CLEAVAGE OF THE LOW-DENSITY LIPOPROTEIN-RECEPTOR-RELATED PROTEIN (LRP) BY MATRIX METALLOPROTEINASES (MMPs) IN A NEURAL CELL MODEL: IDENTIFICATION OF MULTIPLE SOLUBLE LRP (sLRP) CLEAVAGE PRODUCTS.

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DECLARATION

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PUBLICATIONS ARISING FROM THIS THESIS

PEER REVIEWED PUBLICATIONS:

1. **Fitzsimmons, C.,** Johnstone, D., Parsons, C.H., Conant, K., Milward, E.A. 'Soluble lipoprotein receptor-related protein (sLRP) immunoreactive species in cell culture media and serum replacement supplements' (in preparation).

CONFERENCE PUBLICATION:

1. **Fitzsimmons, C.,** Johnstone, D., Conant, K., Milward, E.A. 'Cleavage of the low-density lipoprotein receptor-related protein (LRP) by matrix metalloproteinases (MMPs) in the CNS', The Australian Society for Medical Research (ASMR) XVI NSW Scientific Meeting, Sydney, Australia (2007).

2. **Fitzsimmons, C.,** Johnstone, D., Conant, K., Milward, EA. 'Soluble lipoprotein receptor-related protein (sLRP) immunoreactive species in cell culture media and serum replacement supplements', The Australian Society for Medical Research (ASMR) XVII NSW Scientific Meeting, Sydney, Australia (2008).

3. **Fitzsimmons, C.,** Johnstone, D., Conant, K., Milward, E.A. 'Matrix metalloproteinase (MMP)-mediated cleavage of the low-density lipoprotein receptor-related protein (LRP) in the CNS to generate soluble LRP (sLRP) species with neurodegenerative potential', Alzheimer's Association International Conference on Alzheimer's Disease and Related Disorders (ICAD), Chicago (2008).

4. **Fitzsimmons, C.,** Johnstone, D., Conant, K., Milward, EA. 'Matrix metalloproteinases (MMP)-mediated cleavage of the low-density lipoprotein receptor-related protein (LRP)'. The Australian Society for Medical Research (ASMR) XVIII NSW Scientific Meeting, Sydney, Australia (2009).

ORAL PRESENTATIONS:

1. Invited by A/Prof. Robert D Moir, Harvard Medical School, Mass. General Institute for Neurodegenerative Disease (MIND Institute), Department of Neurology, Massachusetts General Hospital, Charleston, MA 02129. **Fitzsimmons, C.,** Johnstone, D., Conant, K., Milward, E.A. 'Soluble forms of LRP' (2006).

LIST OF COMMONLY USED ABREVIATIONS

A1AT	α1-Antitrypsin
α2M	Alpha-2-macroglobulin
α2M*	Activated alpha-2-macroglobulin
Aβ	Amyloid beta
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APOER2	Apolipoprotein E receptor 2
APP	Amyloid precursor protein
APS	Ammonium persulfate
BACE-1	β-site APP-cleaving enzyme 1
BeWo	Choriocarcinoma cell line, human
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BHK	Baby hamster kidney
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CA1	Hippocampal subregion 1
Ca^{1+}	Cuprous ion
Ca^{2+}	Cupric ion
$CaCl_2$	Calcium chloride
CBX	Cerebellar cortex
CCL2	Chemokine (C-C motif) ligand 2
CD91	Cluster of differentiation 91
cDNA	Complementary DNA
CED 1	*Caenorhabditis elegans* death protein 1
CHO	Chinese hamster ovary
CNS	Central nervous system
C1r	Complement C1r subcomponent

CSF	Cerebral spinal fluid
DAB-1	Disabled-1
DEAE	Diethylaminoethyl cellulose
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL^{+}	Enhanced chemiluminescence solution
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FAD	Familial form of AD
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGFs	Fibroblast growth factors
FLIM	Fluorescence lifetime imaging microscopy
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GPI	glycosylphosphatidylinositol
HBBS	Hanks' balanced salt solution
HB-EGF	Heparin-binding epidermal growth factor
HCl	Hydrochloric acid
HEK 293	Embryonic kidney cell line, human
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethane sulfonic acid
HepG2	Hepatocellular carcinoma, human
HIV	Human immunodeficiency virus
HPF	Hippocampal formation
HRP	Horseradish peroxidase
HRV2	Human rhinovirus-2
HSP	Heat shock protein
HSPG	Heparin sulfate proteoglycan
HTI1080	Fibrocarcinoma cell line, human
IGFBP-5	Insulin-like growth factor binding protein 5

IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-6 α	Interleukin 6 receptor alpha subunit
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
ISH	*in situ* hybridization
JIP	JNK (Jun N-terminal Kinase) interacting protein
KA	Kainate
KD	Knockdown
kDa	Kilodalton
KO	Knockout
KPI	Kunitz protease inhibitor domain.
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LIF	Leukaemia inhibitory factor
L-LTP	Late-phase long term potentiation
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein 1
LRP ICD	LRP intracellular domain
LTP	Long term potentiation
mAb	Monoclonal antibody
MafB	v-maf Musculoaponeurotic fibrosarcoma oncogene homologue
MALDI-ToF	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MAP	Mitogen-activated protein
MC7	Recombinant breast carcinoma cell lines, human
MCP-1	Monocyte chemoattractant protein-1
MEF	Mouse embryonic fibroblast
MegF7	Multiple epidermal growth factor-containing protein 7
MEM	Minimum essential medium
MES	2 (N-morpholino) ethanesulfonic acid
MMP	Matrix metalloproteinase
MOPS	n-(3-sulfopropyl morpholine) propanesulfonic acid
mRNA	Messenger RNA

MS	Multiple sclerosis
MT-MMP	Membrane-type matrix metalloproteinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
$NaPO_4$	Sodium phosphate
Neurobasal+N2	Neurobasal medium supplemented with N2
NMDAR	N-methyl-d-aspartic acid receptor
NOS	Nitric acid synthase
Notch-3	Neurogenic locus notch homolog protein 3
NPxY	A tetra-amino-acid motif
NTC	Non-transfected control
OD	Optical density
pAb	Polyclonal antibody
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + 0.1% Tween 20
PC12	Pheochromocytoma
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	*Pseudomonas exotoxin* A
pI	isoelectric point
PS1	Presenilin protein 1
PS2	Presenilin protein 2
PSD-95	Postsynaptic density protein-95
PTB	Phosphotyrosine binding
PTC	*pseudotumour cerebri*
PVDF	Polyvinylidene fluoride
RAGE	Receptor for advanced glycation end products
RAP	Receptor-associated protein
RISC	Argonaut-dependent RNA-induced silencing complex
RIP	Regulated intramembranous proteolysis
RNA	Ribonucleic acid
RNAi	RNA interference

PrPc	Cellular prion protein
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SAP	Sphingolipid activator protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
SHRSP	Stroke-prone spontaneously hypersensitive rats
SH-SY5Y	Neuroblastoma cell line, human
siRNA	Short interfering RNA
sLRP	Soluble LRP
SORLA	Sortilin-related receptor, low-density lipoprotein receptor class A repeat-containing protein
SREC1	Scavenging receptor 1 of endothelial cells
SYBR	Synergy Brands
TAPI-1	TNF-α Protease Inhibitor-1
TACE	TNF-α converting enzyme
TAT	Transactivator of Transcription
TEMED	*NNN'N'*-tetramethylethylene-diamine
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-α
TNFR1	Tumour necrosis factor receptor-1
tPA	Tissue plasminogen activator
TSP	Thrombospondin
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
UV	Ultraviolet
VLDL	Very low-density lipoprotein
WB	Western immunoblot
WKY	Wistar Kyoto rats
Wnt	Wingless-int

ABSTRACT

The low-density lipoprotein receptor-related protein (LRP) is a large transmembrane scavenger and signalling receptor. Binding over 50 ligands in the extracellular environment LRP has a wide range of physiological and pathological functions. For example, in the brain, LRP ligands include the Alzheimer's disease amyloid beta peptide (Aβ) and apolipoprotein E (APOE). Cellular LRP can be cleaved by proteolytic enzymes to generate soluble fragments (sLRP). Some sLRP fragments retain one or more ligand-binding domains and may exert antagonistic effects on cellular LRP. Previous studies using broad spectrum matrix metalloproteinase (MMP) inhibitors suggest one or more MMPs may be implicated in sLRP generation. The MMPs are a group of zinc-dependent endopeptidases that are best characterised for their ability to degrade extracellular matrix (ECM) proteins and remodel existing cell-matrix boundaries.

The broad hypothesis underlying this project is that MMPs can contribute to the generation of sLRP species in a cellular system relevant to the CNS.

The SH-SY5Y cell line, derived from a human neuroblastoma, was established as a useful cell model. The cell line was found to endogenously release several putative sLRP α-chain (~500 kDa) and β-chain (~275 kDa, ~150-100kDa, ~85 kDa, ~75 kDa, ~65 kDa and ~55 kDa) species *in vitro*. While developing this model under serum-free conditions, substantial quantities of sLRP immunoreactivity were detected in the B27 supplement that was not listed among the components. The unrecognised presence of sLRP, and possibly other undefined serum proteins, has the potential to introduce experimental artefacts and interfere with experimental results and interpretation in cell culture studies involving LRP or any of its ligands. Neurobasal medium supplemented with N2 was identified as an alternative serum-free medium suitable for investigating sLRP production *in vitro*.

Treatment with synthetic MMP inhibitors reduced the release of soluble LRP species into the culture medium. It was found that sLRP is generated primarily by MMP-2 in this system and possibly also by MMP-9 dependent activity. The data provided evidence that LRP contains at least two MMP cleavage sites: at least one located within the β-chain and one in the α-chain.

N-terminal sequencing using Edman degradation and MALDI-ToF did not generate sequence data due to N-terminal blockage. However all the soluble species, with the exception of the ~75 kDa and ~65 kDa species, were no longer detectable after RNA interference was used to silence LRP gene function. This confirms that these species are soluble forms of cellular LRP. This also shows that the ~75 kDa and ~65 kDa species are not in fact derived from LRP.

RNA interference was also utilised as an alternative approach to reduce the levels of specific MMP transcripts within the cell model. In accordance with the MMP inhibitor studies it was found that sLRP production is MMP-2 and MMP-9 mediated. As MT1-MMP is required for the activation of pro-MMP and potentially also pro-MMP-9, the effect of down-regulating MT1-MMP gene function was also investigated. Production of the major ~500 kDa sLRP-α species and the major ~85 kDa and ~55 kDa sLRP-β species was almost completely abolished, proving evidence that MT-MMP has important roles in sLRP generation in this system.

In summary, the findings presented within this thesis provide evidence for the first time that several sLRP species are endogenously produced in cells relevant to the CNS. Furthermore, data is also presented that identifies MT1-MMP, as well as MMP-2 and MMP-9, as the key matrix metalloproteinase able to affect sLRP production in a neural cell system. Understanding the mechanisms by which LRP is processed may help develop new treatment strategies for Alzheimer's disease or related neurodegenerative disorders.