biomarkers for the differential diagnosis between the two conditions. C3a was among 28 proteins identified which can distinguish between the two pathologies[109], suggesting C3a is among a collection of mediators orchestrating this pathology. Interestingly, amyloid plaques contain C1q and activated C3 fragments[110], implying that amyloid plaque themselves can invoke complement activation via the classical pathway. One study examined this further by applying normal serum depleted of C3 to $A\beta_{1-42}$ aggregates in vitro. C3 activity on the $A\beta_{1-42}$ was detected, and this was associated with binding of C1 and activation of C4 on the aggregated peptide. Thus $A\beta_{1-42}$ is sufficient to serve as a substrate for complement activation, and the subsequently produced C3a may be involved directly the inflammation seen in AD brains[111]. Similarly to AD, the overall level of complement activation in the Huntington's disease (HD) brain is 2-5 times higher than in normal brains[112]. Notably, neurons and astrocytes in the striatum from Huntington's disease (HD) patients stain positively for C3 and iC3b, C3 was also shown to localise to microglia in the internal capsule, and C3aR mRNAs is strongly expressed in HD caudate[112]. It was suggested that local complement production by glia contributes to HD inflammatory pathology and possibly neuronal necrosis.

C3aR expression has also been shown to be elevated on microglia and astrocytes in the animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE)[11,113]. $C3aR^{-/-}$ mice develop attenuated experimental autoimmune encephalomyelitis (EAE) in the chronic phase of disease, which was associated with reduced demyelination and cellular infiltration of macrophages and T cells compared to wild-type mice[114]. In contrast, mice that express C3a exclusively in the CNS (C3a/GFAP transgenic mice) develop severe and exacerbated EAE compared to wild-type mice. Surviving C3a/GFAP mice exhibited profound macrophage and $CD4^+$ T cell infiltrate throughout the meninges of the spinal cord, in Virchow-Robins spaces, and around blood vessels in the parenchyma. The recovery of C3a/GFAP x $C3aR^{-/-}$ mice was comparable to that of $C3aR^{-/-}$ mice, demonstrating that the phenotype of C3a/GFAP mice is mediated via C3aR[115]. Interestingly, there may be some cross-modulation between the anaphylatoxin receptors in this pathology, as mice deficient in both anaphylatoxin receptors ($C3aR^{-/-}C5aR^{-/-}$ mice) do not have attenuated disease severity compared to wild-type mice, even though $C5aR^{-/-}$ and C5a/GFAP mice have identical pathology compared to wild-type mice[114,116,117]. Overall, this data suggests however that C3a, via C3aR, is a key modulator of EAE pathology rather than a bystander fragment.

This data is consistent with that observed in cuprizone-mediated demyelination and remyelination. C3a/GFAP expressing mice have worse demyelination severity, increased cellular infiltration and thickness of the inflammatory lesion within and surrounding the corpus callosum, greater astrogliosis and microglial infiltration than WT mice. Interestingly, C3a/GFAP mice also showed increased numbers of $Olig2^+$ oligodendrocytes during the early stages of remyelination[118]. This was suggested to be a compensatory mechanism for the increased severity of demyelination in the corpus callosum. C3a was also shown to alter cytokine and chemokine production in glia cultures, including CCL4(MIP1β), CCL5, CCL11, IL-6 and IL-13[118]. It was concluded that C3a worsens this demyelinating pathology via stimulating upregulation of chemokine production, which induced migration of supplementary reactive astrocytes and microglia to the demyelinating corpus callosum.

Emerging Novel Roles

The notion of a system of combatting infection as being important for embryonic development or tissue regeneration appears initially antithetical. However, this may be due to the fact that the first ascribed role for complement, that of a player in immunity, has stuck so tenaciously to our ideas of the system that it is impossible to tease the two apart. And so, most recent articles on C3aR outside of the immune system begin with the proclamation that the proceeding article will outline a novel role for the receptor. This is, justifiably, an area of excitement within complementology, drawing

developmental biologists to observe complement in a new light. In actual fact, the link between tissue damage and repair is not as disjointed as it may first appear. After injury, apoptotic cells must be cleared and new cells must fill their place; is it inconceivable that the key mediators of both processes could be the same set of proteins?

An early report of this duality was the role of C3aR in liver regeneration after injury or toxic insult. It has been shown that mice deficient in C3 exhibit reduced proliferation and a corresponding delayed gain in liver weight after partial hepatectomy, an effect that can be rescued by C3a administration[7]. Although these experiments do not directly demonstrate a role for C3aR in liver regeneration, in a model of chemically-induced liver injury, C3aR$^{-/-}$ mice exhibit reduced proliferation, supporting the hypothesis that the effects observed in partial hepatectomy are C3aR dependent[7,119,120]. In addition, C3$^{-/-}$ mice have increased necrosis and long term liver damage following both toxic injury and partial hepatectomy. This phenotype can be rescued through the administration of C3-sufficient sera[7,119,120]. The authors of these papers suggest that C3aR-mediated hepatocyte proliferation after injury is an indirect effect, via Kupffer cell C3aR signalling causing the release of pro-mitotic factors[7,121]. Although C3aR Kuppfer cells that can trigger the release of regenerative factors, the importance of C3aR signalling on the hepatocytes themselves has however, not been thoroughly investigated[7]. As an organ that undergoes sustained challenge and constantly regenerates, the authors postulated that the scope of this discovery was limited solely to the liver. However, it may be tentatively said that the idea of C3aR promoting tissue growth and differentiation may also apply to the embryo, and that research in this area is starting to unlock a whole new world of complement biology.

In the developing *Xenopus* embryo, C3 and C3aR are expressed throughout organogenesis[5]. There is expression in the liver diverticulum and posterior gastric endoderm, which may be expected given the traditional functions of both C3 and C3aR[5,122]. However, the expression of C3 and C3aR is particularly striking during neurulation, where the transcript is localised, in both *Xenopus* and zebrafish models, to the neural crest and neural groove[5,9]. Given our laboratory's recent report of the protective effect of C5aR signalling in folate-deficient neural tube closure, similar expression of C3aR during neurulation may lead us to hypothesise a role for C3aR in neural tube closure. Expression of both ligand and receptor follows the migration routes of the neural crest cells, particularly evident within the brachial migration routes[5,9,122].

It would be tempting to hypothesise that the function of C3aR in the neural crest may be similar to its novel role in the liver, promoting cellular proliferation. However, the C3a-C3aR axis, at least in

zebrafish, functions in this area as a regulator of collective cell migration. Migration of neural crest cells is dependent on a balance between contact inhibition and coattration, leading to the orderly migration of cells through exquisitely defined pathways. Disruption of the C3a-C3aR axis with C3aR-targetted morpholinos or anti-C3a antibodies leads to disorderly and attenuated neural crest cell migration[9]. In the case of the C3aR morpholinos, this effect can be rescued through the delivery of C3aR mRNA to the affected embryos[9]. These experiments have demonstrated that the chemoattractive effect of C3a on neural crest cells is imperative for correct zebrafish development. However, what role does the C3a-C3aR axis have in mammalian development? The existence of C3aR$^{-/-}$ animals that do not exhibit any gross developmental anomalies may have clouded our vision surrounding that question. Should we be considering the work of compensatory mechanisms in the knockouts? Would conditional knockouts be better genetic tools for the investigation of this receptor in development? Certainly, the answer to the latter question must be a resounding, yes. There have been just a few studies on C3aR in relation to neural development and regeneration, but each has demonstrated that modulation of this receptor can dramatically affect the development of the brain.

This developmental aspect of C3aR function must be inferred from research surrounding post-natal neurogenesis. The affect that C3aR signalling appears to have on the neural progenitor population can be described as a complex one. C3aR is expressed on these progenitors, both in *in vitro* models and in the embryonic and adult brain[8,28,123]. Indeed this expression of C3aR can be traced back to the neuroepithelial cells in early neural tube formation (Angela Jeanes, *personal communication*). Signalling through C3aR has been shown to modulate stromal derived factor-1α (SDF-1α), leading to either an amplification or attenuation of SDF-1α-dependent migration[28]. SDF-1α is a known chemoattractant for neural progenitors and other stem cells, acting through its receptor Cxcr4[124]. C3aR modulates this attraction through acting as an inhibitor at high SDF-1α concentrations (500ng/mL) and augments the effect at low concentrations (4ng/mL)[28]. C3aR or Cxcr4 signalling alone promotes neuronal differentiation of the progenitors, however this effect is annulled when both agonists are applied simultaneously[28].

It is difficult to speculate what this means for neural progenitor biology, without knowing when and where C3a is present in the brain. Is it that the influence of C3a only becomes apparent under conditions of insult and injury, where the complement system is activated, or do these results suggest a more subtle, developmental role for the C3a-C3aR axis? This question has been partially answered through the observation that, in C3$^{-/-}$ and C3aR$^{-/-}$ mice, basal neurogenesis is reduced, indicating that C3aR is a player in the normal physiology of neurogenesis[8]. This effect can also be

elicited with administration of the controversial C3aR antagonist, SB290157[8,56]. In rat postnatal cerebellar development C3a is also a chemoattractant for cerebellar granule neurons of the external granule cell layer and this effect can be blocked by SB290157[125]. The use of this antagonist, albeit one of controversy, supports the assumption that the effects demonstrated through the application of C3a to neural progenitors is indeed through C3aR, a question that was not fully answered in the work of Shinjyo and colleagues (2009). *In vivo*, the subdural administration of C3a caused a reduction in the thickness of the proliferative external granule cell layer and a increase in thickness of the post-mitotic internal granule cell layer, suggesting that C3a acts to promote exit from the cell cycle and migration to form functional neurons[125]. In addition to a purported role in basal neurogenesis, C3aR also functions to accelerate neurogenesis in mouse models of stroke[8]. C3 deficient mice demonstrate reduced numbers of progenitor cells in both the infarct area and penumbra after middle cerebral artery occlusion, this translates into a reduced number of newly formed neurons during recovery[8]. It is postulated that this decrease in progenitors could be ascribed to the previously demonstrated function *in vitro* of C3aR as a chemoattractive mediator. In models such as stroke, which involve a large inflammatory component and glial infiltration, it is easy to comprehend how and where C3a is generated in order to promote neurogenesis. The question for future research in this area will be how C3aR is stimulated in basal neurogenesis without the inflammatory sequalae.

Therapeutic Potential

As a pivotal regulator of the innate immune response, modulation of C3aR signalling is a valuable target in the therapeutic treatment of immune mediated inflammatory diseases. Whilst studies using C3aR receptor knockout mice are important in the investigation of C3aR mediated processes, a pharmacological approach to C3aR modulation is vital for progression of C3aR based therapies from the laboratory to the clinical environment. Though holding vast therapeutic potential, the number of C3aR targeted drugs available is limited, in particular, there is currently no specific C3aR antagonist available for use.

C3aR Antagonists

The report of the chemical compound, N^2-[(2,2-diphenylethoxy)acetyl]-L-arginine (SB 290157) as an antagonist of C3aR (IC50 reported between 30-200nM) created an avenue for the study of pharmacological inhibition of C3aR[126]. Use of this antagonist lead to reports for the potential of C3aR antagonism in therapeutic applications such as in the reduction of injury in models of cerebral ischemia reperfusion and lupus[92,94], and treating metabolic dysfunction[127]. Whilst these results were promising for C3aR therapeutics, other studies, which used SB290157, found unusual or conflicting

results bringing into question the antagonistic activity of the compound. In the study of C3aR in ischemia reperfusion injuries, our laboratory demonstrated high doses of SB caused transient neutropenia and hypertension[100], both of which have been associated with C3aR activity[128]. Furthermore, in models of allergic asthma SB290157 was found to have no effect, a result which conflicted previous reports for a critical role of C3aR in the pathogenesis of asthma[129-131]. Due to such results, Mathieu and colleagues (2005) examined the compound and, in opposition to the reports of C3aR antagonism, demonstrated that SB290157 was a C3aR agonist in a number of *in vitro* assays[56]. Such a finding explained the unusual results that have been observed when using SB290157, and raises critical concerns over the validity of previous results, which have used the compound to attribute C3aR antagonism to disease therapy. Whilst the previously mentioned reports which used SB290157 may have observed an effect due to C3aR inhibition, they are just as equally likely to have been due to C3aR activation, demonstrating that, though not an ideal model for potential therapies, that C3aR knockout animal studies are necessary for demonstrating the validity of such results. More importantly, this highlights the necessity for further research and development of C3aR antagonists.

Recently, Scully and colleagues (2010) reported the discovery of the hexapeptide antagonist, FLTChaAR (Binding IC50 = 240nM)[132]. This peptide was developed through the modification of the C5aR agonist, FKP(dCha)(Cha)r, and subsequent screening for C3aR activity. Due to the high similarity between C5aR and C3aR, it was hypothesised that modifications to this peptide would lead to a shift in 30specificity towards C3aR. No further studies have reported the use of this peptide and, with following publications by the group choosing to use SB290157 over their own peptide, it is probable that FLTCHaAR is not suitable for animal and therapeutic studies[127].
Whilst no further reports of C3aR antagonists have been made, the application of a de novo protein design framework, using computational modelling to predict amino acid sequences which are favourable for receptor interactions, was utilised to produce two partial agonist peptides with full C3aR antagonist activity[133]. Though these peptides are not favourable for antagonist studies, they demonstrate the potential for this approach in the production of C3aR specific antagonists.

C3aR Agonists

Due to the multiple functions of C3aR, not only inhibition, but context specific activation of C3aR may have important therapeutic potential. Using receptor knockout mice, it has been demonstrated that C3aR loss worsens tissue injury following intestinal ischemia reperfusion (IR)[54]. This effect was attributed to the role of C3aR in preventing neutrophil mobilisation and subsequent inflammatory mediated injury following ischemia. The therapeutic potential of C3a activation was

further demonstrated with the use of the specific peptide agonist of C3aR, WWGKKYRASKLGLAR, which abated IR induced neutrophil mobilisation and tissue injury[54].

Development of the C3aR agonist, WWGKKYRASKLGLAR, was based upon the modelling of the C-terminus sequence of C3a, the region implicated in C3aR activation[134]. This peptide contained the highly-conserved LGLAR sequence that has been identified to be essential for receptor activation, as well as a hydrophobic N-terminal group (WW), which, in support of the two site binding hypothesis for C3aR activation, is postulated to be required to bind to the secondary activation site on C3aR. As well as both groups being required for peptide activity, distance between the two was essential for binding of the receptor sites, and was optimised for maximal potency of the peptide. The combination of these properties yielded a peptide with a reported 15-fold increase in activity compared to C3a. WWGKKYRASKLGLAR is an unconstrained, natural amino acid, and linear peptide, all of which are undesirable properties when considering therapeutic applications, due to a predicted poor oral bioavailability and rapid breakdown[135]. When considered for use during surgical procedures known to result in ischemic injury, treatment with an acute, intravenous dose of WWGKKYRASKLGLAR is feasible for reduction of IR tissue damage. Instability of the compound may even be preferred as, when considering the potential for C3aR to mediate chronic inflammatory disease, it would be preferable to only prevent immediate neutrophil mediated damage following surgery.

In a recent study, Scully and colleagues (2010) utilised modelling of the C5aR agonist, FKP(dCha)(Cha)r, to develop a number of C3aR agonist peptides along with the previously mentioned antagonist, FLTChaAR[132]. Substitution of the (dCHa)(Cha)r for the C-terminal C3a sequence, LAR, and subsequent modifications of the N-terminus lead to the identification of a number of short hexapeptide agonists with specific activity for C3aR, of which the most potent were FLPLAR (0.42 uM), FIPLAR (0.95 uM), FWTLAR (0.3 uM) and FLTLAR (0.32 uM). Further investigation of FLTLAR identified a bend in the NMR structure in a similar manner as to what is seen in C3a[132]. This suggests a requirement of this conformation for the binding and activation of C3aR, and potentially why the shorter peptides were able to activate C3aR in contrast to the previously reported necessity for a longer sequence such as WWGKKYRASKLGLAR. It may be equally possible that the hypothesised necessity for binding of two C3aR sites is not necessary for full receptor activation. Identification of shorter sequences that are able to functionally bind C3aR provides valuable insights into further C3aR ligand development. The use of such hexapeptides may also be therapeutically advantageous over the longer peptide,

WWGKKYRASKLGLAR, reducing production cost and potentially increasing stability and tissue penetrance.

Whilst there is clear evidence for the therapeutic application of C3aR targeting, there is a desperate need for research into developing specific and potent C3aR ligands. From animal studies, it is apparent that context specific antagonism or agonism of C3aR is a viable option in treating a number of diseases. The realisation of such clinical applications is significantly hindered by the lack of molecular tools for targeting C3aR. Screening of chemical libraries resulted in the discovery of SB290157, a compound widely used as a C3aR antagonist. Recent studies have demonstrated the agonist activity of this compound[56], highlighting the need for cessation of SB290157 use and publication as a C3aR antagonist. Worryingly, there is continued use of SB290157 as a C3aR antagonist, with recent publications utilising this compound as the sole method for formulating conclusions about C3aR functions in disease progression[127,136,137,139]. This continued use hinders research into C3aR mediated pathologies, and highlights the importance for development of a specific, potent, C3aR antagonist, which to date has remained elusive.

Conclusion

The functions of C3aR are an evolving field of study, novel functions for this receptor exist beyond the traditional confines of innate immunity. Additionally, even within its roles in immunity there is debate as to whether the receptor operates, in an overarching manner, to promote or inhibit inflammation[138]. C3aR offers a promising target for modulation in pathologies, especially given its ‘bilingualism’ in the context of inflammation. However, currently there are no stable and clinically translatable pharmaceuticals available, and this is most acute with regards to the antagonists. This review has aimed to summarise the current state of knowledge for C3aR with the view of stimulating interest in this receptor as a potential target in inflammatory pathologies.

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