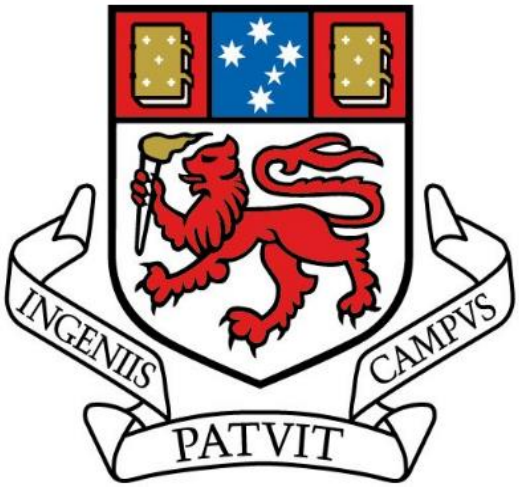

Localisation of LRP1 in neurons and glia of the central nervous system

Rosalind Peta Herbert

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Statements

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Abstract

The low density lipoprotein receptor-related protein-1 (LRP1) receptor modulates neuronal survival, neurite outgrowth, regeneration and calcium signaling. Its effects are thought to be ligand and cell specific, but how LRP1 is able to differentiate between different signals to produce specific responses is unclear. The subcellular localisation of LRP1 in neurons and glia of the central nervous system (CNS) was investigated to gain insight into the receptor's role in specific cell types and during maturation. The role of LRP1 in calcium signaling and regeneration following injury was assessed using two of its ligands, metallothionein (MT) and emtinB.

Expression and localisation of LRP1 *in vivo* and *in vitro* was determined using western blot analysis and immunochemistry. LRP1 was expressed in neurons and glia of post-natal day 2 (P2), P7 and adult rat brains. Greater immunoreactivity was demonstrated in P2 compared to adult brain, mainly due to increased expression in neurons and oligodendrocytes. Neuronal expression was greatest in cell bodies of hippocampal and cortical neurons in P2 and adult brain. Glial expression of LRP1 was greatest in oligodendrocytes of the corpus callosum and hippocampal fimbria, but LRP1-positive astrocytes and microglia were also present throughout the brain at both time points. These data suggest that LRP1 has a role in neuronal and glial function and that this changes during maturation.

LRP1 was localised to cell bodies of hippocampal neurons 3, 7, 14 and 21 days *in vitro* (DIV). The receptor was also expressed on dendrites at all time points, and did not co-localise with synaptophysin, PSD-95 or the NMDA receptor subunit, NR2a, but did partially co-localise with NR2b. Addition of MT and emtinB did not induce calcium influx in live neurons demonstrating that LRP1 mediated synaptic regulation is not modulated by these ligands in this cell type. LRP1 was also expressed along and at the ends of 3DIV and some 7DIV axons, suggesting a role in neurite outgrowth. This

hippocampal neuron model was used to investigate the role of LRP1 in response to injury. LRP1 was expressed at the ends of extending neurites 24 hours after scratch injury, suggesting that it is involved in neurite regeneration. 10μg/mL MT did not promote neurite extension but 25μM emtinB significantly increased the number of neurites that extended along or into the scratch injury site (57.8 ± 4.6 neurites/cm injury site) compared to saline controls (27.7 ± 3.3 neurites/cm injury site; $p<0.005$) and cultures treated with both emtinB and the LRP1 inhibitor, receptor associated protein (36.5 ± 3.2 neurites/cm injury site; $p<0.005$). These findings demonstrate the ability for LRP1 to promote neurite extension following injury through stimulation by emtinB.

This thesis presents the temporal, regional and subcellular expression profile of LRP1 in neurons and glia of the CNS and how this may influence the receptor's function in specific cell types during normal and injury conditions. The data support a role for LRP1 in neuronal biology and provide the foundation for further investigation into how LRP1 regulates neuron function.

Contents

Chapter 1: Introduction

List of tables

List of figures

Abbreviations

α2-M: α2-macroglobulin

ApoE: apolipoprotein E

BBB: blood brain barrier

BDNF: brain derived neurotrophin factor

CA: cornu ammonis region of the hippocampus

CC: corpus callosum

CNS: central nervous system

Cx: cerebral cortex

DAB: diaminobenzidine

DG: dentate gyrus

DIV: days *in vitro*

DRG: dorsal root ganglia

F: fluorescence

HBSS: Hank's buffered saline solution

Hp Fi: hippocampal fimbriae

Hp SR: stratum radiatum of the hippocampus

HRP: horse radish peroxidase

HyT: hypothalamus

IHC: immunohistochemistry

IP: immunoprecipitation

ISH: *in situ* hybridisation

LDL: low density lipoprotein

LRP1: low density lipoprotein receptor-related protein-1

LTP: long term potentiation

MAG: myelin associated glycoprotein

MMP-9: matrix metalloproteinase-9

MT: metallothionein

NA: not applicable

NGF: nerve growth factor

NMDA: N-methyl D-aspartate

NS: not specified

NT-3/4: neurotrophin-3/4

NthB: northern blot

NY: nuclear yellow

P2/7: post-natal day 2/7

$p75^{NTR}$: p75 neurotrophin receptor

PBS: phosphate buffered saline

PBS-Tw: phosphate buffered saline with 0.05% Tween-20

PNS: peripheral nervous system

PSD-95: post-synaptic density-95

RAP: receptor associated protein

RIPA: radio-immunoprecipitation assay

RPA: RNase protection assay

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Th: thalamus

tPA: tissue-type plasminogen activator

Trk: tropomyosin-related kinase

WB: western blot

1 Chapter 1: Introduction

1.1 GENERAL DEVELOPMENT OF NEURAL CONNECTIONS IN THE BRAIN

There are billions of neurons in the brain responsible for our ability to sense, think and move. Without the correct regulation and maintenance of these cells, life would be impossible. Control of neuronal function in development and maturity is complex and affected by both intrinsic and extrinsic factors. Chemical transmission is fundamental to the function in the central nervous system (CNS) and is dependent on the formation of correct connections between neurons during embryonic development. Developing neurons detect attractive or repulsive cues from the extracellular environment. These cues activate intracellular pathways involved in modulating cytoskeleton rearrangement and axon movement (Yamada et al., 1970). This allows the extending axon to maneouver through the brain to reach its specific destination (reviewed by (Mueller, 1999)). The axon then forms and stabilises a synaptic connection that allows neurons to receive, interpret and forward chemical signals through the CNS (reviewed by (Vaughn, 1989)). Neurons continue to receive a range of extracellular signals through maturity in order to maintain cell integrity and function appropriately.

The interaction of extracellular and intracellular elements to produce a response is highly precise. Extracellular proteins interact with specific membrane bound receptors that modulate intracellular protein activation and distribution. This in turn activates or inhibits transcription factor activity and gene and protein expression (reviewed in (Kadonaga, 2004)). For all neuron functions, the cell must receive extracellular cues that mediate intracellular changes and responses.

1.2 ROLE OF CELL SURFACE RECEPTORS IN INTEGRATING EXTRACELLULAR AND INTRACELLULAR ENVIRONMENTS

One key link between extracellular and intracellular environments is the membrane receptor. Extracellular ligands bind to their specific surface receptors to induce a conformational change that elicits an intracellular chemical response (Elliott and Elliott, 2004). This enables cells to detect signals in their surrounding environment and to respond appropriately. These signals are not only receptor-ligand specific but also cell specific. Receptors and their intracellular signaling targets are only present in certain cell types or on specific subcellular locations within that cell. In addition, signals from other receptors can promote or inhibit responses (Nelson and Cox, 2008) (Figure 1.1). This complex mix of ligand, receptor and cell expressed proteins ensures that responses are tightly regulated and appropriate.

1.2.1 EXAMPLE OF A WELL UNDERSTOOD BUT COMPLEX NEURONAL SIGNALING PATHWAY: THE NEUROTROPHINS

An example of, and one of the best understood, systems that displays the complex interactions between ligand, receptor and intracellular proteins to produce neuronal responses is the neurotrophin signaling pathway. Four neurotrophins have been identified in the mammalian system: nerve growth factor (NGF) (Cohen and Levi-Montalcini, 1956), brain derived neurotrophin factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Maisonpierre et al., 1990) and NT-4 (Hallbook et al., 1991). Neurotrophins are ligands for the tropomyosin-related kinase (Trk) receptors, TrkA, TrkB and TrkC, and the p75 neurotrophin receptor ($p75^{NTR}$) (Klein et al., 1991, Squinto et al., 1991, Ip et al., 1992). Each neurotrophin binds to a specific Trk receptor but all neurotrophins are able to bind to $p75^{NTR}$ (Hallbook et al., 1991, Kaplan et al., 1991, Lamballe et al., 1991, Squinto et al., 1991). Upon neurotrophin binding, Trk receptors phosphorylate intracellular proteins that activate pathways to promote neuronal growth,

migration, differentiation and survival (Datta et al., 1997, Xing et al., 1998). In comparison, neurotrophin stimulation of $p75^{NTR}$ often activates pro-apoptotic pathways (Frade et al., 1996, Bamji et al., 1998).

The outcomes of neurotrophin binding are complex and dependent on interactions between the receptor, the ligand that binds it, and other receptors and intracellular proteins expressed by the cell. For example, NGF induces apoptosis in retinal neurons (Frade et al., 1996) but promotes survival in sensory neurons, of which both effects are through activation of $p75^{NTR}$ (Hamanoue et al., 1999). This demonstrates that the same ligand and receptor combination can mediate opposing effects in different cell types. BDNF protects against NGF and $p75^{NTR}$ mediated apoptosis in retinal neurons through TrkB activation (Frade et al., 1997). This demonstrates how the balance of neurotrophins and interactions of their Trk and $p75^{NTR}$ receptors is crucial in determining cell outcomes. Trk receptors can promote neuronal survival through suppression of $p75^{NTR}$ and pro-apoptotic pathways (Yoon et al., 1998). This explains, in part, why $p75^{NTR}$ mediated apoptosis only occurs when Trk receptors are inactive or decreased in expression (Bamji et al., 1998, Davey and Davies, 1998). Interestingly, the affinity of neurotrophins to bind to Trk receptors is stronger and more selective in the presence of $p75^{NTR}$ (Hempstead et al., 1991, Mahadeo et al., 1994, Bibel et al., 1999). The effect of neurotrophin receptor activation and subsequent outcomes is dependent on the identity of the neurotrophin and the proteins expressed by the target cell that can interact with the receptor.

The objective of this thesis is to investigate another complex, but less understood, receptor system. As will be discussed, the low density lipoprotein receptor-related protein-1 (LRP1) is a receptor that, similar to neurotrophin receptors, modulates a diverse range of neuronal responses. The receptor also has an unusually high number

of ligands, giving rise to the hypothesis that it assists neurons to integrate and respond to a range of extracellular cues.

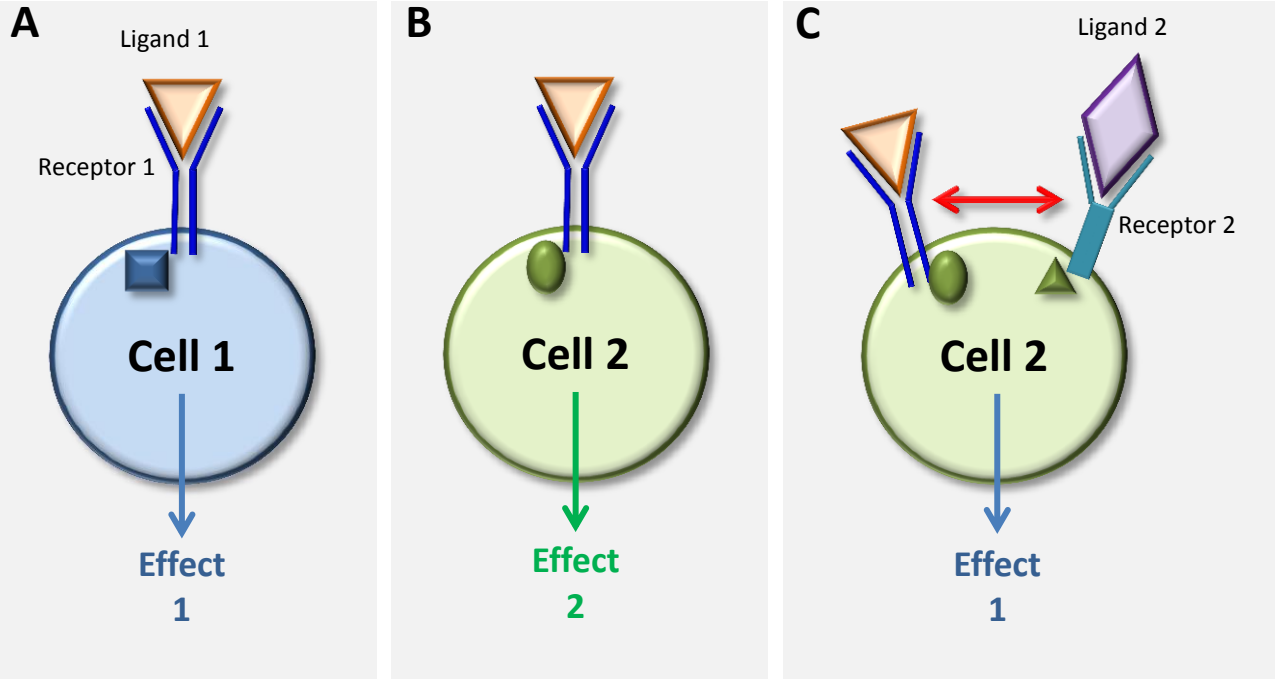

Figure 1.1: Simplified diagram of some receptor-ligand interactions in different cell types. Ligand activation of a receptor leads to activation of intracellular proteins (square, oval and small triangle) to produce an effect (A). The same receptor and ligand in another cell type that expresses a different set of intracellular proteins can produce a different response (B). Interactions between receptors expressed upon the same cell can alter the cellular effect (C).

1.3 LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN-1 (LRP1)

LRP1 was first described in 1988 by Herz and colleagues as a 600kDa receptor found in great abundance in the liver and brain (Herz et al., 1988). The receptor is one of the eleven members of the low density lipoprotein (LDL) receptor family. Like other members of this family, the primary role of LRP1 was initially believed to be in apolipoprotein transport (Herz et al., 1988, Kowal et al., 1989). However, further analysis of the receptor found it to be identical to the α2-macroglobulin receptor, which bound many more ligands and mediated several signaling pathways unrelated to the LDL receptor (Strickland et al., 1990). LRP1 is now known to have over 40 ligands (Table 1.1) and is expressed in cells of the liver, kidney, bone, fat, lung, skin, spleen, ovaries and testes (Herz et al., 1988, Feldman and Sangha, 1992, Moestrup et al., 1992, Zheng et al., 1994, Igdoura et al., 1997, Niemeier et al., 2005). Expression is also present in neurons and glia of human and rodent central nervous systems (discussed below).

LRP1 is highly conserved between bacterial, avian and mammalian species (Springer, 1998) and knock-out mice are embryonically unviable, demonstrating a vital role for the receptor during embryonic development. Though the reason for this lethality is not entirely understood, it could be due to blockage of embryo implantation or abnormalities in liver development (Herz et al., 1992, Roebroek et al., 2006). In genetically altered mice where brain and liver LRP1 expression is decreased by 75%, animals are viable and functionally normal despite increased levels of plasma proteins (Willnow et al., 1995). This indicates that reduced expression of the receptor is sufficient for normal embryonic development. Mouse strains with a conditional knock-out in specific neuronal populations are also viable but display increased neuronal death and cognitive deficits as they age (May et al., 2004, Liu et al., 2010). While the primary

function of LRP1 remains unclear, it is apparent that the receptor has an essential role in mammalian systems during development and maturity.

Ligand	*Reference*
α2-macroglobulin	*(Moestrup and Gliemann, 1989)* *(Gliemann et al., 1989)*
Amyloid-β	*(Deane et al., 2004)*
Apolipoprotein E	*(Kowal et al., 1989)*
- Apolipoprotein E3	*(Nathan et al., 2002)* *(Matsuo et al., 2011)*
- Apolipoprotein E4	*(Nathan et al., 2002)* *(Hashimoto et al., 2000)*
Amyloid precursor protein	*(Kounnas et al., 1995)*
β-very low density lipoprotein	*(Kowal et al., 1989)* *(Handelmann et al., 1992)*
C1s-C1 inhibitor complex	*(Storm et al., 1997)*
Calreticulin	*(Ogden et al., 2001)* *(Gardai et al., 2005)*
Cholesterol	*(Handelmann et al., 1992)*
Complement C3	*(Meilinger et al., 1999)*
Connective tissue growth factor	*(Segarini et al., 2001)*
EmtinB	*(Ambjørn et al., 2008)*
Factor VIII	*(Lenting et al., 1999)* *(Saenko et al., 1999)*
Fibronectin	*(Salicioni et al., 2002)*
Gentamicin	*(Moestrup et al., 1995)*
gp96	*(Binder et al., 2000)*
Hepatic lipase	*(Kounnas et al., 1995)*

Ligand	*Reference*
Insulin-like growth factor binding protein-3	*(Huang et al., 2003)*
Lactoferrin	*(Willnow et al., 1992)*
Leptin	*(Liu et al., 2011)*
Lipoprotein lipase	*(Willnow et al., 1992)*
Matrix metalloproteinase-9	*(Mantuano et al., 2008)*
Myelin basic protein	*(Gaultier et al., 2009)*
Myelin associated glycoprotein	*(Stiles et al., 2013)*
Midkine	*(Muramatsu et al., 2000)*
Metallothionein 1/2	*(Ambjørn et al., 2008)*
Platelet-derived growth factor	*(Loukinova et al., 2002)* *(Boucher et al., 2002)*
Plasminogen activator inhibitor type 1	*(Nykjaer et al., 1994)*
Polymyxin B	*(Moestrup et al., 1995)*
Pseudomonas exotoxin A	*(Kounnas et al., 1992)*
Receptor associated protein	*(Strickland et al., 1990)* *(Herz et al., 1991)*
Transforming growth factor-β	*(Huang et al., 2003)*
Thrombospondin-1	*(Godyna et al., 1995)* *(Chen et al., 1996)*
Thrombospondin-2	*(Chen et al., 1996)*
Tissue-type plasminogen activator	*(Bu et al., 1992)* *(Orth et al., 1992)*
Tissue factor pathway inhibitor	*(Warshawsky et al., 1994)*
Urokinase plasminogen activator	*(Nykjaer et al., 1994)*

Table 1.1: Ligands that bind LRP1. Low density lipoprotein receptor-related protein (LRP1) has over 40 ligands, of which 37 are listed, that bind primarily to regions II and IV of the extracellular domain.

1.3.1 STRUCTURE AND PROCESSING OF LRP1

The human LRP1 gene is located on chromosome 12 (Myklebost et al., 1989) and codes a 4544 amino acid protein (Herz et al., 1988). After translation and transport to the endoplasmic reticulum, LRP1 binds with high affinity to a 39kDa chaperone called receptor associated protein (RAP) (Strickland et al., 1990) (Figure 1.2). This binding inhibits protein interaction and receptor clumping (Herz et al., 1991) and assists with correct protein folding (Bu and Rennke, 1996). The receptor is transported to the Golgi apparatus where it is cleaved by the enzyme furin to form two non-covalently bound subunits of 515kDa and 85kDa. The 515kDa fragment (also known as the α- or heavy chain) contains the majority of the extracellular domain and all ligand binding regions of the receptor. The 85kDa fragment (also known as the β- or light chain) is composed of the membrane anchor and cytoplasmic domain (Herz et al., 1990). RAP dissociates from LRP1 in the lower pH of the Golgi apparatus, leaving the receptor functionally active to be transported to the cell membrane (Bu et al., 1995). The importance of LRP1-RAP interactions during processing is confirmed in RAP deficient mouse models that demonstrate impaired LRP1 expression (Willnow et al., 1995). Conversely, overexpression of RAP leads to its increased secretion from the cell and inhibition of LRP1 on the cell membrane. When applied extracellularly, RAP acts as a competitive antagonist of LDL receptor family members and inhibits receptor-mediated effects (Willnow et al., 1994, Medh et al., 1995).

LRP1 has distinct extracellular, transmembrane and intracellular motifs (Figure 1.3). The extracellular domain is composed of four regions of cysteine rich ligand binding repeats separated by four regions of epidermal growth factor/β-sheet motifs, which are involved in ligand release (Davis et al., 1987). The majority of ligands bind to ligand binding regions II and IV, though binding can involve multiple regions (Neels et al., 1999, Mikhailenko et al., 2001). One of the reasons that the receptor can bind many

ligands is due to the 31 ligand binding repeats contained in its extracellular domain (Mikhailenko et al., 2001). A type 1 transmembrane domain spans the cell membrane, connecting the extracellular and intracellular domains (Herz et al., 1988).

The LRP1intracellular domain contains a YxxL tyrosine motif, two di-leucine motifs and two NPxY tyrosine motifs (proximal and distal). The YxxL and di-leucine motifs are the primary mediators of LRP1 mediated endocytosis (Li et al., 2000). As for other members of the LDL family, this process occurs via clathrin-coated vesicles (Bu et al., 1994a). Upon receptor mediated signaling, clathrin is recruited to form vesicles containing the receptor and ligand that bud from the cell membrane. The receptor is then released and returned to the cell surface while the ligand is degraded in lysosomes ((Van Kerkhof et al., 2005); reviewed by (Schwartz, 1995)). Though NPxY motifs can induce endocytosis (Bonifacino and Traub, 2003), they also associate with intracellular proteins and activate pathway signaling.

The NPxY motifs bind to scaffold and adaptor proteins to activate intracellular signals. The ability of intracellular proteins to associate is dependent upon the motifs phosphorylation status. The majority of intracellular proteins bind to the phosphorylated distal NPxY motif, though the non-phosphorylated form is able to bind different proteins and activate separate pathways (Gotthardt et al., 2000, Guttman et al., 2009). Distal NPxY phosphorylation can occur via association of intracellular proteins (Barnes et al., 2001) or by activated platelet derived growth factor receptor (Boucher et al., 2002, Loukinova et al., 2002) and leads to phosphorylation of the proximal NPxY motif (Betts et al., 2008). The primary role of the proximal NPxY motif appears to be in the synthesis of LRP1 (Reekmans et al., 2010), but when phosphorylated it can bind a number of proteins similar to the distal motif (Guttman et al., 2009). Therefore, the ability of LRP1 to activate intracellular signaling pathways is dependent on both intracellular protein composition and activity of co-receptors.

LRP1 activity is also regulated by cleavage of its extracellular and intracellular domains (Figure 1.2). The extracellular domain of LRP1 can be cleaved by the amyloid precursor protein enzyme β-secretase to release it from the membrane (von Arnim et al., 2005). The soluble LRP1 heavy chain has been found free in blood and can bind free ligands to inhibit activation of the cell bound receptor (Quinn et al., 1997). The formation of this soluble fraction has been conserved between mammalian, avian, reptilian and mollusc species and implies an important role for it in regulating LRP1 activity (Grimsley et al., 1999). Following β-secretase cleavage, the transmembrane domain can be cleaved by a γ-secretase complex (May et al., 2002). This releases the intracellular domain that can bind to adaptor proteins (Trommsdorff et al., 1998, Gotthardt et al., 2000) or enter the nucleus to interact directly with transcription factors (Kinoshita et al., 2003). γ-secretase cleavage is regulated by glycosylation of the receptor, a cell specific mechanism, which modulates LRP1 mediated transcription activation in different tissues (May et al., 2003).

LRP1 can modulate a variety of cellular functions due to a structure that allows it to bind a range of extracellular ligands and intracellular proteins. However, the receptors activity is tightly regulated through cell specific mechanisms including phosphorylation, glycosylation and expression of signaling proteins, co-receptors and enzymes. This ensures that while LRP1 responses are diverse, they are also specific.

Figure 1.2: Processing and cleavage of LRP1.

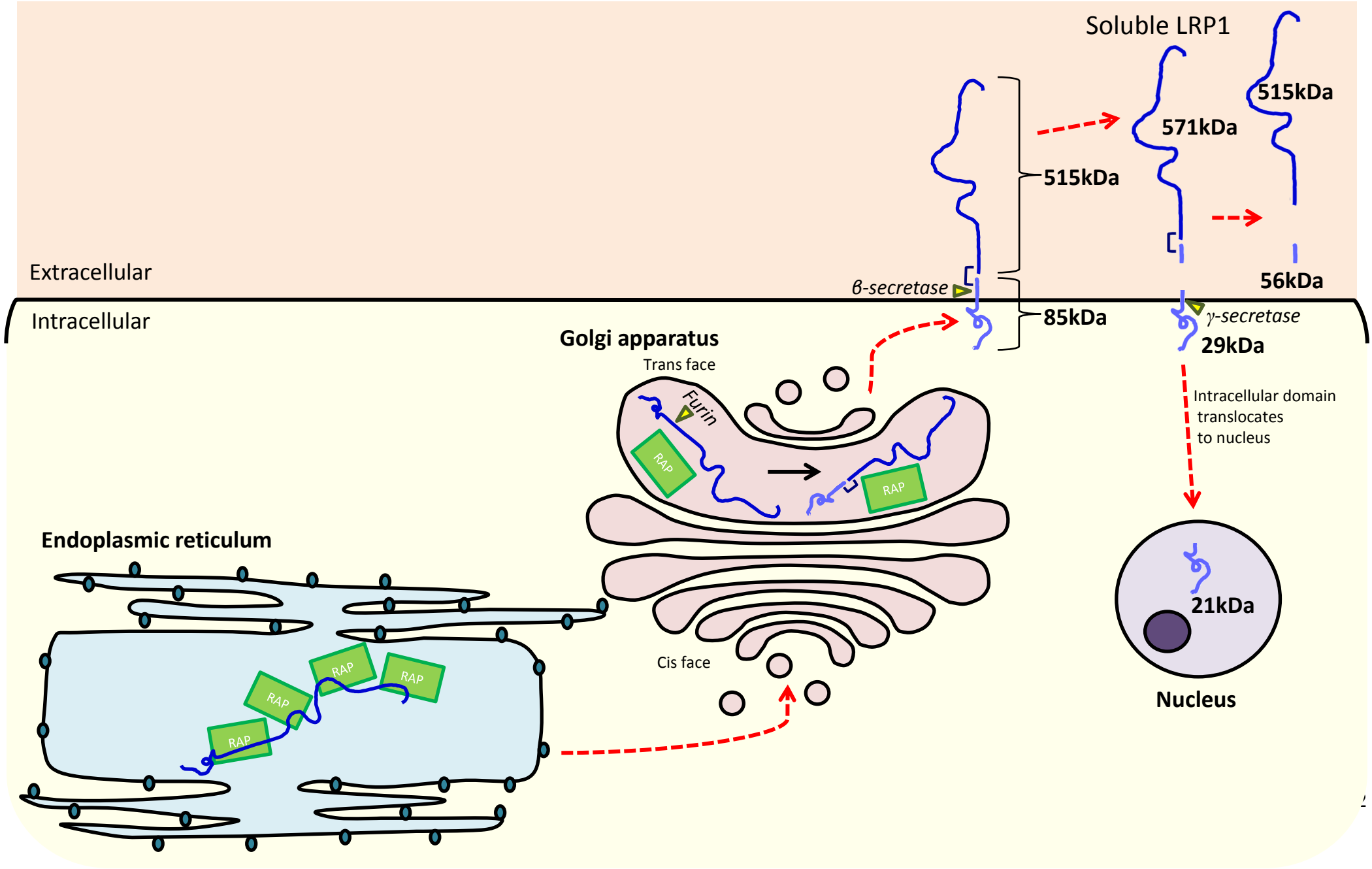

Figure 1.2 continued: Processing and cleavage of LRP1. Low density lipoprotein receptor-related protein (LRP1) is translated and transported to the endoplasmic reticulum where it is bound to receptor associated protein (RAP) to inhibit ligand binding, receptor clumping and to assist with correct protein folding. In the Golgi apparatus the receptor dissociates from RAP and is cleaved by furin to form a 515kDa heavy chain bound non-covalently to an 85kDa light chain. LRP1 is transported to the cell membrane. Extracellular ligands can bind to the receptor for endocytosis or to activate intracellular pathways. Alternatively, the extracellular domain can be cleaved by the amyloid precursor protein enzyme β-secretase to release a 571kDa soluble fraction, which in turn can lose its non-covalent bond to form 515kDa and 56kDa fractions. Soluble LRP1 can antagonize ligand binding in plasma and cerebral spinal fluid. LRP1 can be subsequently cleaved by γ-secretase, releasing the 21kDa intracellular domain which binds intracellular proteins or translocates to the nucleus to regulate transcription events.

Adapted from (Bu, 2009)

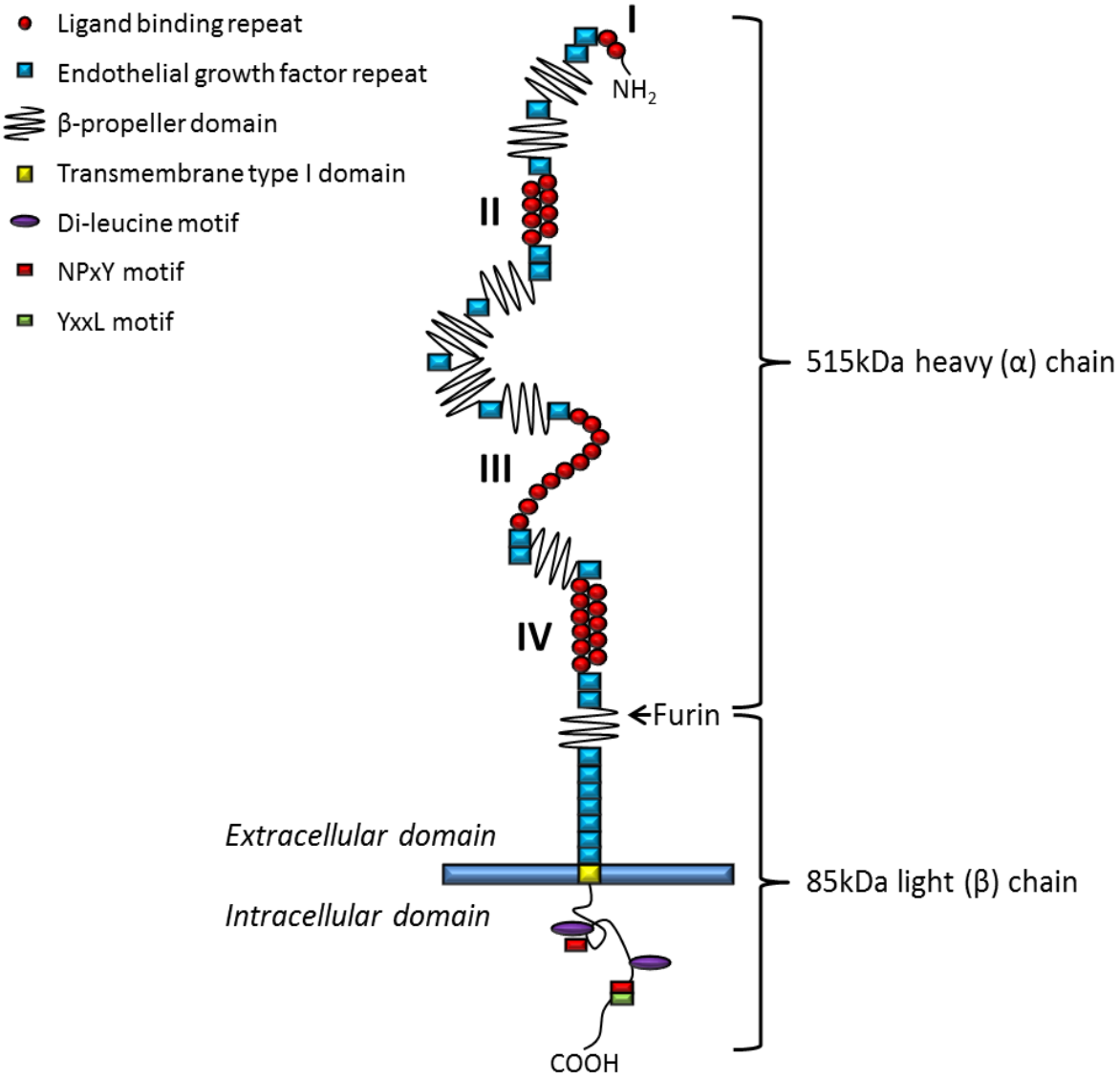

Figure 1.3: Structure, cleavage and binding sites of LRP1. Low density lipoprotein receptor-related protein (LRP1) is a 600kDa receptor that is cleaved by the enzyme furin to form two non-covalently linked subunits, a 515kDa heavy or α-chain and an 85kDa light or β-chain. The extracellular domain of the receptor contains four regions (I, II, II and IV) of cysteine rich ligand binding repeats separated by endothelial growth factor/β-sheet motifs that mediate ligand release. A type I transmembrane domain spans the cell membrane. The intracellular domain contains two NPxY motifs, which bind to and activate intracellular proteins, and a YxxL motif and two di-leucine motifs, which are involved in endocytosis.

Adapted from (Herz et al., 1990)

1.3.2 LRP1 IN THE NERVOUS SYSTEM

LRP1 is expressed in a range of cells of the peripheral and central nervous systems. Peripheral nervous system (PNS) expression of LRP1 has been demonstrated in dorsal root ganglia, the sciatic nerve, Schwann cells and satellite cells (Handelmann et al., 1992, Campana et al., 2006, Shi et al., 2009). In the human and rodent CNS, LRP1 is expressed in a range of cell types throughout the brain (Table 1.2). The primary function of LRP1 in the CNS is not well understood, but it is able to regulate protein transport into the brain, cell survival, neurite outgrowth, regeneration and signaling.

LRP1 is expressed primarily on neuron cell bodies of the cerebral cortex, cerebellum, thalamus, brain stem and hippocampus, though there are some reports of reactivity along proximal and dendritic processes (Wolf et al., 1992, Rebeck et al., 1993, Bu et al., 1994b, Ishiguro et al., 1995, Deane et al., 2004, May et al., 2004, Liu et al., 2007). Further work *in vitro* has supported these *in vivo* findings and allowed more defined identification of LRP1 in these cells. Expression has been reported in hippocampal, cortical, retinal ganglion and cerebellar granule neuron cell bodies and dendritic processes (Brown et al., 1997, May et al., 2004, Hayashi et al., 2007, Ambjørn et al., 2008, Shi et al., 2009). The primary glial cells positive for LRP1 *in vivo* are astrocytes, in which expression is present in cell bodies and processes (Wolf et al., 1992, Rebeck et al., 1993, Zheng et al., 1994, Ishiguro et al., 1995). However, microglia and oligodendrocytes, as well as astrocytes, express the receptor in cell bodies and processes *in vitro* (Marzolo et al., 2000, Gaultier et al., 2009). Much of the interest in LRP1 expression in the CNS has evolved from findings that the receptor is involved in Alzheimer's disease.

LRP1 has been associated with late onset of Alzheimer's disease by a number of mechanisms. The receptor has been genetically linked to the risk of developing the

disease (Saher et al., 2005), and its expression is significantly altered in brains of patients with mild cognitive impairment and Alzheimer's disease (Donahue et al., 2006, Sultana et al., 2010). Forebrain LRP1 knock-out mice display Alzheimer's disease-like symptoms as they age, suggesting a role for the receptor in preventing the disease (Liu et al., 2010). However, overexpression of a LRP1 mini receptor in the brain increases soluble amyloid-β and causes spatial learning and memory deficits typical to Alzheimer's disease (Zerbinatti et al., 2004). In addition, two LRP1 ligands, apolipoprotein E (apoE) and α2-macroglobulin, have been genetically linked to the risk of developing Alzheimer's disease (Eng et al., 1968, Zhou et al., 2000, Paxinos and Watson, 2007, Ashwell and Paxinos, 2008). The receptor also directly associates with and endocytoses amyloid precursor protein (Kounnas et al., 1995) and amyloid-β, which it clears from the brain and circulation via the blood brain barrier (BBB) and liver, respectively (Shibata et al., 2000, Deane et al., 2004, Zlokovic et al., 2010).

LRP1 is expressed on endothelial cells, astrocytic processes, pericytes and at the basal membrane of microvessels through the brain (Moestrup et al., 1992, Wolf et al., 1992, Deane et al., 2004, Polavarapu et al., 2007). The receptor regulates movement of proteins, such as amyloid-β, across the BBB (Shibata et al., 2000, Deane et al., 2004), whilst also maintaining the permeability of this barrier (Yepes et al., 2003). LRP1 is also expressed on ependymal cells of the choroid plexus, where it most likely involved in transport or secretion between the brain and cerebral spinal fluid (Zheng et al., 1994). LRP1 at the BBB and choroid plexus is involved in transport of molecules into and out of the brain. In this way the receptor regulates of the brains microenvironment and is able to indirectly affect the function of neurons and glia. However, LRP1 expressed upon these cells can directly affect their function, such as survival, neurite outgrowth, regeneration or synaptic responses.

Table 1.2: LRP1 is expressed in cells of the human, rat and mouse central nervous systems

	Tissue	Cell	Location on cell	Technique	Animal strain	Reference
HUMAN	Cerebral cortex	Neurons	Cell body	IHC (DAB)	NA	(Moestrup et al., 1992)
		Astrocytes (fibrillary)	Cell body Processes			
		Capillaries	Basal membrane			
	Cerebellum	Granule cells	Cell body			
		Purkinje cells	Cell body			
		Capillaries	Basal membrane			
	Cerebral cortex Thalamus Dentate nucleus	Pyramidal neurons	Cell body Proximal processes	IHC (DAB)	NA	(Wolf et al., 1992)
	Cerebellar cortex	Astrocytes	Perivascular processes			
	Cerebral cortex			WB		
	Temporal cortex	Pyramidal neurons	Cell body Dendritic processes Proximal axons	IHC (DAB)	NA	(Rebeck et al., 1993)
		Astrocytes	Cell body Processes			
	Hippocampus	Pyramidal neurons (CA1, CA3, CA4)	Cell body Dendritic processes			
		Granule neurons (DG)	Cell body Dendritic processes			
	Frontal cortex	Endothelial cells	Cell body	IHC (F)	NA	(Deane et al., 2004)
RAT	Choroid plexus	Ependymal cells	Diffuse throughout	IHC (F)	Lewis	(Zheng et al., 1994)
	CNS (NS)	Neurons (not shown)	Focal			
	CNS (NS)	Astrocytes (not shown)	Focal			
	Cerebral cortex	Neurons		IP	NS (P10)	(Bu et al., 1994a)
	Cerebral cortex Hippocampus Brain stem Cerebellum			RPA	NS (adult)	
	Hippocampus	Neurons (DG, CA3)	NS	ISH		
	Cerebellum	Purkinje cells	Cell body			
	Cerebral cortex	Neurons Glia	Cell body	ISH	Hooded Wistar (E20)	(Ishiguro et al., 1995)
	Hippocampus	Pyramidal neurons (CA3)	Cell body		Hooded Wistar (Adult)	
	Cerebral cortex	Neurons	Cell body			
	Cerebellum	Granule cells Purkinje cells	Cell body			
	Whole brain			NthB	Hooded Wistar (E18 to adult)	
	Whole brain			WB	Sprague Dawley	(Hayashi et al., 2007)

	Tissue	Cell	Location on cell	Technique	Animal strain	Reference
MOUSE	Cerebral cortex	Neurons	Cell body	IHC (DAB)	C57BL/6	(May et al., 2004)
	'Other regions of brain'	Neurons	NS			
	Whole brain			NthB WB		
	Hippocampus CA1	Neurons	Cell body	IHC (F)	C57BL/6	(Liu et al., 2007)
	Frontal cortex	Pyramidal neurons	Cell body			
	Forebrain			WB		
	Forebrain			WB	C57BL/6	(Xu et al., 2008)
	Whole brain			Slot blot	NS	(Herz et al., 1988)
	Microvessels	NS		IHC (DAB	C57BL/6	(Shibata et al., 2000)
	Cerebral cortex Hippocampus Thalamus	Endothelial cells	Cell body	IHC (F)	C57BL/6	(Deane et al., 2004)

Table 1.2 continued: LRP1 is expressed in cells of the human, rat and mouse central nervous systems. Low density lipoprotein receptor-related protein-1 (LRP1) expression is detected in cell bodies and processes of neurons, glia and other cells of human, rat and mouse central nervous systems. Both LRP1 mRNA and protein have been detected using a range of laboratory techniques and rodent strains.

DAB: diaminobenzidine; DG: dentate gyrus; F: fluorescence; IHC: immunohistochemistry; IP: immunoprecipitation; ISH: in situ hybridisation; NA: not applicable; NS: not specified; NthB: northern blot; RPA: RNase protection assay; WB: western blot

1.3.2.1 Regulation of neuronal survival

The development of *in vitro* models of neuron survival, neurite outgrowth, regeneration and signaling has enabled the investigation of LRP1 function in a range of neuron subtypes.

LRP1 regulates neuronal survival in a manner dependent on cell type, ligand and apoptotic stimuli involved (Table 1.3). LRP1 knock-down increases apoptosis of mixed cortical and hippocampal neurons following trophic withdrawal and amyloid-β toxicity *in vitro* (Fuentealba et al., 2009). This demonstrates a role for the receptor in maintaining neuron health, a finding supported by evidence that direct stimulation of LRP1 affects neuron survival. apoE3 with cholesterol protects against amyloid-β mediated apoptosis in hippocampal neurons, an outcome inhibited by addition of antibodies against LRP1 (Sen et al., 2012). In comparison, apoE3 with cholesterol does not inhibit apoptosis of retinal ganglion cells following trophic withdrawal (Hayashi et al., 2007). However, apoE containing lipoproteins prevent apoptosis of these cells following trophic withdrawal and glutamate toxicity, effects that are abolished when LRP1 is inhibited (Hayashi et al., 2007, Hayashi et al., 2012). In comparison, LRP1 induces apoptosis of dorsal root ganglia (DRG) hybrid F11 cells through apoE4, though apoE3 does not affect these cells survival. Both RAP and an anti-sense oligonucleotide that caused 40-70% knock-down of LRP1 inhibited apoE4 induced apoptosis. Interestingly, a high dose of apoE3 (200μg/mL vs 30μg/mL) induced apoptosis in F11 cells (Hashimoto et al., 2000). The binding domain of α2-macroglobulin prevents apoptosis of DRGs, but interestingly, α2-macroglobulin itself does not affect survival of F11 cells (Hashimoto et al., 2000, Yamauchi et al., 2013). These findings demonstrate that LRP1 mediated effects are significantly dependent on cell type and ligand properties.

Ligand competition also affects LRP1 mediated neuron survival. α2-macroglobulin is able to inhibit the protective effects of apoE-containing lipoproteins following glutamate toxicity, though high concentrations of the lipoproteins are able to overcome this antagonism (Hayashi et al., 2012). α2-macroglobulin and apoE3 also inhibit apoE4 mediated apoptosis in DRG hybrid F11 cells (Hashimoto et al., 2000). This shows that the balance of ligand concentrations is important in regulating LRP1 effects.

The type of stress the cell is under also affects LRP1 mediated effects. α2-macroglobulin does not affect retinal ganglion cell outcome following glutamate toxicity (Hayashi et al., 2012) but prevents apoptosis of these cells following trophic withdrawal (Hayashi et al., 2007). ApoE4-lipoproteins are less effective than apoE3-lipoproteins at preventing apoptosis in retinal ganglion cells following trophic withdrawal (49.8% apoptosis vs 24.5% apoptosis; (Hayashi et al., 2007)). However, both ligands prevent apoptosis to a similar degree following glutamate toxicity (both 25-30% apoptosis; (Hayashi et al., 2012)). Therefore, the LRP1 response can depend on the type of stress the cell is under. This may be due to differences in extracellular protein composition following the stress or changes in intracellular protein expression in response to that stress.

1.3.2.2 Regulation of neurite outgrowth and regeneration

LRP1 influences neurite extension in both developing and mature neuron systems (Table 1.3). α2-macroglobulin increases both neurite length and LRP1 expression on cortical neurons (Rogove et al., 1999). The α2-macroglobulin binding domain, in addition to matrix metalloproteinase-9 and apoE containing lipoproteins, also enhances developing DRG neurite outgrowth (Handelmann et al., 1992, Yamauchi et al., 2013). This shows that even structurally distinct ligands can induce the same

response. Furthermore, the binding domain of α2-macroglobulin increases neurite outgrowth of cerebellar granule neurons, an effect that is believed to involve an interaction of LRP1 with Trk receptors (Shi et al., 2009). In comparison, myelin associated glycoprotein inhibits neurite length of these and PC12 cells by a mechanism involving both LRP1 and $p75^{NTR}$ (Stiles et al., 2013). Therefore, LRP1 mediated neurite outgrowth is dependent on the ligand that is present and also involves co-receptor interactions.

Regeneration in the mature nervous system is regulated by LRP1 both *in vivo* and *in vitro*. Axonal sprouting and regeneration is increased in rat spinal cord injury following addition of the α2-macroglobulin binding domain to the injury site (Yoon et al., 1998). Outgrowth of retinal ganglion cell neurites following axotomy is enhanced after addition of apoE containing lipoproteins. This only occurs when the lipoprotein is applied to the distal end of the injured axons rather than the cell body and is inhibited by RAP (Hayashi et al., 2004). Further studies have found that addition of α2-macroglobulin or LRP1 siRNA and antibodies inhibit apoE-lipoprotein mediated neurite regeneration in this model. Regeneration of retinal ganglion cells following axotomy is promoted by apoE3-containing lipoprotein, but not by apoE4-containing lipoprotein or α2-macroglobulin (Matsuo et al., 2011). These findings further indicate that LRP1 functions are dictated by the ligands involved, but also show that the location of the receptor influences its effect.

Table 1.3: Effect of LRP1 activation on neuronal survival, neurite outgrowth and regeneration

	Cell type	Ligand	Treatment	Effect	LRP1 inhibition/evidence	Reference
NEURONAL SURVIVAL	Mixed cortical and hippocampal neurons	NA	Untreated	No effect	Knock-down	Fuentealba et al, 2009
			Trophic withdrawal	Increases apoptosis		
			Amyloid-β toxicity			
	Hippocampal neurons	apoE3 + cholesterol	Amyloid-β toxicity	Prevents apoptosis	RAP LRP1 antibody	Sen et al, 2012
	Retinal ganglion cells	apoE3 + cholesterol	Trophic withdrawal	No effect	NA	Hayashi et al, 2007
		apoE-lipoprotein		Prevents apoptosis	RAP LRP1 antibody LRP1 siRNA	
		apoE3-lipoprotein			Assumed given apoE- lipoprotein effects	
		apoE4-lipoprotein				
		α2-M				
		apoE-lipoprotein	Glutamate cytotoxicity	Prevents apoptosis	LRP1 antibody	Hayashi et al, 2012
		apoE3-lipoprotein			Assumed given apoE- lipoprotein effects	
		apoE4-lipoprotein				
		α2-M		No effect		
	Dorsal root ganglia hybrid F11 cells	apoE3	NA	No effect	RAP LRP1 anti-sense oligonucleotide	Hashimoto et al, 2000
		apoE4		Increases apoptosis		
		apoE3 (high dose)		Increases apoptosis	RAP	
		α2-M		No effect	NA	
	Dorsal root ganglia	α2-M	NA	Prevents apoptosis	RAP	Yamauchi et al, 2013

	Cell type	Ligand	Treatment	Effect	LRP1 inhibition	Reference
NEURITE OUTGROWTH	Dorsal root ganglia	α2-M binding domain	Untreated	Increases neurite length	RAP	Yamauchi et al, 2013
		MMP-9			None	
		apoE-lipoprotein + β-VLDL			LRP1 expressed upon cells	Handelmann et al, 1992
	Cerebellar granule cells	α2-M binding domain	Untreated	Increases neurite length	RAP	Shi et al, 2009
		MAG		Inhibits neurite length	RAP LRP1 siRNA	Stiles et al, 2013
	PC12 cell line	α2-M binding domain		Increases neurite length	RAP	Shi et al, 2009
	Cortical neurons	α2-M	Untreated	Increases neurite length	RAP Increased LRP1 expression	Qiu et al, 2004
REGENERATION	Retinal ganglion cells	apoE-lipoprotein	Axotomy	Increases neurite length	RAP	Hayashi et al, 2004
		apoE-lipoprotein			LRP1 antibody LRP1 siRNA	Matsuo et al, 2011
		apoE3-lipoprotein			Assumed given apoE- lipoprotein effects	
		apoE4-liporotein		No effect		
		α-2M				

Table 1.3 continued: Effect of LRP1 activation on neuronal survival, neurite outgrowth and regeneration. Low density lipoprotein receptor-related protein-1 (LRP1) activation by structurally distinct ligands can promote, inhibit or have no effect on neuronal survival, neurite outgrowth or regeneration. These effects are ligand and cell type dependent. The role of LRP1 in these responses was determined using a number of LRP1 inhibitors.

α2-M: α2-macroglobulin; apoE: apolipoprotein E; MAG: myelin associated glycoprotein; MMP-9: matrix metalloproteinase-9; NA: not applicable; RAP: receptor associated protein; tPA: tissue-type plasminogen activator

1.3.2.3 Regulation of synaptic function

While LRP1 functions to regulate outgrowth and survival in a range of neuron subtypes, it can also affect neuronal synaptic function and calcium signaling (Table 1.4). The distal NPxY motif of LRP1 associates with post-synaptic proteins such as post-synaptic density-95 (PSD-95) (Gotthardt et al., 2000, Martin et al., 2008). During synaptic transmission, N-methyl-D-aspartate (NMDA) activation of the NMDA receptor causes calcium influx. This process can be regulated by LRP1 through acute (5-10 minutes) or chronic (48 hours) incubation of ligands prior to NMDA addition. Acute incubation of tissue-type plasminogen activator (tPA) enhances NMDA mediated calcium influx in cortical neurons and is inhibited by RAP (Samson et al., 2008). In contrast, chronic pre-treatment of α2-macroglobulin or lactoferrin inhibits NMDA mediated calcium responses in hippocampal neurons. These effects were abolished by RAP. An acute treatment of α2-macroglobulin had no effect on NMDA responses (Qiu et al., 2002).

In addition to affecting calcium pathways through ligand pre-treatment, LRP1 can directly induce NMDA receptor mediated calcium influx. This demonstrates that the receptor is able to affect both fast and slow acting calcium pathways. Though pre-treatment with lactoferrin inhibits hippocampal NMDA mediated calcium responses as mentioned above, it has no effect on immediate calcium influx upon addition to cortical neurons. However, α2-macroglobulin and antibodies to one of LRP1's ligand binding domains induce NMDA dependent calcium influx in these cells (Bacskai et al., 2000). In contrast, a similar dose of α2-macroglobulin in hippocampal neurons had no effect on calcium response (Qiu et al., 2002). tPA addition to primary hippocampal neurons and hippocampal derived HT22 cells induces NMDA receptor dependent calcium influx. This is inhibited by RAP and reduced by PSD-95 knock-down, suggesting that LRP1

mediates NMDA receptor function through PSD-95 (Martin et al., 2008). Interestingly, tPA has no effect on calcium influx in cortical neurons, but as mentioned previously, pre-treatment of these cells with tPA promotes NMDA mediated calcium influx (Samson et al., 2008). Once again this shows that ligand concentration and cell type are important in dictating LRP1 responses. In addition to regulating NMDA receptor responses, LRP1 can modulate glutamate receptor responses. ApoE containing lipoproteins inhibit glutamate mediated calcium influx in retinal ganglion cells, an effect inhibited by addition of antibodies against LRP1 (Hayashi et al., 2012). This demonstrates an ability of the receptor to associate with and affect function of multiple other receptors.

LRP1 is also able to affect synaptic activity *in vivo*. Forebrain LRP1 knock-out mice display increased dendritic spine degeneration and decreased synaptophysin, PSD-95, NMDA receptor and glutamate receptor expression in both the cortex and hippocampus at 18 months of age. These mice display motor and memory deficits at 13 months, with hyperactivity and long term potentiation (LTP) deficits at 18 months (Liu et al., 2010). Hyperactivity and abnormalities in motor function are also observed in Cre-Lox LRP1 knock-out in differentiated neurons of the brain, though these effects are first observed at three weeks of age (May et al., 2004). However, brain morphology of these mice shows no signs of neurodegeneration. This could be due to the shorter time point at which mice were analysed (3 to 6 months old) when compared to the extensive neurodegeneration observed in the 18 month old forebrain knock-out mice. The lack of degeneration in the Cre-Lox mice suggests that abnormalities in behaviour are due to changes in synaptic function and neurotransmission in the CNS. Interestingly, LTP was not affected in hippocampal slices taken from Cre-Lox mice (May et al., 2004). This is despite findings that direct addition of RAP to hippocampal slices inhibits both normal

and tPA induced LTP. LRP1 is expressed with tPA in slices and is the primary receptor that tPA bound to, supporting a role for LRP1 in this process (Zhuo et al., 2000). LRP1 knock-out models demonstrate an important role for LRP1 in behavioural and motor functions, a role that involves both protection against neuron death and regulation of neurotransmission.

1.3.2.4 LRP1 mediated signaling

As LRP1 can affect calcium influx in a number of cell models (Table 1.4), and many LRP1 mediated responses are based on calcium events, it is likely that the receptor is able to mediate its effects through calcium signaling mechanisms. There are a range of mechanisms used to control LRP1 function. Not only is the presence of a ligand available to bind to the receptor important, but also the balance of all ligands and their interactions in the environment. Cell specific proteins, such as intracellular signaling proteins and co-receptors, also regulate LRP1 pathway activation upon ligand binding. The regulation of LRP1 is particular important as it is has so many effects, is expressed in a range of tissues and binds an abnormally large number of ligands. One of these ligands of particular interest due to its effects on neuronal biology is metallothionein (MT).

	Cell type	Ligand	Treatment	Response	LRP1 inhibition	Reference
PRE-TREATMENT	Cortical neurons	tPA	Acute incubation (5 minutes)	Enhances NMDA mediated calcium influx	RAP	Samson et al, 2008
	Hippocampal neurons	α2-M	Acute incubation (5-10 minutes)	No effect on NMDA mediated calcium influx	NA	Qiu et al, 2002
			Chronic incubation (48 hours)	Reduces NMDA mediated calcium influx	RAP	
		Lactoferrin	Chronic incubation (48 hours)	Reduces NMDA mediated calcium influx		
IMMEDIATE RESPONSE	HT22 cells	tPA	NA	Calcium influx (NMDA receptor dependent)	RAP LRP1 NPxY mutation	Martin et al, 2008
	Hippocampal neurons	α2-M	NA	No effect	NA	Qiu et al, 2002
	Cortical neurons	tPA	NA	No effect	NA	Samson et al, 2008
		α2-M	NA	Calcium influx (NMDA receptor dependent)	RAP	Bacskai et al, 2000
		LRP1 antibody	NA	Calcium influx (NMDA receptor dependent)	-	
		Lactoferrin	NA	No effect	NA	
	Retinal ganglion cells	apoE-lipoprotein	Glutamate	Inhibits glutamate mediated calcium influx	LRP1 antibody	Hayashi et al, 2012

Table 1.4: Effect of LRP1 on synaptic calcium responses following pre-treatment or immediate addition of ligands. Pre-treatment with low density lipoprotein receptor-related protein-1 (LRP1) ligands can enhance, reduce or have no effect on NMDA mediated calcium influx in neurons. Addition of LRP1 ligand can cause immediate calcium responses including NMDA receptor dependent calcium influx or inhibition of spontaneous calcium oscillations. Some ligands had no immediate effect on calcium responses.

α2-M: α2-macroglobulin; apoE: apolipoprotein E; NA: not applicable; NMDA: N-methyl-D-aspartate; RAP: receptor associated protein; tPA: tissue-type plasminogen activator

1.4 METALLOTHIONEIN

There are four different isoforms in the MT family: MT-I, MT-II, MT-II and MT-IV (Kägi et al., 1974, Lawson et al., 1990, Quaife et al., 1994). The most common and best characterised members of the MT family are MT-I and MT-II. These two isoforms are often referred in combination as MT-I/II as they are so similar in structure, function and regulation in the mouse system (Searle et al., 1984). However, this may not be true in other mammalian species, including humans (Blackstad et al., 1970). For the remainder of this thesis, MT-I/II will be referred to as MT.

MTs possess seven cysteine rich sites that bind metals such as zinc and copper (Kägi et al., 1974, Kojima et al., 1976). MT sequesters heavy metals (Kelly et al., 1996, Seamans et al., 1997), regulates the availability of metals able to interact with other proteins (Udom and Brady, 1980, Jiang et al., 1998) and scavenges free radicals to protect cells from oxidative stress (Hussain et al., 1996, Ye et al., 2003). MT has two known receptors, LRP1 and LRP2 (Fitzgerald et al., 2007) and can activate intracellular pathways through binding these receptors (Klassen et al., 2004, Ambjørn et al., 2008). Through LRP mediated mechanisms, MT is able to regulate neuronal survival, outgrowth and regeneration.

In recent years, peptides modeled after MT subunits have been developed that possess similar properties. These peptides, called emtins, include emtinAn, emtinAc, which are based on the N- and C-terminals of MT's α-domain, respectively (Lopes et al., 1994), and emtinB, which is based on MT's β-domain (Ambjørn et al., 2008). Emtins are able to cross the BBB (Henze et al., 1996) and bind LRPs (Ambjørn et al., 2008). As these peptides do not bind metals or scavenge free radicals (Ambjørn et al., 2008), it is plausible that LRP activation is their primary mode of action and allows them to mediate neurotrophic responses.

1.4.1 METALLOTHIONEIN AND EMTIN REGULATION OF NEURON FUNCTION

MT-IIA, an isoform of MT-II, increases wound healing following *in vivo* cortical needle-stick injury and *in vitro* cortical axon transection (Chung et al., 2003). Addition of MT, emtinAc and emtinB to cerebellar granule neurons increases developing neurite outgrowth and protects against apoptosis triggered by potassium withdrawal. These effects occurred through activation of pro-survival pathways and were inhibited by RAP, demonstrating a LRP dependent mechanism (Lopes et al., 1994, Ambjørn et al., 2008). MT is also able to promote DRG outgrowth and stimulate regeneration of DRG axons *in vitro* after scratch injury through a similar mechanism. The role of LRPs in these outcomes was demonstrated by RAP inhibition of effects (Leung et al., 2011). These findings indicate that many of the neurotrophic effects of MT are mediated through LRP activation.

In addition to acting directly upon neurons, MT can also indirectly affect function through actions on supportive glial cells such as astrocytes. Cultured astrocytes undergo pro-regenerative astrogliosis in the presence of MT leading to activation of pathways that increase cortical neuron axon regeneration following injury (Leung et al., 2010). Astrocytes not only respond to MT but also secrete it to be taken up by surrounding cortical neurons, an action inhibited by RAP (Chung et al., 2008). MT and emtins promote neuronal survival and regeneration in a range of neuron models. LRP mediated intracellular signaling is likely to be involved in this process, though the exact role of LRP1 is currently unknown.

Interestingly, different MT isoform can have opposing effects through LRP activation. MT is a chemoattractive cue in growth cone turning in developing DRGs, whereas MT-III is chemorepulsive. Both these responses are inhibited by siRNA against either or both LRP1 and LRP2 (Landowski et al., 2011). The metal composition of MT also affects its function. While zinc bound MT inhibits LTP in normal hippocampal

slices, non-metal bound MT has no effect in untreated slices and protects against an amyloid-beta mediated inhibition of LTP (Herbert et al., 2012). These findings are consistent with past reports that similar ligands can produce different effects through LRP1 activation, and that the nature of the ligand is crucial in dictating these effects.

1.5 PROJECT HYPOTHESIS AND AIMS

The range of LRP1 expression throughout the body, its many ligands and its ability to dictate numerous cellular processes suggest that this receptor may be a generic sensor of the extracellular environment. In the nervous system, it appears particularly important in maintaining healthy, functional neurons and glia. LRP1 regulation is complex but specific to produce precise effects. Understanding the specificity of LRP1 in different conditions is important in determining how the receptor is able to regulate so many different functions. LRP1 activity is affected by the ligands available to bind it and cell specific proteins that interact with the receptor. These in turn are determined by the cell LRP1 is expressed upon and its location upon that cell. Therefore, the pattern of LRP1 expression and how this changes in development or injury will provide insight into the role of the receptor in different neuronal functions.

MTs and emtins provide an interesting tool through which to study LRP1 function as these proteins act through LRPs, have many effects on neuron function and there are a range of isoforms that can have different effects. Using MT isoforms, we can study the effects of LRP1 activation on neuronal function and gain insight into the receptors' biology.

This thesis forms the groundwork for exploring the proposal that the diversity and specificity of an LRP1 response modulates neuronal function, such as regeneration. In this regard, I hypothesise that 1) LRP1 protein expression *in vivo* and *in vitro* demonstrates developmental and regional differences, and 2) injury will induce changes

in cellular expression of LRP1 and regeneration will be promoted in an LRP dependent manner following addition of MT or emtinB.

Thus, the aims of my project are as follows:

1. Define the expression profile of LRP1 in neurons and glia of the developing and mature brain through western blots and immunohistochemistry
2. Determine the expression profile of LRP1 in cultured hippocampal neurons and glia as they mature through western blots and immunocytochemistry
3. Investigate whether MT and emtinB modulate calcium signaling in hippocampal neurons *in vitro* in an LRP1 dependent manner
4. Develop an *in vitro* scratch injury model to determine whether MT and emtinB promote regeneration of hippocampal neuron axons in an LRP1 dependent manner

2 Chapter 2: *In vivo* characterisation of LRP1 in the maturing rat brain

2.1 *INTRODUCTION*

LRP1 is a multifunctional receptor that binds a wide range of structurally distinct ligands and is expressed in, and regulates the function of, neurons and glia of the brain (Moestrup et al., 1992, Wolf et al., 1992, Rebeck et al., 1993, Zheng et al., 1994, Ishiguro et al., 1995). *In vitro* models demonstrate LRP1 mediated regulation of neuronal survival, neurite outgrowth, regeneration and calcium signaling in a range of neuron subtypes (Bacskai et al., 2000, Qiu et al., 2002, Hayashi et al., 2007, Martin et al., 2008, Fuentealba et al., 2009, Matsuo et al., 2011, Hayashi et al., 2012, Stiles et al., 2013, Yamauchi et al., 2013). LRP1 mediated responses vary depending on both the expression of cell specific intracellular proteins and availability of extracellular ligands present to interact with and activate the receptor (Hashimoto et al., 2000, Qiu et al., 2002, Shi et al., 2009, Matsuo et al., 2011, Stiles et al., 2013). Therefore, LRP1 effects may vary in different regions of the brain due to differences in cell types and extracellular ligands.

Intracellular protein composition can vary at different subcellular locations in the same cell, and therefore LRP1 mediated effects may vary from one subcellular compartment to another. In neurons, the cell body is the primary location of protein synthesis, dendrites receive and interpret synaptic signals and axons transmit outgoing signals (Haines, 2002). Therefore, receptors located in these structures may be involved in these functions. Glia have fundamental roles in maintaining neuron health and function and LRP1 expression in these cells may have implications for neuron health. The glia cell body, similar to the neuron, is the site of protein synthesis, but glial processes can have a range of functions including structurally supporting neurons and

surveying and maintaining the surrounding environment (Haines, 2002, Nimmerjahn et al., 2005). Through investigation of its subcellular location in specific regional populations of neurons and glia, the role of LRP1 in a given cell type may be better understood.

Experimental rodent models allow investigation of LRP1 function *in vivo*. The majority of LRP1 expression studies in rats have been conducted through detection of mRNA or immunoblots of protein (Bu et al., 1994b, Ishiguro et al., 1995, Hayashi et al., 2007). When LRP1 protein was visualised using immunofluorescence in one study it was noted on neurons and astrocytes but specific cellular localisation of the receptor was not commented on (Zheng et al., 1994). Differences in LRP1 expression have also been previously noted between developing and mature brains (Moestrup et al., 1992, Ishiguro et al., 1995). However, specific distribution of the receptor and how this changes during maturation has not been investigated thoroughly.

The function of neurons and glia during development is very different to their function in the mature brain. During embryonic development, neurons proliferate at the subventricular zone of the neural tube (Nicholls et al., 2001). Through binding to subtype specific surface molecules on radial glia processes, different neuron populations are directed to precise brain regions (Rakic, 1972, Nowakowski and Rakic, 1979, Edmondson et al., 1988, Zheng et al., 1996). Once in position, neurons differentiate and develop dendrites and axons (Nowakowski and Rakic, 1979). Extending axons are directed through the brain by attractive or repulsive extracellular cues, extracellular matrix substrates and cell adhesion molecules to form synapses with other neurons (Manthorpe et al., 1983, Neugebauer et al., 1988, Serafini et al., 1994, Luo et al., 1995). Following the formation of a synapse, a large number of neurons undergo apoptosis and synaptic pruning to refine innervation pathways (Hollyday and Hamburger, 1976, Nakamura and O'Leary, 1989, Blaschke et al., 1996). Synaptogenesis continues post-

natally but decreases during maturation, though it can occur in adulthood to a lesser degree (Huttenlocher, 1979). Once functional neuronal networks have formed, healthy neurons and glia must be maintained to allow for chemotransmission (Nicholls et al., 2001).

A healthy and functional neuronal network requires the presence of astrocytes, oligodendrocytes and microglia. During development, astrocytes create an environment permissive for neuron migration, neurite extension and synaptic formation. Astrocytes produce extracellular matrix components such as laminin, express cell adhesion molecules such as neural cell adhesion molecule, and release growth factors such as NGF, all of which aid in neuron and neurite guidance (Liesi and Silver, 1988, Neugebauer et al., 1988, Yoshida and Gage, 1991). As the brain matures, the ability of astrocytes to mediate axon regeneration decreases (Smith et al., 1986). An absence of astrocytes *in vitro* results in the failure of neurons to form functional, mature synapses, demonstrating a critical role of these cells in development and stabilisation of these structures (Ullian et al., 2001). This may be in part through the production of thrombospondins. These proteins promote synaptogenesis but are downregulated in adult brains. It is believed that this decrease in thrombospondins contributes to the decreased synaptogenesis capabilities of adult brains (Christopherson et al., 2005). However, astrocytes in a mature brain continue to regulate synaptic function by direct control of synaptic transmission through release and uptake of neurotransmitters (reviewed by (Perea et al., 2009)). These cells also regulate cerebral blood flow, provide structural and physical support to neurons of the brain and are activated to promote immune responses in response to injury ((Haines, 2002, Zonta et al., 2002); reviewed by (Sofroniew and Vinters, 2010))

Oligodendrocytes are also essential for correct brain development and function through the myelination of neuron axons to aid in conductance. Differentiation of

precursor oligodendrocytes into mature, myelinating cells results in the gradual decline of proliferative and migratory properties and increase in myelination capabilities of these cells (Miller et al., 1985). Precursor oligodendrocyte cells differentiate to progenitor cells, then to pre-oligodendrocytes that begin to associate with axons. These cells form immature non-myelinating cell before becoming mature non-myelinating cells, and eventually differentiating into mature myelinating oligodendrocyte (reviewed by (Baumann and Pham-Dinh, 2001)). Myelination begins at different stages of development in different areas of the brain. Myelination of the rat spinal cord begins at foetal day 19, whereas it occurs in the brain stem and frontal cortex post-natal day 1 and 7, respectively. This process in the brain continues until about 60 days post-natally (Bjelke and Seiger, 1989, Bjartmar et al., 1994, Hamano et al., 1996). Very little myelination occurs in adults and instead oligodendrocytes maintain myelin and replace it during injury. Mature oligodendrocytes are renewed by oligodendrocyte precursors present in the adult brain in cases such as injury (Ffrench-Constant and Raff, 1986). However, oligodendrocyte precursors present in the adult rat differ in morphology and have slower migration and differentiation rates to those found in post-natal day 1 brain. This is believed to be due to differences in the needs for new mature oligodendrocytes in the adult brain compared to the developing brain (Wolswijk and Noble, 1989).

Microglia are the primary initiators of immune responses in the central nervous system. They possess many properties similar to macrophages in that they can phagocytose and present antigens to lymphocytes, and secrete inflammatory mediators such as interleukin-1 that activate astrocytes (Giulian and Baker, 1985, Giulian et al., 1986, Hickey and Kimura, 1988). Microglia are present in the central nervous system in either amoeboid or ramified morphology. The majority of microglia in the foetal brain are amoeboid. These cells phagocytose apoptotic neurons during development and allow functional networks to be established (Ashwell, 1990, Brockhaus et al., 1996).

Amoeboid microglia differentiate to ramified 'resting' microglia during the first 15 days following birth (Ling, 1979, Murabe and Sano, 1982, Wu et al., 1992). Ramified microglia have reduced capacity for proliferation, secretion and phagocytosis (Ilschner and Brandt, 1996). However, upon activation during injury or infection in the adult brain ramified microglia convert back to amoeboid microglia to promote inflammatory responses and remove pathogens and debris (Stence et al., 2001).

The functions of neurons and glia change significantly from the developing to mature brain. This is due to changes in the functional activity of the brain and creation of an environment that accommodates these changes. Therefore, the role of receptors expressed upon these cells could be expected to change as well. This is particularly relevant for a receptor such as LRP1 that can mediate a wide range of effects such as survival, neurite outgrowth and signaling that are essential in both developing and mature brains (Hayashi et al., 2007, Martin et al., 2008, Shi et al., 2009, Stiles et al., 2013).

Through identification of LRP1 expression in cells within specific regions of the brain, and its subcellular location upon those cells, it is possible to gain a greater understanding into the role of the receptor in the brain. I hypothesise that LRP1 will be predominately expressed in cell bodies of neurons and glia of the rat brain, and that this expression will change from pup to adult brain.

2.2 *METHODS*

All experiments involving the use of animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania (ethics number A0011957), and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Sprague Dawley rats were used in all animal experiments.

Buffer recipes and product information for all reagents can be found in appendices. An LRP1 antibody that targeted the C-terminal end of the light chain intracellular domain was used in western blots and immunohistochemistry.

2.2.1 WESTERN BLOT

Adult female rats (250-300g) were euthanised by carbon dioxide asphyxiation and post-natal day 2 and 7 rat pups (P2 and P7 respectively) were decapitated. Brain tissue was harvested from three separate animals for each time point. For each animal, the skull was removed and brain transferred to a petri dish. The cerebellum and part of the brain stem was discarded and the two brain hemispheres separated using scissors. The cortex and hippocampus were selected for identification of LRP1 as these regions have been found to express the receptor previously (Rebeck et al., 1993, Bu et al., 1994b, Ishiguro et al., 1995). The hippocampus is also an interesting tissue as it is one of the sites in the adult brain to undergo neurogenesis (Eriksson et al., 1998). From one hemisphere the midbrain was removed to expose the hippocampus which was dissected out. The remaining thalamus and hypothalamus were removed leaving only cortex. Hippocampal and cortical tissue and the remaining hemisphere were snap frozen, crushed using a mortar and pestle in liquid nitrogen and stored at -80°C.

A sample of tissue was homogenised in 100µL RIPA lysis buffer with 1:100 protease inhibitor (Thermo Scientific, USA) using an ultra-turrax. Homogenate was centrifuged at 13,000g for 10 minutes at 4°C and the supernatant removed. Total protein

concentration was determined using a BCA protein assay (Bio-Rad, USA). A working reagent was prepared as directed by the manufacturer. Briefly, part A and part B solutions were mixed by a 50:1 ratio. 200µL of working reagent was added to each well of a 96 well plate. Bovine serum albumin protein standards at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mg/mL were prepared to calculate a protein standard curve. 1µL of protein standards or 1µL of brain sample were mixed with the BCA reagent and incubated for 30 minutes at 37°C. Triplicates were prepared for each sample. Absorbance at 595nm was measured using a SpectraMax M2 plate reader (Molecular Devices) and SoftMax Pro computer program (v 4.8). A standard curve was calculated and used to determine each sample's concentration.

1mm thick sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared as 10% resolving and 5% stacking gels (see appendices). 5µL of Spectra protein ladder (Thermo Scientific, USA) was loaded into the first lane. 20µg protein was loaded for each sample. Samples were run using a Mini-Protean Tetra electrophoresis system (Bio-Rad, USA) in running buffer at 200 volts for 35-45 minutes. Transfer to nitrocellulose paper (Thermo Scientific, USA) was conducted overnight using an XCell Sure Lock electrophoresis system (Life Technologies, USA) at 20 volts at 4°C. The nitrocellulose membrane was protein blocked using 5% milk powder in phosphate buffered saline with 0.05% Tween-20 (PBS-Tw) for at least 60 minutes. Primary antibodies to the light chain of LRP1 or β-actin (housekeeping protein) were applied in 5% milk powder in PBS-Tw overnight at 4°C (antibody concentrations and combinations in Table 2.1). Controls of whole brain blots omitting the primary antibodies were incubated with 5% milk powder alone. The secondary antibody in PBS-Tw was incubated while shaking for 1 hour at room temperature. Three 10-minute washes with PBS-Tw occurred after each antibody incubation. Signal was detected using a chemiluminescent substrate (Millipore, USA). Peroxide and

luminol components were mixed at a 1:1 ratio and incubated with the membrane for 5 minutes at room temperature. The membrane was exposed for 600 seconds using a Chemi-Smart 5000 imaging system and Chemi-Capt 5000 computer program (v 12.8). Natural light was used to capture the ladder. Image contrast and brightness were uniformly enhanced using Adobe Photoshop (v 11.0.2).

2.2.2 IMMUNOHISTOCHEMISTRY

Three P2 and three P7 rats were decapitated and the skull removed to expose the brain which was dissected out and drop fixed in 4% paraformaldehyde overnight. Three adult male rats (~250g) were intraperitoneally injected with 0.1mL/100g pentobarbitone (60mg/mL; Troy Laboratories, Aus). Reflexes were tested and once negative rats were transcardially perfused with PBS followed by 4% paraformaldehyde. The brain was removed and transferred to 4% paraformaldehyde overnight. All brain tissue was placed sequentially in 10%, 20% and 30% sucrose overnight for cryoprotection.

Brains were frozen in cryomatrix and sectioned coronally at 10μm on a Leica CM 1850 UV cryostat. Sections were washed with PBS and dried overnight at 37°C prior to antigen retrieval with citrate buffer. Sections were immersed in citrate buffer (see appendices) and cooked at maximum pressure in a pressure cooker for 6 minutes. Pressure was decreased to 65% for an additional 14 minutes. Sections were cooled to room temperature and washed for 10 minutes three times in PBS. They were then incubated in 10% goat serum (Vector Laboratories, USA) in 0.03% triton X-100 (Lab-Chem, USA**)** in PBS for 30 minutes at room temperature to block non-specific protein binding. This was followed by incubation in primary antibodies diluted in 10% goat serum at room temperature for 1 hour and overnight at 4°C. LRP1 primary antibody was incubated with primary antibodies against either GFAP (astrocytes), MAP2 (dendrites), NeuN (neuron cell bodies), olig2 (oligodendrocytes), SMI-312 (axons) or tomato lectin (microglia; Table 2.1). Negative controls of sections omitting the primary antibodies

were incubated with 10% goat serum alone. Next, sections were incubated with secondary antibodies diluted in PBS for 1 hour at room temperature. Sections were washed three times by gentle shaking in PBS for 5 minutes following each antibody application. Nuclei were visualised by incubating sections with 10μg/mL DAPI (Molecular Probes, USA) for 5 minutes at room temperature. Glass coverslips were mounted to slides using fluorescent mounting media (Dako, USA) and dried overnight.

To determine regional specificity of LRP1 in P2 and adult rats, sections located between -3.5 and -4.0 to bregma were immunostained for NeuN, GFAP, tomato lectin or olig2 were taken at 20X objective on an Olympus BX50 microscope using a Photomatrix Cool Snap HQ^2 camera. Photographs were taken from the cerebral cortex (P2 = 8 sections; adult = 25 sections), hippocampus (P2 = 10 sections; adult = 12 sections), corpus callosum (P2 = 3 sections; adult = 5 sections), thalamus (P2 = 4 sections; adult = 7 sections) and hypothalamus (P2 = 3 sections; adult = 5 sections; Figures 2.1 and 2.2). Images were taken from the motor, somatosensory, auditory, entorhinal and piriform cortex and as there were no differences in LRP1-positive cells between these regions, values were pooled and recorded collectively as 'cortex'. Similarly, the stratum radiatum and cornu ammonis (CA) regions, CA1, CA2 and CA3, of the hippocampus displayed no difference in positive LRP1 cells and values for these regions were pooled and recorded collectively as 'CA'.

To determine cellular specificity of LRP1, photographs of sections immunolabelled for LRP1 with either GFAP, MAP2, NeuN, olig2, SMI-312 or tomato lectin were taken at 40X objective on an Olympus BX50 microscope using a Photomatrix Cool Snap HQ^2 camera. Images were merged and uniformly enhanced using Adobe Photoshop (v 11.0.2). Values were recorded as 0-1 (0), 1-10 (+), 10-50 (++), 50-100 (+++) or >100 cells/mm^2 (++++).

	Primary	Type	Dilution	Secondary	Conjugate	Dilution
Western blot	LRP1	R	1:1000	Goat anti-rabbit	HRP	1:1000
	β-actin	M	1:20,000	Goat anti-mouse	HRP	1:5000

	Primary	Type	Dilution	Secondary	Conjugate	Dilution	Target
Immunohistochemistry	GFAP	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Astrocytes
	LRP1	R	1:1000	Goat anti-rabbit	AlexaFluor®-594	1:1000	C-terminus of LRP1 light chain
	MAP2	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Dendrites
	NeuN	M	1:100	Goat anti-mouse	AlexaFluor®-488	1:1000	Neuron cell bodies
	Olig2	M	1:500	Goat anti-mouse	AlexaFluor®-488	1:1000	Oligodendrocytes
	SMI-312	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Axons
	Tomato lectin (Biotin)		1:1000	Streptavidin	AlexaFluor®-488	1:1000	Microglia

Table 2.1: A list of working dilutions of primary antibodies, their targets and respective secondary antibodies used for western blot and immunohistochemistry analysis. Antibodies were either polyclonal rabbit (R) or monoclonal mouse (M) antibody.

HRP: horse radish peroxidase

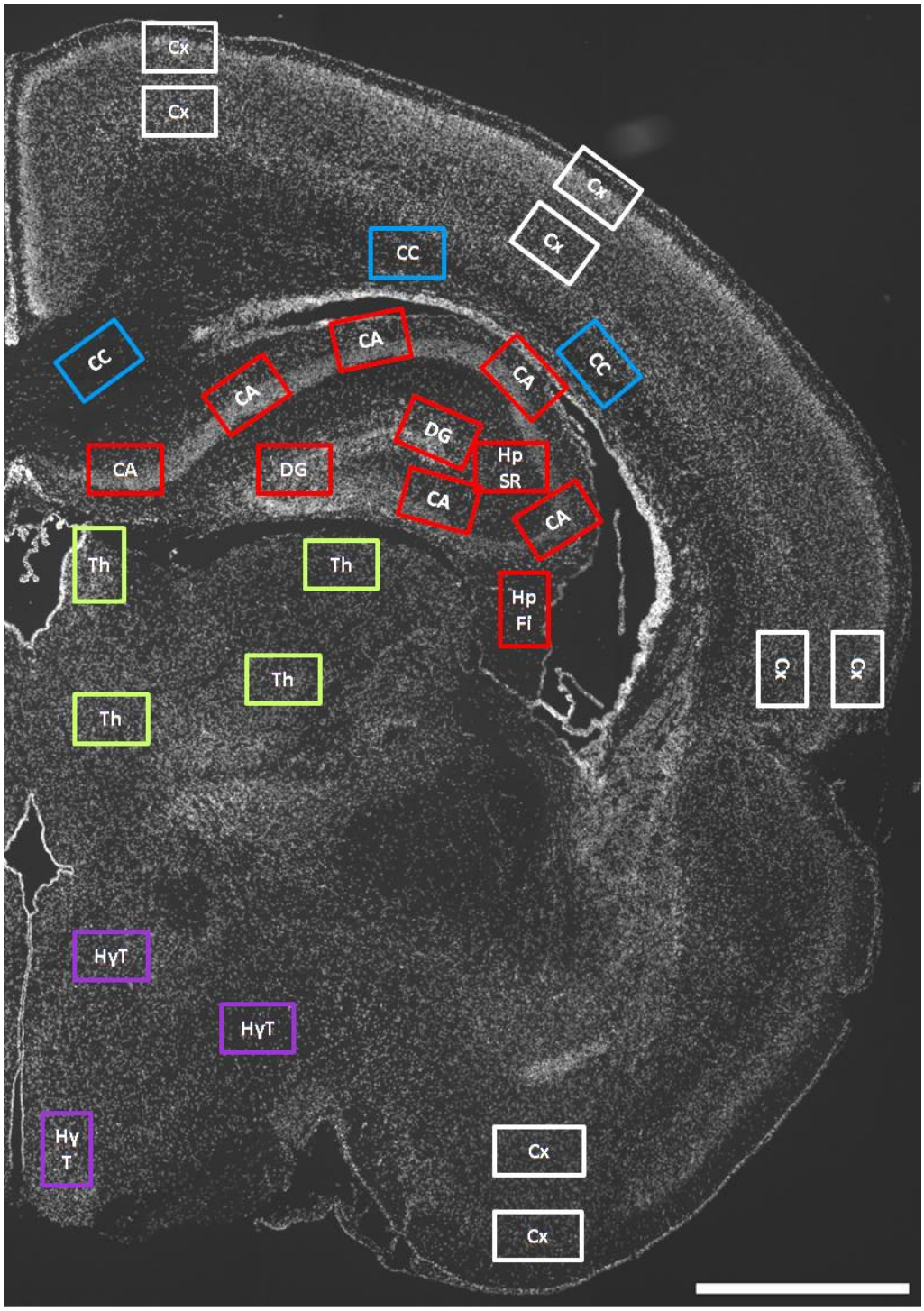

Figure 2.1: Brain regions photographed for semi-quantitative values of LRP1 regional localisation in the post-natal day 2 rat brain. Images of LRP1 with either GFAP, NeuN, olig2 or tomato lectin were taken at 20X objective and LRP1-positive cells counted. Images were taken from regions of the cerebral cortex (Cx), corpus callosum (CC), cornu ammonis region of the hippocampus (CA), stratum radiatum and fimbria of the hippocampus (Hp SR and Hp Fi, respectively), dentate gyrus (DG), thalamus (Th) and hypothalamus (HyT).

Scale bar = 1000μm

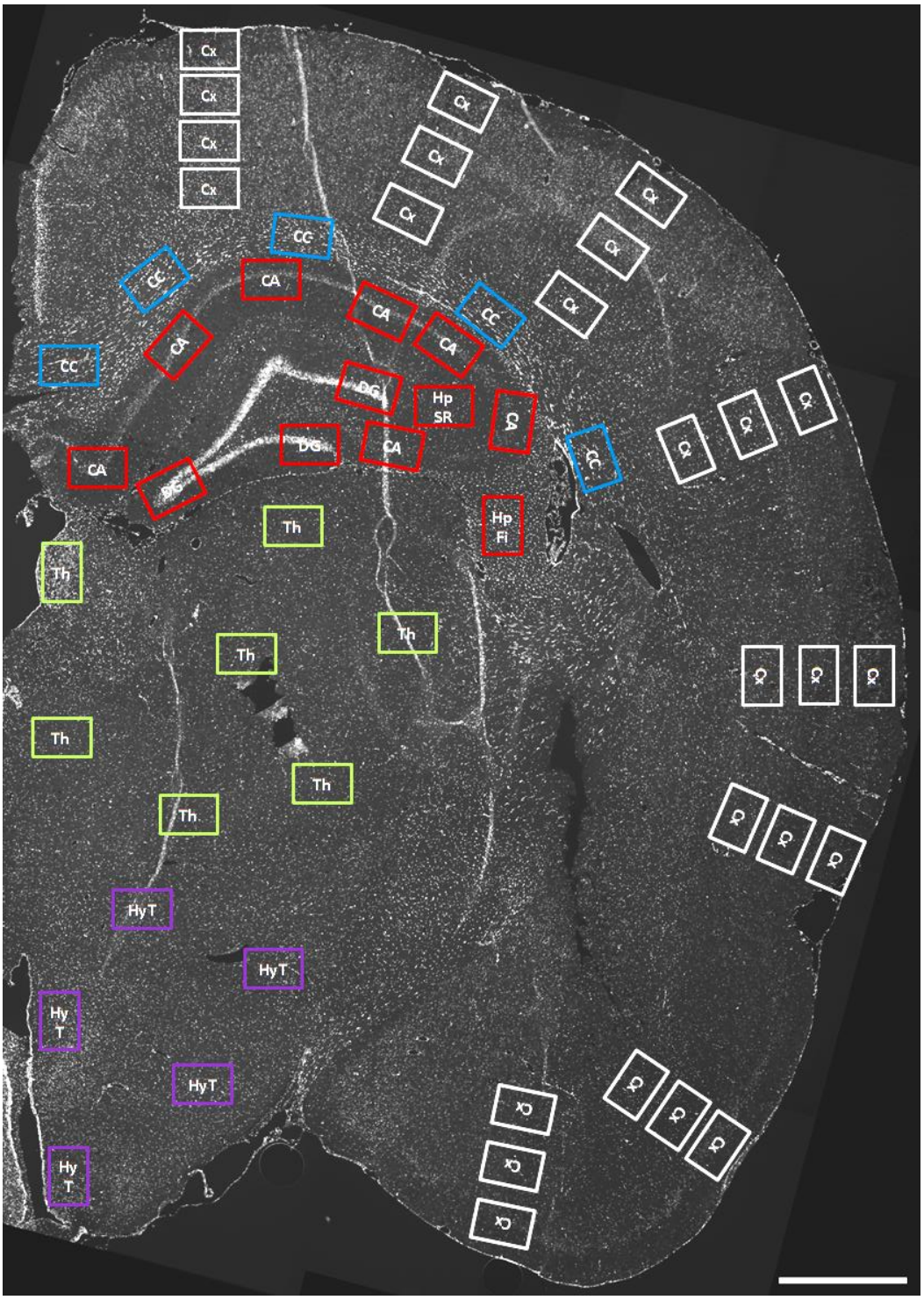

Figure 2.2: Brain regions photographed for semi-quantitative values of LRP1 regional localisation in the adult rat brain. Images of LRP1 with either GFAP, NeuN, olig2 or tomato lectin were taken at 20X objective and LRP1-positive cells counted. Images were taken from regions of the cortex (Cx), corpus callosum (CC), cornu ammonis of the hippocampus (CA), stratum radiatum and fimbria of the hippocampus (Hp SR and Hp Fi, respectively), dentate gyrus (DG), thalamus (Th) and hypothalamus (HyT).

Scale bar = 1000μm

2.3 ***RESULTS***

2.3.1 CLEAVAGE PATTERN OF LRP1 CHANGES DURING DEVELOPMENT

Western blot detection demonstrated that the processing of LRP1 changed during maturation. In whole brain, a ~85kDa band corresponding to the LRP1 light chain was the predominant form, and was expressed at higher levels in P2 and P7 compared to adult tissue (Figure 2.3 A). P2 and P7 brains also expressed a ~130kDa and ~100kDa band that was not present in the adult. Bands at ~80kDa and between ~40-60kDa were present at all time points but decreased in strength from P2 to P7 to adult. Bands at both ~150kDa and ~40kDa were also in all time points but were stronger in adults compared to P2 and P7. A band at ~70kDa was only present in adult whole brain.

The light chain ~85kDa band appeared to be expressed at slightly higher levels in P7 than P2 cortical tissue, though both were stronger than adult (Figure 2.3 B). As for whole brain, the ~130kDa and ~100kDa band observed in P2 and P7 cortical tissue was not present in adult. Faint bands from ~37-60kDa were present in P7 tissue but were stronger in adult. A ~70kDa band was only observed in adult tissue. Similar to whole brain and cortical tissue, hippocampal tissue of P2 and P7 rats showed higher expression of a ~85kDa form than adult tissue (Figure 2.3 C). A ~130kDa band was present in P2 and P7 tissue but not adult. Lower molecular weight bands ranging from ~37-70kDa were more intense in adult than P2 and P7.

Differences in LRP1 expression pattern between whole brain, cortical and hippocampal tissue were observed for each developmental time point. The light chain ~85kDa band was expressed to a similar degree in all tissues of P2 rats (Figure 2.3 A-C, left 3 lanes). A ~130kDa band was present in whole brain and cortex but was present at lower levels in the hippocampus. Whole brain and cortical tissue expressed a ~100kDa band but this was not present in hippocampal tissue. A number of bands between ~40-

70kDa were present in whole brain and these were fainter in hippocampal tissue and absent from cortical tissue. A ~80kDa band was only present in whole brain.

Expression of LRP1 in P7 brain was very similar to P2 expression throughout all tissues (Figure 2.3 A-C, middle 3 lanes). The ~85kDa was present in comparable amounts in all tissue types, and a ~130kDa band was stronger in whole brain and cortical tissue compared to hippocampal tissue. Whole brain and cortex also expressed a ~100kDa band that was absent from the hippocampus. The ~80kDa band was only in whole brain, and bands at ~40-70kDa were also stronger in this tissue, though some of these were faintly positive in cortical and hippocampal tissue.

A similar amount of the ~85kDa band was in all adult tissue (Figure 2.3 A-C, right 3 lanes). Whole brain was the only tissue to express ~150kDa and ~80kDa bands. Bands at ~38kDa, ~40kDa and ~70kDa were present in whole brain and cortical tissue but not hippocampal tissue. A greater number of faint bands between ~45-60kDa were observed in hippocampal tissue, though some of these were also present in whole brain and cortex to a lesser extent. Negative controls of whole brain blots omitting primary antibodies showed no immunoreactivity (Figure 2.3 D).

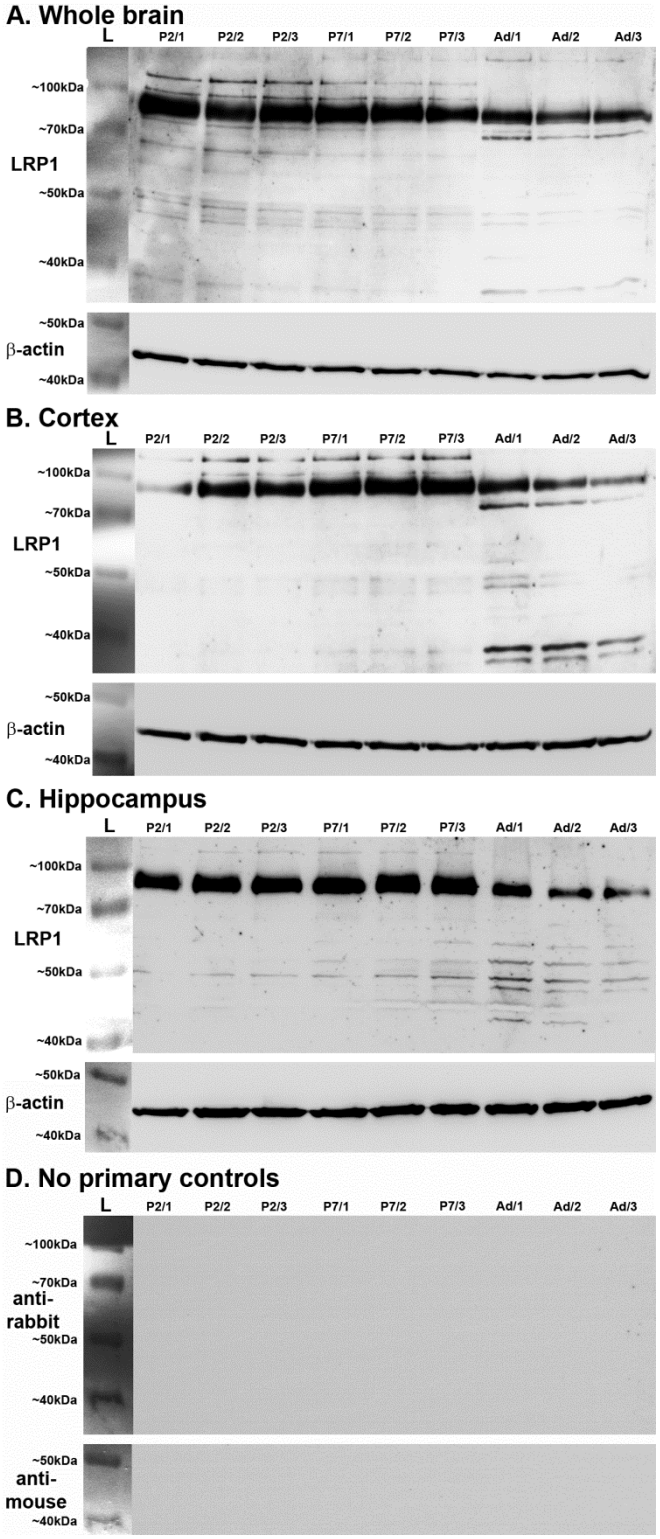

Figure 2.3: LRP1 is expressed in post natal day 2 (P2), day 7 (P7) and adult rat hippocampus. Whole brain (A), cortical (B) or hippocampal tissue (C) was collected from 3 separate animals for either P2 (left 3 lanes), P7 (middle 3 lanes) or adult rats (Ad; right 3 lanes). 20μg total protein for each sample was run on an SDS-PAGE gel and probed with anti-LRP1 or anti-β-actin antibodies. Similar amounts of protein were loaded for each sample (β-actin). Controls of whole brain blots omitting primary antibodies were negative for non-specific secondary antibody binding (D). Molecular weights of bands were determined using a commercial protein ladder (L).

2.3.2 LRP1 IS EXPRESSED IN NEURON AND GLIA CELL BODIES OF P2, P7 AND ADULT RAT BRAINS

Immunohistochemistry showed that LRP1 expression was present on neurons, astrocytes, oligodendrocytes and microglia of P2, P7 and adult rat brains. Punctate immunoreactivity of LRP1 in P2 and P7 rat brains appeared to be both cellular and extracellular throughout the cortex, hippocampus, thalamus, hypothalamus and corpus callosum. LRP1 immunoreactivity in adult brain was punctate and cellular throughout all regions of the brain.

LRP1 was expressed at all time points in a subpopulation of neuronal cell bodies of the cerebral cortex (Figure 2.4 A, C, E) and pyramidal layer of the hippocampus (Figure 2.4 B, D, F), as well as some neurons in the thalamus and hypothalamus (data not shown). A small number of dendrites were LRP1-positive in the P2, but not P7 cerebral cortex brain (Figure 2.5 A, C). The proximal segments of adult apical dendrites were LRP1-positive (Figure 2.5 E) while axons were negative for LRP1 immunoreactivity at all time points and in all brain regions including the cerebral cortex and hippocampus (Figure 2.5 B, D, F).

Subpopulations of astrocytes, oligodendrocytes and microglia express LRP1 in P2, P7 and adult rat brains. LRP1 was present in astrocyte cell bodies and foot processes lining some blood vessels (Figure 2.6 A-C). Cell bodies and processes of oligodendrocytes (Figure 2.7 A-C) and microglia were also LRP1-positive at all time points (Figure 2.8A-C). LRP1 expressing microglia had a ramified appearance. The receptor was expressed on some endothelial cells in P2 and P7 brains (Figure 2.8 A-B) but not in adult brains. The choroid plexus, used as a positive LRP1 control, was strongly LRP1 immunoreactive at all time points (Figure 2.9 A, C, E). Negative controls omitting primary antibodies showed no immunoreactivity (Figure 2.9 B, D, F).

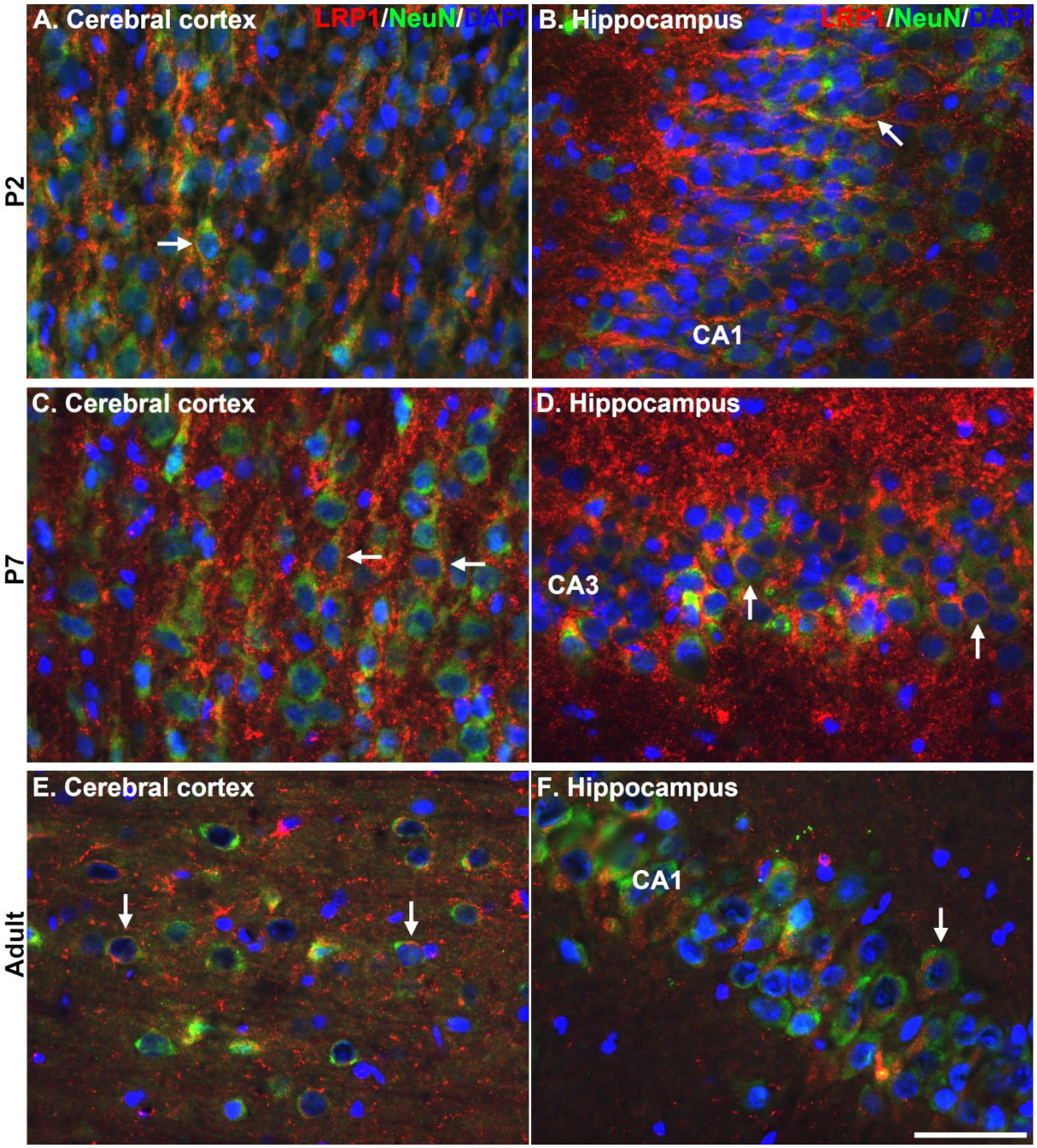

Figure 2.4: LRP1 is expressed on neuron cell bodies of post-natal day 2 (P2), day 7 (P7) and adult rat brain. 3 separate brains at each time point were sectioned at 10µm and immunolabelled for LRP1 (red) and NeuN (neuron cell bodies; green). Cell bodies of a subpopulation of neurons in the cerebral cortex and cornu ammonis regions (CA) of the hippocampus were LRP1-positive in P2 (A and B; arrows), P7 (C and D; arrows) and adult brains (E and F; arrows).

Scale bar = 50µm

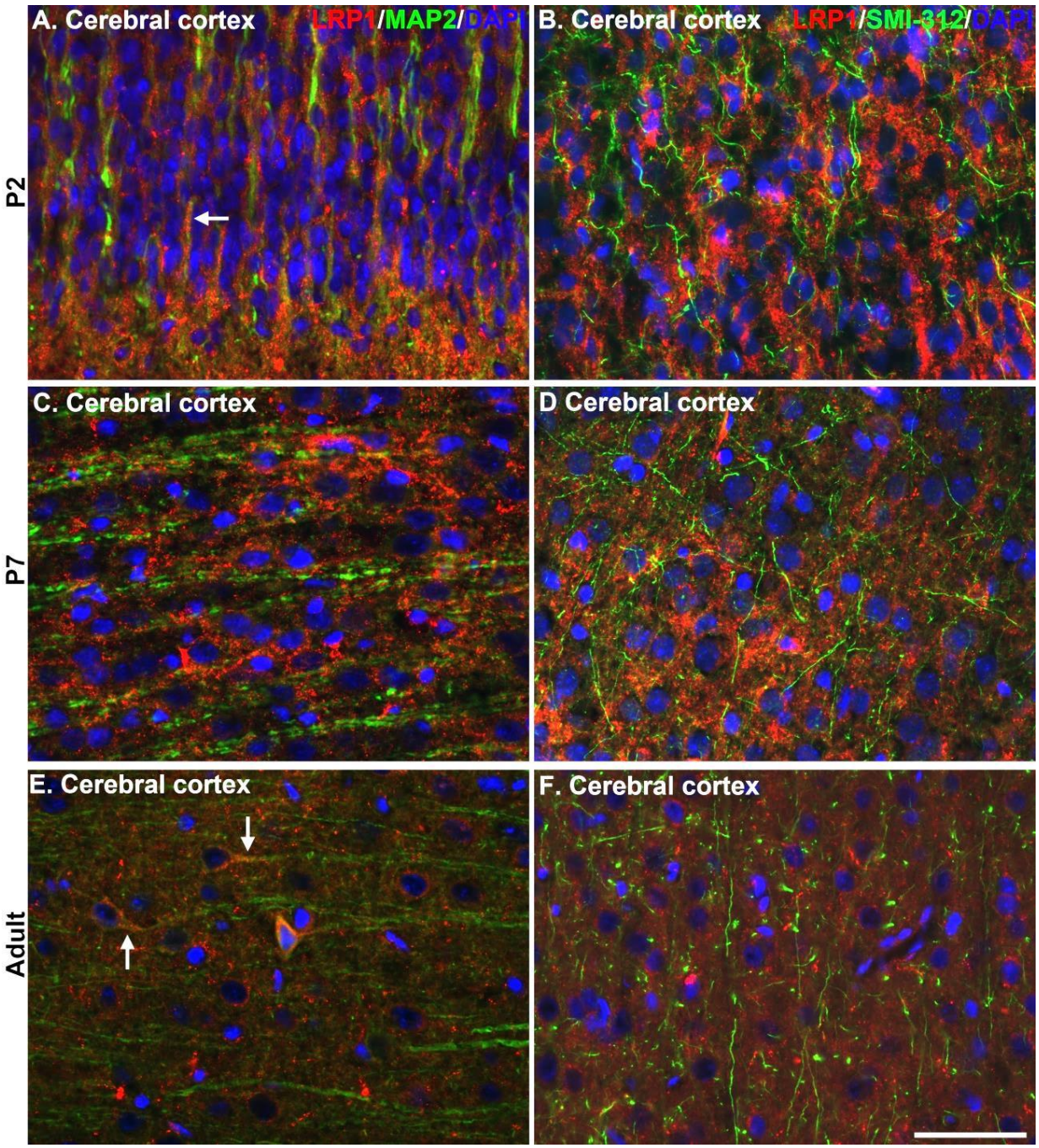

Figure 2.5: LRP1 is expressed upon dendrites of adults and some post-natal day 2 (P2) neurons. 3 separate brains at each time point were sectioned at 10µm and immunolabelled for LRP1 (red) and MAP2 (dendrites) or SMI-312 (axons; both green). Very few dendrites were LRP1-positive in P2 brain (A; arrow), and no dendrites were positive in post-natal day 7 (P7) brains (C). Proximal segments of adult neuron dendrites were LRP1-positive (E; arrows). Axons were not LRP1-positive in P2 (B), P7 (D) or adult brain (F). Nuclei were counterstained with DAPI (blue).

Scale bar = 50µm

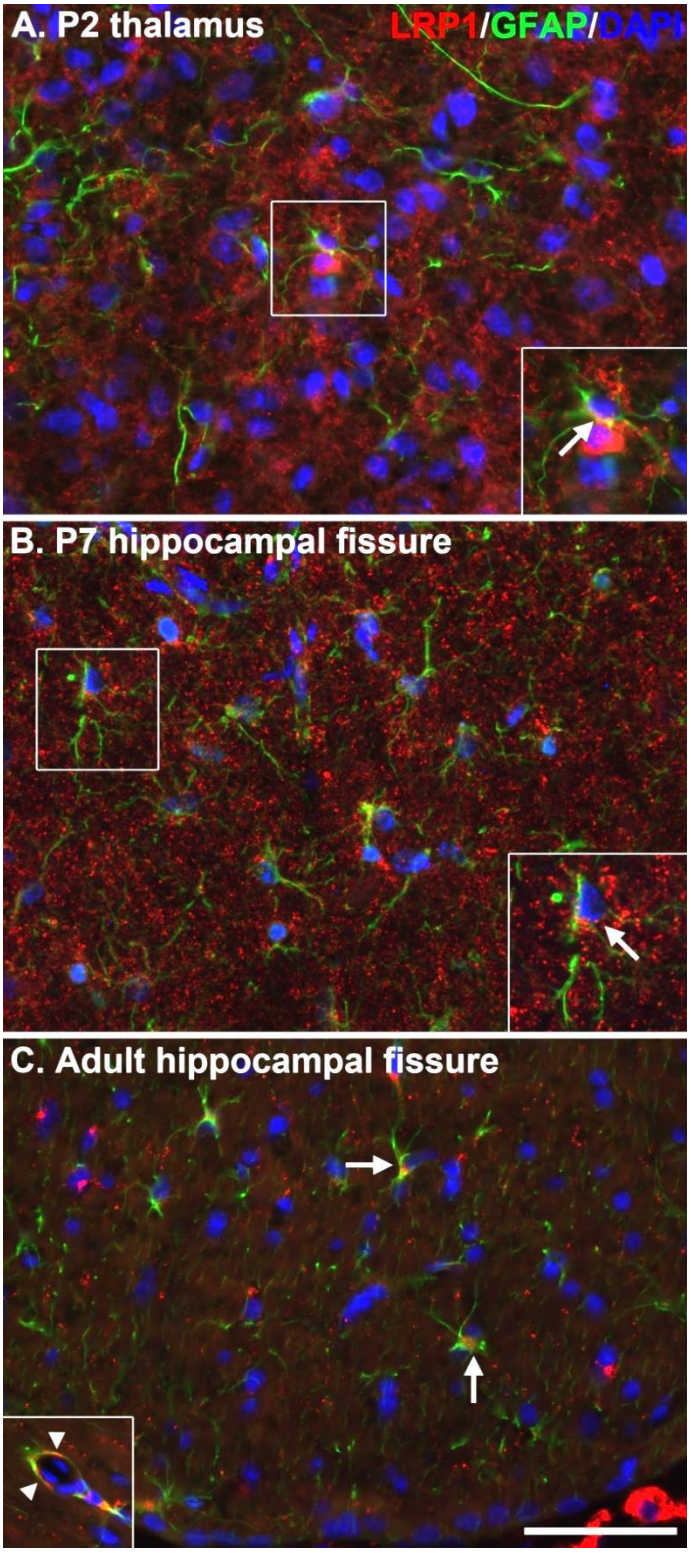

Figure 2.6: LRP1 is expressed on astrocytes of post-natal day 2 (P2), day 7 (P7) and adult rat brain. 3 separate brains at each time point were sectioned at 10µm and immunolabelled for LRP1 (red) and GFAP (green). Astrocyte cell bodies in P2 (A; inset & arrow), P7 (B; inset and arrow) and adult brains (C; arrows), and foot processes around some blood vessels (c; inset and arrowheads), were LRP1-positive. Nuclei were counterstained with DAPI (blue).

Scale bar (A, B, C) and inset in C = 50µm; A and B inset scale bar = 33µm

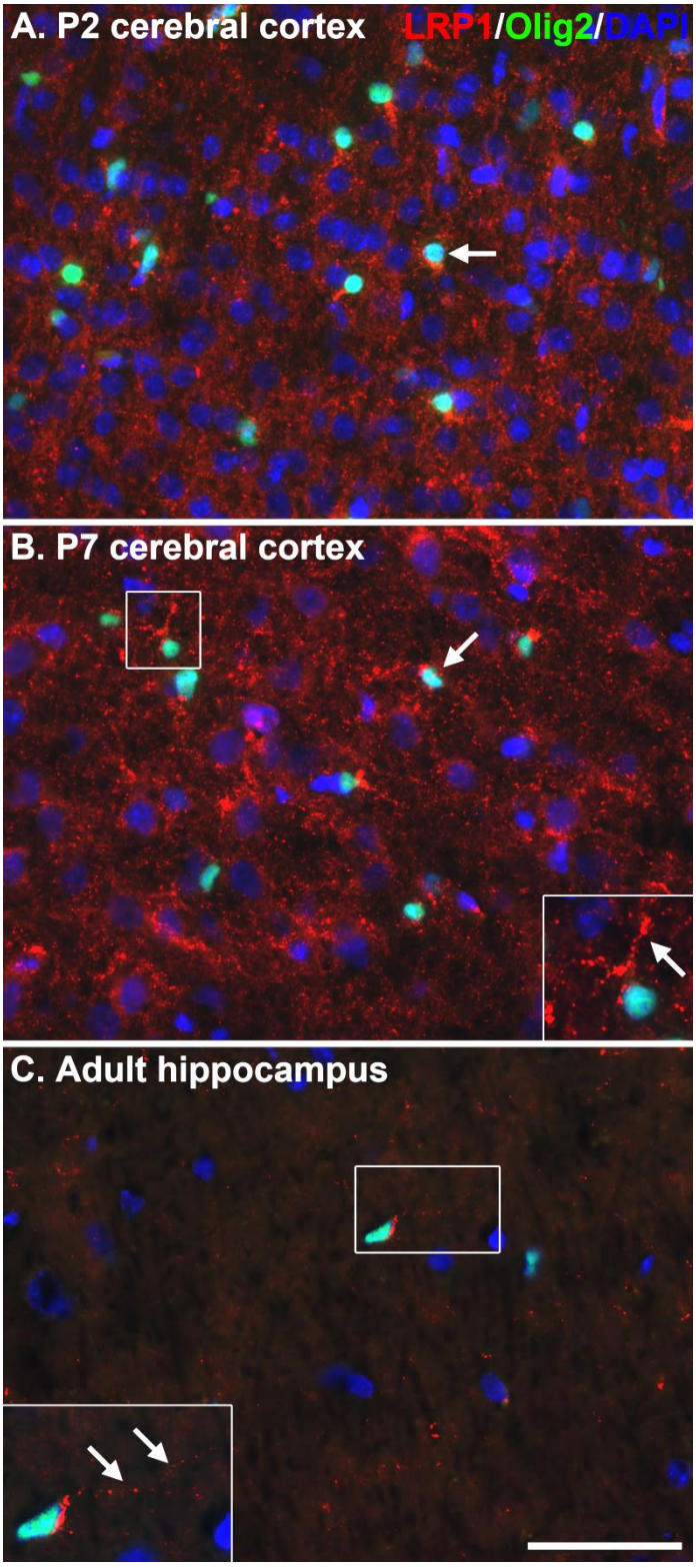

Figure 2.7: LRP1 is expressed on oligodendrocytes of post-natal day 2 (P2), day 7 (P7) and adult rat brain. 3 separate brains at each time point were sectioned at 10μm and immunolabelled for LRP1 (red) and olig2 (green). A subpopulation of oligodendrocytes were LRP1-positive in cell bodies and processes in P2 (A; arrows), P7 (B; arrows and inset) and adult brains (C; arrows and inset). Nuclei were counterstained with DAPI (blue).

Scale bar = 50μm; B inset scale bar = 30μm; C inset scale bar = 33μm

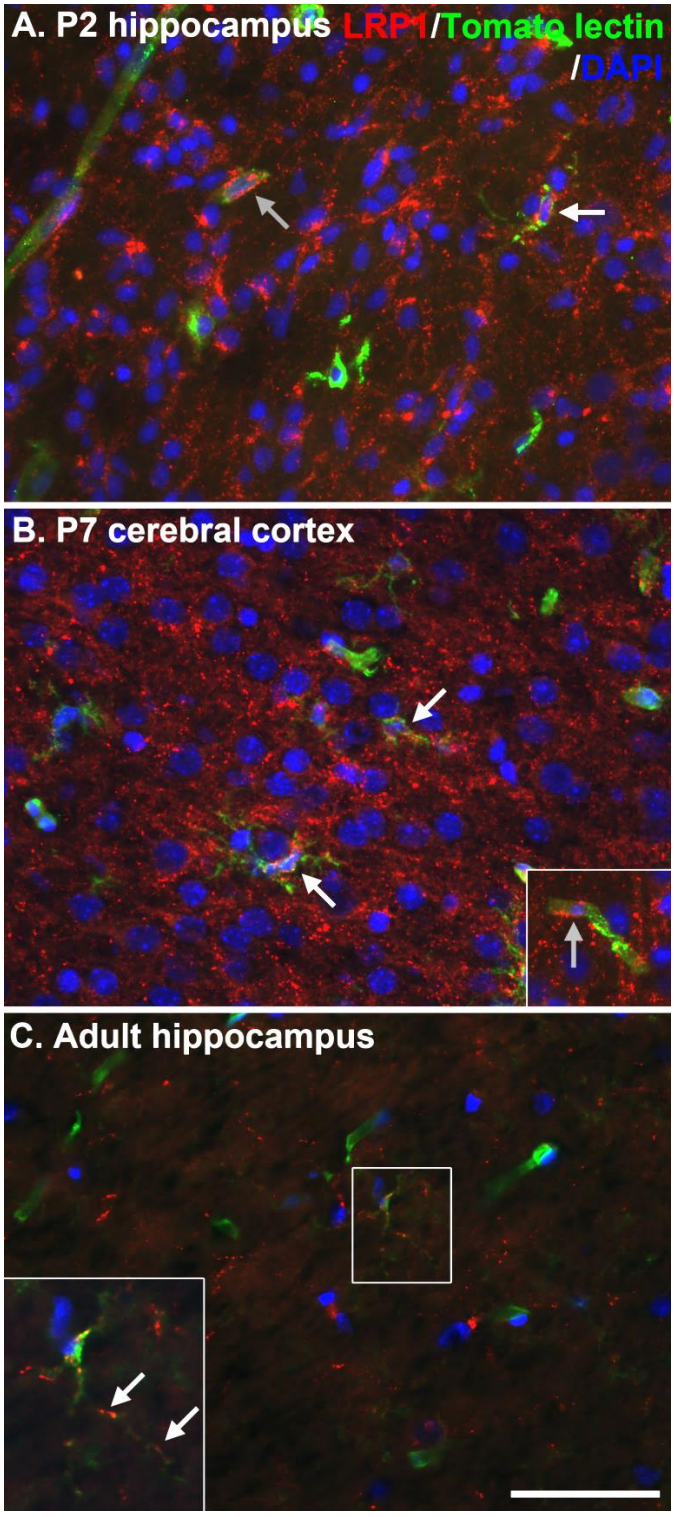

Figure 2.8: LRP1 is expressed on microglia of post-natal day 2 (P2), day 7 (P7) and adult rat brain. 3 separate brains at each time point were sectioned at 10µm and immunolabelled for LRP1 (red) and tomato lectin (green). LRP1 is expressed on a subpopulation of microglia cell bodies and processes in P2 (A; arrows), P7 (B; arrows) and adult brains (C; arrows and inset). Some endothelial cells in P2 and P7 brains were also LRP1-positive (A and B inset; grey arrows). Nuclei were counterstained with DAPI (blue).

Scale bar = 50µm; B inset scale bar = 50µm; C inset scale bar = 25µm

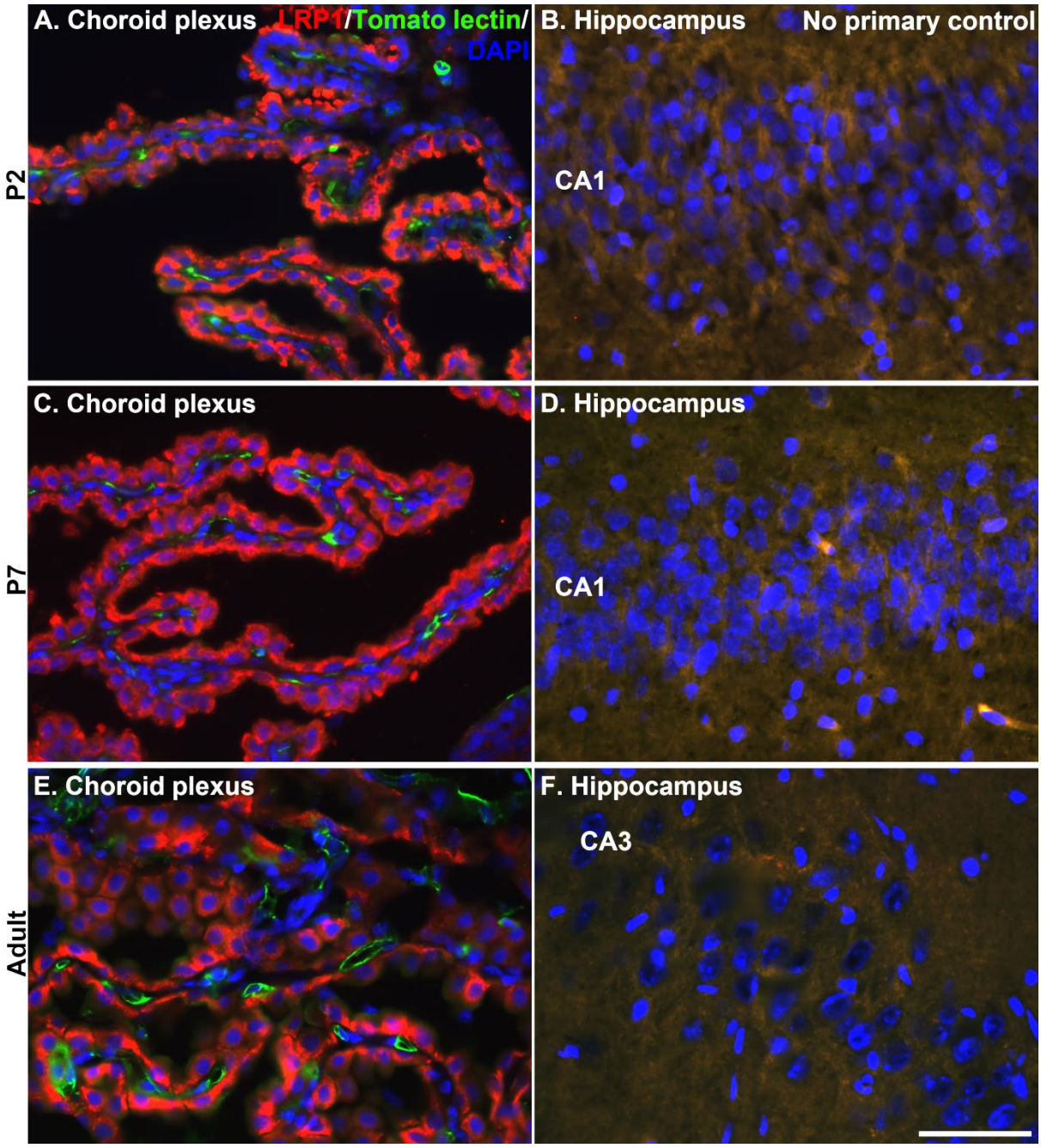

Figure 2.9: Positive and negative controls for LRP1 immunohistochemistry. The choroid plexus was used as an LRP1-positive control for post-natal day 2 (P2; A), day 7 (P7; C) and adult rat brain (E). 3 separate brains at each time point were sectioned at 10μm and immunolabelled for LRP1 (red) and tomato lectin (endothelial cells; green). Ependymal cells of the choroid plexus were strongly positive for LRP1 at every time point. Negative controls of sections omitting primary antibodies were immunonegative for non-specific secondary binding in P2 (B), P7 (D) and adult rat brains (F). Nuclei were counterstained with DAPI (blue).

Scale bar = 50μm

2.3.3 LRP1-POSITIVE NEURONS AND GLIA ARE LOCATED THROUGHOUT THE P2 AND ADULT RAT BRAIN

Semi-quantitative analysis of sections examined by immunohistochemistry showed that LRP1 expressing cells were present at higher levels in P2 brains compared to adults (Table 2.2; 38.9 ± 5.6 vs 22.3 ± 4.2 cells/mm^2 respectively). The most abundant LRP1-positive cells in P2 brains were neurons throughout grey matter, except for the thalamus. The regions with the greatest number of LRP1-positive neurons were the cortex and CA region of the hippocampus. Of these two areas, the CA region of the hippocampus had about two and a half times as many positive neurons as the cortex (273 ± 17 vs 105 ± 7 cells/mm^2 respectively). In comparison, there were fewer positive neurons in the dentate gyrus and hypothalamus, and the thalamus had the least number of LRP1-positive neurons. Oligodendrocytes were the most abundant LRP1-positive glia cells in white matter regions of the corpus callosum and hippocampal fimbria. There were LRP1-positive oligodendrocytes throughout the grey matter, though to a lesser extent. LRP1-positive astrocytes and microglia were located throughout both grey and white matter regions of the brain.

LRP1-positive neurons of the adult rat brain were predominately located in the CA region and dentate gyrus of the hippocampus. Similar numbers of LRP1 expressing neurons were observed in the cortex and hypothalamus. The region with the least number of LRP1-positive neurons was the thalamus. The white matter tracts of the corpus callosum and hippocampal fimbria were the region's most prolific in LRP1-positive astrocytes and oligodendrocytes. As for P2 brains, microglia were the glial type that displayed the least number of total LRP1-positive cells, and this was consistent across all regions of the brain.

The number and regional location of LRP1-positive neurons, astrocytes and oligodendrocytes changed from P2 to adult rat brains. There were more LRP1-positive

neurons in the cortex, thalamus and hypothalamus of the P2 brain compared to the adult brain. There were almost twice as many positive neurons of the P2 CA region compared to the adult (P2 = 273 ± 17 cell/mm^2; adult = 162 ± 35 cell/mm^2). However, there were more LRP1 expressing neurons in the adult dentate gyrus than the P2.

P2 rats had more LRP1-positive astrocytes in the dentate gyrus of the hippocampus compared to the adult. However, there were more of these cells positive for LRP1 in the adult CA regions and fimbria of the hippocampus, thalamus, hypothalamus and corpus callosum compared to the P2 brain. Similar numbers of LRP1-positive astrocytes were observed in the cerebral cortex. There were greater numbers of LRP1 expressing astrocytes in the adult than P2 brain in all other regions, except the dentate gyrus of the hippocampus where there were more in the P2 brain. Similar numbers of LRP1-positive oligodendrocytes were observed in the grey matter of P2 and adult rat brains. P2 rat brains had more LRP1-positive oligodendrocytes in white matter regions of the corpus callosum and hippocampal fimbria than the adult. No differences were observed in LRP1-positive microglia between P2 and adult brains except for the hippocampal fimbria where there were slightly greater numbers of LRP1-positive cells in the P2.

Overall there were more LRP1-positive cells in P2 rat brain compared to adult rat brain. This was largely due to greater numbers of LRP1-positive neurons and oligodendrocytes, and is consistent with western blot data that demonstrate greater LRP1 immunoreactivity in P2 brains compared to adult brains.

			Hippocampus													
	Cortex		CA		Fimbria		DG		Thalamus		Hypothalamus		Corpus callosum		Total	
	P2	Adult	P2	Adult	P2	Adult	P2	Adult	P2	Adult	P2	Adult	P2	Adult	P2	Adult
NeuN	++++	++	++++	++++	NA	NA	+++	++++	++	+	+++	++	NA	NA	**+++**	**++**
GFAP	+	+	+	++	++	+++	++	+	+	++	+	++	++	+++	**++**	**++**
Olig2	++	++	++	++	++++	+++	++	++	++	++	++	++	++++	+++	**+++**	**++**
T-L	+	+	+	+	++	+	+	+	+	+	+	+	+	+	**+**	**+**
Total (cells/mm²)	++	++	++	++	+++	+++	++	++	++	+	++	++	+++	++	**++ (38.9)**	**++ (22.3)**

Table 2.2: LRP1-positive cells are present in different brain regions of the post-natal day 2 (P2) and adult rat brain. LRP1 was immunolabelled in 3 separate P2 or adult rat brains with either NeuN (neuron cell bodies), olig2 (oligodendrocytes), GFAP (astrocytes) or tomato-lectin (T-L; microglia). Photographs were taken of the cerebral cortex, hippocampus (cornu ammonis regions (CA), hippocampal fimbria and dentate gyrus (DG)), thalamus, hypothalamus and corpus callosum and LRP1-positive cells counted. Cell counts were recorded as 0-1 (0), 1-10 (+), 10-50 (++), 50-100 (+++) or >100 (++++) cells/mm^2.

NA: not applicable

2.4 *DISCUSSION*

Western blot and immunohistochemical analysis have demonstrated that LRP1 is expressed in the brain of P2, P7 and adult rats. The expression pattern of the receptor changes during maturation and is predominately located on cell bodies of a subpopulation of neurons, astrocytes and oligodendrocytes throughout the cortex, hippocampus, hypothalamus, thalamus and corpus callosum. A small subset of microglia were also LRP1-positive.

2.4.1 THE CLEAVAGE PATTERN OF LRP1 CHANGES DURING MATURATION OF THE RAT BRAIN

A number of bands ranging from ~37kDa to ~150kDa were observed throughout whole brain, cortical and hippocampal tissue of P2, P7 and adult rat brain. The antibody used for these experiments binds C-terminal end of the intracellular domain of the LRP1 light chain. The LRP1 light chain undergoes a number of post-translational modifications, including cleavage by β- and γ-secretase (May et al., 2002, von Arnim et al., 2005), phosphorylation of NPxY intracellular domains (Barnes et al., 2001, Guttman et al., 2009) and glycosylation of the light chain (Quinn et al., 1999, May et al., 2003) (Figure 2.10). These modifications allow for the tight regulation of LRP1 function.

Bands of a molecular weight larger than the ~85kDa light chain were observed at 100kDa and 130kDa of P2 and P7 brains, and at 150kDa of adult brain. These forms of LRP1 most likely correspond to glycosylated or phosphorylated forms of this domain. Wolf and colleagues (1992) observed a 130kDa band in western blots of human cerebral cortex using antibodies targeting both the heavy and light chain of LRP1. They hypothesised that this corresponded to digestion products of the LRP1 heavy chain. However, as the antibody used in the present experiments labels the C-

terminal of the light chain, the product observed here is unlikely to be a heavy chain product.

The main band detected for all time points in whole brain, cortical and hippocampal tissue was at ~85kDa. This corresponded to the light chain, which is normally non-covalently linked to the heavy chain of LRP1. It is likely that the non-covalently bound heavy and light chains of the LRP1 receptor dissociate during reduction in preparation for western blot analysis. Previous studies have demonstrated the detection of similar molecular weight bands (Herz et al., 1990, Wolf et al., 1992, Ambjørn et al., 2008).

Bands of a lower molecular weight than the ~85kDa light chain most likely correspond to cleaved forms of the light chain. These bands ranged from 30kDa to 80kDa in P2, P7 and adult tissues. The extracellular segment of the LRP1 light chain can be cleaved by β-secretase to produce a ~30kDa product (von Arnim et al., 2005). It is possible that the ~37 to ~80kDa bands observed correspond to the cleaved fraction of the LRP1 light chain that has been glycosylated or phosphorylated. Previous data demonstrate alterations in light chain LRP1 band sizes between ~50kDa to ~80kDa due to glycosylation (Quinn et al., 1999, Ambjørn et al., 2008). Therefore, the different sized bands observed here could correspond to different stages of LRP1 post-translational modifications.

There was greater expression of the ~85kDa light chain band in P2 and P7 brains compared to adult in whole brain, cortical and hippocampal tissue. This demonstrates that overall expression of LRP1 is greater in the younger brain, consistent with findings from previous studies (Moestrup et al., 1992, Ishiguro et al., 1995). ~100kDa and ~130kDa bands observed in P2 and P7 whole brain and cortical tissue were not observed in any adult tissue, suggesting that these LRP1 forms have a developmental role. Adult cortical and hippocampal tissue showed a number of lower molecular weight

bands between ~37-70kDa that were absent or faint in P2 and P7 samples. These fractions, therefore, may have a role in the mature brain but not in the developing brain. These findings demonstrate that the post-translational regulation of LRP1 changes from the developing to mature brain.

Hippocampal tissue in P2 and P7 brain did not express the ~100kDa band and only faintly expressed the ~130kDa observed in whole brain and cortical tissue. This suggests that higher molecular weight forms do not have as an important role in development of this region of the brain. However, hippocampal tissue did express a number of lower molecular weight bands that were absent from the cortex in P2 brain, and reduced in P7. This demonstrates that the cleavage and maturation of LRP1 is regionally specific during the first week of post-natal development.

Adult whole brain expressed a ~150kDa band that was not observed in cortical or hippocampal tissues, suggesting that this form of LRP1 is expressed in a brain region other than these. A ~70kDa band observed in whole brain and cortical tissue was not present in hippocampal tissue. There were also stronger bands at ~37kDa and ~40kDa in cortical tissue than hippocampal, but the latter tissue expressed more lower weighted bands between ~40-70kDa. These data provide evidence that post-translational modifications and regulation of LRP1 in cortical tissue differ from that of hippocampal tissue in adult tissue.

The identity of each LRP1-positive band in whole brain, cortical and hippocampal rat tissue is currently unknown. However, these findings demonstrate that post-translational modifications of LRP1 differ between rat brain regions and stages of maturity. This suggests that regulation, and therefore function, of LRP1 is dependent on the tissue in which it is expressed and its stage of development.

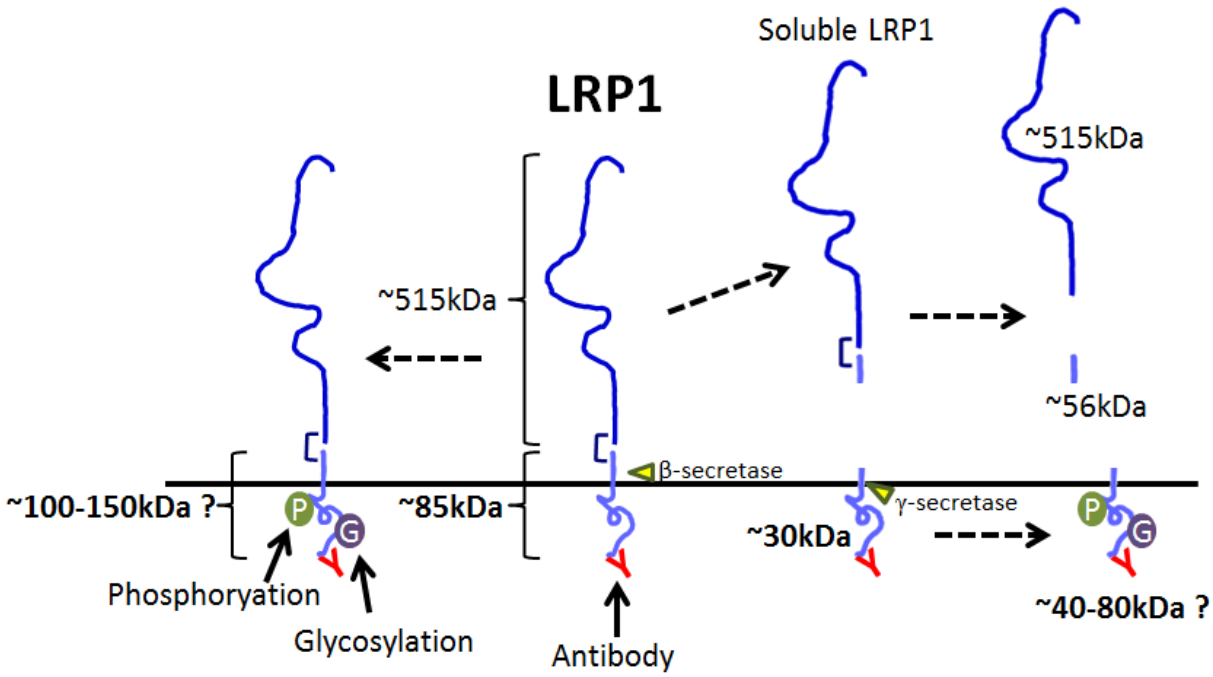

Figure 2.10: Post-translational modifications of the LRP1 light chain that may correspond to bands detected on western blots. The LRP1 antibody binds the C-terminal of the intracellular domain of the light chain. The ~85kDa light chain of LRP1 can be phosphorylated or glycosylated to produce a larger molecular weight form. This form dissociates from the LRP1 heavy chain that during western blot processing and is detected at ~100-150kDa. Alternatively, the ~85kDa light chain can be cleaved by β-secretase to yield a ~30kDa form of LRP1 and the soluble heavy chain. This form of LRP1 can be phosphorylated or glycosylated to produce a ~40-80kDa form.

Adapted from (Bu, 2009)

2.4.2 LRP1 IS EXPRESSED IN CELL BODIES AND PROCESSES OF NEURONS AND GLIA

Immunoreactivity of LRP1 appeared more widespread in P2 and P7 rat brains compared to adult brain, supporting western blot data. Immunohistochemical analysis demonstrated that LRP1 is localised to cell bodies of a subset of P2 and P7 neurons, and cell bodies and proximal segments of apical dendrites of adult neurons. The receptor was also expressed in the cell bodies of a subset of astrocytes, and cell bodies and processes of a subset of oligodendrocytes and microglia. There appeared to be extracellular, punctate staining of LRP1 throughout all regions of the P2 and P7 but not in the adult brain. The antibody used in these experiments targets the intracellular domain of the receptor, so this LRP1 product cannot be soluble LRP1 as this fragment is only composed of the extracellular domain (von Arnim et al., 2005). It is currently unclear what this apparent extracellular staining seen in the work shown here is, though this pattern of staining has been noted before in human foetal tissue (Moestrup et al., 1992) and suggests a role for this LRP1 fragment in development.

The choroid plexus was consistently abundant in LRP1 between pups and adults and corresponded to expression patterns previously reported (Zheng et al., 1994). Interestingly, LRP1 immunoreactivity has been found to be absent in the choroid plexus of human brain (Wolf et al., 1992, Lopes et al., 1994). Whether these discrepancies are due to species or technical differences is currently unknown.

2.4.2.1 LRP1 expression in neurons

LRP1 regulates the survival and signaling of healthy neurons *in vitro* (Bacskai et al., 2000, Qiu et al., 2002, Hayashi et al., 2007, Martin et al., 2008, Fuentealba et al., 2009, Sen et al., 2012). Though LRP1 was not expressed in axons and therefore is unlikely to be involved in pre-synaptic activity, it was present at the proximal segment

of apical dendrites. This site possesses specific synaptic characteristics that LRP1 may have a role in. Mossy fibres of the dentate gyrus synapse at the proximal end of apical dendrites on CA3 pyramidal neurons of the hippocampus (Blackstad et al., 1970, Henze et al., 1996). In addition, synapses on proximal apical dendrites can amplify excitatory post-synaptic potentials initiated in distal segments of prefrontal cortical neurons (Seamans et al., 1997). Calcium accumulation also varies between proximal and distal segments of apical dendrites suggesting different roles at these sites (Yuste et al., 1994). LRP1 is able to associate with synaptic proteins including PSD-95 and NMDA receptor, and affects neurotransmitter mediated calcium influx (Gotthardt et al., 2000, May et al., 2004, Martin et al., 2008, Hayashi et al., 2012). Therefore, LRP1 may have a role in regulation of synaptic activity at proximal segments of apical dendrites, and therefore the signaling potential of LRP1 expressing neurons in the adult brain.

Numbers of LRP1 expressing neurons decreased from P2 to adult in the CA region of the hippocampus, cerebral cortex, thalamus and hypothalamus, suggesting that there may be a role of LRP1 in the development of neurons in these regions. Following synaptogenesis, which occurs embryonically and post-natally, a large proportion of neurons undergo apoptosis to refine neuronal networks (Huttenlocher, 1979, Blaschke et al., 1996). However, many neurons need to remain alive and functional and this process can be regulated by neurotrophins (reviewed by (Yuan and Yankner, 2000)). LRP1 can interact with neurotrophin receptors (Shi et al., 2009) and activate the same intracellular pathways as neurotrophins to promote neuronal survival (Fuentealba et al., 2009). The role of LRP1 in developing neurons may be in the regulation of their apoptosis during this critical stage.

LRP1-positive neurons of the adult brain were found predominately in the CA regions and dentate gyrus of the hippocampus. There were more LRP1-positive neurons in the adult dentate gyrus of the hippocampus compared to the P2 brain. This suggests

that LRP1 has a function specific to mature hippocampal neurons. The hippocampus is a region of the brain involved primarily in learning and memory (Scoville and Milner, 1957). Significant memory impairments and hippocampal neuron loss occur following events such as ischaemia and Alzheimer's disease, suggesting that it is a region of the brain that is particularly vulnerable to injury and disease (Zola-Morgan et al., 1986, Kesslak et al., 1991, Wood et al., 1993, Laakso et al., 1995). Different regions of the hippocampus also display specific vulnerabilities. For example, granule cells of the dentate gyrus undergo degeneration following removal of the adrenal glands and corticosterone, though neurons of the CA regions of the hippocampus are unaffected (Sloviter et al., 1989). In comparison, ischaemia causes significant loss of CA1 neurons but the CA3 region and dentate gyrus are unaffected (Zola-Morgan et al., 1986). LRP1 may be expressed in specific neuron populations to protect them from apoptosis. LRP1 knock-down both *in vivo* and *in vitro* increases the vulnerability of cortical and hippocampal neurons to apoptosis. Knock-out of LRP1 in mouse forebrain neurons causes neurodegeneration of the cerebral cortex and hippocampus in a manner similar to Alzheimer's disease (Liu et al., 2010). Hippocampal and cortical neurons *in vitro* also undergo increased apoptosis during trophic withdrawal and amyloid-β toxicity when LRP1 is knocked-down (Fuentealba et al., 2009). These studies support the idea that LRP1 could protect neurons from apoptosis. This may be particularly important in a region such as the hippocampus that appears already vulnerable to injury and disease.

2.4.2.2 LRP1 expression in oligodendrocytes

The majority of LRP1-positive oligodendrocytes were located in white matter of P2 and adult rat brain, though some cells were located throughout grey matter. LRP1 mRNA has been identified in rat brain cells likely to be oligodendrocytes (Ishiguro et al., 1995, Saher et al., 2005) but expression of the LRP1 protein has not been definitively identified in these cells previously. *In vitro* work has demonstrated greater

LRP1 mRNA and protein expression in oligodendrocytes than both astrocytes and microglia (Gaultier et al., 2009). Interestingly, studies in human tissue have reported the absence of LRP1 immunoreactivity in oligodendrocytes of human brain (Moestrup et al., 1992, Wolf et al., 1992, Lopes et al., 1994). These disparities may be due to species or technical differences.

LRP1 was expressed in a subset of oligodendrocytes and establishing what maturity these cells are could aid in determining whether the receptor is involved in the myelination process. Olig2 is a transcription factor expressed by oligodendrocytes throughout maturity (Zhou et al., 2000). Oligodendrocytes mature over a number of steps that can be identified by specific antigenic phenotypes. Oligodendrocyte precursor cells differentiate to progenitor cells, pre-oligodendrocytes, immature non-myelinating oligodendrocytes, mature non-myelinating oligodendrocytes and finally to mature myelinating oligodendrocytes (reviewed by (Baumann and Pham-Dinh, 2001)). By using specific markers for different stages of oligodendrocyte maturity it would be possible to identify whether LRP1 expression is specific to one or more of these stages of maturity. As oligodendrocytes mature, their capability to proliferate and migrate decreases while their ability to myelinate increases (Miller et al., 1985). Silencing of the LRP1 gene in Schwann cells inhibits migration of these cells (Mantuano et al., 2010). Therefore, there is a possibility that if expressed in precursor oligodendrocyte cells, LRP1 could regulate migration of these cells in the CNS. It may also support oligodendrocyte survival. LRP1 silencing in Schwann cells increases their susceptibility to TNF-α and serum deprivation induced apoptosis, suggesting a role for the receptor in pro-survival mechanisms in these cells (Campana et al., 2006). *In vivo*, Schwann cell death following sciatic nerve crush injury is increased from <3% for wild type mice to over 35% when LRP1 is knocked out of these cells (Orita et al., 2013). It is possible that

LRP1 expression in both the developing and adult brain may be involved in maintaining oligodendrocyte health.

There were more LRP1-positive oligodendrocytes in P2 brain than adult brain. Myelination of the central nervous system begins foetally in the spinal cord but extends through to the frontal cortex during the first week following birth (Bjelke and Seiger, 1989, Bjartmar et al., 1994, Hamano et al., 1996). One major component of myelin is cholesterol (Saher et al., 2005) which can bind to and be endocytosed by LRP1 (Handelmann et al., 1992). Though the bulk of cholesterol used in myelin is synthesised by oligodendrocytes, it can also be transferred to these cells from other CNS cell types when cholesterol is depleted (Saher et al., 2005). Oligodendrocytes of the developing brain may require LRP1 for myelin production during this stage if cholesterol is low. LRP1 also regulates phagocytosis of myelin vesicles, as a model of degraded myelin, in oligodendrocytes *in vitro* (Gaultier et al., 2009). In addition, LRP1 may be involved in direct myelination of axons. Myelin thickness around axons of the sciatic nerve in LRP1 knockout in mice is significantly reduced when compared to wild type mice. Sciatic nerve crush injury in knockout mice also demonstrate accelerated myelin sheath degeneration and decreased re-myelination (Orita et al., 2013). Therefore, the receptor may have a role in clearance of myelin in injury and replacement of it through cholesterol uptake.

2.4.2.3 LRP1 expression in astrocytes

Expression of LRP1 was evident in a subpopulation of astrocytes in both pup and adult rat brain. The presence of morphologically and regionally distinct astrocyte subpopulations has been recognised for over a century. Protoplasmic astrocytes are branched in morphology and located primarily in grey matter, whereas fibrous astrocytes have long processes and are located in white matter (Ramon and Cajal, 1909). These two population of astrocytes not only vary in morphology and regional

location, but also in protein expression and developmental history (Miller and Raff, 1984). The greatest number of LRP1-positive astrocytes were in the corpus callosum and hippocampal fimbria of both P2 and adult rats. This suggests that the LRP1 may be expressed primarily on fibrous astrocytes in both the developing and mature brain. There were more LRP1-positive astrocytes in white matter of the adult compared to P2 brain, suggesting that there is a greater role for LRP1 in astrocytes of the mature brain in these regions. Astrocytes in grey matter also expressed LRP1, suggesting that the receptor may have a function in a subpopulation of protoplasmic astrocytes. Recent microarray studies have begun to reveal that there is even greater astrocyte heterogeneity that is region specific, but studies into functional and phenotypic differences between these subpopulations are limited (Bachoo et al., 2004, Sofroniew and Vinters, 2010). Further investigation is required to determine whether LRP1 is expressed on a specific subpopulation of astrocytes.

Astrocytes have a complex range of roles in the healthy brain in addition to providing structural and metabolic support to neurons (Haines, 2002). These glia are not only essential for the correct formation and functioning of neuron synapses (Ullian et al., 2001) but also regulate subsequent activity at these sites. Upon calcium stimulation, astrocytes release neurotransmitters such as glutamate to promote neuron transmission (Parpura et al., 1994). LRP1 can regulate calcium signaling (Bacskai et al., 2000, Martin et al., 2008, Hayashi et al., 2012) and through similar mechanisms could have a role in astrocyte neurotransmitter release.

2.4.2.4 LRP1 expression in microglia

Microglia expression of LRP1 has not been noted in rodent brains previously but these cells have been reported as being negative for LRP1 in human brain (Wolf et al., 1992). However, rat microglia are LRP1-positive *in vitro* (Marzolo et al., 2000, Gaultier et al., 2009). Though LRP1 immunoreactivity was present at both the cell body and

some processes of ramified microglia, only a small subset of these cells was positive for the receptor and there did not appear to be regional specificity. The identification of microglia in the brain can be difficult due to their scattered distribution and relatively low population in the brain compared to other glia (Lawson et al., 1990, Marzolo et al., 2000). Therefore, it may be that previous reports have overlooked this co-localisation due to the difficultly in identifying the small proportion of cells positive for LRP1.

While the majority of microglia appear quiescent in healthy brain they actually have an active role in maintaining normal brain function and homeostasis. Ramified microglia processes are surprisingly dynamic and continually survey the brain's microenvironment to clear accumulations of metabolic products and degraded tissue (Nimmerjahn et al., 2005). However, these cells also have an important role in immune responses in the brain (reviewed by (Kreutzberg, 1996)). LRP1 may have a role in the detection of necrotic cells in infection or injury and the subsequent immune response regulated by microglia.

Upon necrotic cell death, heat shock proteins are released and associate with cell derived antigens before binding to antigen presenting cells such as macrophages to induce cytokine release and antigen presentation to T-cells (Basu et al., 2000). This process has been found to be mediated by LRP1. Peritoneal macrophages and bone marrow-derived dendritic cells present antigen bound heat shock protein gp-90 to T-cells. These effects were inhibited with antibodies against LRP1 (Basu et al., 2001). In addition, α2-macroglobulin complexes with antigens such as parasite derived proteinases enhance macrophage antigen uptake and presentation to T-cells through LRP1 (Morrot et al., 1997). Macrophages incubated with an α2-macroglobulin-lysozyme complex require 200-250 times less lipopolysaccharide for effective antigen presentation to T-cells (Chu and Pizzo, 1993). Furthermore, LRP1 can mediate phagocytosis of apoptotic cells in both human monocyte-derived macrophages and

alveolar macrophages (Ogden et al., 2001, Vandivier et al., 2002) and lipopolysaccharide or amyloid-β activated microglia express LRP1 that binds to calreticulin on cerebellar granule neurons to mediate their phagocytosis (Fricker et al., 2012). LRP1 present on ramified microglia that survey the brain environment may be involved in immune responses to cell death. Binding of heat shock proteins and α2-macroglobulin complexes to LRP1 on microglia may induce their activation and contribute to microglia mediated phagocytosis of these cells.

2.4.2.5 LRP1 expression at the BBB

LRP1 was expressed in a subset of endothelial cells in P2 and P7 brain, though this has only been reported in adult human brain previously (Deane et al., 2004). Endothelial cells have specialised tight junctions to limit the ability of molecules to move in and out of the brain (reviewed by (Abbott et al., 2006)). Previous work demonstrates that LRP1 regulates permeability and transport of molecules across the BBB (Shibata et al., 2000, Yepes et al., 2003, Deane et al., 2004). It is likely that this process is mediated by endothelial-expressed LRP1. The presence of LRP1 in endothelial cells of the pup but not in the adult suggests that there is a greater role for the receptor in transport and BBB permeability during development.

LRP1 was also expressed on astrocytic foot processes lining blood vessels from pup through to adult, which are also part of the BBB. This has previously been observed in human and rat brain (Moestrup et al., 1992, Wolf et al., 1992, Rebeck et al., 1993, Zheng et al., 1994). Astrocytes are able to affect cerebral blood flow through neurotransmitter mediated calcium increase. Glutamate and noradrenaline induce calcium oscillations in astrocyte end-feet causing vasodilation and vasoconstriction, respectively (Zonta et al., 2002, Mulligan and MacVicar, 2004). LRP1 is able to regulate NMDA and glutamate mediated calcium influx (Bacskai et al., 2000, Martin et al., 2008, Hayashi et al., 2012) and therefore, may be involved in astrocyte mediated

vascular flow in the brain. By regulating BBB permeability and cerebral blood flow LRP1 may have an important role in the maintaining the microenvironment of the brain.

2.5 *CONCLUSIONS*

LRP1 is able to regulate survival and function of a range of neuron and glial subtypes *in vitro* and its expression upon neurons and glia in the rat brain suggests that it regulates the function of these cells *in vivo*. Post-translational modifications aid in regulating the function of LRP1 and appear to change during maturation and between rat brain regions suggesting that the receptors function changes from development to maturity.

LRP1 expression was present on neuronal cell bodies of P2, P7 and adult rats where it may be involved in regulating survival through both development and maturity. The presence of the receptor on apical proximal dendrites of adult rat neurons suggests it may also be involved in neuronal signaling. Expression upon oligodendrocyte cell bodies and processes indicate a role for LRP1 in myelin synthesis through cholesterol endocytosis, cell survival and migration of precursor cells. The receptor was present on astrocyte cell bodies where it may affect astrocyte mediated neurotransmitter release and immune responses. LRP1 expressed on microglia could potentially be involved in antigen presentation and phagocytosis. The receptor was also located on endothelial cells and astrocytic end-feet of the BBB, suggesting that it may regulate the microenvironment of the brain through transport of molecules in and out of it.

Subcellular and regional expression of LRP1 in the rat brain indicates a role of the receptor in mediating neuronal survival through direct effects on these cells. However, it may also indirectly affect function of these cells through maintaining survival and function of glia and modulating the microenvironment of the brain via the BBB. While identification of the subcellular and regional localisation of LRP1 allows speculation into its function in subsets of neurons and glia of the brain, it does not definitively explain the function of the receptor in these cells. *In vivo* observations

provide direction for what to investigate in *in vitro* models of neuron and glia to determine the specific functions that LRP1 regulates in these cells.

3 Chapter 3: *In vitro* characterisation of LRP1 expression in hippocampal neurons

3.1 *INTRODUCTION*

LRP1 is expressed in neurons, astrocytes, oligodendrocytes and microglia throughout the grey and white matter regions of the rat brain (Chapter 2.3.2). The extensive expression of LRP1 in the CNS suggests that it has a role in maintaining a healthy, functional brain by regulating neuron and glial survival and function. However, the precise function of LRP1 in these cell populations *in vivo* is unknown. Simpler *in vitro* neuron models have demonstrated that LRP1 can regulate neuron functions such as survival, neurite outgrowth and calcium signaling (Qiu et al., 2002, Qiu et al., 2004, Hayashi et al., 2007, Martin et al., 2008, Fuentealba et al., 2009, Hayashi et al., 2012, Sen et al., 2012, Stiles et al., 2013). Culture systems investigating LRP1 functions in CNS glia have been more limited. However, LRP1 expression has been demonstrated in astrocytes, oligodendrocytes and microglia *in vitro* (Marzolo et al., 2000, Gaultier et al., 2009). These *in vitro* models allow investigation into the effects of injury or LRP1 ligand addition on cell biology that can be investigated further *in vivo*.

LRP1 is expressed on a range of neuron subtypes *in vitro* including hippocampal, cortical, cerebellar granule and retinal ganglionic neurons (Brown et al., 1997, May et al., 2004, Hayashi et al., 2007, Ambjørn et al., 2008, Shi et al., 2009). Hippocampal neurons have been well characterised in culture and develop well defined dendrites and axons (Caceres et al., 1986, Dotti et al., 1987, Dotti et al., 1988). These neurons also express functional synaptic networks that begin to form at 10-14 days *in vitro* (DIV) and are fully established by 21DIV (Fletcher et al., 1991, Rao et al., 1998). This makes them a useful culture model for studying neuronal functions such as survival, neurite outgrowth, regeneration and synaptic signaling.

Investigating the regulation of hippocampal neuron survival and function is particularly relevant for neurodegenerative conditions such as Alzheimer's disease. The hippocampus undergoes significant neurodegeneration in Alzheimer's disease. This pathology corresponds to the short and long-term memory dysfunction associated with the condition (Kesslak et al., 1991, Laakso et al., 1995). LRP1 and some of its ligands, including apoE and α2-macroglobulin, have been genetically linked to the risk of developing Alzheimer's disease (Strittmatter et al., 1993, Kang et al., 1997, Blacker et al., 1998). LRP1 expression is altered in the hippocampus of patients with early and late stages of the disease (Donahue et al., 2006, Sultana et al., 2010). Animal models further support a role of LRP1 in Alzheimer's disease. LRP1 knock-out mouse models display Alzheimer's disease-like pathology as they age, such as neurodegeneration of the hippocampus (Liu et al., 2010). Investigating the role of LRP1 in hippocampal neurons may aid in understanding how to promote survival and function of these cells and to limit the degeneration that occurs in conditions such as Alzheimer's disease.

LRP1 is expressed in cell bodies, axons and dendrites of rat hippocampal neurons in the first week of culture. After 7DIV it becomes restricted to cell bodies and dendrites (Brown et al., 1997). This correlates to observations made *in vivo* where LRP1 is expressed on a subset of neuron cell bodies and proximal segments of their dendrites but absent from axons (Chapter 2.3.2). Determining subcellular localisation of LRP1 in hippocampal neurons will aid in understanding how this receptor regulates neuron function. Confirming previous *in vivo* and *in vitro* work will allow further investigation into the receptor's function at specific cellular sites.

Evidence that LRP1 is involved in synaptic function is increasing through both *in vivo* and *in vitro* work. LRP1 is expressed on the proximal segment of apical dendrites of hippocampal neurons in adult rats (Chapter 2.3.2). This is a site in which specific neurons can synapse, post-synaptic potentials can be amplified and has distinct

calcium accumulation characteristics (Blackstad et al., 1970, Yuste et al., 1994, Seamans et al., 1997). *In vitro*, LRP1 co-localises with the NR2a subunit of NMDA receptor in hippocampal neurons (May et al., 2004). The receptor regulates NMDA receptor dependent calcium influx of hippocampal neurons, demonstrating an ability to modulate synaptic activity. Knock-in of a mutant distal NPxY motif to LRP1 inhibits tPA mediated NMDA receptor dependent calcium influx in hippocampal neurons (Martin et al., 2008). This demonstrates that LRP1 regulated calcium influx is mediated by this motif. It is believed that the mechanism by which this occurs is through an interaction with the post-synaptic protein, PSD-95.

PSD-95 binds to and anchors the NMDA receptor to post-synaptic sites as well as regulating its calcium signaling (Kornau et al., 1995, Sattler et al., 1999). Yeast two-hybrid screening and pull-down assays have demonstrated the ability for the distal NPxY motif of LRP1 to associate with PSD-95 (Gotthardt et al., 2000). However, direct co-localisation of LRP1 and PSD-95 has not been identified in hippocampal neurons. Whether the receptor associates with pre-synaptic proteins such as synaptophysin has not been investigated previously. But given the expression of LRP1 on dendrites not axons after 7DIV (Brown et al., 1997) when the neurons are becoming synaptically active, it is unlikely that the receptor is expressed upon pre-synaptic sites.

LRP1 ligands are able to regulate calcium influx in an NMDA receptor dependent manner. tPA and α2-macroglobulin addition induce an immediate calcium influx in hippocampal and cortical neurons, respectively. Both these effects are ablated by the NMDA receptor inhibitor, MK801 (Bacskai et al., 2000, Martin et al., 2008). Another LRP1 ligand, MT, regulates calcium influx and signaling pathways to mediate growth cone turning of dorsal root ganglia. siRNA against LRP1 inhibits the growth turning effects of MT (Landowski et al., 2012). These findings demonstrate the potential for this ligand to regulate the calcium signaling function of LRP1. The effect

of MT on calcium signaling in hippocampal neurons, and whether this is NMDA receptor dependent, is unknown. In addition, the effects of a peptide modeled after the C-terminus of the MT beta chain, emtinB, on calcium signaling has not been studied previously.

In addition to neuronal expression, LRP1 was identified on cell bodies and processes of astrocytes, oligodendrocytes and microglia in the P2, P7 and adult rat brain (Chapter 2.3.2). The receptor is expressed in glia of pure astrocyte, oligodendrocyte and microglia cultures and regulates myelin vesicle phagocytosis of these cells (Gaultier et al., 2009). Microglia also phagocytose cerebellar granule neurons following toxicity and cell debris from heat shock treated oligodendrocytes by LRP1 dependent mechanisms (Fricker et al., 2012, Fernandez-Castaneda et al., 2013). Expression of LRP1 in microglia is also affected by inflammatory mediators, suggesting that the receptor has a function in immune responses of these cells (Marzolo et al., 2000). Less work has been conducted investigating the function of LRP1 in astrocytes and oligodendrocytes. Investigation of LRP1 in glia of mixed cultures provides an environment more closely related to what is observed *in vivo*. Therefore, determining whether LRP1 is expressed in astrocytes, oligodendrocytes and microglia in the presence of hippocampal neurons may further support previous *in vivo* and *in vitro* findings.

I hypothesise that LRP1 is expressed on hippocampal neuron cell bodies and dendrites of hippocampal neurons in culture. As the neurons become synaptically mature, LRP1 will co-localise with post-synaptic proteins, such as PSD-95 and NMDA receptor subunits, but not pre-synaptic proteins, such as synaptophysin. LRP1 ligands, MT and emtinB, will induce calcium influx in synaptically mature neurons in an LRP1 and NMDA receptor dependent manner. LRP1 will be expressed upon cell bodies and processes of contaminating astrocytes, oligodendrocytes and microglia within hippocampal neuron cultures.

3.2 *METHODS*

All experiments involving the use of animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania (ethics number A0011957), which is in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Sprague Dawley rats were used in all animal work.

Buffer recipes and product information for all reagents can be found in appendices.

3.2.1 PRIMARY HIPPOCAMPAL NEURON CULTURE

Primary hippocampal neuron cultures were prepared from embryonic day 17/18 Sprague Dawley rats. Embryos were dissected from mothers' euthanised by carbon dioxide asphyxiation, removed from embryonic sacs, placed on ice and decapitated. The brain was removed by cutting through the midline of the skull and squeezing the sides of the head gently. Tissue was transferred to Hank's buffered saline solution (HBSS; Sigma, USA) where the cerebellum and part of the brain stem were discarded and the hemispheres separated. Under a dissection microscope the thalamus and hypothalamus were removed and the meninges pulled away to reveal the hippocampus which was dissected from the brain and placed in chilled HBSS. Cells were trypsinised using 0.1% trypsin (Sigma, USA) at 37°C for 5 minutes. Trypsin was removed and cells were resuspended and dissociated by pipette in 1mL Neurobasal media with 10% foetal calf serum. Neurobasal refers to Neurobasal media (Gibco, USA) with 0.1% B27 (Gibco, USA), 0.5mM L-glutamine (Sigma, USA) and 1% gentamicin (Gibco, USA) unless otherwise stated.

Cell count was performed using Trypan Blue (Sigma, USA) exclusion. Cells used for western blot were plated at $1x10^5$ cells/well in 24 well plates (BD Falcon, USA) coated with 1:10 poly-L-lysine (Sigma, USA). Cells used for

immunohistochemistry were plated at $5x10^4$ cells/well on 13mm glass coverslips in 24 well plates. Cells used for live calcium imaging were plated at $5x10^4$ cells/well on 18mm glass coverslips in 12 well plates (BD Falcon, USA). Glass coverslips were treated with >69% nitric acid (Fluka, USA) for four hours, washed in tap water overnight, ethanol cleaned and sterilised by exposure to ultraviolet light for 30 minutes. All coverslips were coated overnight with neat poly-L-lysine. Cultures were maintained at 37°C in humidified air with 5% carbon dioxide. A full media change to serum free Neurobasal media was conducted 24 hours after plating and every three to four days thereafter.

3.2.2 WESTERN BLOT

Hippocampal neuron cultures were grown to 3, 7, 14 and 21DIV. 3 separate cultures were used for each time point and $5x10^5$ cells were collected for each culture. Cultures were washed twice with HBSS free of B27 or serum. While on ice, cells were scraped from wells by pipette using 150µL RIPA lysis buffer with 1:100 protease inhibitor (Thermo Scientific, USA) and triturated 15-20 times. Samples were centrifuged in 4°C at 13,000rpm for 10 minutes. The supernatant was removed, snap frozen in liquid nitrogen and stored at -80°C.

Total protein concentration was determined using a BCA protein assay (Bio-Rad, USA). Briefly, part A and part B solutions were mixed by a 50:1 ratio. 200µL of working reagent was added to each well of a 96 well plate. Protein standards at 0.1, 0.2, 0.5, 0.7 and 1.0mg/mL were prepared to calculate a protein standard curve. 20µL of protein standards and 20µL of cell sample were mixed with the BCA reagent and incubated for 30 minutes at 37°C. Triplicates were prepared for each sample. Absorbance at 595nm was measured using a SpectraMax M2 plate reader (Molecular Devices) and SoftMax Pro computer program (v 4.8). A standard curve was calculated and used to determine each sample's concentration.

1mm thick SDS-PAGE gels were prepared as 10% resolving and 5% stacking gels (see appendices). 5μL of Spectra protein ladder (Thermo Scientific, USA) was loaded into the first lane. 5μg protein was loaded for each sample. Samples were run using a Mini-Protean Tetra electrophoresis system (Bio-Rad, USA) in running buffer at 200 volts for 35-45 minutes. Transfer to nitrocellulose paper (Thermo Scientific, USA) was conducted overnight using an XCell Sure Lock electrophoresis system (Life Technologies, USA) at 20 volts in 4°C. The nitrocellulose membrane was protein blocked using 5% milk powder in 0.05% PBS-Tw for at least 60 minutes. Primary antibodies to the LRP1 light chain or β-actin (housekeeping protein) were applied in 5% milk powder in PBS-Tw overnight at 4°C (antibody concentrations and combinations in Table 3.1). Controls omitting primary antibodies were incubated with 5% milk powder alone. Secondary antibodies in PBS-Tw were incubated while shaking for 1 hour at room temperature. Three 10-minute washes with PBS-Tw occurred after each antibody incubation. Signal was detected using a chemiluminescent substrate (Millipore, USA). Peroxidase and luminol components were mixed at a 1:1 ratio and incubated with the membrane for 5 minutes at room temperature. The membrane was exposed for 600 seconds using a Chemi-Smart 5000 imaging system and Chemi-Capt 5000 computer program (v 12.8). Natural light was used to capture the ladder. Image contrast and brightness were uniformly enhanced using Adobe Photoshop (v 11.0.2).

3.2.3 IMMUNOCYTOCHEMISTRY

Cellular and subcellular specificity of LRP1 was determined by incubating anti-LRP1 antibody with antibodies against either MAP2 (dendrites), SMI-312 (axons), synaptophysin (pre-synaptic site), PSD-95 (post-synaptic site), GFAP (astrocytes), olig2 (oligodendrocytes) or isolectin (microglia). 3 separate cultures were grown for each antibody combination at each time point. Culture purity was determined by immunostaining cultures with antibodies against GFAP (astrocytes) and tau (neurons). 4

separate coverslips, each from a different culture, were prepared from each time point. Cultures were grown to 3, 7, 14 or 21DIV and fixed with 4% paraformaldehyde for 15-20 minutes at room temperature and stored at 4°C in PBS with 0.01% sodium azide. Cells were protein blocked using 10% goat serum (Vector Laboratories, USA) in 0.03% triton X-100 (Lab-Chem, USA) in PBS for 30 minutes at room temperature to inhibit non-specific antibody binding. Primary antibodies were diluted in 10% goat serum and incubated for 1 hour at room temperature and overnight at 4°C. Cultures omitting the primary antibody were incubated with 10% goat serum alone to ensure no non-specific binding of secondary antibodies. Next, cultures were incubated with secondary antibodies for one hour at room temperature before counterstaining with 1μg/ml nuclear yellow (Invitrogen, USA). Coverslips were washed three times by gentle shaking in PBS for 5 minutes following each antibody incubation. Coverslips were mounted to slides using fluorescent mounting media (Dako, USA) and dried overnight.

Cells from 3 separate 14DIV cultures to be immunolabelled for NMDA receptor subunits were fixed with methanol for 10 minutes at -20°C and stored in PBS with 0.01% sodium azide at 4°C. Cells were protein blocked using 10% goat serum (Vector Laboratories, USA) in 0.03% triton X-100 (Lab-Chem, USA) in PBS for 30 minutes at room temperature. Primary antibodies to either NR2a or NR2b were diluted in 10% goat serum and incubated for 1 hour at room temperature and overnight at 4°C. Cultures were incubated with anti-rabbit secondary antibodies conjugated with AlexaFluor®-488 (Molecular Probes, USA) for one hour at room temperature. Cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed three times in PBS. Primary antibodies to LRP1 were diluted in 10% goat serum and incubated for 1 hour at room temperature and overnight at 4°C. Next, cultures were incubated with anti-rabbit secondary antibodies conjugated with AlexaFluor®-594 (Molecular Probes, USA) for one hour at room temperature before counterstaining with 1μg/ml nuclear

yellow (Invitrogen, USA). Coverslips were washed three times by gentle shaking in PBS for 5 minutes following each antibody incubation. Cultures omitting the primary antibody were also prepared to ensure no non-specific binding of secondary antibodies. Coverslips were mounted to slides using fluorescent mounting media (Dako, USA) and dried overnight.

Representative photographs for LRP1 cellular and subcellular localisation were taken at 40X objective. To determine culture purity, 11 random frames from each coverslip were photographed at 20X objective. Tau positive (neurons), GFAP positive (astrocytes) and tau and GFAP negative cells (determined by nuclei and classed as 'other') were counted. All images were captured using an Olympus BX50 microscope and Photomatrix Cool Snap HQ^2 camera. Images were uniformly enhanced using Adobe Photoshop (v11.0.2).

	Primary	Type	Dilution	Secondary	Conjugate	Dilution
Western blot	LRP1	R	1:1000	Goat anti-rabbit	HRP	1:1000
	β-actin	M	1:20,000	Goat anti-mouse	HRP	1:5000

	Primary	Type	Dilution	Secondary	Conjugate	Dilution	Target
Immunocytochemistry	GFAP	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Astrocytes
	LRP1	R	1:1000	Goat anti-rabbit	AlexaFluor®-594	1:1000	C-terminus of LRP1 light chain
	MAP2	M	1:1000	Goat anti-mouse		1:1000	Dendrites
	Olig2	M	1:500	Goat anti-mouse	AlexaFluor®-594	1:1000	Oligodendrocytes
	PSD-95	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Post-synaptic site
	SMI-312	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Axons
	Synaptophysin	M	1:1000	Goat anti-mouse	AlexaFluor®-488	1:1000	Pre-synaptic site
	Isolectin (488)		1:500	NA			Microglia
	Tau	R	1:1000	Goat anti-rabbit	AlexaFluor®-594	1:1000	Neuron cell body, dendrites and axons
	NR2a	R	1:100	Goat anti-rabbit	AlexaFluor®-488	1:1000	NMDA receptor 2a subunit
	NR2b	R	1:100	Goat anti-rabbit	AlexaFluor®-488	1:1000	NMDA receptor 2b subunit

Table 3.1: A list of working dilutions of primary antibodies, their targets and respective secondary antibodies used for western blots and immunocytochemistry analysis. Antibodies were either polyclonal rabbit (R) or monoclonal mouse (M) antibody.

HRP: horse radish peroxidase; NMDA: N-methyl-D-aspartate

3.2.4 HIPPOCAMPAL NEURON SYNAPTIC ACTIVITY ASSAY

Hippocampal neuron cultures were grown to 14-15DIV and incubated with 5μM Fluo-4 in serum free Neurobasal media for 15 minutes at 37°C in humidified air with 5% carbon dioxide. Cells were washed once with warm imaging buffer (see appendices). Coverslips were mounted onto the mounting chamber and 300μL imaging buffer was pipetted gently on top. Imaging was conducted in a microscope incubator (TCH885-5; Clear State Solutions Pty Ltd) at 37°C using a Nikon Eclipse Ti microscope.

To determine whether cells were responsive to glutamate or NMDA, 4 regions containing healthy and relatively isolated hippocampal neurons were selected based on morphology under phase contrast (pyramidal shape with a smooth, raised surface and defined nucleus and processes). Cells were imaged using an Evolve™ 512 EMCCD camera (Photometrics) every 10 seconds using a 488nm wavelength at 40X objective. A phase contrast image was taken only at the start and end of the experiment to limit photo bleaching. Baseline fluorescence to determine basal intracellular calcium was established by imaging cells for 2 minutes at the start of every experiment. Imaging was paused as 0.9% saline, used as a vehicle control, was added and imaging resumed for an additional 2 minutes. Imaging was paused again and 10μM NMDA or 10μM glutamate added before imaging continued for another eight minutes. At least 6 separate coverslips from 3 different cultures were used for each treatment.

MK801, a NMDA receptor inhibitor, was added to cells to determine that NMDA responses were specific. Basal calcium was measured for 2 minutes before the experiment was paused, 10μM NMDA applied and imaging continued for 3 minutes. Cells were washed for 2 minutes by flowing imaging buffer over the coverslip which was vacuum suctioned off. The wash was stopped and imaging buffer replaced with 300μL of either 10μM MK801 or the equivalent volume of saline in imaging buffer.

Cells were left for 7.5 minutes to allow for MK801 inhibition before 10μM NMDA was added again and imaging continued for an additional 6 minutes. 2-3 separate coverslips, each from a different culture, were used for each experiment.

The effects of the LRP1 ligands, MT (Bestenbalt LLC, Estonia) and emtinB (Schafer-N, Denmark), on NMDA receptor dependent calcium influx were investigated. Cells were incubated with Fluo-4 as above and baseline fluorescence determined by imaging cells for 2 minutes at the start of the experiment. Imaging was paused and 10μM NMDA added to determine whether neurons were synaptically active. After 2-3 minutes of imaging, cultures were washed for 2 minutes by flowing fresh imaging buffer onto the coverslip which was vacuum suctioned off to remove any remaining NMDA. The wash was stopped and cells allowed to settle for 2 minutes before imaging was paused and either 10μg/mL MT (equivalent to 1.5μM) or 25μM emtinB (equivalent to 75μg/mL) added and imaging resumed for 6.5 minutes. 6 coverslips from 3 cultures were used to determine MT effects, while 2 coverslips from 1 culture were used to determine emtinB effects.

Video of live imaging fluorescence was analysed using the NIS Elements AR computer program (v 3.21.00). A region of interest was manually drawn around the soma of each cell using the phase contrast image. Raw fluorescence was measured and corrected for background. Data were transferred to Microsoft Excel (v14.0.7015.1000) and baseline fluorescence calculated as the average fluorescence of the two minute baseline period. Raw fluorescence was converted to actual fluorescence using the following equation:

$$Fluorescence = \frac{F - F0}{F0}$$

where F = the raw fluorescence at a the time point being measured and F0 = baseline fluorescence (reviewed by (Takahashi et al., 1999)). Results for each cell were presented in graph form. Graphs in Figure 3.12 represent the response of a typical cell. A synaptic response was classed as an increase of fluorescence above a value of 1 that occurred immediately after addition of the neurotransmitter and which quickly reduced to near baseline level.

3.3 ***RESULTS***

3.3.1 LRP1 IS EXPRESSED IN HIPPOCAMPAL NEURON CULTURES FROM 3 TO 21DIV

Western blot analysis demonstrated that the processing of LRP1 changes during maturation in culture. The strongest band observed in 3DIV cultures corresponded to the ~85kDa band representing the LRP1 light chain. A faint ~120kDa band was also present at this time point (Figure 3.1 A, lanes 1-3). Both the ~85kDa and ~120kDa bands became stronger from 3 to 7DIV (Figure 3.1 A, lanes 4-6). Expression of the ~85kDa band remained similar between 7 and 14DIV cultures, but the ~120kDa band appeared fainter at 14DIV compared to 7DIV. 14DIV cultures also faintly expressed ~50kDa, ~130kDa and ~150kDa bands not present at 3 or 7DIV (Figure 3.1 A, lanes 7-9). 21DIV cultures expressed the ~85kDa band similar to 7 and 14DIV cultures. The ~130kDa and ~150kDa were fainter at 21DIV than 14DIV, but the ~50kDa band was stronger. A ~37kDa band was present at 21DIV that was not observed at any other time point (Figure 3.1 A, lanes 10-12).

A similar amount of protein was loaded for each sample (β-actin). Negative controls of hippocampal culture blots omitting primary antibodies showed no immunoreactivity (Figure 3.1 B).

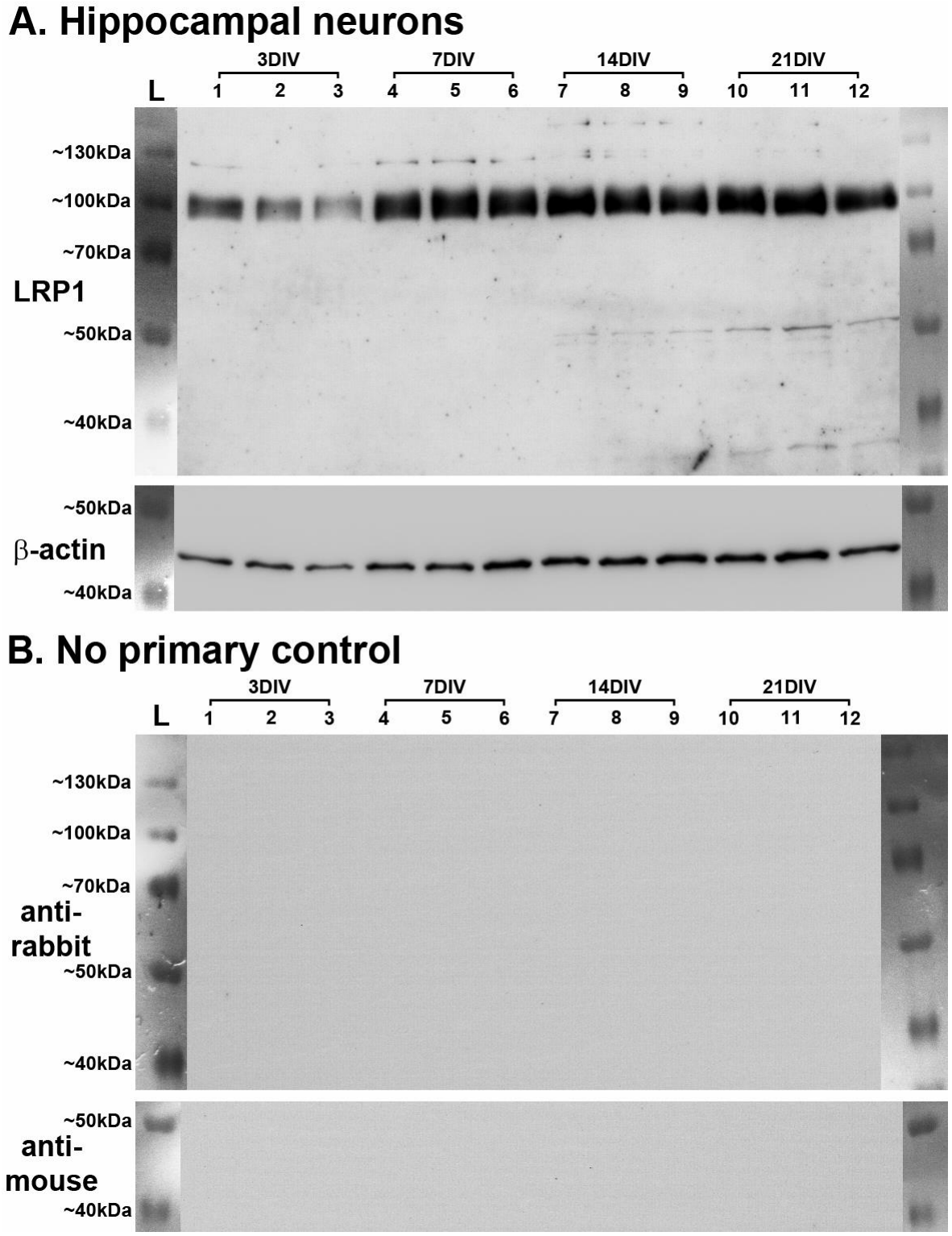

Figure 3.1: LRP1 is expressed in hippocampal neuron cultures at 3, 7, 14 and 21 days *in vitro* (DIV). $5x10^5$ cells from hippocampal neuron cultures (A) were collected at 3DIV (lanes 1-3), 7DIV (lanes 4-6), 14DIV (lanes 7-9) and 21DIV (lanes 10-12). 5µg total protein for each sample was run on an SDS-PAGE gel and probed with anti-LRP1 or anti-β-actin antibodies. Similar amounts of protein were loaded for each sample (β-actin). Controls blots omitting primary antibodies were negative for non-specific secondary antibody binding (D). Molecular weights of bands were determined using a commercial protein ladder (L).

3.3.2 LRP1 IS LOCALISED TO CELL BODIES AND DENDRITES OF HIPPOCAMPAL NEURONS IN VITRO

Hippocampal neuron culture purity was determined using markers for neurons, astrocytes and nuclei of unstained cells (other). Purity was determined and presented in Figure 3.2.

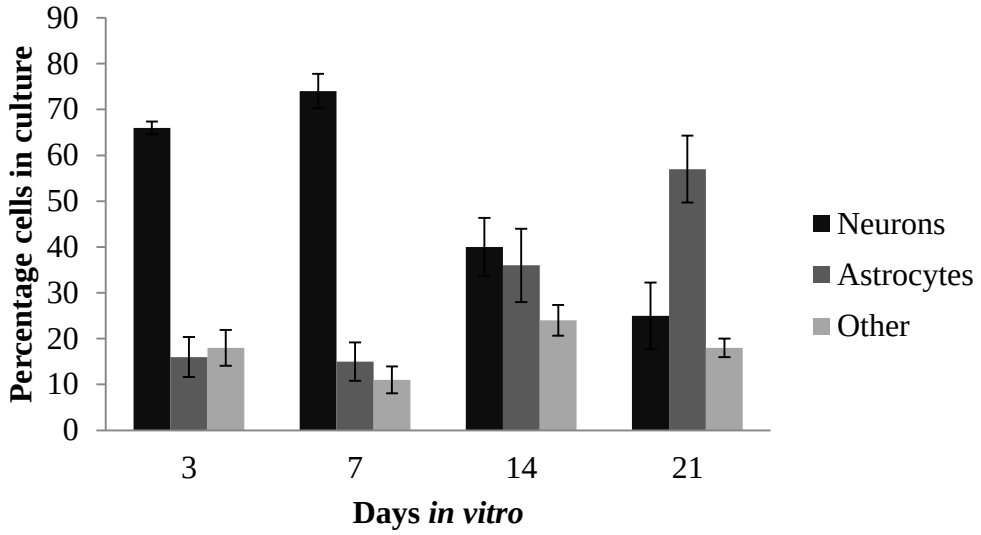

Figure 3.2: Purity of neurons, astrocytes and other glia in hippocampal neuron cultures. Cultures were immunolabelled for tau (neurons), GFAP (astrocytes) and nuclear yellow (nuclei). Tau positive, GFAP positive and tau/GFAP negative cells (determined by nuclei) were counted. Error bars = standard error

Percentage of neurons in cultures decreased due to proliferating glia and apoptosis of neurons, evidenced from an increased number of pyknotic nuclei (data not shown). Tau positive neurons had pyramidal or round cell bodies with multiple prominent neurites that branched as they extended away from the cell body. GFAP positive astrocytes appeared flatter than neurons and had larger nuclei and cell bodies with thicker, shorter processes. Other glia nuclei were either much larger and less condensed than hippocampal neuron nuclei, or were smaller and more condensed.

LRP1 was expressed on astrocyte cell bodies and processes from 3 to 21DIV (Figure 3.3 A-D). Oligodendrocytes were positive for LRP1 on cell bodies and processes from 7 to 21DIV (Figure 3.4 A-C). Oligodendrocytes had small cell bodies with condensed nuclei. The oligodendrocyte marker, olig2, was not detected in cultures at 3DIV. Microglial expression of LRP1 was heterogeneous. Some cells were strongly positive for the receptor throughout the cell body, but others only expressed the receptor faintly. This occurred at 3, 7, 14 and 21DIV (Figure 3.5 A-D). Microglia were noted as either flat and round or elongated with processes. Differences in LRP1 expression did not appear to correspond to specific cell morphology.

LRP1 immunoreactivity was observed in all hippocampal neurons from 3 to 21DIV (Figures 3.6 – 3.9). Expression was particularly strong at the cell body of hippocampal neurons. The receptor was present along and at growth cones at the ends of extending axons of 3DIV neurons (Figure 3.6 A). This expression was reduced in 7DIV neurons and was absent from axons of 14DIV or 21DIV hippocampal neurons (Figure 3.6 B-D). At these later time points LRP1 positive processes were observed alongside axons but not upon the axons themselves. LRP1 reactive processes were dendrites, which were strongly positive for the receptor from 3DIV to 21DIV (Figure 3.7 A-D). LRP1 did not co-localise with or PSD-95 (Figure 3.8 A-C) or synaptophysin from 7 to 21DIV (Figure 3.9 A-C). Though partial co-localisation with PSD-95 and synaptophysin may have appeared to occur in some instances, higher magnification demonstrated that this was not the case. Rather it appeared that due to the high density of staining, LRP1 immunoreactivity randomly crossed-over with PSD-95 or synaptophysin. Controls omitting primary antibodies were incubated with goat anti-rabbit conjugated to AlexaFluor®-594 and goat anti-mouse conjugated to AlexaFluor®-488 secondary antibodies and were immunonegative (Figure 3.10 A-D).

Cultures at 14DIV were immunolabelled for LRP1 and the NMDA receptor subunits, NR2a and NR2b. Hippocampal neurons begin to form mature synapses at about 12DIV, with complete synaptic activity reached at 21DIV (Rao et al., 1998). 14DIV was chosen as healthy cells were relatively easy to grow to this age and synaptically mature neurons should be present. LRP1 did not co-localise with NR2a (Figure 3.11 A) but did partially co-localise with NR2b (Figure 3.11 B). No primary controls for methanol and 4% paraformaldehyde fixed cultures were immunonegative (Figure 3.11 C).

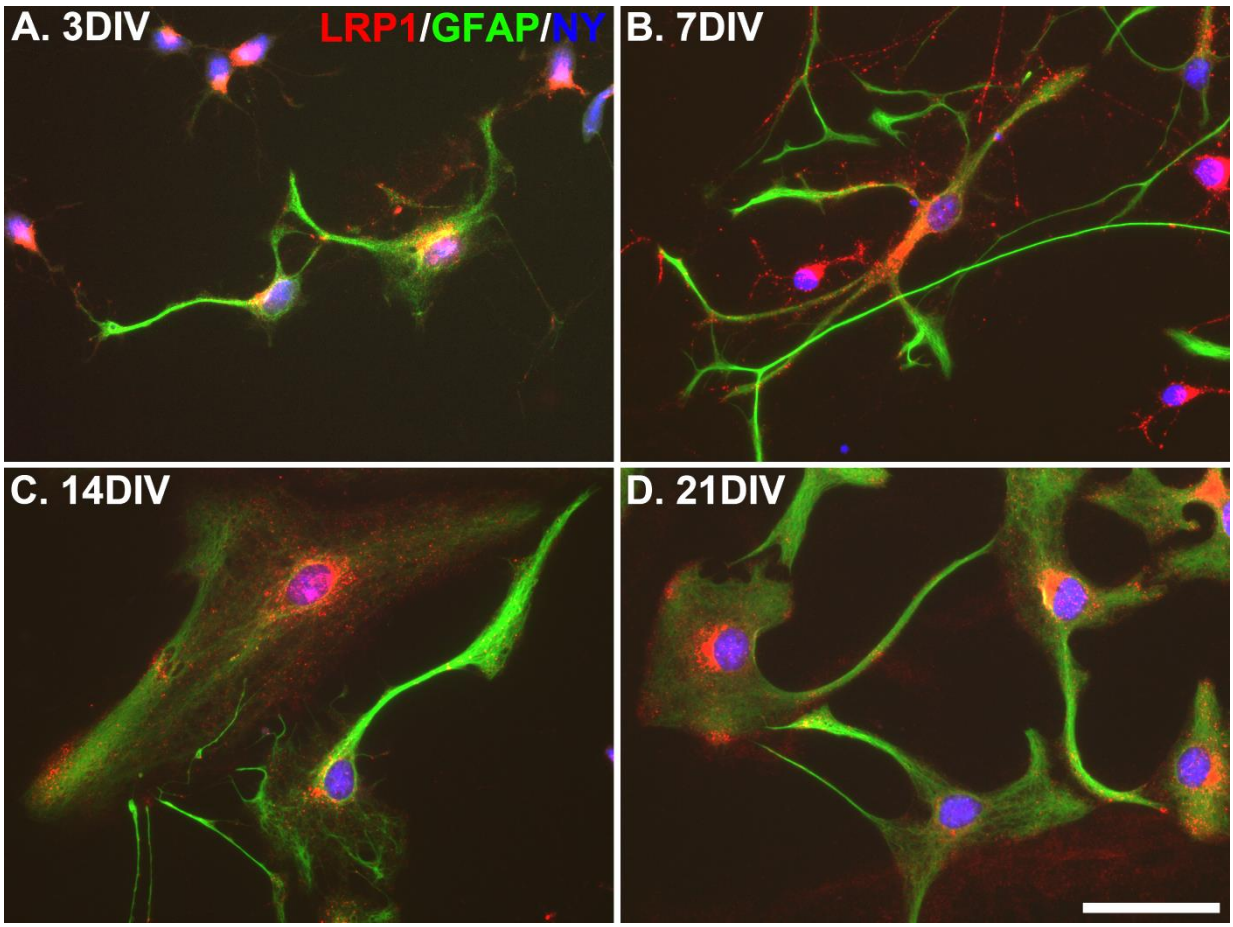

Figure 3.3: LRP1 is expressed on astrocytes in hippocampal neuron cultures. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 3 (A), 7 (B), 14 (C) and 21 days *in vitro* (D) and immunolabelled for LRP1 (red) and GFAP (green). Nuclei were counterstained with 1μg/mL nuclear yellow (NY; blue).

Scale bar = 50μm

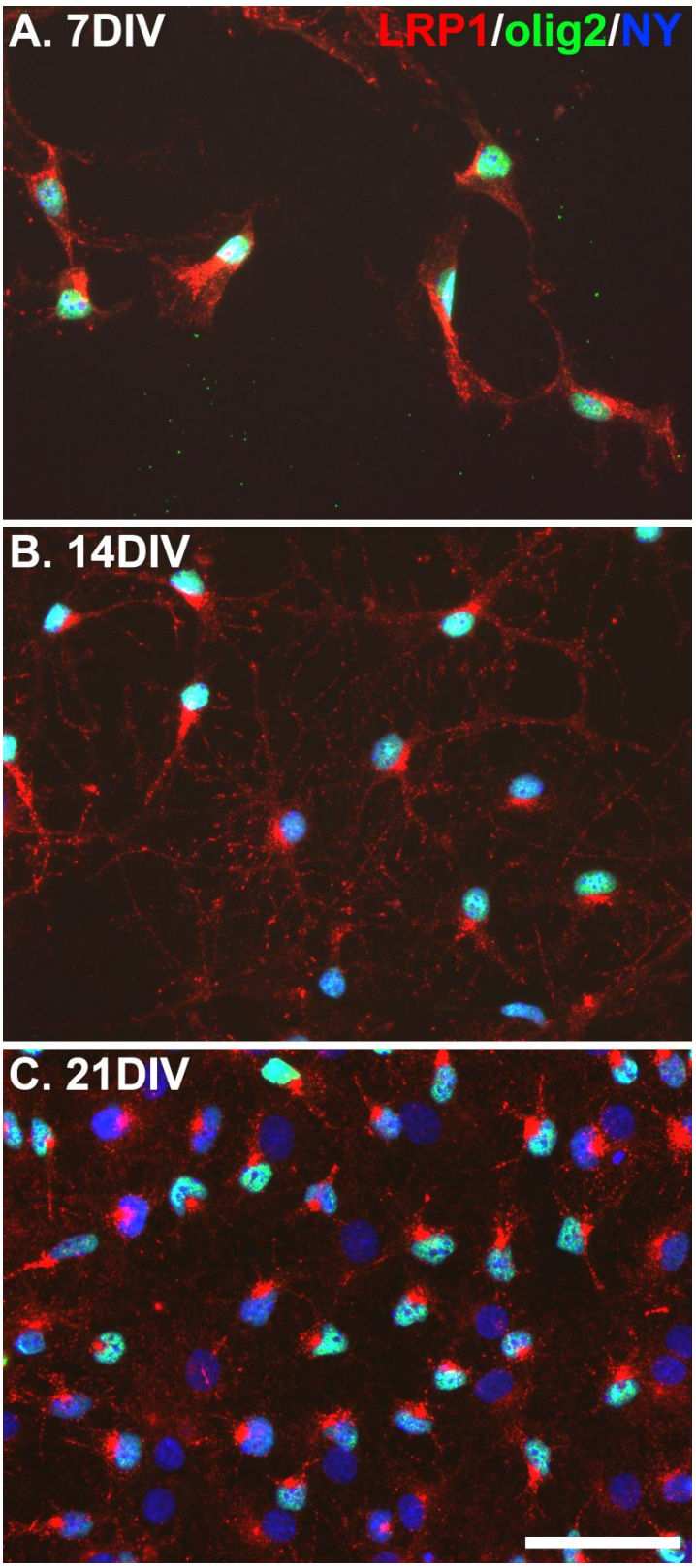

Figure 3.4: LRP1 is expressed on oligodendrocytes in hippocampal neuron cultures. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 7 (A), 14 (B) and 21 days *in vitro* (DIV; C) and immunolabelled for LRP1 (red) and olig2 (green). Nuclei were counterstained with 1μg/mL nuclear yellow (NY; blue).

Scale bar = 50μm

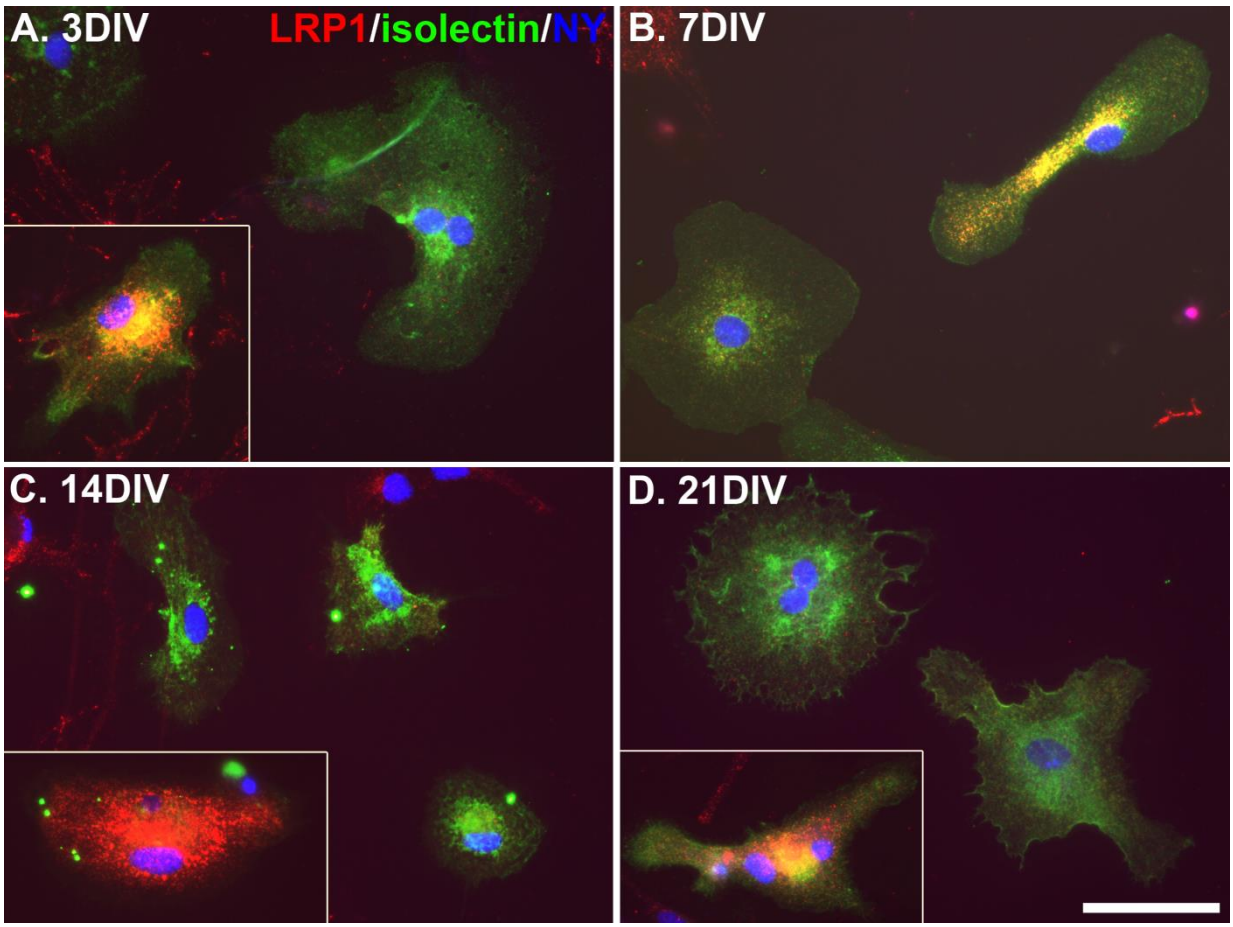

Figure 3.5: LRP1 is expressed on microglia in hippocampal neuron cultures. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 3 (A), 7 (B), 14 (C) and 21 days *in vitro* (D) and immunolabelled for LRP1 (red) and isolectin (green). Nuclei were counterstained with 1µg/mL nuclear yellow (NY; blue). LRP1 expression in microglia was heterogeneous and while some microglia were strongly immunreactive for LRP1 (insets A, and D) others were only faintly positive for LRP1.

Scale bar = 50µm; A, C, D inset scale bar = 50µm

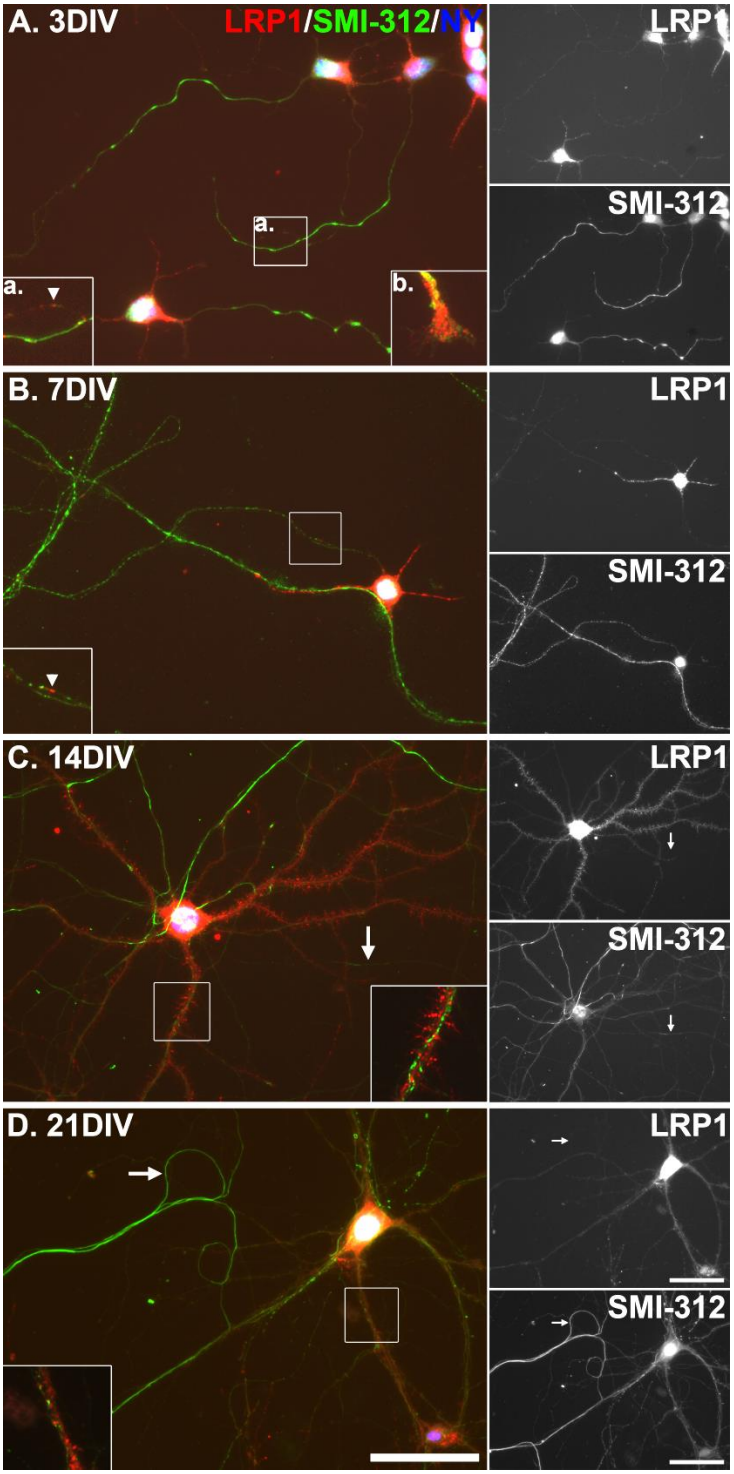

Figure 3.6: LRP1 is expressed along axons of neurons at 3 and 7 days *in vitro* (DIV) but not at 14 or 21DIV. Hippocampal neuron cultures were grown to 3 (A), 7 (B), 14 (C) or 21DIV (D) and fixed with 4% paraformaldehyde. Cells were immunolabelled with antibodies against LRP1 (red) and SMI-312 (green; axonal marker). Nuclei were counterstained with 1µg/mL nuclear yellow (NY; blue). At 3DIV, LRP1 was expressed along (A; inset a) and at the ends (A; inset b) of extending axons. Some 7DIV axons were LRP1 positive, (B; inset) but 14 and 21DIV axons were negative for LRP1 (C and D; arrows). Axons appeared to follow alongside LRP1 positive dendrites (C and D insets).

Scale bar = 50µm; A.a., A.b., B, C and D inset scale bar = 25µm

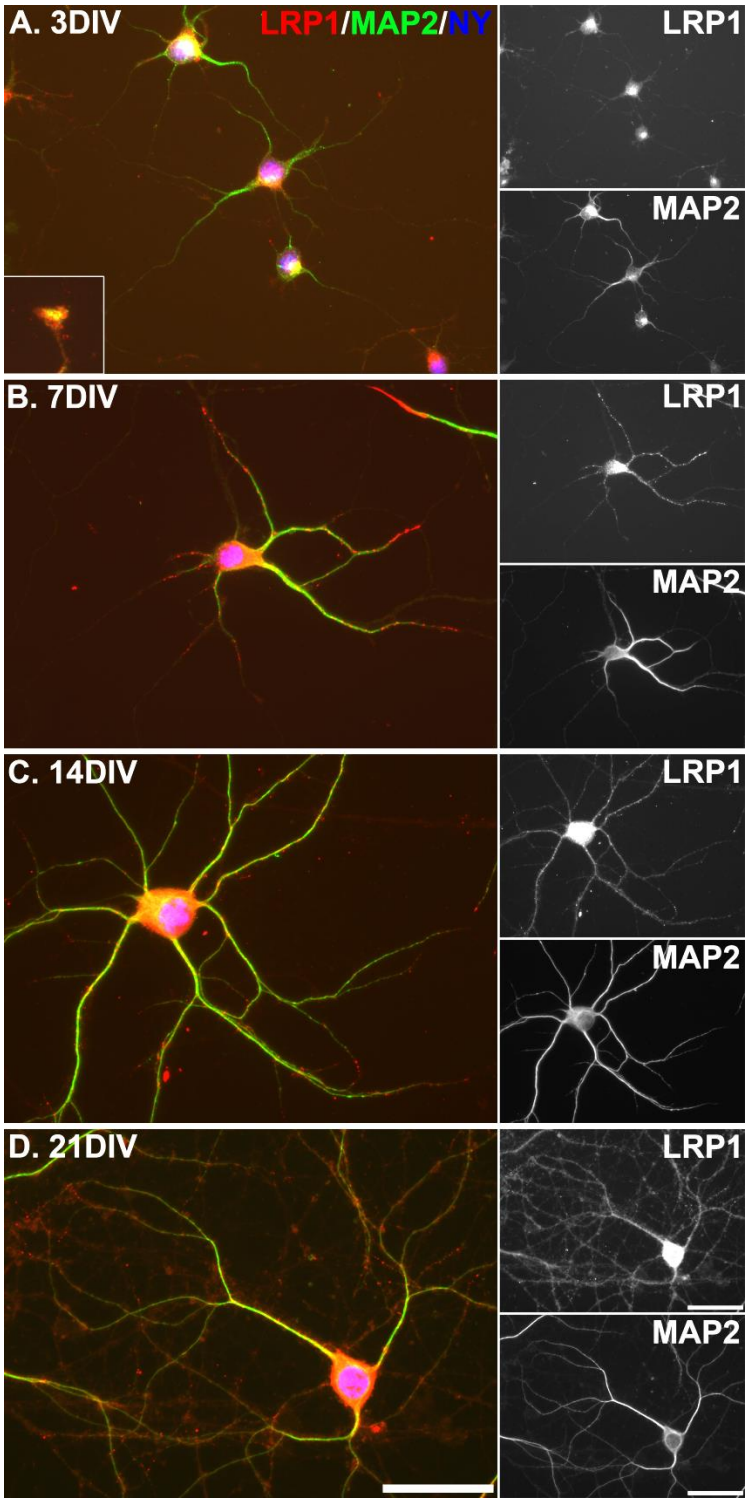

Figure 3.7: LRP1 is expressed on dendrites of hippocampal neurons *in vitro*. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 3 (A), 7 (B), 14 (C) and 21 days *in vitro* (DIV; D) and immunolabelled for LRP1 (red) and MAP2 (green). Nuclei were counterstained with 1µg/mL nuclear yellow (NY; blue). LRP1 was expressed along MAP2 positive processes at every time point and was also present at the ends of dendrites in 3DIV neurons (inset).

Scale bar = 50µm; A inset scale bar = 25µm

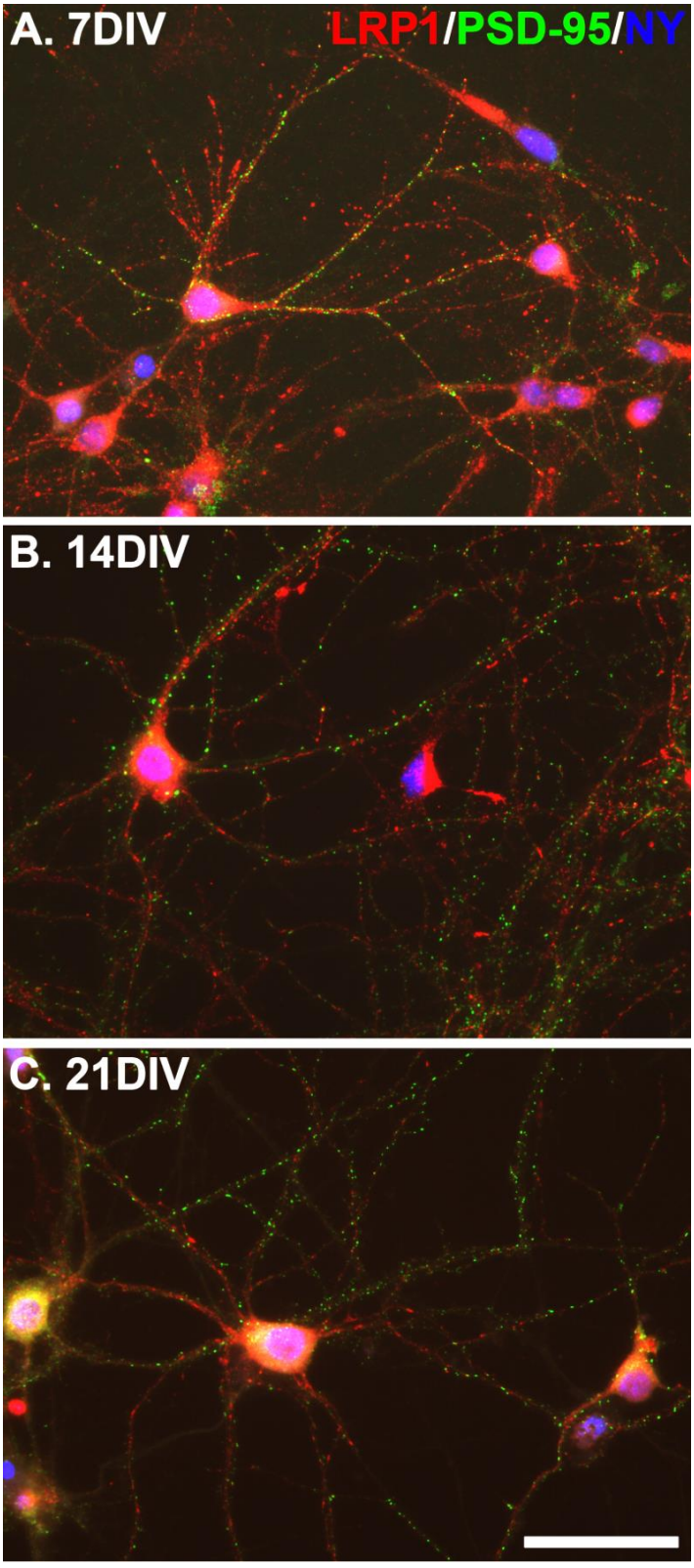

Figure 3.8: LRP1 does not co-localise with PSD-95 in hippocampal neurons *in vitro*. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 7 (A), 14 (B) and 21 days *in vitro* (DIV; C) and immunolabelled for LRP1 (red) and the post-synaptic protein, post-synaptic density-95 (PSD-95; green), and counterstained with 1µg/mL nuclear yellow (NY; blue).

Scale bar = 50µm

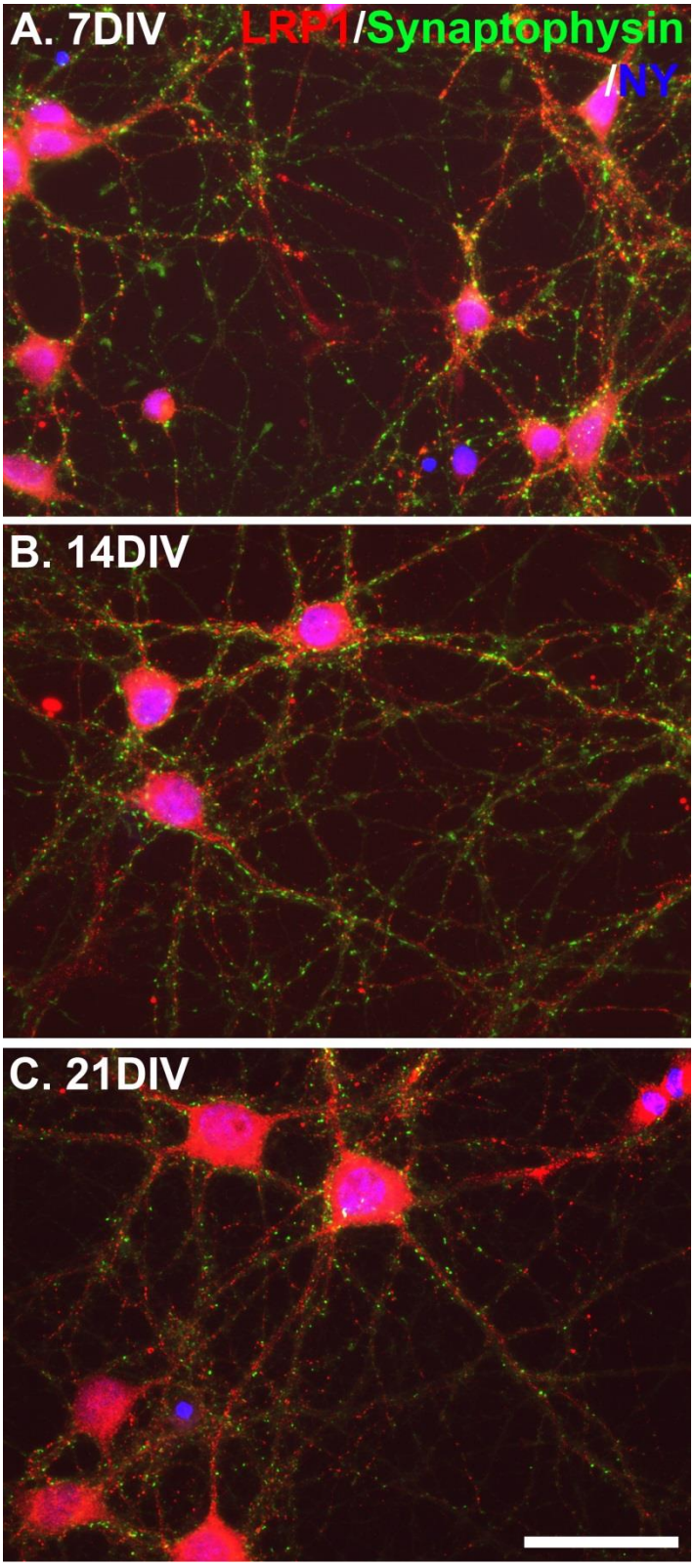

Figure 3.9: LRP1 does not co-localise with synaptophysin in hippocampal neurons *in vitro*. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 7 (A), 14 (B) and 21 days *in vitro* (DIV; C) and immunolabelled for LRP1 (red) and the pre-synaptic protein, synaptophysin (green), and counterstained with 1µg/mL nuclear yellow (NY; blue).

Scale bar = 50µm

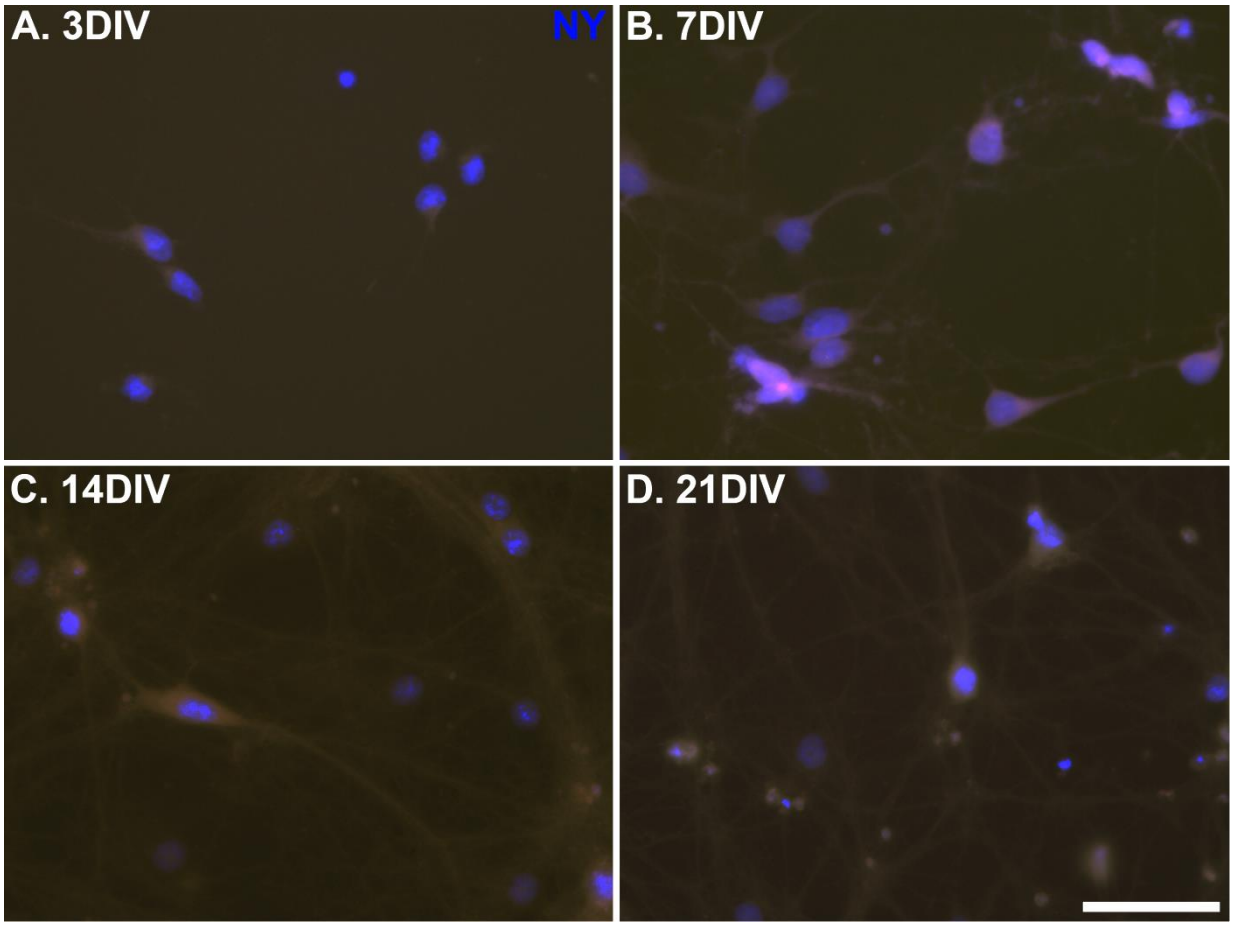

Figure 3.10: Cultures omitting primary antibodies are immunonegative. Hippocampal neuron cultures were fixed with 4% paraformaldehyde at 3 (A), 7 (B), 14 (C) and 21 days *in vitro* (D). Cultures were incubated with goat serum omitting primary antibodies followed by goat anti-rabbit conjugated to AlexaFluor®-594 and goat anti-mouse conjugated to AlexaFluor®-488 secondary antibodies. Nuclei were counterstained with 1µg/mL nuclear yellow (NY; blue). Secondary antibodies did not bind non-specifically to hippocampal neurons.

Scale bar = 50µm

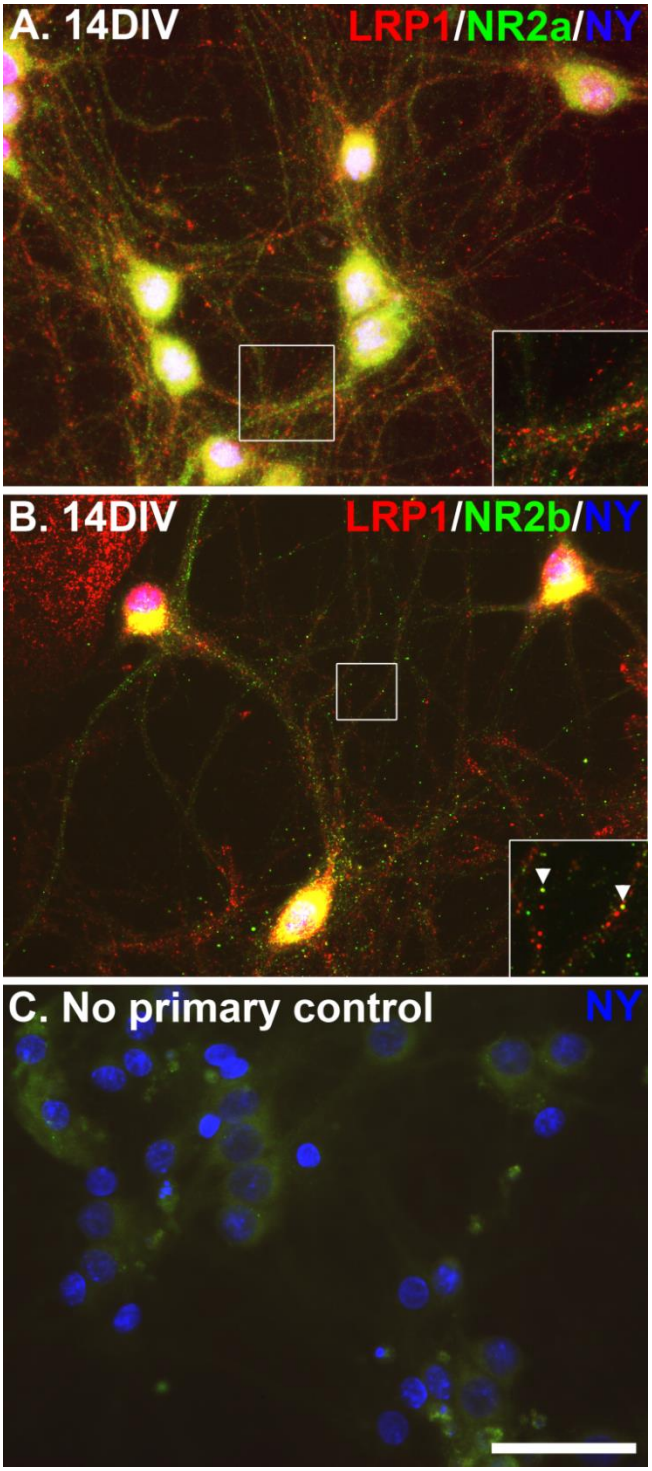

Figure 3.11: LRP1 co-localises with NR2b but not NR2a *in vitro*. Hippocampal neurons cultures were fixed with methanol at 14 days *in vitro* (DIV) and immunolabelled for NMDA receptor subunits, NR2a (A) or NR2b (B; both green). Cells were then fixed with 4% paraformaldehyde and immunolabelled for LRP1 (red). Nuclei were counterstained with 1µg/mL nuclear yellow (NY; blue). LRP1 did not co-localise with NR2a (A inset) but did partially co-localise with NR2b (B inset and arrowheads). Cultures omitting primary antibodies were immunonegative (C).

Scale bar = 50µm; A inset scale bar = 30µm; B inset scale bar = 20µm

3.3.3 14-15DIV HIPPOCAMPAL NEURONS RESPOND TO NMDA AND GLUTAMATE

14DIV hippocampal neurons express the NMDA receptors subunits NR2a and NR2b (Figure 3.10 A and B). Live cell calcium imaging was used to determine whether hippocampal neurons were synaptically mature and able to respond to NMDA or glutamate. 52% (23 of 44 cells) and 54% (19 of 35 cells) of 14-15DIV hippocampal neurons responded to NMDA and glutamate, respectively. This response was characterised by an immediate and large increase in intracellular calcium following neurotransmitter addition, with a subsequent decrease in calcium near to baseline (Figure 3.12 A and B). Addition of 10μM MK801 inhibited NMDA responses in cells that had previously responded to the neurotransmitter (14 cells; Figure 3.12 C). A saline control did not inhibit NMDA responses (11 cells; Figure 3.12 D).

Live imaging was used to determine whether the LRP1 ligands, MT and emtinB, affect calcium signaling in hippocampal neurons via an NMDA receptor dependent mechanism. 10μM NMDA was added to neurons following loading of the calcium fluorophore and neurons that responded with an increased uptake of calcium were further exposed to MT or emtinB. Addition of either MT or emtinB failed to induce calcium influx in neurons (Figure 3.13 A and B; MT: 17 NMDA responsive neurons; emtinB: 6 NMDA responsive neurons).

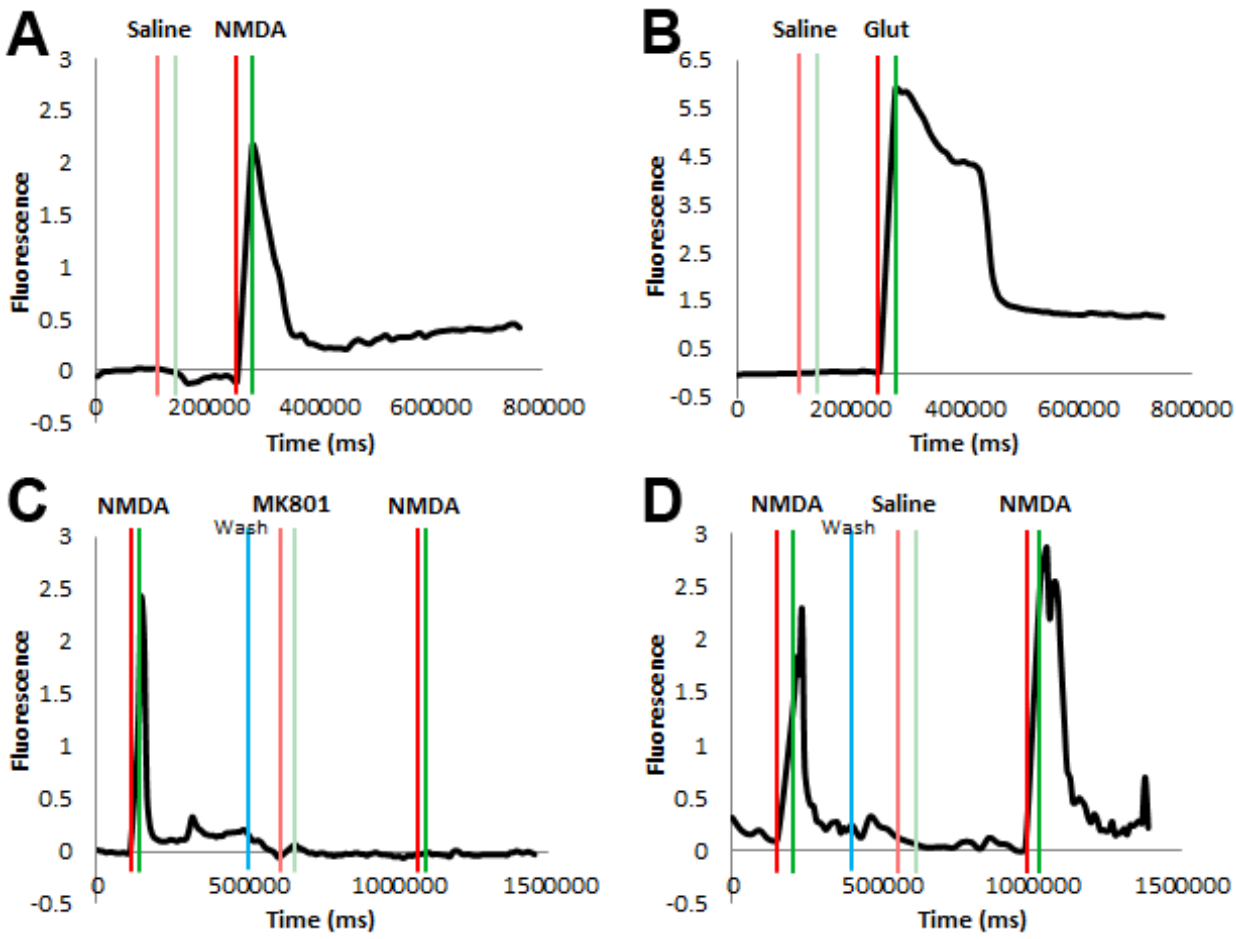

Figure 3.12: Hippocampal neurons respond to NMDA and glutamate at 14-15 days *in vitro*. Hippocampal neurons were loaded with 5μM Fluo-4 calcium indicator at 14-15 days *in vitro* and imaged every ten seconds. Baseline fluorescence was determined by imaging cells for two minutes at the start of every experiment. Imaging was paused (faded red line) as a saline control was added and imaging resumed (faded green line) for an additional 2 minutes. Imaging was paused again (red line) and either 10μM NMDA (A) or 10μM glutamate (B) added and imaging resumed (green line) for 8 minutes. The NMDA receptor inhibitor, MK801, was added to determine that NMDA responses were specific. 10μM NMDA was added and cells imaged for 3 minutes before a 2 minute wash (blue line). Imaging was paused as the wash was stopped (faded red line) and imaging buffer replaced with 10μM MK801 (C) or a saline control (D) and imaging resumed (faded green line) for 7.5 minutes. NMDA was added again and imaged for another 6 minutes. NMDA and glutamate caused an immediate increase in intracellular calcium in 23 of 44 (52%) and 19 of 25 (54%) cells, respectively. The NMDA response was inhibited by MK801 but not by the saline control. Representative traces demonstrate the typical response from a single responding neuron.

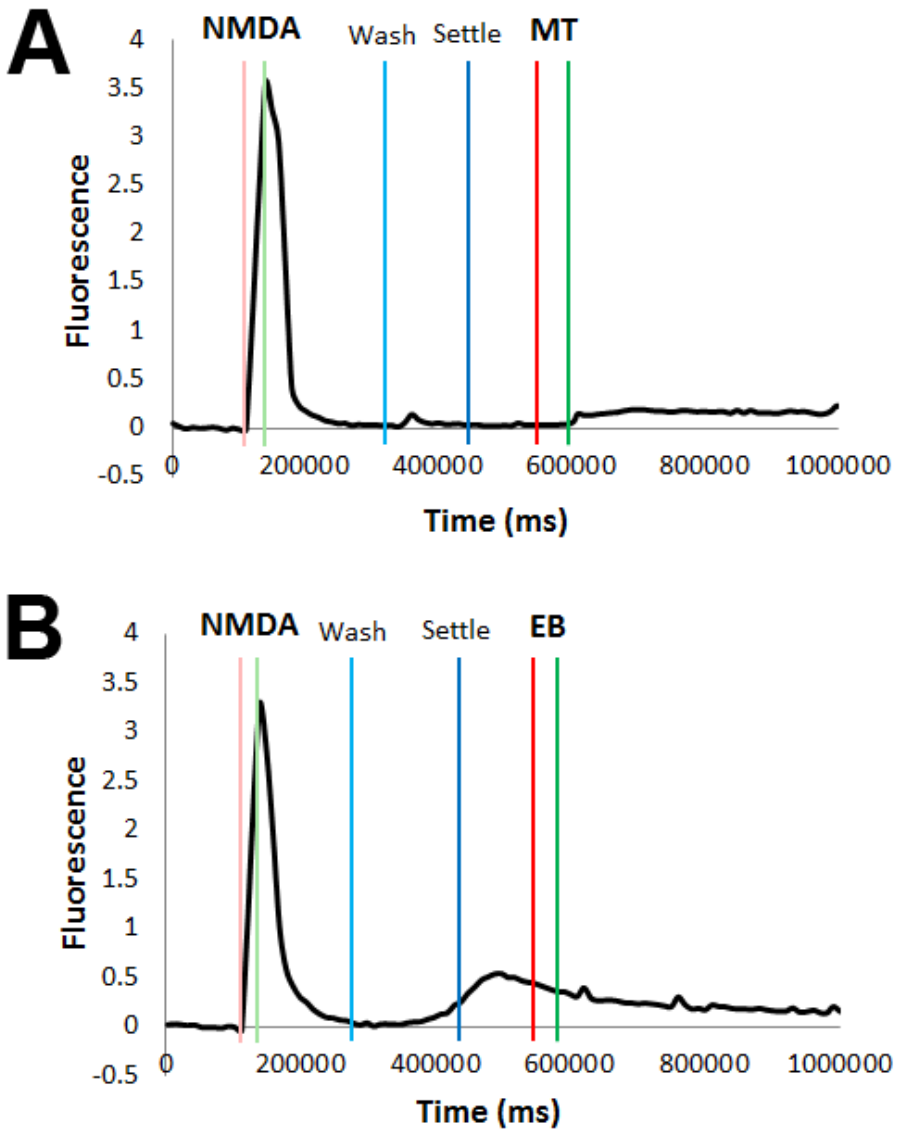

Figure 3.13: 10µg/mL MT or 25µM emtinB do not induce calcium influx in NMDA responsive hippocampal neurons at 14-15 day *in vitro*. Hippocampal neurons were loaded with 5µM Fluo-4 calcium indicator and imaged every ten seconds. Baseline fluorescence was determined by imaging cells for two minutes at the start of the experiment. Imaging was paused (faded red line) as 10µM NMDA was added and imaging resumed (faded green line) for an additional 2-3 minutes. Cells were washed for 2 minutes (light blue line) before the wash was stopped (dark blue) and cells allowed to settle for 2 minutes. Imaging was paused again (red line) and 10µg/mL MT (A) or 25µM emtinB (B) added and imaging resumed (green line) for 6.5 minutes. NMDA caused an immediate increase in intracellular calcium that quickly returned to near baseline, but MT or emtinB did not cause any change in intracellular calcium.

3.4 ***DISCUSSION***

Western blot and immunocytochemistry analysis have demonstrated expression of LRP1 in hippocampal neurons cultures at 3, 7, 14 and 21DIV. The receptor was expressed upon cell bodies and dendrites of all neurons at all time points. Expression of LRP1 was present along and at the ends of axons of 3DIV neurons. Some axons at 7DIV were also LRP1 positive, but axons at 14DIV and 21DIV did not express the receptor. LRP1 did not co-localise with pre- or post-synaptic proteins from 7DIV to 21DIV or with the NMDA receptor subunit NR2a at 14DIV. However, it did appear to co-localise partially with the NR2b NMDA receptor subunit at 14DIV. About 50% of hippocampal neurons at 14-15DIV responded to NMDA and glutamate by demonstrating an immediate calcium influx following neurotransmitter addition and were therefore deemed to be synaptically active. LRP1 ligands, MT and emtinB, did not induce calcium influx in NMDA responsive neurons. LRP1 was also expressed on cell bodies and processes of astrocytes, oligodendrocytes and microglia in hippocampal neuron cultures.

3.4.1 THE CLEAVAGE PATTERN OF LRP1 CHANGES DURING MATURATION OF HIPPOCAMPAL NEURON CULTURES

A number of LRP1 positive bands between ~37kDa and ~150kDa were detected in hippocampal neuron cultures at 3, 7, 14 and 21DIV. As for *in vivo* western blots (Chapter 2.3.1), the LRP1 antibody used targeted the C-terminal end of the intracellular domain. Therefore, the different molecular weighted bands correspond to LRP1 light chain forms that have undergone different post-translational modifications. Post-translational modifications of LRP1 can include cleavage by β- and γ-secretase (May et al., 2002, von Arnim et al., 2005), phosphorylation of NPxY motifs (Barnes et al., 2001, Guttman et al., 2009) and glycosylation (Quinn et al., 1999, May et al., 2003).

Modification of LRP1 by these mechanisms regulates the receptors' function by affecting intracellular proteins ability to bind to it and the ability of the intracellular domain to translocate to the nucleus (Trommsdorff et al., 1998, Gotthardt et al., 2000, Kinoshita et al., 2003, Guttman et al., 2009).

Previous *in vitro* work with cerebellar granule neurons demonstrated immunoreactivity on western blots at ~85kDa which corresponds to the LRP1 light chain (Ambjørn et al., 2008). The ~85kDa band was the strongest band expressed in hippocampal neuron cultures at every time point. Though the LRP1 light and heavy chains are normally non-covalently bound to form a 600kDa product, these domains most likely dissociate during western blot preparation, resulting in the detection of the light chain. Bands at ~120kDa, ~130kDa and ~150kDa were observed in hippocampal neuron cultures during maturation. As these bands have higher molecular weight than the ~85kDa LRP1 light chain it is reasonable to assume that these forms of the intracellular domain have undergone phosphorylation or glycosylation. Molecular weight bands of LRP1 greater than ~85kDa have been observed *in vivo* previously (Wolf et al., 1992). However, very few studies have used western blots to identify LRP1 expression *in vitro*. Of the studies that have used western blot analysis, the presence of higher molecular weight bands was not mentioned (Ambjørn et al., 2008) or the molecular weight of LRP1 positive bands observed were not mentioned at all (Hayashi et al., 2007, Fuentealba et al., 2009).

LRP1 positive bands observed between ~37kDa and ~50kDa, being lower molecular weight than the ~85kDa, are likely to be cleaved forms of the LRP1 light chain. Cleavage of the extracellular domain of LRP1 by β-secretase releases a ~30kDa fragment (von Arnim et al., 2005). This fragment can be further modified by phosphorylation of the distal NPxY motif or glycosylation (Barnes et al., 2001, Guttman et al., 2009). Glycosylation of the LRP1 light chain has been reported previously to

yield LRP1 positive fragments of ~55kDa from trophoblast cell lines (Quinn et al., 1999). Therefore, glycosylation, and possibly phosphorylation, of the cleaved light chain fragment could produce the bands observed between ~37kDa and ~50kDa.

Expression of the ~85kDa LRP1 band appeared weakest at 3DIV, but increased and remained consistent from 7 to 21DIV. This is in contrast to *in vivo* data that demonstrated greater expression of the ~85kDa fragment in P2 and P7 hippocampi when compared to the adult rat hippocampus (Chapter 2.3.1). Hippocampal neurons were cultured from embryonic day 17-18 rats. It may be that LRP1 expression is lower at this stage of development than in P2 or P7 rats. However, northern blot analysis demonstrates greater expression of LRP1 mRNA in embryonic day 18 and 21 rat brains than in post-natal day 3 rat brain (Ishiguro et al., 1995). Whether this corresponds to protein expression in hippocampal neurons is unknown. While mRNA expression of the receptor throughout whole brain may be greater during embryonic development, protein expression may be lower. In addition, the expression of LRP1 in the hippocampus specifically may be lower in the embryo. Alternatively, the trauma of dissection and dissociation during culture preparation may cause a reduction in LRP1 expression that is recovered during the first week of culture.

A ~120kDa band was present in 3DIV and 7DIV cultures, but this band became fainter in 14DIV and 21DIV cultures. The function of this fragment could be in initial neurite outgrowth and establishment of neuronal networks throughout the culture. LRP1 can regulate neurite outgrowth in other neuron subtypes. Outgrowth of cerebellar granule neurons is inhibited following 48 hours treatment with myelin associated glycoprotein, an effect abolished by siRNA against LRP1 (Stiles et al., 2013). In contrast, α2-macroglobulin promotes neurite outgrowth and increases LRP1 expression in cortical neuron cultures, which is inhibited by RAP (Qiu et al., 2004, Shi et al.,

2009). Therefore, the ~120kDa LRP1 positive band in developing cultures may regulate neurite outgrowth of hippocampal neurons.

At 14DIV there was the appearance of bands at ~37kDa, ~50kDa, ~130kDa and ~150kDa. The ~37kDa and ~50kDa bands became stronger at 21DIV though the ~130kDa and ~150kDa bands were weaker when compared to 14DIV. The appearance of lower weight molecular bands in more mature hippocampal neuron cultures corresponds to *in vivo* data. The presence of lower weight bands was observed in the adult hippocampus but not in P2 or P7 hippocampus (Chapter 2.3.1). Hippocampal neurons become increasingly synaptically mature from 14DIV to 21DIV and these fragments may be involved in synaptic function of these neurons. Addition of tPA to hippocampal neuron cultures induces NMDA receptor dependent calcium influx, which is inhibited when LRP1 is dysfunctional (Martin et al., 2008). 48 hour treatment of hippocampal neurons with α2-macroglobulin and lactoferrin inhibits NMDA mediated calcium influx. This was abolished by RAP (Qiu et al., 2002). These findings demonstrate the ability of LRP1 to regulate NMDA receptor activity. The low molecular weight fragments present in 14DIV and 21DIV cultures that are not present at 3DIV or 7DIV may be involved in this function. However, from 14DIV the proportion of neurons to glia drops significantly (14DIV: 40% neurons, 60% glia; 21DIV: 25% neurons, 75% glia). This is due to proliferation of glia and apoptosis of neurons. It is possible that these lower molecular weight forms are expressed in glia and become stronger as the percentage of these cells increase. LRP1 regulates the ability of astrocytes, oligodendrocytes and microglia to phagocytose myelin vesicles (Gaultier et al., 2009). Microglia are also able to phagocytose apoptotic cerebellar granule neurons following lipopolysaccharide and β-amyloid treatment (Fricker et al., 2012). Therefore, low molecular weight fractions of LRP1 may be expressed in glia that are involved in phagocytosis of apoptotic neurons as cultures age.

Changes in the post-translational modification of LRP1 during maturation in culture partially reflect what is observed in the hippocampus *in vivo*. Though the precise function of these LRP1 fractions is unknown, these data demonstrate a change in function of the receptor as cultures develop. This indicates that investigation into the role of LRP1 during neuronal maturation is possible in culture.

3.4.2 LRP1 IS EXPRESSED IN CELL BODIES AND PROCESSES OF HIPPOCAMPAL NEURONS FROM 3 TO 21DIV

LRP1 was expressed upon cell bodies and dendrites of all hippocampal neurons from 3DIV to 21DIV. The receptor was present along and at the ends of extending axons at 3DIV but axonal expression was reduced in 7DIV neurons and absent in 14DIV and 21DIV cultures. This pattern of expression was consistent with previous reports. Brown and colleagues (1997) demonstrated the presence of LRP1 on rat hippocampal neuron cell bodies and processes from 1DIV. Axonal expression of LRP1 was present on axons of 3DIV, but only residual expression was found on 5DIV cultures and was absent by 7-8DIV. From this time onwards LRP1 was restricted to neuronal cell bodies, dendrites and dendritic spines. While Brown's study found no expression at 7DIV, the present data did demonstrate this, though to a lesser degree than at 3DIV. These discrepancies could be due to technical differences such as the antibody used. Otherwise, the expression pattern of LRP1 is consistent between the two studies and demonstrates reproducibility of LRP1 expression in hippocampal neuron cultures.

LRP1 is expressed on hippocampal neuron cell bodies and apical segments of dendrites in the adult rat brain (Chapter 2.3.2). Similarly, LRP1 is identified on hippocampal neuron cell bodies and dendrites *in vitro*. However, dendritic expression is also located at the distal ends of dendrites *in vitro* in both developing and mature cultures. Hippocampal neurons in P2 rat brains displayed little dendritic LRP1 immunoreactivity, and P7 neurons had none. Cultures at 3DIV represent a stage of

development younger than P2 brains when neurites are extending through their environment to form neuronal connections. This better represents embryonic development and suggests that the receptor may be expressed along and at the ends of neurites as they extend processes through the brain during this stage of development. The expression of LRP1 at growth cones of extending hippocampal neuron dendrites at 3DIV suggests that the receptor may be involved in neurite extension.

LRP1 mediated neurite extension has been demonstrated in DRGs, cerebellar granule cells and cortical neurons (Qiu et al., 2004, Stiles et al., 2013, Yamauchi et al., 2013). Cortical neuron outgrowth is increased by 71% following 24 hours incubation with α2-macroglobulin. Cerebellar granule neuron neurite outgrowth is almost 3 times greater following 48 hour treatment with the ligand binding region of α2-macroglobulin. Outgrowth in both these cell types was inhibited by RAP (Qiu et al., 2004, Shi et al., 2009). In contrast, 48 hour treatment with myelin-associated glycoprotein inhibits cerebellar granule neuron neurite length to 40% of untreated controls. These effects were inhibited by RAP and siRNA against LRP1 (Stiles et al., 2013). These studies demonstrate that LRP1 is able to both promote and inhibit neurite outgrowth. Both these processes need to occur in order to establish functional neuronal connections and suggest a role for LRP1 in regulation of developmental neurite outgrowth.

Expression of LRP1 *in vitro* was most prominent at the cell body, consistent with *in vivo* observations. Its role at this subcellular location could be in neuronal survival, in which it has been implicated previously. Hippocampal neurons are protected from amyloid-β toxicity through treatment with apoE bound cholesterol, effects that are inhibited by RAP and antibodies against LRP1 (Sen et al., 2012). ApoE3 and apoE4 bound lipoproteins promote retinal ganglionic cell survival following trophic withdrawal and glutamate toxicity. Antibodies or siRNA against LRP1 suppressed this protection (Hayashi et al., 2007, Hayashi et al., 2012). In addition, LRP1 knock-down in

mixed cortical and hippocampal neuron culture results in increased susceptibility to trophic withdrawal mediated apoptosis and amyloid-β toxicity. However, knock-down did not affect untreated neuron viability (Fuentealba et al., 2009). Therefore, LRP1 may not be essential for survival of hippocampal neurons in healthy conditions, but becomes vital in cases of stress. LRP1 may be expressed upon hippocampal neurons in culture to inhibit their apoptosis in response to stressful or toxic stimuli.

In addition to expression at the cell body, LRP1 was immunoreactive along dendrites at all time points *in vitro.* Dendrites receive and interpret incoming neuronal signals, and LRP1 expression along these structures suggests that the receptor may be involved in this process. The formation of mature hippocampal synapses *in vitro* begins at about 12DIV and continues to 21DIV, though the construction of synapses begins as early as 2DIV (Rao et al., 1998). The function of dendritic LRP1 could be in synaptic function.

3.4.3 LRP1 DOES NOT CO-LOCALISE WITH PSD-95, SYNAPTOPHYSIN OR NR2A BUT DOES PARTIALLY CO-LOCALISE WITH NR2B

LRP1 has been implicated in regulation of synaptic activity due to its ability to associate with synaptic proteins and modulate calcium influx in neurons in response to neurotransmitters. Previous studies have used immunoprecipitation and pull down assays to demonstrate an association between LRP1 and PSD-95 (Gotthardt et al., 2000, May et al., 2004), an interaction that may occur through binding of the LRP1 distal NPxY motif to PSD-95. This in turn might lead to the association of LRP1 and the NMDA receptor and may be how LRP1 regulates NMDA and glutamate mediated calcium influx (Bacskai et al., 2000, Martin et al., 2008, Hayashi et al., 2012). In the present study, LRP1 did not co-localise with PSD-95 or the pre-synaptic protein, synaptophysin, in hippocampal neurons at 7, 14 or 21DIV, though this latter observation was expected as LRP1 is not present on axons where pre-synaptic sites are located. The

association of LRP1 with PSD-95 is believed to lead to the interaction of LRP1 and the NMDA receptor. May and colleagues (2004) found that LRP1 co-localised directly with the NMDA receptor subunit, NR2a, in hippocampal neurons *in vitro*, though the age of these cultures was not specified. LRP1 did not co-localise with NR2a in 14DIV hippocampal neurons in the current culture model, which may be expected as it did not associate with PSD-95. 14DIV was chosen as a time point to investigate LRP1 and NMDA receptor interactions as hippocampal neurons begin to become synaptically mature at about 12DIV, and full synaptic maturity occurs between 15-21DIV (Rao et al., 1998). The age of May and colleagues' (2004) hippocampal neurons in which co-localisation occurred may have been at a later stage of synaptic maturity. Alternatively, co-localisation of LRP1 with post-synaptic proteins may only occur when stimulated by either a ligand or neurotransmitter such as NMDA or glutamate.

In hippocampal neurons, α2-macroglobulin has no immediate effect on calcium influx upon addition, but 48 hours incubation with this ligand reduces NMDA mediated calcium influx, effects inhibited by RAP (Qiu et al., 2002, Qiu et al., 2004). In contrast, tPA directly stimulates NMDA receptor mediated calcium influx in hippocampal neurons. This is inhibited by both RAP and mutation to the distal NPxY LRP1 motif (Martin et al., 2008). These findings demonstrate that LRP1 regulation of NMDA receptor mediated calcium influx is ligand specific and may provide some insight as to why LRP1 and PSD-95 do not co-localise in unstimulated neurons. This interaction may only occur when stimulated by a specific ligand. However, despite lack of co-localisation with PSD-95 or NR2a, LRP1 did partially co-localise with the NR2b subunit.

The NMDA receptor is a heterotetramic complex consisting of at least one NR1 subunit and one or more of the NR2 subunits, NR2a, NR2b, NR2c or NR2d (Moriyoshi et al., 1991, Monyer et al., 1992, Ishii et al., 1993). NR2 subunits are defined by

different calcium kinetics and pharmacological properties and display development and regional specificity (Ishii et al., 1993, Monyer et al., 1994, Vicini et al., 1998). During development, NR2b is the predominant NR2 subunit expressed throughout the brain. During post-natal maturation, expression of NR2a at synapses increases while NR2b expression decreases at these sites (Liu et al., 2004). Excitatory post-synaptic currents (EPSCs) produced by NR1/NR2a channels are shorter in duration than NR1/NR2b channels. As the ratio of NR2a to NR2b channels increases during early post-natal development, EPSC duration decreases (Flint et al., 1997, Stocca and Vicini, 1998, Vicini et al., 1998, Lu et al., 2001). The longer duration EPSCs mediated by NR2b channels are believed to be through association of the subunit with CaMKII. CaMKII is activated by calcium influx following NMDA receptor activation and is essential for initiation of long-term potentiation (Reymann et al., 1988, Malenka et al., 1989, Giese et al., 1998). CaMKII has a lower affinity for binding to NR2a than NR2b (Leonard et al., 1999). The shift in EPSC potential between NR2a and NR2b channels may be associated with the decrease in synaptic plasticity between the immature and mature brain (Sheng et al., 1994). The partial co-localisation of LRP1 with NR2b subunits suggests that the receptor could have a role in developmental synaptic activity and plasticity. However, *in vivo* data demonstrate expression of LRP1 at the apical segments of dendrites in the adult brain but very little expression in dendrites of P2 and P7 hippocampal neurons (Chapter 2.3.2). In addition, co-localisation *in vitro* occurred at 14DIV when neuronal networks were reasonably mature. These findings argue against a role for LRP1 in developmental synaptic function. However, it may have a role in NR2b receptor function in the mature brain. Though NR2b is primarily expressed in developing brains, it is present both synaptically and extrasynaptically in the mature brain (Thomas et al., 2006).

Extrasynaptic NMDA receptors demonstrate different pharmacological and signaling properties to synaptic receptors. Extrasynaptic NMDA receptors preferentially bind glycine and are involved in propagation of long-term depression, whereas synaptic receptors have a higher affinity for D-serine and modulate long-term potentiation (Papouin et al., 2012). Astrocytes also preferentially activate extrasynaptic NMDA receptors to produce EPSCs. These display slow EPSC kinetics, suggesting that extrasynaptic NMDA receptors are composed predominately of NR1/NR2b rather than NR1/NR2a subunits (Fellin et al., 2004). LRP1 co-localisation with NR2b *in vitro* could be specific for either synaptic or extrasynaptic sites and may regulate the function of NMDA receptors at these sites. If the association is specific for one of these sites it may account for the partial co-localisation of LRP1 and NR2b. Alternatively, LRP1 may only associate with NR2b at mature, active synapses.

About 50% of hippocampal neurons were determined to be synaptically active at 14DIV as they demonstrated calcium influx consistent with synaptic activation following NMDA or glutamate addition and this may account for the partial co-localisation of LRP1 and NR2b. If left for longer *in vitro* the percentage of NMDA and glutamate responsive neurons would most likely increase and the co-localisation of LRP1 with NR2b may increase. In addition, the receptor may also be found to associate with other synaptic proteins such as PSD-95 or NR2a as the neurons become more synaptically mature.

3.4.4 LRP1 LIGANDS, MT AND EMTINB, DO NOT INDUCE CALCIUM INFLUX IN HIPPOCAMPAL NEURONS

A number of ligands induce NMDA receptor dependent calcium influx through LRP1. Immediately following addition of tPA and α2-macroglobulin, intracellular calcium concentration increases in hippocampal and cortical neurons, respectively. In both cases, RAP and the NMDA receptor inhibitor, MK801, abolished calcium

responses (Bacskai et al., 2000, Martin et al., 2008). Another LRP1 ligand, MT, is also able to regulate calcium signaling via the receptor. Calcium concentration in dorsal root ganglion growth cones is increased following addition of MT, effects inhibited by siRNA against LRP1 (Landowski et al., 2012). However, both ligands failed to induce any calcium response in NMDA responsive hippocampal neurons.

The effects of LRP1 ligands on calcium signaling appear to be both ligand and cell specific. tPA induces an immediate LRP1 and NMDA receptor dependent calcium response in hippocampal neurons but has no immediate effect on cortical neurons (Martin et al., 2008, Samson et al., 2008). A similar effect is observed for α2-macroglobulin which induces an immediate calcium influx following addition to cortical neurons but not hippocampal neurons (Bacskai et al., 2000, Qiu et al., 2002). Interestingly, chronic treatment with α2-macroglobulin for up to 48 hours reduced NMDA mediated calcium influx in hippocampal neurons (Qiu et al., 2002). Lactoferrin also inhibits hippocampal neuron NMDA receptor dependent calcium influx following 48 hour incubation, but has no direct effect on calcium concentration in cortical neurons (Bacskai et al., 2000, Qiu et al., 2002). Therefore, while MT and emtinB may not induce an immediate calcium response in hippocampal neurons, they could directly affect calcium influx in other neurons such as cortical neurons.

3.4.5 LRP1 EXPRESSION IN ASTROCYTES, OLIGODENDROCYTES AND MICROGLIA OF HIPPOCAMPAL NEURON CULTURES

LRP1 was expressed in the cell bodies and processes of astrocytes, oligodendrocytes and microglia in hippocampal neuron cultures from 3 to 21DIV. This is consistent with data demonstrating that LRP1 is expressed on glia *in vivo* throughout development (Chapter 2.3.2). Astrocytes, oligodendrocytes and microglia have been found to express LRP1 *in vitro* previously (Marzolo et al., 2000, Gaultier et al., 2009).

The few studies that have investigated the role of LRP1 in glia *in vitro* have provided evidence that the receptor regulates phagocytosis and immune responses in these cells.

Astrocytes, oligodendrocytes and microglia are all able to phagocytose myelin vesicles by a LRP1 dependent mechanism (Gaultier et al., 2009). A microglial cell line phagocytoses heat shock treated necrotic oligodendrocytes, an effect that is reduced by 35% when microglia were pre-treated with RAP. In addition, LRP1 is able to bind components of myelin normally located intracellularly (Fernandez-Castaneda et al., 2013). This demonstrates a role of the receptor in recognising and removing necrotic cell debris from the extracellular environment. The function of the receptor on microglia *in vitro* could be in the removal of cell debris caused by culture preparation and apoptotic neurons. This is supported by data that demonstrate the ability for microglia to phagocytose cerebellar granule neurons in response to lipopolysaccharide or amyloid-β induced apoptosis. These effects were inhibited by both RAP and antibodies against LRP1 (Fricker et al., 2012).

The expression of LRP1 in microglia in hippocampal neuron cultures was heterogeneous and did not appear to be specific to different cell morphologies. Some cells expressed LRP1 strongly while others only expressed the receptor faintly. Heterogeneous expression of LRP1 in microglia *in vivo* was not observed, but it may be that immunohistochemistry was not sensitive enough to detect this. The expression of LRP1 in microglia *in vitro* is in agreement with previous work that demonstrates heterogeneous expression of LRP1 in pure microglia cultures. In this same study, treatment with the anti-inflammatory agent dexamethasone increased LRP1 expression, which subsequently became homogeneous. Treatment with pro-inflammatory agents lipopolysaccharide and interferon-γ reduced LRP1 expression (Marzolo et al., 2000). These findings demonstrate the ability of LRP1 to respond to inflammatory factors and possibly contribute to an immune response. Microglia activation is response to

lipopolysaccharide can also be promoted by the LRP1 ligand tPA. Activation of tPA-deficient microglia in cortical neuron cultures is inhibited, demonstrated by decreased activated morphology and tumour necrosis factor-α secretion (Rogove et al., 1999). These data demonstrate that microglial LRP1 is able to respond to inflammatory mediators *in vitro*, and that in some instances this could be through stimulation by ligands such as tPA. These *in vitro* findings support a role of LRP1 in microglial mediated immune responses.

Few studies have investigated the function of LRP1 in astrocytes previously. Expression of LRP1 is decreased in astrocytes when incubated with lipopolysaccharide and interferon-γ in combination. However, there was no change in expression following treatment with either of these inflammatory agents alone or with dexamethasone (Marzolo et al., 2000). Therefore, LRP1 could be involved in astrocyte responses in strong pro-inflammatory conditions. Astrocytes are also involved in neurotransmission and release glutamate following calcium influx (Parpura et al., 1994). Though LRP1 is able to regulate calcium influx in neurons (Bacskai et al., 2000, Qiu et al., 2002, Martin et al., 2008, Hayashi et al., 2012), whether it does this in astrocytes to modulate glutamate release of these cells is unknown.

The function of LRP1 in oligodendrocytes of hippocampal neuron cultures may not be in phagocytosis. Oligodendrocytes that phagocytosed myelin vesicles in previous work were identified as CNPase positive (Gaultier et al., 2009). CNPase is an enzyme involved in myelin synthesis (Kim and Pleasure, 1978), and is expressed at a more maturate stage of differentiation than the oligodendrocyte appear to be in the present culture model. The establishment of hippocampal neuron and oligodendrocyte co-culture to study myelination requires the addition of oligodendrocytes to 14DIV neurons and the subsequent maturation of cells for up to 21 days (Gardner et al., 2012). It is therefore unlikely that oligodendrocytes in the current culture model are actively

myelinating hippocampal neurons. By determining the maturation stage of oligodendrocytes using markers other than olig2, such as CNPase, and labelling cultures for myelin would confirm whether this is the case. Oligodendrocytes appear to proliferate as cultures mature, suggesting that the majority of them are progenitor cells as more mature oligodendrocytes do not proliferate (reviewed by (Baumann and Pham-Dinh, 2001)). Whether LRP1 is involved in regulating proliferation of these cells is unknown. Given that LRP promotes Schwann cell migration (Mantuano et al., 2010) it is more likely that the receptor is involved in oligodendrocyte progenitor cell migration, an important function of these cells if they are to connect to and myelinate neurons.

3.5 *CONCLUSIONS*

LRP1 is expressed in hippocampal neuron cultures at 3, 7, 14 and 21DIV. The post-translational modifications of the receptor changes as the cultures mature. These differences may reflect changes in regulation of the receptor during neuron and glia maturation.

Immunoreactivity is present along and at the ends of axons and dendrites of 3DIV neurons, suggesting a function of the receptor in developmental neurite outgrowth. LRP1 expression is reduced in axons of 7DIV and absent from 14DIV and 21DIV cultures. Localisation of LRP1 remains at the cell body and along dendrites at all time points where it may have a role in neuronal survival or synaptic signaling. However, LRP1 did not co-localise with PSD-95 or synaptophysin at any time point, or with NR2a at 14DIV. Partial co-localisation with NR2b was observed at 14DIV, suggesting it may be involved in synaptic activity of these NMDA receptors. 50% of hippocampal neurons were synaptically active, demonstrated by responses to NMDA and glutamate. Older cultures that are completely synaptically active may demonstrate greater co-localisation of LRP1 with NR2b and possibly with other synaptic proteins. In addition, stimulating neurons with neurotransmitters or LRP1 ligands may induce co-localisation with other synaptic proteins. LRP1 was also expressed upon astrocytes, oligodendrocytes and microglia *in vitro*, supporting *in vivo* data and implicating a function for the receptor in these cells.

LRP1 ligands have been found to induce calcium influx in cortical and hippocampal neurons previously in an NMDA receptor dependent manner. However, two LRP1 ligands that affect calcium signaling in dorsal root ganglion growth cones, MT and emtinB, did not induce calcium influx in hippocampal neurons.

The subcellular expression of LRP1 in hippocampal neurons adds further support for a role of the receptor in neuronal biology. This may be in neurite outgrowth,

neuronal survival or functioning of NR2b containing NMDA receptors. Establishing and characterising an *in vitro* neuron model of LRP1 expression allows further investigation into the receptor's function in processes such as neurite outgrowth, injury, toxicity or synaptic function.

4 Chapter 4: Effects of the LRP1 ligands, MT and emtinB, on hippocampal neurite extension following scratch injury

4.1 *INTRODUCTION*

LRP1 is expressed on rat hippocampal neurons both *in vivo* and *in vitro* as reported in chapters 2 and 3 respectively. The receptor is expressed in the cell body and dendrites of hippocampal neurons at 3, 7, 14 and 21DIV. LRP1 expression on axons is present at 3DIV, reduced at 7DIV and absent from 14DIV and 21DIV cultures. The presence of LRP1 at the ends of extending neurites in 3DIV cultures suggests that it is involved in hippocampal neurite outgrowth during development (Chapter 3.3.2). Given that LRP1 is expressed at ends of developing neurites, it could also be expressed in regenerating neurites following injury. This is suggested by previous work which demonstrates that LRP1 is necessary for apoE containing lipoprotein mediated axon extension of axotomised retinal ganglion cells. Addition of apoE containing lipoproteins promoted axon extension by 50%, but only when applied to axons. When applied to cell bodies axon extension was the same as for controls (Hayashi et al., 2004, Matsuo et al., 2011). This is evidence that LRP1 located on injured axons mediates neurite extension.

Another LRP1 ligand, MT, is also able to promote neurite regeneration following injury. MT increases the average length of cortical neurites 12 hours after scratch injury from 41.5 ± 1.7μm to 70.1 ± 3.4μm. Regeneration of *in vivo* cortical needlestick injury after 4 days is also enhanced with application of MT (Chung et al., 2003). MT also increases regenerative sprout length of dorsal root ganglion cells by 66% when applied to axons following scratch injury. Addition of MT to cell bodies produced an even greater increase in sprout length by almost fourfold (Leung et al., 2011). Whether MT promotes regeneration of hippocampal neurites following scratch injury is unknown. 24 hour treatment with MT enhances hippocampal neurite extension

by 1.6 times that of controls (Køhler et al., 2003). Therefore, it is conceivable that it may have an effect on hippocampal outgrowth following injury.

EmtinB, a synthetic peptide modeled after the C-terminus of the MT β-domain (Figure 4.1), also promotes neurite outgrowth, but whether it promotes regeneration following injury is unknown. 24 hour treatment with emtinB increases neurite length of hippocampal and cerebellar granule neurons by 7 and 3 times that of controls, respectively (Ambjørn et al., 2008, Sonn et al., 2010). Investigating whether this peptide has an effect on neurite regeneration may be of therapeutic importance as emtinB is able to cross the blood brain barrier (Sonn et al., 2010) and could be used to promote neuron function following brain injury.

I hypothesise that (1) LRP1 is expressed at the ends of regenerating neurites following scratch injury in hippocampal neuron cultures; and (2) the LRP1 ligands, MT and emtinB, promote extension of neurites following scratch injury.

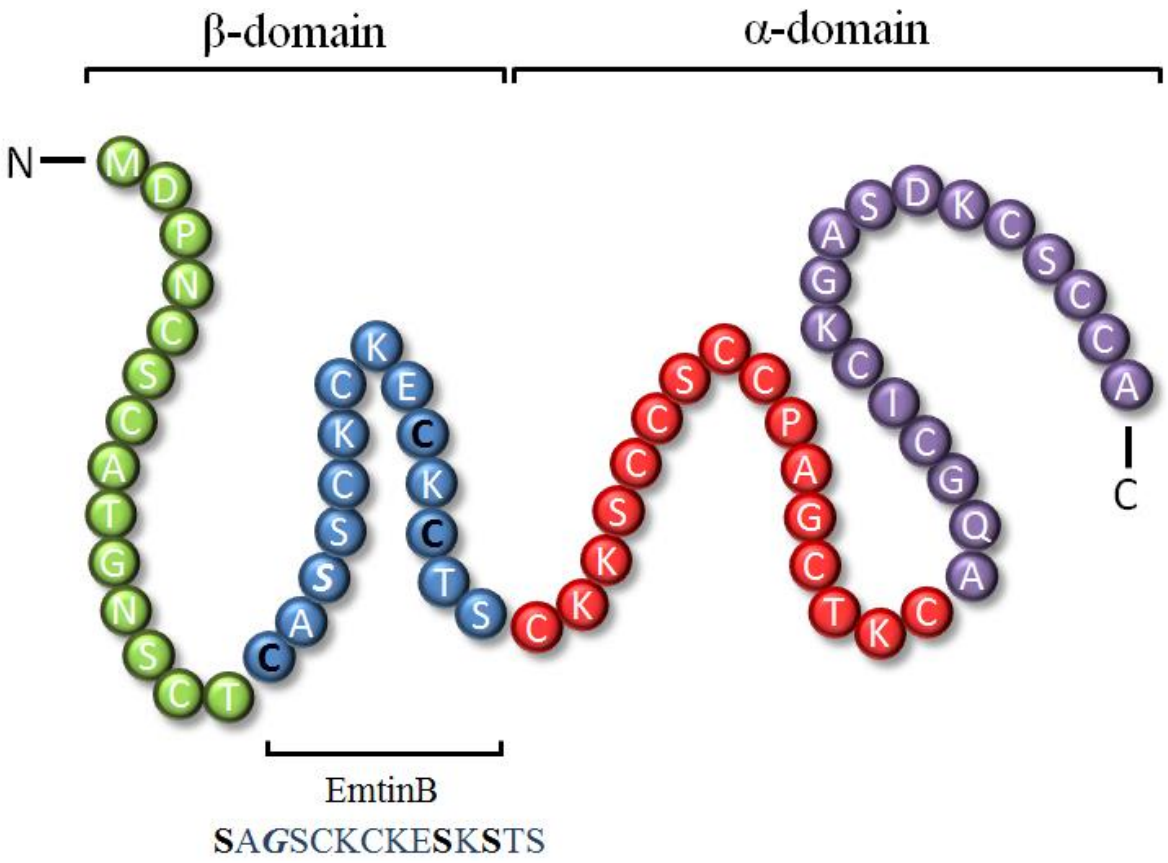

Figure 4.1: Simplified schematic and amino acid sequence of rabbit MT and the synthetic peptide, emtinB. Rabbit MT, used in the experiments of this thesis, is composed of a β- and α-domain. EmtinB is modeled after the C-terminal end of the human β-domain of MT, which possesses a serine residue (***S***) instead of a glycine residue (***G***) that is present in rabbit MT. Some cysteine residues (bold black C's) in the MT sequence were substituted with serine (bold black S's) in emtinB to inhibit inappropriate peptide aggregation.

Adapted from (Asmussen et al., 2009)

Rabbit MT GenBank accession number: 1515428A

4.2 *METHODS*

All experiments involving the use of animals were approved by the Animal Experimentation Ethics Committee of the University of Tasmania (ethics number A0011957), which is in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Buffer recipes and product information for all reagents can be found in appendices.

4.2.1 PRIMARY HIPPOCAMPAL NEURON CULTURE

Primary hippocampal neuron cultures were prepared from embryonic day 17/18 Sprague Dawley rats as described previously (Chapter 3.2.1). Briefly, hippocampi were dissected from embryos and trypsinised using 0.1% trypsin in HBSS at 37°C for 5 minutes. Following trypsin removal, cells were resuspended in 1mL Neurobasal with 10% foetal calf serum and dissociated by pipette. Cells were counted using Trypan Blue exclusion and plated at $5x10^4$ cells/well on 13mm nitric acid etched glass coverslips coated overnight with neat poly-L-lysine in 24 well plates. A full media change to serum free Neurobasal media was conducted 24 hours after plating and every three to four days thereafter. Cultures were maintained at 37°C in humidified air with 5% carbon dioxide.

4.2.2 SCRATCH INJURY AND IMMUNOCYTOCHEMISTRY

Scratch injury was performed on hippocampal neuron cultures at 6DIV and 13DIV using a fine goniotomy knife (Kaisers). Two scratches were made to form an 'X' across each coverslip (Figure 4.2). Cells were fixed either immediately after injury or 24 hours later at 7DIV or 14DIV with 4% paraformaldehyde for 15-20 minutes at room temperature. All coverslips were stored at 4°C in PBS with 0.01% sodium azide.

Cells were protein blocked using 10% goat serum in 0.03% triton X-100 in PBS for 30 minutes at room temperature to inhibit non-specific antibody binding. To assess

whether LRP1 was expressed on regenerating neurites, rabbit polyclonal anti-LRP1 antibodies were incubated with mouse monoclonal antibodies against axons and dendrites. These were either anti-tau, which labels hippocampal neuron cell bodies, dendrites and axons (Dotti et al., 1987), anti-SMI-312, which specifically labels phosphorylated axons (Masliah et al., 1993), or anti-MAP2 antibodies, which specifically labels dendrites (Matus et al., 1981). To determine whether tau positive neurites that extended into or along the scratch injury site were primarily axons or dendrites, rabbit polyclonal anti-tau antibodies and mouse monoclonal anti-SMI-312 or anti-MAP2 antibodies were incubated with cultures. All primary antibodies were diluted together in 10% goat serum in PBS with 0.03% triton X-100 and incubated with cultures for 1 hour at room temperature and overnight at 4°C. Next, cultures were incubated with goat anti-rabbit AlexaFluor®-594 and goat anti-mouse AlexaFluor®-488 secondary antibodies (both Molecular Probes, USA) for one hour at room temperature before counterstaining with 1μg/ml nuclear yellow. Coverslips were washed three times by gentle shaking in PBS for 5 minutes following each antibody incubation. Coverslips were mounted to slides using fluorescent mounting media and dried overnight. At least 1 coverslip from 3 separate cultures were prepared for each antibody combination.

Photographs of scratch injuries were taken using an Olympus BX50 microscope and Photomatrix Cool Snap HQ^2 camera. Photographs of 7DIV and 14DIV cultures 24 hours after scratch injury were taken at 60X objective to assess LRP1 expression at extending neurites. Representative images of cultures 0 or 24 hours after injury at 6DIV or 7DIV, respectively, were captured at 20X objective. All images were uniformly enhanced and analysed using Adobe Photoshop (v11.0.2).

Whether the LRP1 ligands MT and emtinB promote neurite extension following injury was investigated. 10μg/mL MT has been shown to promote neurite outgrowth

previously (Køhler et al., 2003, Fitzgerald et al., 2007, Ambjørn et al., 2008, Chung et al., 2008). Our lab has demonstrated that 25μM emtinB ameliorates β-amyloid toxicity in 7DIV hippocampal neuron cultures (Eaton et al., 2012). Concentrations between 200-500nM RAP have been used previously to inhibit LRP1 mediated neurite outgrowth and regeneration (Hayashi et al., 2004, Qiu et al., 2004, Stiles et al., 2013). Therefore, 6DIV cultures were treated with 10μg/mL MT (equivalent to 1.5μM), 25μM emtinB (equivalent to 75μg/mL) or the equivalent volume of saline with or without 500nM RAP (Progen Biotechnik, Germany) immediately prior to the injury. For each treatment 2-3 coverslips were prepared from 3 separate cultures.

Cultures were protein blocked and underwent immunocytochemistry as described above. Briefly, rabbit polyclonal anti-LRP1 and mouse monoclonal anti-tau antibodies were diluted together in 10% goat serum in PBS with 0.03% triton X-100 for 1 hour at room temperature and then overnight at 4°C. Cultures were then incubated with goat anti-rabbit AlexaFluor®-594 and goat anti-mouse AlexaFluor®-488 secondary antibodies, counterstained with nuclear yellow, mounted and dried overnight. Counts for tau positive neurites were analysed from 3 photographs taken at 10X objective in each direction extending from where the two scratch injuries intersected (Figure 4.2). All images were uniformly enhanced and analysed using Adobe Photoshop (v11.0.2).

The majority of neurites were cleanly cut immediately after scratch injury (Figure 4.6 A), and therefore neurites that extended along or entered the injury site 24 hours later were counted as regenerating neurites. It is likely that poly-L-lysine substrate was removed from the coverslip during scratch injury, inhibiting neurite extension across the injury site. As growth across the injury site was impeded, neurites instead extended along the side of the injury. It was difficult to accurately track and measure most individual neurites as they joined and clumped, so quantitation of neurite

extension was conducted by counting the numbers of neurites that extended along or entered the injury site. Neurites were counted blind by one observer and converted to neurites per cm of injury site. Data analysis was performed using Microsoft Excel (v14.0.7015.1000). Data for cultures treated with MT and emtinB were pooled separately and statistics performed using single factor ANOVA. Statistically significant results were indicated by $p<0.05$. Data were expressed as averages ± standard error of the mean.

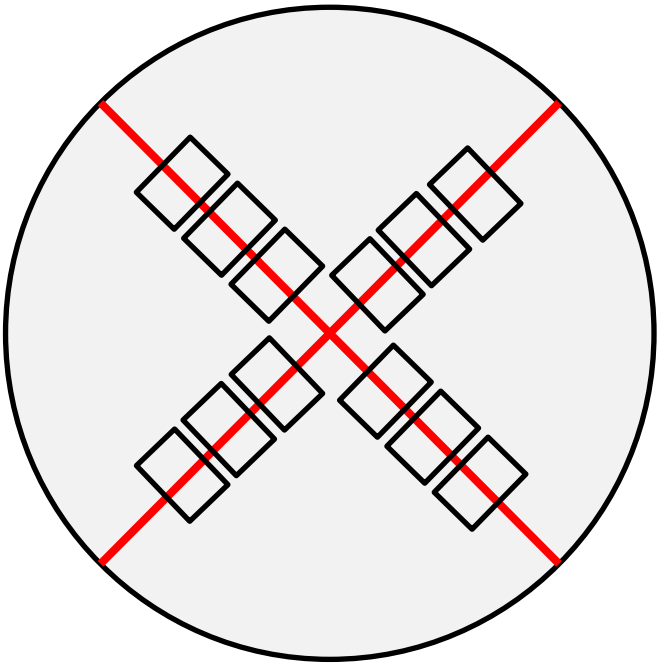

Figure 4.2: Pattern of photographs taken for scratch injury analysis. Three photographs (black boxes) were captured in each direction from where the two scratch injuries (red lines) intersected. Neurites that extended along or into the injury site were counted.

4.3 ***RESULTS***

4.3.1 THE MAJORITY OF REGENERATING TAU POSITIVE NEURITES ARE AXONS AND EXPRESS LRP1

The majority of tau positive neurites were cut cleanly immediately following scratch injury at 6DIV, though some did appear to be 'dragged' slightly along the injury site (Figure 4.3 A). 24 hours after injury some neurites touched, extended along or entered the injury site (Figure 4.3 B). To determine whether these neurites were axons or dendrites, cells were immunolabelled for tau and SMI-312 or MAP2 respectively. The majority of tau positive neurites that extended along or into the injury site were SMI-312 positive (Figure 4.4 A), but very few were immunoreactive for MAP2 (Figure 4.4 B). These results demonstrate that the majority of tau positive neurites that extended along or into the scratch injury site 24 hours after injury were axons.

LRP1 was expressed along and at the ends of most, but not all, tau and SMI-312 positive neurites that extended along or into the scratch injury site at 7DIV (Figure 4.5 A and B). LRP1 immunoreactivity was similar in both tau and SMI-312 positive neurites. Intensity of LRP1 staining was faint along most extending axons, similar to immunoreactivity observed in axons of uninjured cultures. Expression at the growth cone was strong and comparable to LRP1 immunoreactivity detected in dendrites of uninjured cultures. A greater number of axons appeared to express LRP1 in injured cultures compared to uninjured cultures. Some extending tau positive neurites demonstrated LRP1 immunoreactivity at 14DIV (Figure 4.5 C), but the majority did not. Neurites that were LRP1 positive demonstrated faint staining when compared to cell body and dendritic staining of uninjured 14DIV neurons. LRP1 positive neurites that extended across the injury site were not MAP2 positive at 7DIV (Figure 4.6). These results demonstrate that LRP1 is expressed at growth cones of regenerating hippocampal neuron axons following injury.

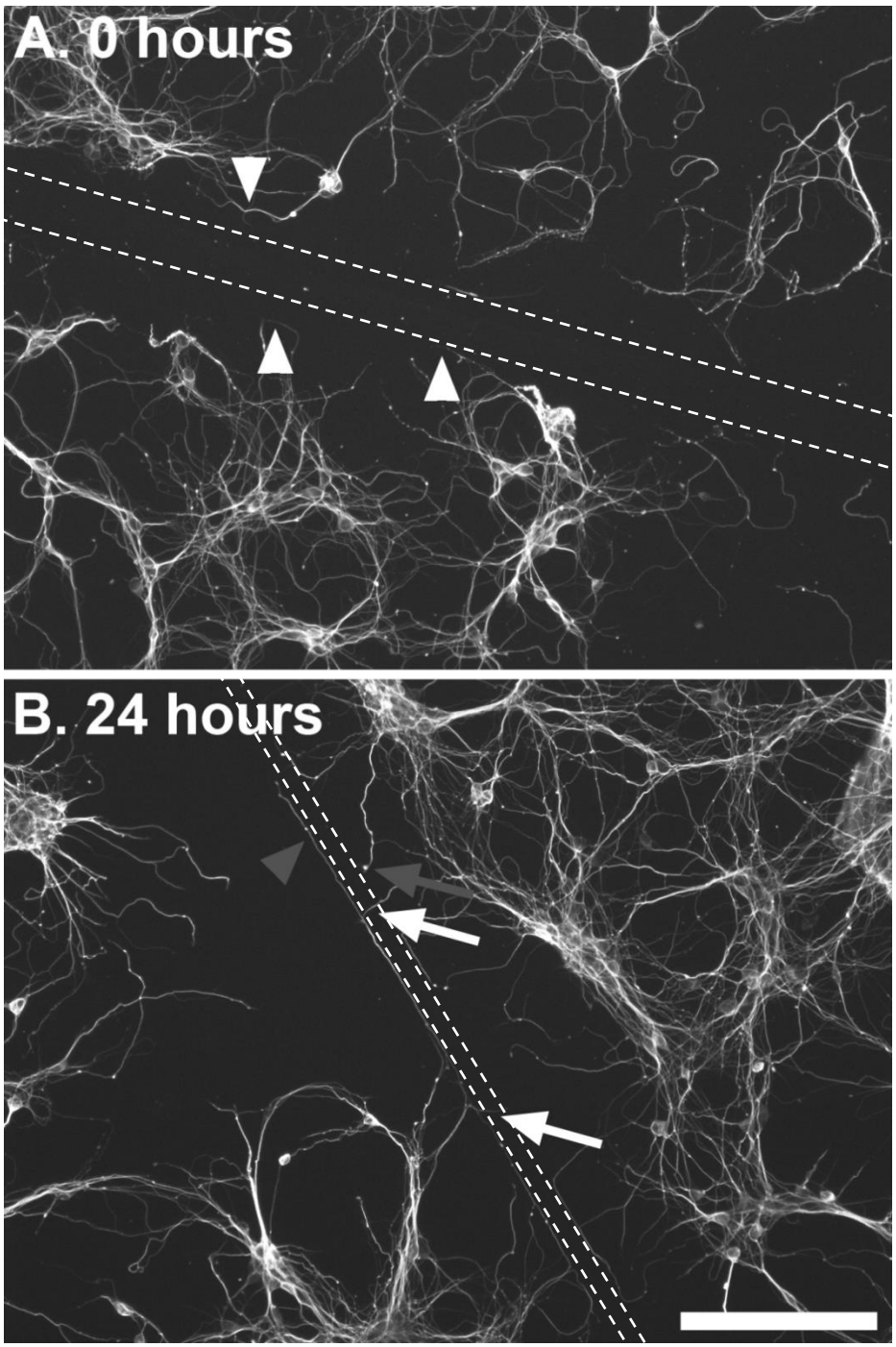

Figure 4.3: The majority of neurites are cut during scratch injury. Hippocampal neurons were grown to 6 days *in vitro* (DIV), fixed at either 0 (A) or 24 hours (B) following scratch injury (white dashed lines) and immunolabelled for tau. Some neurites appeared to be 'pulled' by the scratch injury (A, white arrowheads) but the majority were cut. 24 hours after injury, some neurites appeared to touch (B, grey arrow), follow (B, grey arrowhead) or extend into the injury site (B, white arrow).

Scale bar = 500μm

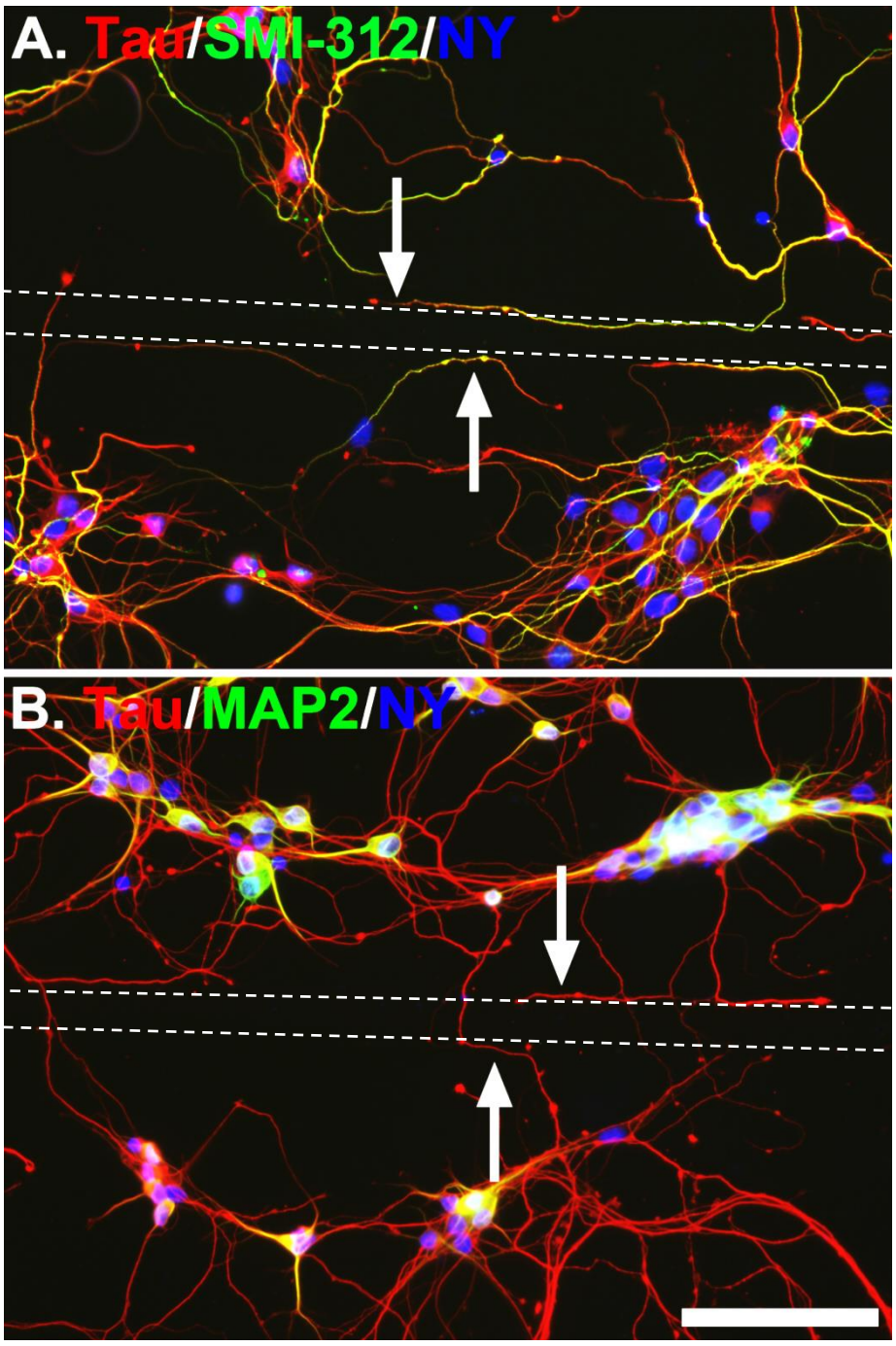

Figure 4.4: The majority of neurites that extend into or along the injury site 24 hours after scratch injury at 7 days *in vitro* (DIV) are axons. Scratch injury was performed on 6DIV hippocampal neuron cultures that were fixed 24 hours later at 7DIV. Cultures were immunolabelled for tau, which labels axons and dendrites (red) and either the axon marker SMI-312 (red; A) or the dendritic marker MAP2 (red; B) and counterstained with nuclear yellow (blue). Tau positive neurites that followed or extended into the injury site (dashed lines) were SMI-312 positive (A arrows), but very few were MAP2 positive (B arrows).

Scale bar = 100µm

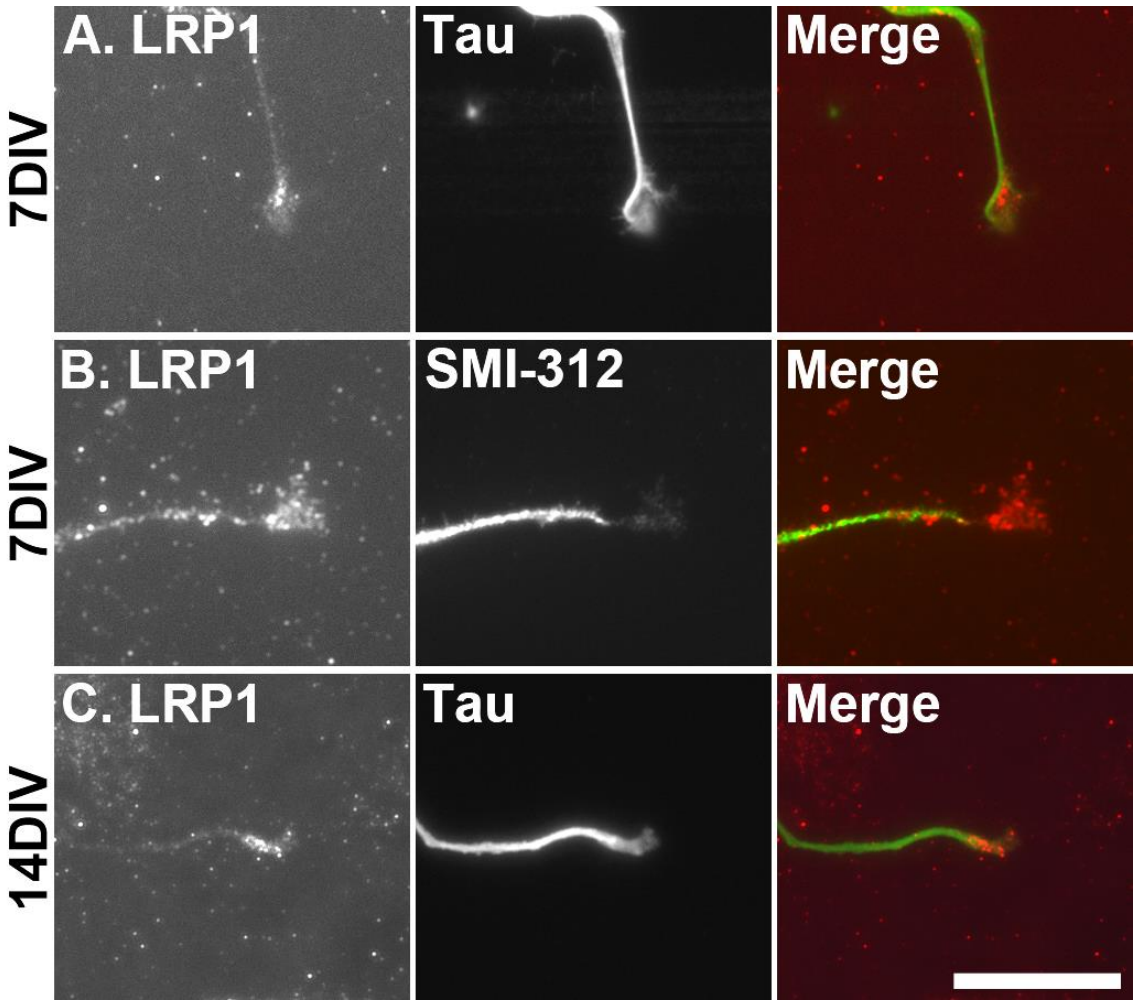

Figure 4.5: LRP1 is expressed at the ends of extending hippocampal neurites 24 hours after scratch injury at 7 and 14 days *in vitro* (DIV). Scratch injury was performed on hippocampal neuron cultures at 6DIV which were fixed 24 hours later at 7DIV and immunolabelled for LRP1 and tau (A) or SMI-312 (B). Hippocampal neuron cultures grown to 13DIV were scratch injured, fixed 24 hours later at 14DIV and immunolabelled for LRP1 and tau (C). LRP1 was expressed at the ends of most tau and SMI-312 positive neurites extending towards and into the scratch injury site at 7DIV, but only some tau positive neurites at 14DIV.

Scale bar = 25μm

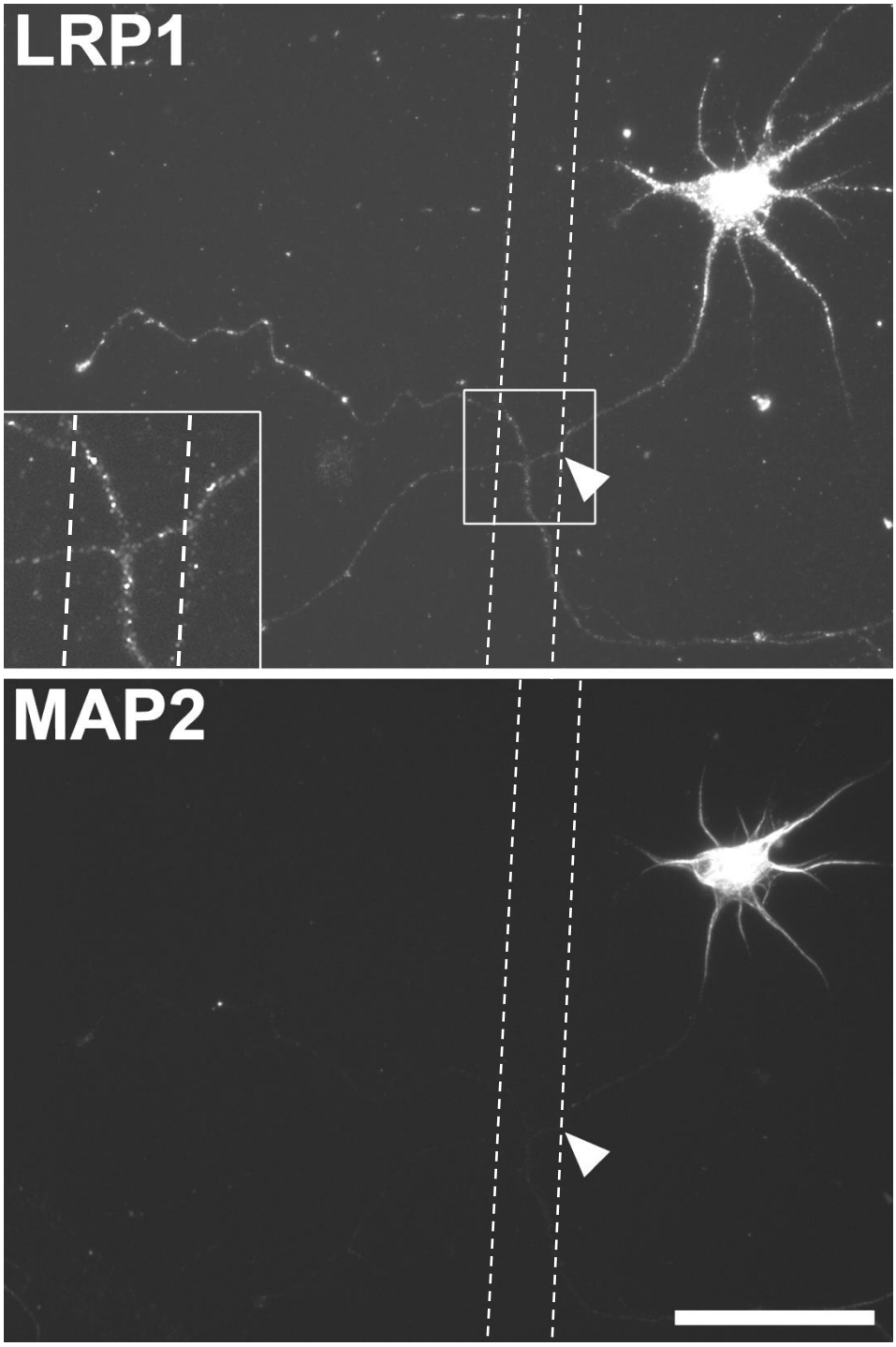

Figure 4.6: Most LRP1 positive processes that extend across or along the injury site are not dendrites. Hippocampal neuron cultures were fixed at 7 days *in vitro* (DIV), 24 hours after scratch injury (dotted lines). Cultures were immunolabelled for LRP1 and the dendritic marker MAP2. LRP1 positive processes that extended across the injury site were negative for MAP2 (inset and arrowheads).

Scale bar = 50μm; inset scale bar = 25μm

4.3.2 EMTINB INCREASES THE NUMBER OF NEURITES THAT EXTEND ALONG OR INTO THE SCRATCH INJURY SITE BUT MT DOES NOT

Neurites that touched, extended along or entered the injury site were observed in all cultures treated with saline, MT and emtinB with or without RAP (Figure 4.7 A-F). The numbers of neurites that extended along or entered the injury site were counted per cm of injury site. No significant difference in the number of neurites that extended along or into the injury site were observed when MT, RAP or MT + RAP were added to cultures when compared to saline controls (Figure 4.8 A). EmtinB significantly increased the number of neurites that extended along or entered the injury site (57.8 ± 4.6 neurites/cm injury site) compared to both saline (27.7 ± 3.3 neurites/cm injury site; $p<0.005$) and emtinB + RAP treated cultures (36.5 ± 3.2 neurites/cm injury site; $p<0.005$; Figure 4.8 B). No difference in the number of neurites that extended along or into the injury site was observed between saline treated controls and cultures treated with RAP or emtinB in combination with RAP. Therefore, emtinB appears to promote hippocampal neurite extension in an LRP1 dependent manner 24 hours following scratch injury but MT does not.

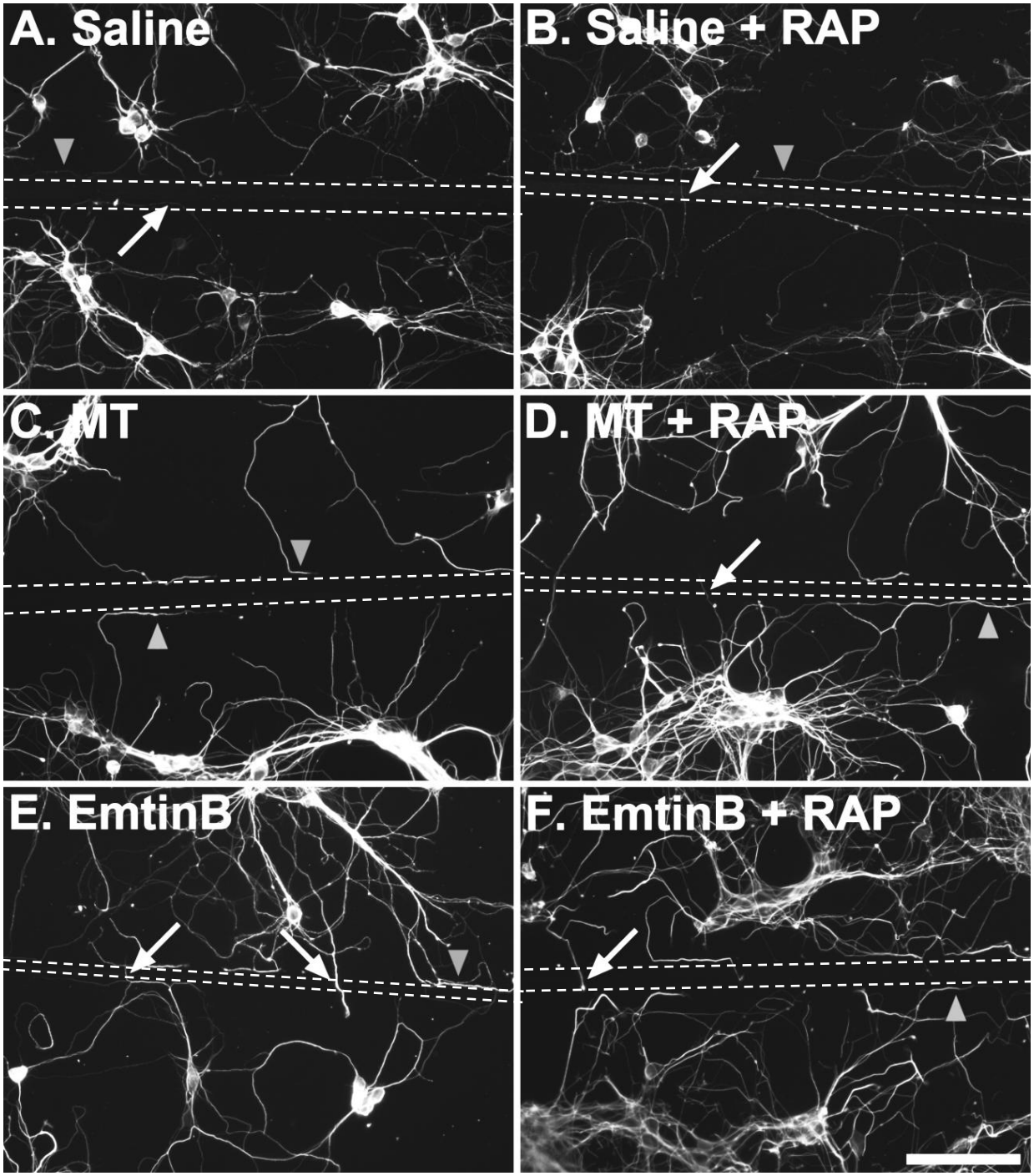

Figure 4.7: Representative images of neurite extension 24 hours after scratch injury with saline, MT or emtinB with or without RAP. Hippocampal neurons were grown to 6 days *in vitro* (DIV) and either saline, 10μg/mL MT or 25μM emtinB alone (A, C, E, respectively) or with 500nM RAP (B, D, F, respectively) was added immediately prior to scratch injury (white dashed lines). Cultures were fixed 24 hours later at 7DIV and immunolabelled for tau. Neurites appeared to touch and follow the injury site (grey arrowheads) or extended into or across it (white arrows).

Scale bar = 100μm

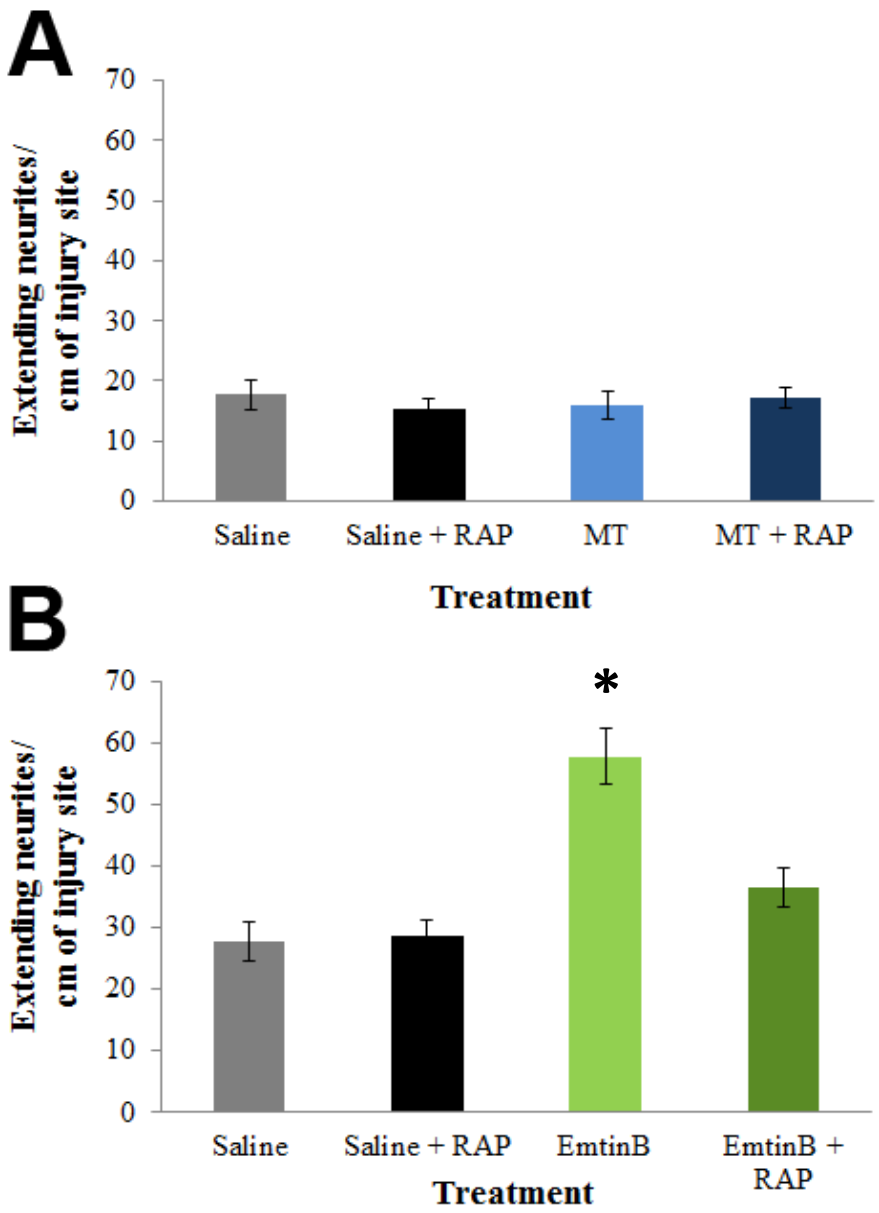

Figure 4.8: Effect of MT and emtinB on the number of neurites that extended along or into the scratch injury site. Saline, 10µg/mL MT or 25µM emtinB with or without 500nM RAP was added to hippocampal neuron cultures immediately prior to scratch injury at 6 days *in vitro* (DIV). Cells were fixed and immunolabelled for tau 24 hours later at 7DIV. Neurites that extended along or into the injury site were counted and converted to extending neurites per cm of injury site length. Addition of MT had no effect on the number of neurites that extended along or into the injury site (A). EmtinB increased the number of neurites that extended along or entered the injury site when compared to saline treated controls (B). RAP inhibited emtinB mediated neurite extension. RAP by itself did not increase the neurite extension when compared to the saline control.

* = $p<0.005$ compared to all other treatments; error bars = standard error of the mean

4.4 *DISCUSSION*

LRP1 is expressed at the ends of extending neurites 24 hours following scratch injury in both 7DIV and 14DIV hippocampal neuron cultures. The majority of these neurites are axons which are cleanly cut following scratch injury at 6DIV and appear to touch, extend into or enter the scratch injury site after 24 hours at 7DIV. MT did not promote neurite extension, but emtinB increased the number of neurites that extended along or into the injury site by two-fold when compared to the saline control. RAP inhibited this effect, demonstrating that emtinB promotes neurite extension through an LRP1 mechanism.

4.4.1 LRP1 IS EXPRESSED AT THE ENDS OF EXTENDING HIPPOCAMPAL NEURITES AFTER SCRATCH INJURY

24 hours following scratch injury, LRP1 immunoreactivity is present at the ends of most 7DIV and some 14DIV extending tau positive neurites. This demonstrates the potential for LRP1 to direct neurite extension in relatively immature neurons (7DIV) as well as in some mature, synaptically active neurons (14DIV). The majority of tau positive neurites that expressed LRP1 and extended into or along the injury site were determined to be axons. Very few axons in uninjured 7DIV cultures were LRP1 positive (Chapter 3.3.2). However, most extending axons 24 hours after scratch injury at 7DIV were immunoreactive for LRP1, particularly at growth cones. Therefore it appears that expression of the receptor changes in response to injury and suggests that it may be involved in axon extension.

The ability of LRP1 to promote axon extension has been demonstrated both *in vitro* and *in vivo* previously. ApoE containing lipoprotein enhances retinal ganglion cell axon extension by 50% following axotomy (Hayashi et al., 2004). An increase in axon extension only occurred when the ligand was added to axons, not cell bodies, and was inhibited by addition of RAP, siRNA and antibodies against LRP1 (Hayashi et al., 2004,

Matsuo et al., 2011). *In vivo* work demonstrates that the receptor binding domain of α2-macroglobulin, another LRP1 ligand, also promotes axon regeneration following dorsal column lesion in rats. Intrathecal injection of the ligand into the lesion increases the total proportion of axons into the site by 71% and the length of axons by 3.6-fold when compared to vehicle infused controls, presumably through LRP1 (Yoon et al., 2013). These studies demonstrate that LRP1 is located at the ends of severed axons and that it mediates regeneration of these processes. It is possible then that LRP1 located at the ends of hippocampal axons also promotes neurite extension following scratch injury of this neuronal subtype.

4.4.2 MT DOES NOT APPEAR TO PROMOTE HIPPOCAMPAL NEURITE EXTENSION FOLLOWING INJURY

Hippocampal neurite length during development is enhanced by 1.6 times following 24 hours treatment with 10μg/mL MT (Køhler et al., 2003). In injury models, MT addition to both cortical neurons and dorsal root ganglion cells increased neurite length by 60-70% when compared to vehicle treated controls following scratch injury (Chung et al., 2003, Leung et al., 2011). Given these reports, it was unexpected that MT did not promote the number of neurites that extended along or into the scratch injury site of 7DIV hippocampal neurons. However, these current findings are not comparable to previous literature that report regeneration as neurite length (Chung et al., 2003, Leung et al., 2011) as opposed to the number of neurites/unit length of injury site. Previous work has demonstrated that while MT promotes neurite length it does not enhance the number of reactive cortical processes per 100μm of the scratch injury site (Chung et al., 2003). Therefore, it appears that counting the number of neurites extending into or along the injury site could be a less sensitive measurement of regeneration and it cannot be definitively concluded that MT does not promote neurite regeneration based on current data. Measurement of neurite length was not possible in

the current hippocampal neuron injury model as it was difficult to accurately track and measure individual neurites that joined and clumped with other neurites. Using compartmentalised chambers, such as microfluidic chambers, to sever all axons from a specific point would enable precise measurement of neurite length and regeneration (Taylor et al., 2005). This may reveal regenerative effects of MT masked using the current analysis.

Additional explanations as to why MT did not appear to promote neurite extension may include masking of its effects by endogenous MT production in response to scratch injury. MT is expressed in astrocytes both *in vivo* and *in vitro* and its expression is significantly upregulated from 1 to 24 hours following scratch injury in mixed cortical neuron and astrocyte cultures (Young et al., 1991, Chung et al., 2004). It has been demonstrated that astrocyte MT expression is induced specifically by neuron injury. The total concentration of secreted MT detected in media of uninjured cultures was less than 0.1μg/mL, but local concentrations of MT that occur between astrocytes and neurons *in vivo* or in co-cultures were likely to be much higher (Chung et al., 2008). The amount of MT secreted by astrocytes following scratch injury in neuron cultures is likely to be even higher (Chung et al., 2004). At 7DIV, 15% of cells in hippocampal neuron cultures were astrocytes (Chapter 3.3.2). These astrocytes may secrete MT in response to the hippocampal neuron injury and mask effects of exogenous MT. Though addition of 5μg/mL MT to cortical neuron cultures significantly promoted the length of regenerating neurites following injury (Chung et al., 2003), astrocytes may behave differently in hippocampal neuron cultures to secrete greater amounts of MT. However, the lack of an MT mediated increase in hippocampal neurite extension following injury may also be due to experimental procedure and analysis.

In the current study, hippocampal neurons were analysed for neurite extension 24 hours following injury. Though MT mediated cortical neuron and dorsal root

ganglion cell neurite outgrowth was evident 12 and 16 hours post-scratch injury respectively (Chung et al., 2003, Leung et al., 2011), hippocampal neurite extension may take longer to occur. Length of retinal ganglion cell neurite extension significantly increased 4 days after injury following treatment with apoE3 containing lipoproteins through an LRP1 dependent mechanism (Hayashi et al., 2004). Therefore, increasing the time after injury before analysis may reveal effects of MT in this cell model. This would also increase the number of hippocampal neurites that extend into the injury site, which at 24 hours was relatively low. The number of reactive cortical processes per cm of injury site observed 16 hours after injury were up to 300 times greater than that observed for hippocampal neurites (Chung et al., 2003). With so few neurites contacting the injury site, significant differences may be masked between treatments.

4.4.3 EMTINB INCREASES THE NUMBER OF HIPPOCAMPAL NEURITES THAT EXTEND ALONG OR ENTER THE INJURY SITE

EmtinB doubled the number of hippocampal neurites that extended along or into the scratch injury site when compared to the saline control. It cannot be definitely concluded whether these neurites are severed neurites regenerating or newly formed sprouting neurites. Previous work has demonstrated that 24 hour emtinB treatment increases developmental hippocampal neurite outgrowth by 7 times that of controls (Sonn et al., 2010). The peptide also enhances neurite outgrowth of cerebellar granule neurons by up to 3 times that of controls following 24 hours incubation, effects inhibited by RAP (Ambjørn et al., 2008). These findings support a role for emtinB in promotion of neurite extension following injury. By measuring neurite length it can be determined whether these neurites are newly formed or regenerating after being cut. As discussed previously, measurement of neurite length was not possible in this culture model but can be conducted using compartmentalised chambers and severing all axons from a precise point (Taylor et al., 2005). Measuring neurite length over a number of

time points would also aid in determining that regeneration is occurring as opposed to sprouting. However, this current work has provided evidence that emtinB may be involved in neurite extension following injury and that this needs to be confirmed.

The emtinB induced increase in the number of hippocampal neurites that extended into or along the injury site was abolished by treatment with RAP. These results demonstrate that neurite extension mediated by emtinB is through LRP1. However, MT and emtinB are also ligands for the LDL receptor family member LRP2, which binds to and is inhibited by RAP (Orlando et al., 1992, Ambjørn et al., 2008). Therefore, the emtinB effects observed in these current experiments may not be specifically through LRP1 but also through LRP2. Specific inhibition of LRP2 with antibodies or siRNA abolishes MT mediated increases in retinal ganglion cell axon formation and cortical neurite length (Fitzgerald et al., 2007, Chung et al., 2008). Therefore, emtinB mediated neurite extension may be through LRP2. However, LRP1 and LRP2 can mediate their effects by associating with each other and forming a signaling domain (Landowski et al., 2012). The two receptors co-localise in growth cones of developing dorsal root ganglion cells and modulate MT mediated growth cone attraction by working synergistically with each other. siRNA against either LRP1 or LRP2, as well as in combination, inhibits MT mediated growth turning (Landowski et al., 2012). EmtinB mediated neurite extension may occur through a similar mechanism in which LRP1 and LRP2 recruit each other to mediate signaling.

4.4.4 DIFFERENT EFFECTS OF LRP1 LIGANDS ON NEURITE EXTENSION

The different effect of MT and emtinB on hippocampal neurite extension following injury further supports the importance of ligand composition in LRP1 mediated responses. This has been demonstrated with regard to neurite extension previously. EmtinB modeled after the C-terminus of the MT beta chain increases neurite outgrowth of cerebellar granule neurons but emtinB modeled after the N-terminus does

not (Ambjørn et al., 2008, Asmussen et al., 2009). Retinal ganglion cell axon extension following axotomy is enhanced by apoE3 containing lipoproteins by 28%, but apoE4 containing lipoproteins and α2-macroglobulin have no effect (Matsuo et al., 2011). It was assumed that the apoE3 containing lipoproteins acted through LRP1 as a mix of apoE containing lipoproteins promoted similar neurite extension and these effects were inhibited by siRNA and antibodies against LRP1 (Matsuo et al., 2011). These studies support the current findings that even structurally similar ligands, such as MT and emtinB, can have different effects on LRP1 function in neurite extension.

4.5 *CONCLUSIONS*

LRP1 is expressed at the ends of extending hippocampal axons 24 hours following scratch injury. EmtinB increased the number of neurites that extended along or into the injury site, but MT did not. RAP inhibited emtinB mediated neurite extension, demonstrating that the effect of emtinB was through an LRP1 dependent mechanism.

Chapter 5: Conclusions and future directions

The aim of this thesis was to establish a model to investigate the diversity and specificity of LRP1 on neuron function. Subcellular localisation of the receptor in neurons and glia *in vivo* and *in vitro* provides evidence that LRP1 is involved in these cell's activity. Potential roles may be in survival, signaling or outgrowth, which the receptor has been demonstrated to modulate in previous literature (Hashimoto et al., 2000, Hayashi et al., 2007, Shi et al., 2009, Matsuo et al., 2011, Hayashi et al., 2012, Stiles et al., 2013, Yamauchi et al., 2013). By establishing an *in vitro* neuron model that recapitulated aspects of *in vivo* LRP1 expression patterns, it was possible to further investigate the role of LRP1 in calcium signaling and regeneration following injury through the use of its ligands, MT and emtinB.

LRP1 is expressed in the brains of P2, P7 and adult rats. Different molecular weight LRP1 fragments were detected in whole brain, cortical and hippocampal tissue at each time point, suggesting that regulation of LRP1 may be region specific. P2 and P7 whole brain, cortex and hippocampus exhibited different LRP1 profiles by western blotting when compared to the same brain regions in adults. LRP1 fragments detected in hippocampal neuron cultures also differed from 3 to 21DIV. These findings demonstrate that regulation, and possibly function, of LRP1 changes during maturation. To identify the composition of each LRP1 positive fragment, brain lysate can be run on 2-dimensional gels to further separate protein fractions and stained to identify LRP1 molecules that correspond to the same size detected with western blots. Following digestion and by referring to the LRP1 structure, mass spectrometry can be used to determine fragment composition (Shevchenko et al., 1996, Guttman et al., 2009). Cultured cells can then be transfected with a LRP1 receptor possessing knock-in mutations in each specific fragment to determine whether these fractions are required for LRP1 functions such as ligand binding, endocytosis or intracellular protein

signaling. For example, transfection of the mutant receptor into the LRP1-null Chinese hamster ovary cell line can be conducted to evaluate the endocytic capabilities of LRP1 (FitzGerald et al., 1995, Li et al., 2000). Co-immunoprecipitation of fragments with specific intracellular proteins or ligands can be conducted using pull down assays to determine whether these fractions associate with signaling proteins and may be involved in their activation (Gotthardt et al., 2000). By identifying these fractions and determining their function, it may be possible to better understand LRP1 functions and how they are regulated.

LRP1 was expressed in the cell bodies of a subset of neurons in the cortex, hippocampus, thalamus and hypothalamus of P2, P7 and adult brains. This correlated with the immunoreactivity of LRP1 in cultured hippocampal neurons, which was particularly evident at the cell body. Previous data have demonstrated the ability of LRP1 to promote neuronal survival (Hayashi et al., 2007, Fuentealba et al., 2009, Hayashi et al., 2012, Sen et al., 2012). As the cell body is the site involved primarily in protein synthesis for processes such as survival (Haines, 2002), LRP1 expressed on neuronal cell bodies may be involved in this process. *In vivo*, mice with an LRP1 knock-out in forebrain neurons have increased neurodegeneration in the cortex and hippocampus compared to wild type mice (Liu et al., 2010). Similar knock-out models could be used to investigate the role of LRP1 in neuronal survival in conditions such as excitotoxicity and ischaemia. These conditions can be induced *in vivo* by intraperitoneal kainic acid injection (Schwob et al., 1980, Yang et al., 1997) or cerebral artery occlusion (Longa et al., 1989), respectively. If neuronal LRP1 knock-out mice are more susceptible to neurodegeneration it would support a role for the receptor in promoting neuron survival. Alternatively, LRP1 ligands such as MT could be injected following *in vivo* excitotoxicity or ischaemia to determine whether stimulation of the receptor can protect against neurodegeneration.

Cortical and hippocampal neurons in the adult brain also expressed LRP1 on apical dendrites where it may be involved in synaptic signaling. LRP1 immunoreactivity was also present on dendrites of cultured hippocampal neurons at 3, 7, 14 and 21DIV. However, LRP1 did not co-localise with PSD-95 or the NMDA receptor subunit NR2a. It may be that neurons require stimulation either by NMDA or glutamate, or LRP1 ligands, such as tPA, that induce NMDA receptor dependent calcium influx to induce LRP1 to physically associate with synaptic proteins. This can be tested *in vitro* by adding agents to synaptically mature hippocampal neuron cultures and fixing cells in 30 second intervals from 0 up to 2 minutes afterwards. Neurons can then be co-immunolabelled for LRP1 and synaptic proteins to observe whether the receptor associates with these proteins to induce calcium responses. In unstimulated neurons, LRP1 did partially co-localise with the NMDA receptor subunit NR2b which is expressed at synapses of the developing brain but also synaptically and extrasynaptically in mature brain (Malenka et al., 1989, Giese et al., 1998, Stocca and Vicini, 1998, Vicini et al., 1998, Liu et al., 2004). LRP1 expression was present at apical dendrites of adult brains, but there was very little LRP1 expression in dendrites of P2 and P7 brains. These findings suggest that the receptor may regulate NDMA receptor dependent calcium influx through interaction with the NR2b subunit in the mature rather than in the developing brain. LRP1 co-localisation with NR2b or other synaptic proteins *in vivo* can be conducted to investigate whether the receptor may be involved in synaptic signaling in the brain.

The LRP1 ligands, MT and emtinB, did not induce calcium influx in hippocampal neurons expressing functional NMDA receptors. MT and emtinB may mediate NMDA receptor calcium influx through long term pathways that require chronic incubation, similar to those reported for the LRP1 ligand α2-macroglobulin (Qiu et al., 2002). This can be investigated by incubating MT and emtinB with

hippocampal neurons for a longer time course, such as 48 hours, and then assessing calcium influx in response to NMDA. The potential ability of MT and emtinB to induce calcium influx may also be neuron specific similar to the scenario reported for other LRP1 ligands, such as tPA and α2-macroglobulin (Bacskai et al., 2000, Qiu et al., 2002, Martin et al., 2008, Samson et al., 2008). Analysing live cell calcium influx in cortical neurons, or other neuron subtypes, following MT and emtinB addition would determine whether this is the case.

Axonal expression of LRP1 was absent from P2, P7 and adult brain but was present along and at the ends of 3DIV and some 7DIV hippocampal axons. These disparities are unlikely to be due to 3DIV and 7DIV cultures better representing embryonic rather than post-natal hippocampal neurons as the majority of hippocampal cytogenesis and synaptogenesis occurs within the first two post-natal weeks (Crain et al., 1973, Bayer and Altman, 1974, Fricke and Cowan, 1978, Pokorný and Yamamoto, 1981). The differences between *in vivo* and *in vitro* axonal expression of LRP1 may be due to differences between the systems involved. This thesis demonstrates that LRP1 expression is increased at the ends of axons following injury in mature neurons, and suggests that localisation of the receptor changes in response to traumatic stimuli. Therefore, hippocampal neurons may express axonal LRP1 in response to the stress of culture preparation. Stressing hippocampal neurons in other manners, such as through glutamate excitotoxicity or trophic withdrawal, and identifying localisation of the receptor may aid in understanding whether this is the case. This can be taken further *in vivo* to investigate whether localisation of LRP1 changes in response to stressful stimuli such as ischaemia or excitotoxicity, as described above.

MT did not promote the number of hippocampal neurites that extended along or into the injury site following scratch injury. This is despite previous studies demonstrating the ability of the protein to promote neurite outgrowth following injury

in cortical neurons and dorsal root ganglion cells (Chung et al., 2003, Leung et al., 2011). This may be due differences in hippocampal neuron responses compared to other neuron models or alternatively, it may reflect the methods used to quantitate regeneration. Previous studies have measured regeneration as neurite length rather than the number of neurites/unit length of injury site, which may be a more sensitive method to quantitate regeneration (Chung et al., 2003, Leung et al., 2011). Culturing hippocampal neurons in compartmentalised chambers, such as microfluidic chambers, and severing axons from the axon compartment would enable measurement of neurite length following injury (Taylor et al., 2005). Additionally, other studies have demonstrated that the MT mediated increase of neuron outgrowth and survival is dose dependent with an effective range usually between 1 to 10μg/mL (Køhler et al., 2003, Fitzgerald et al., 2007, Ambjørn et al., 2008). However, differences in the optimal dose are observed between cortical neurons, retinal ganglion cells, dopaminergic neurons and hippocampal neurons (Køhler et al., 2003, Fitzgerald et al., 2007, Ambjørn et al., 2008). Therefore, 10μg/mL MT may not be the optimal concentration to promote hippocampal neurite outgrowth following injury. Conducting a dose response ranging from 0.1 to 10μg/mL MT following scratch injury may reveal the best concentration of MT to use to stimulate neurite regeneration.

EmtinB doubled the number of neurites that extended along or entered the injury site, but it was not possible to conclude that these were regenerating as opposed to sprouting neurites in the current injury model. Definitively determining that both emtinB and MT promote hippocampal neurite regeneration following injury can be conducted by measuring neurite length of severed axons in compartmentalised chambers, as mentioned previously, over a number of time points. The current finding that emtinB promoted the number of neurites extending into or along the injury site was abolished by RAP suggesting that its effects were through an LRP1 mechanism.

However, emtinB also binds to LRP2 and may induce its effects through a synergistic mechanism between LRP1 and LRP2 (Landowski et al., 2012). To determine whether this is the case, hippocampal neurons can be immunolabelled for LRP2 to evaluate whether they express the receptor. If so, specifically inhibiting LRP1 or LRP2 with siRNA would prove whether one or both of the receptors are required for emtinB mediated neurite outgrowth.

LRP1 was expressed in oligodendrocytes, astrocytes and microglia in P2, P7 and adult brain and in hippocampal neuron cultures at 3, 7, 14 and 21DIV. Expression of LRP1 in astrocytes has been reported *in vivo* and *in vitro* previously (Rebeck et al., 1993, Zheng et al., 1994, Ishiguro et al., 1995, Marzolo et al., 2000, Gaultier et al., 2009). However, this is the first time that *in vivo* LRP1 expression has been reported in oligodendrocytes and microglia despite being identified *in vitro* (Marzolo et al., 2000, Gaultier et al., 2009). Using *in vitro* models, the specific function of LRP1 on glia can be further investigated by inhibiting it using siRNA. Through the use of transgenic mice that express Cre recombinase under the control of oligodendrocyte, astrocyte or microglial specific promoters (Niwa-Kawakita et al., 2000, Uhlmann et al., 2002, Brockschnieder et al., 2004, Garcia et al., 2004, Willemen et al., 2010) LRP1 can be specifically knocked out of these cell populations *in vivo* (Rohlmann et al., 1996, Liu et al., 2010). Myelination and migration of oligodendrocytes, neurotransmitter release of astrocytes or phagocytosis and antigen presentation of microglia can be evaluated in both these *in vivo* and *in vitro* models to determine whether LRP1 has a role in these glial functions.

LRP1 was also expressed on endothelial cells and astrocytic end-feet of the BBB, consistent with previous work that show LRP1 expression on these structures and that the receptor regulate transport of molecules of the brain (Moestrup et al., 1992, Wolf et al., 1992, Shibata et al., 2000, Yepes et al., 2003, Deane et al., 2004). LRP1

may also modulate cerebral blood flow by regulating glutamate mediated calcium signaling in astrocytic end-feet to induce vasodilation or vasoconstriction (Zonta et al., 2002, Hayashi et al., 2012). Through regulating transport of molecules in and out of the brain and cerebral blood flow, LRP1 can aid in controlling the brains' microenvironment and indirectly promote the survival and function of neurons. Inhibiting LRP1 function by injection of antibodies against the receptor can be conducted *in vivo* to assess whether it increases vascular permeability and blood flow or transport of molecules across the BBB (Yepes et al., 2003).

The work in this thesis has provided the groundwork to further explore the function of LRP1 in neurons and glia, both *in vivo* and *in vitro*. It is apparent through previous work and the current data that the role of LRP1 in neurons and glia is ligand and cell specific, and changes during development. This thesis demonstrates that the expression pattern of LRP1 *in vitro* resembles that observed *in vivo*. Therefore, culture models are a useful and relevant tool to investigate the specific functions of the receptor in neuron and glia subtypes. The confirmation of these effects *in vivo* may lead to a better understand of mechanisms underlying neurodegenerative disease or injury and possible therapeutic targets.

References

Abbott NJ, Rönnbäck L, Hansson E (2006) Astrocyte–endothelial interactions at the blood–brain barrier. Nature Reviews Neuroscience 7:41-53.

Ambjørn M, Asmussen JW, Lindstam M, Gotfryd K, Jacobsen C, Kiselyov VV, Moestrup SK, Penkowa M, Bock E, Berezin V (2008) Metallothionein and a peptide modeled after metallothionein, EmtinB, induce neuronal differentiation and survival through binding to receptors of the low density lipoprotein receptor family. Journal of Neurochemistry 104:21-37.

Ashwell K (1990) Microglia and cell death in the developing mouse cerebellum. Developmental Brain Research 55:219-230.

Ashwell KW, Paxinos G (2008) Atlas of the developing rat nervous system: Academic Press.

Asmussen JW, Ambjørn M, Bock E, Berezin V (2009) Peptides modeled after the α-domain of metallothionein induce neurite outgrowth and promote survival of cerebellar granule neurons. European journal of cell biology 88:433-443.

Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH (2004) Molecular diversity of astrocytes with implications for neurological disorders. Proceedings of the National Academy of Sciences 101:8384-8389.

Bacskai B, Xia M, Strickland D, Rebeck G, Hyman B (2000) The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. Proceedings of the National Academy of Sciences 97:11551-11556.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. Journal of Cell Biology 140:911-923.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. The EMBO Journal 1:549-553.

Barnes H, Larsen B, Tyers M, van der Geer P (2001) Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (Lrp1) associates with the adaptor protein SHC in SRC-transformed cells. Journal of Biological Chemistry 276:19119-19125.

Basu S, Binder RJ, Ramalingam T, Srivastava PK (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14:303-313.

Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. International Immunology 12:1539-1546.

Baumann N, Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiological Reviews 81:871-927.

Bayer SA, Altman J (1974) Hippocampal development in the rat: Cytogenesis and morphogenesis examined with autoradiography and low-level X-irradiation. Journal of Comparative Neurology 158:55-79.

Betts GN, van der Geer P, Komives EA (2008) Structural and functional consequences of tyrosine phosphorylation in the LRP1 cytoplasmic domain. Journal of Biological Chemistry 283:15656-15664.

Bibel M, Hoppe E, Barde YA (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. The EMBO Journal 18:616-622.

Binder RJ, Han DK, Srivastava PK (2000) CD91: a receptor for heat shock protein gp96. Nature Immunology 1:151-155.

Bjartmar C, Hildebrand C, Loinder K (1994) Morphological heterogeneity of rat oligodendrocytes: Oligodendrocytes: Electron microscopic studies on serial sections. Glia 11:235-244.

Bjelke B, Seiger A (1989) Morphological distribution of MBP-like immunoreactivity in the brain during development. International Journal of Developmental Neuroscience 7:145-164.

Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B, Bassett SS, McInnis MG (1998) Alpha-2 macroglobulin is genetically associated with Alzheimer disease. Nature Genetics 19:357-360.

Blackstad T, Brink K, Hem J, June B (1970) Distribution of hippocampal mossy fibers in the rat. An experimental study with silver impregnation methods. Journal of Comparative Neurology 138:433-449.

Blaschke AJ, Staley K, Chun J (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development 122:1165-1174.

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annual Review of Biochemistry 72:395-447.

Boucher P, Liu P, Gotthardt M, Hiesberger T, Anderson RGW, Herz J (2002) Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae. Journal of Biological Chemistry 277:15507-15513.

Brockhaus J, Möller T, Kettenmann H (1996) Phagocytozing ameboid microglial cells studied in a mouse corpus callosum slice preparation. Glia 16:81-90.

Brockschnieder D, Lappe-Siefke C, Goebbels S, Boesl MR, Nave K-A, Riethmacher D (2004) Cell depletion due to diphtheria toxin fragment A after Cre-mediated recombination. Molecular and Cellular Biology 24:7636-7642.

Brown MD, Banker GA, Hussaini IM, Gonias SL, VandenBerg SR (1997) Low density lipoprotein receptor-related protein is expressed early and becomes restricted to a somatodendritic domain during neuronal differentiation in culture. Brain Research 747:313-317.

Bu G (2009) Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. Nature Reviews Neuroscience 10:333-344.

Bu G, Geuze HJ, Strous GJ, Schwartz AL (1995) 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. The EMBO Journal 14:2269-2280.

Bu G, Maksymovitch EA, Geuze H, Schwartz AL (1994a) Subcellular localization and endocytic function of low density lipoprotein receptor-related protein in human glioblastoma cells. Journal of Biological Chemistry 269:29874-29882.

Bu G, Maksymovitch EA, Nerbonne JM, Schwartz AL (1994b) Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. Journal of Biological Chemistry 269:18521-18528.

Bu G, Rennke S (1996) Receptor-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. Journal of Biological Chemistry 271:22218-22224.

Bu G, Williams S, Strickland DK, Schwartz AL (1992) Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. Proceedings of the National Academy of Sciences 89:7427-7431.

Caceres A, Banker G, Binder L (1986) Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. Journal of Neuroscience 6:714-722.

Campana WM, Li X, Dragojlovic N, Janes J, Gaultier A, Gonias SL (2006) The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. Journal of Neuroscience 26:11197-11207.

Chen H, Strickland DK, Mosher DF (1996) Metabolism of thrombospondin 2. Journal of Biological Chemistry 271:15993-15999.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120:421-433.

Chu CT, Pizzo SV (1993) Receptor-mediated antigen delivery into macrophages. Complexing antigen to alpha 2-macroglobulin enhances presentation to T cells. Journal of Immunology 150:48-58.

Chung RS, Adlard PA, Dittmann J, Vickers JC, Chuah MI, West AK (2004) Neuron-glia communication: metallothionein expression is specifically up-regulated by astrocytes in response to neuronal injury. Journal of Neurochemistry 88:454-461.

Chung RS, Penkowa M, Dittmann J, King CE, Bartlett C, Asmussen JW, Hidalgo J, Carrasco J, Leung YKJ, Walker AK (2008) Redefining the role of metallothionein within the injured brain. Journal of Biological Chemistry 283:15349-15358.

Chung RS, Vickers JC, Chuah MI, West AK (2003) Metallothionein-IIA promotes initial neurite elongation and postinjury reactive neurite growth and facilitates healing after focal cortical brain injury. Journal of Neuroscience 23:3336-3342.

Cohen S, Levi-Montalcini R (1956) A nerve growth-stimulating factor isolated from snake venom. Proceedings of the National Academy of Sciences 42:571-574.

Crain B, Cotman C, Taylor D, Lynch G (1973) A quantitative electron microscopic study of synaptogenesis in the dentate gyrus of the rat. Brain Research 63:195-204.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

Davey F, Davies AM (1998) TrkB signalling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. Current Biology 8:915-918.

Davis CG, Van Driel I, Russell D, Brown MS, Goldstein JL (1987) The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. Journal of Biological Chemistry 262:4075-4082.

Deane R, Wu Z, Sagare A, Davis J, Du Yan S, Hamm K, Xu F, Parisi M, LaRue B, Hu HW (2004) LRP/Amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. Neuron 43:333-344.

Donahue JE, Flaherty SL, Johanson CE, Duncan JA, Silverberg GD, Miller MC, Tavares R, Yang W, Wu Q, Sabo E (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. Acta Neuropathologica 112:405-415.

Dotti C, Banker G, Binder L (1987) The expression and distribution of the microtubule-associated proteins tau and microtubule-associated protein 2 in hippocampal neurons in the rat *in situ* and in cell culture. Neuroscience 23:121-130.

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. Journal of Neuroscience 8:1454-1468.

Eaton E, Bennett B, Berezin V, West A, Chung R (2012) Do metallothionein and its derivatives protect against Aβ toxicity by an LRP mediated mechanism? Program number 852.01. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

Edmondson JC, Liem R, Kuster JE, Hatten ME (1988) Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures. Journal of Cell Biology 106:505-517.

Elliott WH, Elliott DC (2004) Biochemistry and Molecular Biology: Oxford University Press.

Eng LF, Chao F-C, Gerstl B, Pratt DR, Tavaststjerna M (1968) Maturation of human white matter myelin. Fractionation of the myelin membrane proteins. Biochemistry 7:4455-4465.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn A-M, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nature Medicine 4:1313-1317.

Feldman S, Sangha N (1992) Immunohistochemical localization of alpha 2-macroglobulin receptors in human skin. Acta Dermato-venereologica 72:331-333.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G (2004) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729-743.

Fernandez-Castaneda A, Arandjelovic S, Stiles TL, Schlobach RK, Mowen KA, Gonias SL, Gaultier A (2013) Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. Journal of Biological Chemistry 288:4538-4548.

Ffrench-Constant C, Raff MC (1986) Proliferating bipotential glial progenitor cells in adult rat optic nerve. Nature 319:499-502.

FitzGerald DJ, Fryling CM, Zdanovsky A, Saelinger CB, Kounnas M, Winkles JA, Strickland D, Leppla S (1995) Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. Journal of Cell Biology 129:1533-1541.

Fitzgerald M, Nairn P, Bartlett CA, Chung RS, West AK, Beazley LD (2007) Metallothionein-IIA promotes neurite growth via the megalin receptor. Experimental Brain Research 183:171-180.

Fletcher T, Cameron P, De Camilli P, Banker G (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. Journal of Neuroscience 11:1617-1626.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. Journal of Neuroscience 17:2469-2476.

Frade JM, Bovolenta P, Martínez-Morales JR, Arribas A, Barbas JA, Rodríguez-Tébar A (1997) Control of early cell death by BDNF in the chick retina. Development 124:3313-3320.

Frade JM, Rodríguez-Tébar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166-168.

Fricke R, Cowan W (1978) An autoradiographic study of the commissural and ipsilateral hippocampo-dentate projections in the adult rat. Journal of Comparative Neurology 181:253-269.

Fricker M, Oliva-Martín MJ, Brown GC (2012) Primary phagocytosis of viable neurons by microglia activated with LPS or Abeta is dependent on calreticulin/LRP phagocytic signalling. Journal of Neuroinflammation 9:196.

Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G (2009) Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. Journal of Biological Chemistry 284:34045-34053.

Garcia ADR, Doan NB, Imura T, Bush TG, Sofroniew MV (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nature Neuroscience 7:1233-1241.

Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell 123:321-334.

Gardner A, Jukkola P, Gu C (2012) Myelination of rodent hippocampal neurons in culture. Nature Protocols 7:1774-1782.

Gaultier A, Wu X, Le Moan N, Takimoto S, Mukandala G, Akassoglou K, Campana WM, Gonias SL (2009) Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. Journal of Cell Science 122:1155-1162.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr286 of the α calcium-calmodulin kinase II in LTP and learning. Science 279:870-873.

Giulian D, Baker TJ (1985) Peptides released by ameboid microglia regulate astroglial proliferation. Journal of Cell Biology 101:2411-2415.

Giulian D, Baker TJ, Shih L-C, Lachman LB (1986) Interleukin 1 of the central nervous system is produced by ameboid microglia. Journal of Experimental Medicine 164:594-604.

Gliemann J, Davidsen O, Moestrup SK (1989) Characterization, size estimation and solubilization of [alpha]-macroglobulin complex receptors in liver membranes. Biochimica et Biophysica Acta 980:326-332.

Godyna S, Liau G, Popa I, Stefansson S, Argraves WS (1995) Identification of the low density lipoprotein receptor-related protein (LRP) as an endocytic receptor for thrombospondin-1. Journal of Cell Biology 129:1403-1410.

Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. Journal of Biological Chemistry 275:25616-25624.

Grimsley PG, Quinn KA, Chesterman CN, Owensby DA (1999) Evolutionary conservation of circulating soluble low density lipoprotein receptor-related protein-like ("LRP-like") molecules. Thrombosis Research 94:153-164.

Guttman M, Betts GN, Barnes H, Ghassemian M, van der Geer P, Komives EA (2009) Interactions of the NPXY microdomains of the low density lipoprotein receptor-related protein 1. Proteomics 9:5016-5028.

Haines DE (2002) Fundamental neuroscience: Churchill Livingstone.

Hallbook F, Ibanez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Hamano K, Iwasaki N, Takeya T, Takita H (1996) A quantitative analysis of rat central nervous system myelination using the immunohistochemical method for MBP. Developmental Brain Research 93:18-22.

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-κB activation enhances the survival response of developing

sensory neurons to nerve growth factor. Molecular and Cellular Neuroscience 14:28-40.

Handelmann GE, Boyles JK, Weisgraber KH, Mahley RW, Pitas RE (1992) Effects of apolipoprotein E, beta-very low density lipoproteins, and cholesterol on the extension of neurites by rabbit dorsal root ganglion neurons in vitro. Journal of Lipid Research 33:1677-1688.

Hashimoto Y, Jiang H, Niikura T, Ito Y, Hagiwara A, Umezawa K, Abe Y, Murayama Y, Nishimoto I (2000) Neuronal apoptosis by apolipoprotein E4 through low-density lipoprotein receptor-related protein and heterotrimeric GTPases. Journal of Neuroscience 20:8401-8409.

Hayashi H, Campenot RB, Vance DE, Vance JE (2004) Glial lipoproteins stimulate axon growth of central nervous system neurons in compartmented cultures. Journal of Biological Chemistry 279:14009-14015.

Hayashi H, Campenot RB, Vance DE, Vance JE (2007) Apolipoprotein E-containing lipoproteins protect neurons from apoptosis via a signaling pathway involving low-density lipoprotein receptor-related protein-1. Journal of Neuroscience 27:1933-1941.

Hayashi H, Eguchi Y, Fukuchi-Nakaishi Y, Takeya M, Nakagata N, Tanaka K, Vance JE, Tanihara H (2012) A potential neuroprotective role of apolipoprotein E-containing lipoproteins through low density lipoprotein receptor-related protein 1 in normal tension glaucoma. Journal of Biological Chemistry 287:25395-25406.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678-683.

Henze DA, Cameron WE, Barrionuevo G (1996) Dendritic morphology and its effects on the amplitude and rise-time of synaptic signals in hippocampal CA3 pyramidal cells. Journal of Comparative Neurology 369:331-344.

Herbert RP, Chuah MI, Chung RS, Leung YKJ, Adlard PA, West AK (2012) How do LRPs and their ligands affect LTP in the hippocampus? 32nd Annual Meeting of the Australian Neuroscience Society Abstract, Pos-Tue-020.

Herz J, Clouthier DE, Hammer RE (1992) LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell 71:411-421.

Herz J, Goldstein JL, Strickland D, Ho Y, Brown MS (1991) 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. Journal of Biological Chemistry 266:21232-21238.

Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK (1988) Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. The EMBO Journal 7:4119-4127.

Herz J, Kowal RC, Goldstein JL, Brown MS (1990) Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. The EMBO Journal 9:1769-1776.

Hickey WF, Kimura H (1988) Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. Science 239:290-292.

Hollyday M, Hamburger V (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. Journal of Comparative Neurology 170:311-320.

Huang SS, Ling TYEN, Tseng WENF, Huang YENHWA, Tang FENMEI, Leal SM, San Huang J (2003) Cellular growth inhibition by IGFBP-3 and TGF-ß1 requires LRP-1. The FASEB Journal 17:2068-2081.

Hussain S, Slikker Jr W, Ali SF (1996) Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection. Neurochemistry International 29:145-152.

Huttenlocher PR (1979) Synaptic density in human frontal cortex—developmental changes and effects of aging. Brain Research 163:195-205.

Igdoura S, Argraves W, Morales C (1997) Low density lipoprotein receptor-related protein-1 expression in the testis: regulated expression in Sertoli cells. Journal of Andrology 18:400-410.

Ilschner S, Brandt R (1996) The transition of microglia to a ramified phenotype is associated with the formation of stable acetylated and detyrosinated microtubules. Glia 18:129-140.

Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R, Squinto SP (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proceedings of the National Academy of Sciences 89:3060-3064.

Ishiguro M, Imai Y, Kohsaka S (1995) Expression and distribution of low density lipoprotein receptor-related protein mRNA in the rat central nervous system. Molecular Brain Research 33:37-46.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. Journal of Biological Chemistry 268:2836-2843.

Jiang LJ, Maret W, Vallee BL (1998) The glutathione redox couple modulates zinc transfer from metallothionein to zinc-depleted sorbitol dehydrogenase. Proceedings of the National Academy of Sciences 95:3483-3488.

Kadonaga JT (2004) Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell 116:247-257.

Kägi J, Himmelhoch S, Whanger P, Bethune J, Vallee BL (1974) Equine hepatic and renal metallothioneins. Journal of Biological Chemistry 249:3537-3542.

Kang D, Saitoh T, Chen X, Xia Y, Masliah E, Hansen L, Thomas R, Thal L, Katzman R (1997) Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), and apolipoprotein E receptor, with late-onset Alzheimer's disease. Neurology 49:56-61.

Kaplan DR, Martin-Zanca D, Parada LF (1991) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-160.

Kelly EJ, Quaife CJ, Froelick GJ, Palmiter RD (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. The Journal of Nutrition 126:1782-1790.

Kesslak JP, Nalcioglu O, Cotman CW (1991) Quantification of magnetic resonance scans for hippocampal and parahippocampal atrophy in Alzheimer's disease. Neurology 41:51-54.

Kim SU, Pleasure DE (1978) Tissue culture analysis of neurogenesis: Myelination and synapse formation are retarded by serum deprivation. Brain Research 145:15-25.

Kinoshita A, Shah T, Tangredi MM, Strickland DK, Hyman BT (2003) The intracellular domain of the low density lipoprotein receptor-related protein modulates transactivation mediated by amyloid precursor protein and Fe65. Journal of Biological Chemistry 278:41182-41188.

Klassen RB, Crenshaw K, Kozyraki R, Verroust PJ, Tio L, Atrian S, Allen PL, Hammond TG (2004) Megalin mediates renal uptake of heavy metal metallothionein complexes. American Journal of Physiology 287:F393-F403.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Køhler LB, Berezin V, Bock E, Penkowa M (2003) The role of metallothionein II in neuronal differentiation and survival. Brain Research 992:128-136.

Kojima Y, Berger C, Vallee BL, Kägi J (1976) Amino-acid sequence of equine renal metallothionein-1B. Proceedings of the National Academy of Sciences 73:3413-3417.

Kornau H-C, Schenker LT, Kennedy MB, Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737-1740.

Kounnas M, Morris RE, Thompson M, FitzGerald D, Strickland D, Saelinger C (1992) The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. Journal of Biological Chemistry 267:12420-12423.

Kounnas MZ, Chappell DA, Wong H, Argraves WS, Strickland DK (1995) The cellular internalization and degradation of hepatic lipase is mediated by low density lipoprotein receptor-related protein and requires cell surface proteoglycans. Journal of Biological Chemistry 270:9307-9312.

Kowal R, Herz J, Goldstein J, Esser V, Brown M (1989) LRP mediates uptake of cholesteryl esters derived from apoE-enriched lipoproteins. Proceedings of the National Academy of Sciences 86:5810-5814.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends in Neurosciences 19:312-318.

Laakso M, Soininen H, Partanen K, Helkala E-L, Hartikainen P, Vainio P, Hallikainen M, Hänninen T, Riekkinen Sr P (1995) Volumes of hippocampus, amygdala and frontal lobes in the MRI-based diagnosis of early Alzheimer's disease: correlation with memory functions. Journal of Neural Transmission 9:73-86.

Lamballe F, Klein RÃ, Barbacid M (1991) *trk* C, a new member of the *trk* family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Landowski L, Gasperini R, Small DH, Taylor BV, West AK, Foa L (2011) Metallothionein I/II and metallothionein III induce growth cone chemotaxis via disparate mechanisms. 31st Annual Meeting of the Australian Neuroscience Society Abstract, Pos-Wed-043.

Landowski L, Gasperini R, Taylor B, West A, Foa L (2012) Metallothionein I/II induces growth cone chemotaxis via an LRP-1 and LRP-2 dependent mechanism. Program number 233.16. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

Lawson L, Perry V, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151-170.

Lenting PJ, Neels JG, van den Berg BMM, Clijsters PPFM, Meijerman DWE, Pannekoek H, van Mourik JA, Mertens K, van Zonneveld AJ (1999) The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. Journal of Biological Chemistry 274:23734-23739.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proceedings of the National Academy of Sciences 96:3239-3244.

Leung JYK, Bennett WR, Herbert RP, West AK, Lee PR, Wake H, Fields RD, Chuah MI, Chung RS (2011) Metallothionein promotes regenerative axonal sprouting of dorsal root ganglion neurons after physical axotomy. Cellular and Molecular Life Sciences 69:809-817.

Leung Y, Pankhurst M, Dunlop S, Ray S, Dittmann J, Eaton E, Palumaa P, Sillard R, Chuah M, West A (2010) Metallothionein induces a regenerative reactive astrocyte phenotype via JAK/STAT and RhoA signalling pathways. Experimental Neurology 221:98-106.

Li Y, Paz Marzolo M, van Kerkhof P, Strous GJ, Bu G (2000) The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein. Journal of Biological Chemistry 275:17187-17194.

Liesi P, Silver J (1988) Is astrocyte laminin involved in axon guidance in the mammalian CNS? Developmental Biology 130:774-785.

Ling E (1979) Transformation of monocytes into amoeboid microglia and into microglia in the corpus callosum of postnatal rats, as shown by labelling monocytes by carbon particles. Journal of Anatomy 128:847-858.

Liu Q, Trotter J, Zhang J, Peters MM, Cheng H, Bao J, Han X, Weeber EJ, Bu G (2010) Neuronal LRP1 knockout in adult mice leads to impaired brain lipid metabolism and progressive, age-dependent synapse loss and neurodegeneration. Journal of Neuroscience 30:17068-17078.

Liu Q, Zerbinatti CV, Zhang J, Hoe H-S, Wang B, Cole SL, Herz J, Muglia L, Bu G (2007) Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. Neuron 56:66-78.

Liu Q, Zhang J, Zerbinatti C, Zhan Y, Kolber BJ, Herz J, Muglia LJ, Bu G (2011) Lipoprotein receptor LRP1 regulates leptin signaling and energy homeostasis in the adult central nervous system. PLoS Biology 9:e1000575.

Liu X-B, Murray KD, Jones EG (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. Journal of Neuroscience 24:8885-8895.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84-91.

Lopes MBS, Bogaev CA, Gonias SL, VandenBerg SR (1994) Expression of α2-macroglobulin receptor/low density lipoprotein receptor-related protein is increased in reactive and neoplastic glial cells. FEBS Letters 338:301-305.

Loukinova E, Ranganathan S, Kuznetsov S, Gorlatova N, Migliorini MM, Loukinov D, Ulery PG, Mikhailenko I, Lawrence DA, Strickland DK (2002) Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Journal of Biological Chemistry 277:15499-15506.

Lu H-C, Gonzalez E, Crair MC (2001) Barrel cortex critical period plasticity is independent of changes in NMDA receptor subunit composition. Neuron 32:619-634.

Luo Y, Shepherd I, Li J, Renzi MJ, Chang S, Raper JA (1995) A family of molecules related to collapsin in the embryonic chick nervous system. Neuron 14:1131-1140.

Mahadeo D, Kaplan L, Chao M, Hempstead B (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. Journal of Biological Chemistry 269:6884-6891.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989) An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature 340:554-557.

Manthorpe M, Engvall E, Ruoslahti E, Longo FM, Davis GE, Varon S (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. Journal of Cell Biology 97:1882-1890.

Mantuano E, Inoue G, Li X, Takahashi K, Gaultier A, Gonias SL, Campana WM (2008) The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. Journal of Neuroscience 28:11571-11582.

Mantuano E, Jo M, Gonias SL, Campana WM (2010) Low density lipoprotein receptor-related protein (LRP1) regulates Rac1 and RhoA reciprocally to control Schwann cell adhesion and migration. Journal of Biological Chemistry 285:14259-14266.

Martin AM, Kuhlmann C, Trossbach S, Jaeger S, Waldron E, Roebroek A, Luhmann HJ, Laatsch A, Weggen S, Lessmann V (2008) The functional role of the second NPXY motif of the LRP1 -chain in tissue-type plasminogen activator-mediated activation of N-methyl-d-aspartate receptors. Journal of Biological Chemistry 283:12004-12013.

Marzolo MP, von Bernhardi R, Bu G, Inestrosa NC (2000) Expression of α2-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. Journal of Neuroscience Research 60:401-411.

Masliah E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry R (1993) An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. American Journal of Pathology 142:871-882.

Matsuo M, Campenot RB, Vance DE, Ueda K, Vance JE (2011) Involvement of low density lipoprotein receptor-related protein and ABCG1 in stimulation of axonal extension by Apo E-containing lipoproteins. Biochimica et Biophysica Acta 1811:31-38.

Matus A, Bernhardt R, Hugh-Jones T (1981) High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. Proceedings of the National Academy of Sciences 78:3010-3014.

May P, Bock HH, Nimpf J, Herz J (2003) Differential glycosylation regulates processing of lipoprotein receptors by γ-secretase. Journal of Biological Chemistry 278:37386-37392.

May P, Reddy YK, Herz J (2002) Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. Journal of Biological Chemistry 277:18736-18743.

May P, Rohlmann A, Bock HH, Zurhove K, Marth JD, Schomburg ED, Noebels JL, Beffert U, Sweatt JD, Weeber EJ (2004) Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. Molecular and Cellular Biology 24:8872-8883.

Medh JD, Fry GL, Bowen SL, Pladet MW, Strickland DK, Chappell DA (1995) The 39-kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL receptors. Journal of Biological Chemistry 270:536-540.

Meilinger M, Gschwentner C, Burger I, Haumer M, Wahrmann M, Szollar L, Nimpf J, Huettinger M (1999) Metabolism of activated complement component C3 is mediated by the low density lipoprotein receptor-related protein/α2-macroglobulin receptor. Journal of Biological Chemistry 274:38091-38096.

Mikhailenko I, Battey FD, Migliorini M, Ruiz JF, Argraves K, Moayeri M, Strickland DK (2001) Recognition of α2-macroglobulin by the low density lipoprotein receptor-related protein requires the cooperation of two ligand binding cluster regions. Journal of Biological Chemistry 276:39484-39491.

Miller RH, David S, Patel R, Abney ER, Raff MC (1985) A quantitative immunohistochemical study of macroglial cell development in the rat optic nerve: In vivo evidence for two distinct astrocyte lineages. Developmental Biology 111:35-41.

Miller RH, Raff MC (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. Journal of Neuroscience 4:585-592.

Moestrup S, Gliemann J (1989) Purification of the rat hepatic alpha 2-macroglobulin receptor as an approximately 440-kDa single chain protein. Journal of Biological Chemistry 264:15574-15577.

Moestrup SK, Cui S, Vorum H, Bregengård C, Bjørn S, Norris K, Gliemann J, Christensen EI (1995) Evidence that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs. Journal of Clinical Investigation 96:1404-1413.

Moestrup SK, Gliemann J, Pallesen G (1992) Distribution of the 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. Cell and Tissue Research 269:375-382.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529-540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256:1217-1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31-37.

Morrot A, Strickland DK, Higuchi ML, Reis M, Pedrosa R, Scharfstein J (1997) Human T cell responses against the major cysteine proteinase (cruzipain) of Trypanosoma cruzi: role of the multifunctional alpha 2-macroglobulin receptor in antigen presentation by monocytes. International Immunology 9:825-834.

Mueller BK (1999) Growth cone guidance: first steps towards a deeper understanding. Annual Review of Neuroscience 22:351-388.

Mulligan SJ, MacVicar BA (2004) Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. Nature 431:195-199.

Murabe Y, Sano Y (1982) Morphological studies on neuroglia VI. Postnatal development of microglial cells. Cell and Tissue Research 225:469-485.

Muramatsu H, Zou K, Sakaguchi N, Ikematsu S, Sakuma S, Muramatsu T (2000) LDL receptor-related protein as a component of the midkine receptor. Biochemical and Biophysical Research Communications 270:936-941.

Myklebost O, Arheden K, Rogne S, Kessel AG, Mandahl N, Herz J, Stanley K, Heim S, Mitelman F (1989) The gene for the human putative apoE receptor is on chromosome 12 in the segment q13-14. Genomics 5:65-69.

Nakamura H, O'Leary D (1989) Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. Journal of Neuroscience 9:3776-3795.

Nathan BP, Jiang Y, Wong GK, Shen F, Brewer GJ, Struble RG (2002) Apolipoprotein E4 inhibits, and apolipoprotein E3 promotes neurite outgrowth in cultured adult mouse cortical neurons through the low-density lipoprotein receptor-related protein. Brain Research 928:96-105.

Neels JG, van den Berg BMM, Lookene A, Olivecrona G, Pannekoek H, van Zonneveld AJ (1999) The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. Journal of Biological Chemistry 274:31305-31311.

Nelson DL, Cox MM (2008) Lehninger principles of biochemistry: W.H Freeman and Company.
Neugebauer KM, Tomaselli KJ, Lilien J, Reichardt LF (1988) N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes in vitro. Journal of Cell Biology 107:1177-1187.
Nicholls JG, Martin AR, Wallace BG, Fuchs PA (2001) From neuron to brain: Sinauer Associates Sunderland, MA.
Niemeier A, Kassem M, Toedter K, Wendt D, Ruether W, Beisiegel U, Heeren J (2005) Expression of LRP1 by human osteoblasts: a mechanism for the delivery of lipoproteins and vitamin K1 to bone. Journal of Bone and Mineral Research 20:283-293.
Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.
Niwa-Kawakita M, Abramowski V, Kalamarides M, Thomas G, Giovannini M (2000) Targeted expression of Cre recombinase to myelinating cells of the central nervous system in transgenic mice. Genesis 26:127-129.
Nowakowski RS, Rakic P (1979) The mode of migration of neurons to the hippocampus: a Golgi and electron microscopic analysis in foetal rhesus monkey. Journal of Neurocytology 8:697-718.
Nykjaer A, Kjøller L, Cohen RL, Lawrence DA, Garni-Wagner BA, Todd R, Van Zonneveld A, Gliemann J, Andreasen PA (1994) Regions involved in binding of urokinase-type-1 inhibitor complex and pro-urokinase to the endocytic alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Evidence that the urokinase receptor protects pro-urokinase against binding to the endocytic receptor. Journal of Biological Chemistry 269:25668-25676.
Ogden CA, DeCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, Henson PM (2001) C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. Journal of Experimental Medicine 194:781-795.
Orita S, Henry K, Mantuano E, Yamauchi K, De Corato A, Ishikawa T, Feltri ML, Wrabetz L, Gaultier A, Pollack M (2013) Schwann cell LRP1 regulates remak bundle ultrastructure and axonal interactions to prevent neuropathic pain. Journal of Neuroscience 33:5590-5602.
Orlando RA, Kerjaschki D, Kurihara H, Biemesderfer D, Farquhar MG (1992) gp330 associates with a 44-kDa protein in the rat kidney to form the Heymann nephritis antigenic complex. Proceedings of the National Academy of Sciences 89:6698-6702.
Orth K, Madison EL, Gething MJ, Sambrook JF, Herz J (1992) Complexes of tissue-type plasminogen activator and its serpin inhibitor plasminogen-activator inhibitor type 1 are internalized by means of the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. Proceedings of the National Academy of Sciences 89:7422-7426.
Papouin T, Ladépêche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP, Oliet SHR (2012) Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. Cell 150:633-646.
Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte–neuron signalling. Nature 369:744-747.
Paxinos G, Watson C (2007) The Rat Brain in Stereotaxic Coordinates 6th edn (New York: Academic): Academic Press.
Perea G, Navarrete M, Araque A (2009) Tripartite synapses: astrocytes process and control synaptic information. Trends in Neurosciences 32:421-431.

Pokorný J, Yamamoto T (1981) Postnatal ontogenesis of hippocampal CA1 area in rats. I. Development of dendritic arborisation in pyramidal neurons. Brain Research Bulletin 7:113-120.

Polavarapu R, Gongora MC, Yi H, Ranganthan S, Lawrence DA, Strickland D, Yepes M (2007) Tissue-type plasminogen activator–mediated shedding of astrocytic low-density lipoprotein receptor–related protein increases the permeability of the neurovascular unit. Blood 109:3270-3278.

Qiu Z, Hyman BT, Rebeck GW (2004) Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. Journal of Biological Chemistry 279:34948-34956.

Qiu Z, Strickland DK, Hyman BT, Rebeck GW (2002) α2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons. Journal of Biological Chemistry 277:14458-14466.

Quaife CJ, Findley SD, Erickson JC, Froelick GJ, Kelly EJ, Zambrowicz BP, Palmiter RD (1994) Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. Biochemistry 33:7250-7259.

Quinn KA, Grimsley PG, Dai YP, Tapner M, Chesterman CN, Owensby DA (1997) Soluble low density lipoprotein receptor-related protein (LRP) circulates in human plasma. Journal of Biological Chemistry 272:23946-23951.

Quinn KA, Pye VJ, Dai Y-P, Chesterman CN, Owensby DA (1999) Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP). Experimental Cell Research 251:433-441.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. Journal of Comparative Neurology 145:61-83.

Ramon Y, Cajal S (1909) Histologie du systeme nerveux de l'homme et des vertebres. Maloine, Paris 774-838.

Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. Journal of Neuroscience 18:1217-1229.

Rebeck WG, Reiter JS, Strickland DK, Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. Neuron 11:575-580.

Reekmans SM, Pflanzner T, Gordts PLSM, Isbert S, Zimmermann P, Annaert W, Weggen S, Roebroek AJM, Pietrzik CU (2010) Inactivation of the proximal NPXY motif impairs early steps in LRP1 biosynthesis. Cellular and Molecular Life Sciences 67:135-145.

Reymann KG, Kase H, Matthies H (1988) Inhibitors of calmodulin and protein kinase C block different phases of hippocampal long-term potentiation. Brain Research 461:388-392.

Roebroek AJM, Reekmans S, Lauwers A, Feyaerts N, Smeijers L, Hartmann D (2006) Mutant Lrp1 knock-in mice generated by recombinase-mediated cassette exchange reveal differential importance of the NPXY motifs in the intracellular domain of LRP1 for normal fetal development. Molecular and Cellular Biology 26:605-616.

Rogove AD, Siao C, Keyt B, Strickland S, Tsirka SE (1999) Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system. Journal of Cell Science 112:4007-4016.

Rohlmann A, Gotthardt M, Willnow TE, Hammer RE, Herz J (1996) Sustained somatic gene inactivation by viral transfer of Cre recombinase. Nature Biotechnology 14:1562-1565.

Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG (1999) Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. Journal of Biological Chemistry 274:37685-37692.

Saher G, Brügger B, Lappe-Siefke C, Möbius W, Tozawa R-i, Wehr MC, Wieland F, Ishibashi S, Nave K-A (2005) High cholesterol level is essential for myelin membrane growth. Nature Neuroscience 8:468-475.

Salicioni AM, Mizelle KS, Loukinova E, Mikhailenko I, Strickland DK, Gonias SL (2002) The low density lipoprotein receptor-related protein mediates fibronectin catabolism and inhibits fibronectin accumulation on cell surfaces. Journal of Biological Chemistry 277:16160-16166.

Samson AL, Nevin ST, Croucher D, Niego B, Daniel PB, Weiss TW, Moreno E, Monard D, Lawrence DA, Medcalf RL (2008) Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function. Journal of Neurochemistry 107:1091-1101.

Sattler R, Xiong Z, Lu W-Y, Hafner M, MacDonald JF, Tymianski M (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science 284:1845-1848.

Schwartz AL (1995) Receptor cell biology: receptor-mediated endocytosis. Pediatric Research 38:835-843.

Schwob J, Fuller T, Price J, Olney J (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of kainic acid: a histological study. Neuroscience 5:991-1014.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. Journal of Neurology, Neurosurgery & Psychiatry 20:11-21.

Seamans JK, Gorelova NA, Yang CR (1997) Contributions of voltage-gated Ca2+ channels in the proximal versus distal dendrites to synaptic integration in prefrontal cortical neurons. Journal of Neuroscience 17:5936-5948.

Searle P, Davison B, Stuart G, Wilkie T, Norstedt G, Palmiter R (1984) Regulation, linkage, and sequence of mouse metallothionein I and II genes. Molecular and Cellular Biology 4:1221-1230.

Segarini PR, Nesbitt JE, Li D, Hays LG, Yates JR, Carmichael DF (2001) The low density lipoprotein receptor-related protein/α2-macroglobulin receptor is a receptor for connective tissue growth factor. Journal of Biological Chemistry 276:40659-40667.

Sen A, Alkon DL, Nelson TJ (2012) Apolipoprotein E3 (ApoE3) but Not ApoE4 Protects against Synaptic Loss through Increased Expression of Protein Kinase Cϵ. Journal of Biological Chemistry 287:15947-15958.

Serafini T, Kennedy TE, Gaiko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell 78:409-424.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368:144-147.

Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Mann M (1996) Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proceedings of the National Academy of Sciences 93:14440-14445.

Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL (2009) Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. Science Signaling 2:ra18.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J (2000) Clearance of Alzheimer's amyloid-peptide from brain by LDL receptor–related protein-1 at the blood-brain barrier. Journal of Clinical Investigation 106:1489-1499.

Sloviter RS, Valiquette G, ABRAmS GM, Ronk EC, Sollas AL, Paul LA, Neubort S (1989) Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. Science 243:535-538.

Smith GM, Miller RH, Silver J (1986) Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. Journal of Comparative Neurology 251:23-43.

Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathologica 119:7-35.

Sonn K, Pankratova S, Korshunova I, Zharkovsky A, Bock E, Berezin V, Kiryushko D (2010) A metallothionein mimetic peptide protects neurons against kainic acid-induced excitotoxicity. Journal of Neuroscience Research 88:1074-1082.

Springer TA (1998) An extracellular [beta]-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components1. Journal of Molecular Biology 283:837-862.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Blanco SM, RadzieJewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuela DM (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65:885-893.

Stence N, Waite M, Dailey ME (2001) Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices. Glia 33:256-266.

Stiles TL, Dickendesher TL, Gaultier A, Fernandez-Castaneda A, Mantuano E, Giger RJ, Gonias SL (2013) LDL receptor-related protein-1 is a sialic-acid-independent receptor for myelin-associated glycoprotein that functions in neurite outgrowth inhibition by MAG and CNS myelin. Journal of Cell Science 126:209-220.

Stocca G, Vicini S (1998) Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. Journal of Physiology 507:13-24.

Storm D, Herz J, Trinder P, Loos M (1997) C1 inhibitor-C1s complexes are internalized and degraded by the low density lipoprotein receptor-related protein. Journal of Biological Chemistry 272:31043-31050.

Strickland DK, Ashcom J, Williams S, Burgess W, Migliorini M, Argraves W (1990) Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. Journal of Biological Chemistry 265:17401-17404.

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proceedings of the National Academy of Sciences 90:1977-1981.

Sultana R, Banks WA, Butterfield DA (2010) Decreased levels of PSD95 and two associated proteins and increased levels of BCl2 and caspase 3 in hippocampus from subjects with amnestic mild cognitive impairment: Insights into their potential roles for loss of synapses and memory, accumulation of A , and neurodegeneration in a prodromal stage of Alzheimer's disease. Journal of Neuroscience Research 88:469-477.

Takahashi A, Camacho P, Lechleiter JD, Herman B (1999) Measurement of intracellular calcium. Physiological Reviews 79:1089-1125.

Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL (2005) A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nature Methods 2:599-605.

Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. Journal of Neurophysiology 95:1727-1734.

Trommsdorff M, Borg JP, Margolis B, Herz J (1998) Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. Journal of Biological Chemistry 273:33556-33560.

Udom AO, Brady FO (1980) Reactivation in vitro of zinc-requiring apo-enzymes by rat liver zinc-thionein. Biochemical Journal 187:329-335.

Uhlmann EJ, Wong M, Baldwin RL, Bajenaru ML, Onda H, Kwiatkowski DJ, Yamada K, Gutmann DH (2002) Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. Annals of neurology 52:285-296.

Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. Science 291:657-661.

Van Kerkhof P, Lee J, McCormick L, Tetrault E, Lu W, Schoenfish M, Oorschot V, Strous GJ, Klumperman J, Bu G (2005) Sorting nexin 17 facilitates LRP recycling in the early endosome. The EMBO Journal 24:2851-2861.

Vandivier RW, Ogden CA, Fadok VA, Hoffmann PR, Brown KK, Botto M, Walport MJ, Fisher JH, Henson PM, Greene KE (2002) Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. Journal of Immunology 169:3978-3986.

Vaughn JE (1989) Review: fine structure of synaptogenesis in the vertebrate central nervous system. Synapse 3:255-285.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. Journal of Neurophysiology 79:555-566.

von Arnim CAF, Kinoshita A, Peltan ID, Tangredi MM, Herl L, Lee BM, Spoelgen R, Hshieh TT, Ranganathan S, Battey FD (2005) The low density lipoprotein receptor-related protein (LRP) is a novel β-secretase (BACE1) substrate. Journal of Biological Chemistry 280:17777-17785.

Warshawsky I, Broze GJ, Schwartz AL (1994) The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. Proceedings of the National Academy of Sciences 91:6664-6668.

Willemen HL, Eijkelkamp N, Wang H, Dantzer R, Dorn II GW, Kelley KW, Heijnen CJ, Kavelaars A (2010) Microglial/macrophage GRK2 determines duration of peripheral IL-1β-induced hyperalgesia: contribution of spinal cord CX3CR1, p38 and IL-1 signaling. Pain 150:550-560.

Willnow TE, Armstrong SA, Hammer RE, Herz J (1995) Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. Proceedings of the National Academy of Sciences 92:4537-4541.

Willnow TE, Goldstein J, Orth K, Brown M, Herz J (1992) Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. Journal of Biological Chemistry 267:26172-26180.

Willnow TE, Sheng Z, Ishibashi S, Herz J (1994) Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. Science 264:1471-1474.

Wolf B, Lopes M, VandenBerg S, Gonias S (1992) Characterization and immunohistochemical localization of alpha 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. American Journal of Pathology 141:37-42.

Wolswijk G, Noble M (1989) Identification of an adult-specific glial progenitor cell. Development 105:387-400.

Wood ER, Mumby DG, Pinel JP, Phillips AG (1993) Impaired object recognition memory in rats following ischemia-induced damage to the hippocampus. Behavioral Neuroscience 107:51-62.

Wu C, Wen C, Shieh J, Ling E (1992) A quantitative and morphometric study of the transformation of amoeboid microglia into ramified microglia in the developing corpus callosum in rats. Journal of Anatomy 181:423.

Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME (1998) Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Molecular and Cellular Biology 18:1946-1955.

Xu G, Karch C, Li N, Lin N, Fromholt D, Gonzales V, Borchelt DR (2008) Receptor-associated protein (RAP) plays a central role in modulating Aβ deposition in APP/PS1 transgenic mice. PLoS One 3:e3159.

Yamada KM, Spooner BS, Wessells NK (1970) Axon growth: roles of microfilaments and microtubules. Proceedings of the National Academy of Sciences 66:1206-1212.

Yamauchi K, Yamauchi T, Mantuano E, Murakami K, Henry K, Takahashi K, Campana WM (2013) Low density lipoprotein receptor related protein-1 (LRP1)-dependent cell signaling promotes neurotrophic activity in embryonic sensory neurons. PLoS One 8:e75497.

Yang DD, Kuan C-Y, Whitmarsh AJ, Rinócn M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389:865-870.

Ye G, Metreveli NS, Ren J, Epstein PN (2003) Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. Diabetes 52:777-783.

Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA (2003) Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. Journal of Clinical Investigation 112:1533-1540.

Yoon C, Van Niekerk EA, Henry K, Ishikawa T, Orita S, Tuszynski MH, Campana WM (2013) Low-density lipoprotein receptor-related protein 1 (LRP1)-dependent cell signaling promotes axonal regeneration. Journal of Biological Chemistry 288:26557-26568.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. Journal of Neuroscience 18:3273-3281.

Yoshida K, Gage FH (1991) Fibroblast growth factors stimulate nerve growth factor synthesis and secretion by astrocytes. Brain Research 538:118-126.

Young JK, Garvey JS, Huang P (1991) Glial immunoreactivity for metallothionein in the rat brain. Glia 4:602-610.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Yuste R, Gutnick MJ, Saar D, Delaney KR, Tank DW (1994) Ca2+ accumulations in dendrites of neocortical pyramidal neurons: An apical band and evidence for two functional compartments. Neuron 13:23-43.

Zerbinatti CV, Wozniak DF, Cirrito J, Cam JA, Osaka H, Bales KR, Zhuo M, Paul SM, Holtzman DM, Bu G (2004) Increased soluble amyloid- peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. Proceedings of the National Academy of Sciences 101:1075-1080.

Zheng C, Heintz N, Hatten ME (1996) CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. Science 272:417-419.

Zheng G, Bachinsky D, Stamenkovic I, Strickland D, Brown D, Andres G, McCluskey R (1994) Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpha 2MR, and the receptor-associated protein (RAP). Journal of Histochemistry and Cytochemistry 42:531-542.

Zhou Q, Wang S, Anderson DJ (2000) Identification of a novel family of oligodendrocyte lineage-specific basic helix–loop–helix transcription factors. Neuron 25:331-343.

Zhuo M, Holtzman DM, Li Y, Osaka H, DeMaro J, Jacquin M, Bu G (2000) Role of tissue plasminogen activator receptor LRP in hippocampal long-term potentiation. The Journal of Neuroscience 20:542-549.

Zlokovic BV, Deane R, Sagare AP, Bell RD, Winkler EA (2010) Low density lipoprotein receptor-related protein 1: a serial clearance homeostatic mechanism controlling Alzheimer's amyloid β peptide elimination from the brain. Journal of Neurochemistry 115:1077-1089.

Zola-Morgan S, Squire LR, Amaral D (1986) Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. Journal of Neuroscience 6:2950-2967.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann K-A, Pozzan T, Carmignoto G (2002) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nature Neuroscience 6:43-50.

Appendix 1: Solution and buffer recipes

GENERAL SOLUTIONS AND BUFFERS

0.9% SALINE

0.45g sodium chloride was dissolved in 50mL milliQ water and filter sterilized.

4% PARAFORMALDEHYDE

40g prilled paraformaldehyde was dissolved in 500mL 60°C milliQ water with 10M sodium hydroxide added until solution became clear. Solution was then added to 500mL 2X PBS and stored at 4°C.

PHOSPHATE BUFFERED SALINE

10mL 200mM sodium dihydrogen orthophosphate, 40mL 200mM sodium phosphate dibasic dihydrate and 100mL 0.9% saline were mixed together in 850mL milliQ water.

TISSUE PROCESSING

CITRATE BUFFER

1.92g citric acid was added to 900mL milliQ water, pH-ed to 6.0 and made up to 1L. Buffer was stored at 4°C and could be reused five times for antigen retrieval.

SUCROSE

1g, 2g or 3g sucrose was dissolved in 10mL milliQ water to make sucrose solutions of 10%, 20% and 30% respectively.

NEURON CULTURE

HANK'S BUFFERED SALINE SOLUTION

One vial of Hank's buffered saline solution and 0.35g sodium bicarbonate was dissolved in 1L distilled water and filtered through a 0.22μm bottle top filter in a sterile environment. Stored at 4ºC.

SERUM FREE MEDIA (NEUROBASAL)

500mL neurobasal was supplemented with 0.1% B27, 0.5mM L-glutamine, 0.1% gentamicin.

WESTERN BLOTTING

0.05% PBS-TWEEN

500μL Tween-20 was added to 1L PBS.

4X REDUCING BUFFER

5mL 1M Tris hydrochloride (pH 6.8), 2mL 20% sodium dodecyl sulfate, 2mL glycerol, 2mL β-mercaptoethanol and 0.02% bromophenol blue were mixed together and stored at -20°C.

5% MILK POWDER

5g skim milk powder was dissolved in 100mL PBS-Tween.

5% STACKING GEL

3.55mL milliQ, 833μL 30% acrylamide-bis, 625μL 1M Tris hydrochloride (pH 6.8) and 50μL 10% sodium dodecyl sulfate were mixed together. 150μL 10% ammonium persulfate and 6μL N,N,N,N-tetramethylethylenediamine were added immediately prior to pouring gel into cast.

10% RESOLVING GEL

4.4mL milliQ water, 5mL 30% acrylamide-bis, 5.6mL 1M Tris hydrochloride (pH 8.7) and 150µL 10% sodium dodecyl sulfate were mixed together. 150µL 10% ammonium persulfate and 22.5µL N,N,N,N-tetramethylethylenediamine were added immediately prior to pouring gel into cast.

10X RUNNING BUFFER

5g SDS, 15g Trizma base and 72g glycine were mixed together in 500mL milliQ water. 1X running buffer was prepared by mixing 80mL 10X running buffer in 720mL milliQ water.

30% ACRYLAMIDE-BIS

150g acrylamide and 4g bis were mixed together in 300mL milliQ water. Total volume was brought to 500mL and allowed to warm to room temperature before being stored at 4°C.

RADIO IMMUNOPRECIPITATION ASSAY (RIPA) BUFFER

0.394g Tris hydrochloride, 0.4383g sodium chloride, 0.0186g EDTA, 500µL Triton X-100, 0.5g sodium deoxycholate and 0.05g sodium dodecyl sulfate was added to 50mL milliQ and mixed on an orbital shaker overnight. Buffer was pH-ed to 7.4.

TRANSFER BUFFER

11.5g glycine and 2.4g Trizma base were dissolved in 160mL methanol and 640mL milliQ water at 4°C.

LIVE CELL CALCIUM IMAGING

IMAGING BUFFER STOCKS

The following amounts were dissolved in 50mL milliQ to make 1M stocks:

Calcium chloride: 7.35g dihydrous calcium chloride

Magnesium chloride: 10.17g hexahydrous magnesium chloride

Glucose: 9.00g D-(+)-glucose

IMAGING BUFFER

100mL buffer was made up with sterile milliQ water containing 2mM calcium chloride (200µL of 1M), 1mM magnesium chloride (100µL of 1M stock), 10mM glucose (1mL of 1M stock), 10mM HEPES (1mL of 1M stock) and 10mM HBSS (10mL of 10X stock), adjusted to a pH of 7.2-7.4 and filter sterilised.

Appendix 2: Reagent and equipment information

Reagent	Catalogue number	Supplier
General		
Hydrochloric acid	1367-2.5L GL	Ajax
Methanol	M3641	Sigma
Paraformaldehyde	441244	Sigma
Sodium azide	71289	Fluka
Sodium chloride	S9625	Sigma
Sodium dihydrogen orthophosphate	31463	Ajax
Sodium hydroxide	S5881	Sigma
Sodium phosphate dibasic dishydrate	30412	Sigma
Peptides		
EmtinB Modeled after the MT-II β-domain C-terminus (Swiss Protein Database P02795)		Schafer-N (Denmark)
Metallothionein-II (zinc bound) >98% HPLC purified protein from rabbit liver		Bestenbalt LLC (Estonia)
Receptor associated protein	62321	Progen Biotechnik
Tissue dissection and processing		
Sucrose	S5391	Sigma
Cryomatrix resin	6769006	Thermo Shandon
Pentobarbitone	61108	Ilium
Neuron culture		
B27 supplement	17504-044	Life Technologies/Gibco
Foetal calf serum	10099-141	Gibco
Gentamicin	15710-064	Gibco
Hank's buffered saline solution	H2387-1L	Sigma
HEPES powder	H3375	Sigma
L-glutamine	21051-024	Sigma
Neurobasal	10888-022	Life Technologies
Poly-L-lysine	P4832	Sigma
Sodium bicarbonate	S-5761	Sigma
Trypan blue	T8154	Sigma
Trypsin	T4549	Sigma
Immunochemistry		
Citric acid	C-0759	Sigma
DAPI	D3571	Molecular Probes
Fluorescence mounting media	S3023	Dako
Goat serum	S-1000	Vector Laboratories
Goat α-mouse (488 conjugate)	A11029	Molecular Probes
Goat α-mouse (HRP conjugate)	P0447	Dako
Goat α-rabbit (594 conjugate)	A11012	Molecular Probes
Goat α-rabbit (HRP conjugate)	P0448	Dako

Reagent	Catalogue number	Supplier
Immunochemistry continued		
Isolectin (488)	I-21411	Molecular Probes
Monoclonal mouse anti-GFAP	MAB360	Millipore
Monoclonal mouse anti-MAP2	MAB3418	Millipore
Monoclonal mouse anti-NeuN	MAB377	Millipore
Monoclonal mouse anti-olig2	MABN50	Millipore
Monoclonal mouse anti-PSD-95	120-27230	Abcam
Monoclonal mouse anti-SMI-312	SMI-312R	Covance
Monoclonal mouse anti-synaptophysin	sc-17750	Santa Cruz
Monoclonal mouse anti-tau	sc-32274	Santa Cruz
Nitric acid >69.0%	84385	Fluka
Nuclear yellow	N21485	Invitrogen
Polyclonal rabbit anti-LRP1	L1270	Sigma
Polyclonal rabbit anti-NR2a	AB1555P	Millipore
Polyclonal rabbit anti-NR2b	AB1557P	Millipore
Polyclonal rabbit anti-tau	A0024	Dako
Tomato lectin (biotin conjugate)	L0651	Sigma
Triton-X-100	LC262801	LabChem
β-actin	A2228	Sigma
Live cell calcium imaging		
10X Hank's buffered saline solution	14185-052	Life Technologies/Gibco
D-(+)-Glucose	G5767-500G	Sigma
Dihydrous calcium chloride	10070.0500	Merck
Fluo-4 AM	F-14217	Invitrogen
HEPES (1M)	15630-080	Life Technologies/Gibco
Hexahydrous magnesium chloride	M2670	Sigma
L-Glutamate	11048-014	Gibco
MK801	M107	Sigma
N-methyl-D-aspartate	M3262	Sigma
Western blots		
Acrylamide	A3553	Sigma
Albumin from bovine serum	A2153	Sigma
Ammonium persulfate	A3678-25G	Sigma
BCA protein kit	23225	Thermo Scientific
Bis (N,N-methylenebisacrylamide)	M7279	Sigma
Bromophenol blue	B5525	Sigma
Ethelenediaminetetra acetic acid	ED2SC	Sigma
Glycerol	G5516	Sigma
Glycine	26634	Thermo Scientific
Immobilon Western Chemiluminescent Substrate	WBKLS0050	Millipore
Methanol	2500-S	Millipore
N,N,N,N-tetramethyletheylenediamine	T9281-25ML	Sigma
Re-blot Plus	2500-S	Chemicon

Reagent	Catalogue number	Supplier
Western blots continued		
Instant skim milk powder		Woolworths Homebrand
Sodium deocycholate	D6750	Sigma
Sodium dodecyl sulfate	1610302	Bio-Rad
Spectra™ protein ladder	26634	Thermo Scientific
Trizma base	T1503-500G	Sigma
Tris hydrochloride	161-0719	Bio-Rad
Tween-20	P1379-500	Sigma
β-mercaptoethanol	M7154	Sigma

Equipment	Catalogue number	Supplier
General		
Eppendorf tubes 1.5mL	TUBE-170-C	Extra Gene
Falcon tubes 15mL	352096	BD Biosciences
Falcon tubes 50mL	352070	BD Biosciences
Neuron culture		
0.22µm bottle top filter	SCGPT05RE	Millipore
0.22µm syringe filter	SLGP033RS	Millipore
12 well plate	351143	BD Falcon
13mm glass coverslips	CB00130RA1	Menzel-Gläser
18mm glass coverslips	CB00180RA1	Menzel-Gläser
24 well plate	353047	BD Falcon
30mL syringe	SS*30LE1	Terumo
90mm petri dish with lid	S9014S20	Technoplas/Interpath
Goniotomy knife		Kaiser
Immunochemistry		
Cover glasses	HDLD22501.01PO	HD Scientific
Frosted 1 end glass slides	7107-PPN	Livingstone
MX35 Premier plus microtome blades	3051835	Thermo Scientific
Superfrost Ultra Plus® glass slides	J3800AHD#	Menzel-Gläser
Western blots		
Filter paper		
Nitrocellulose membrane	88018	Thermo Scientific